BIOPROSPECTING OF THERMOTOLERANT BACTERIA FROM HOT WATER SPRINGS OF HIMACHAL PRADESH FOR PRODUCTION OF THERMOSTABLE PULLULANASE ENZYME

Thesis

by

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Submitted in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE

MOLECULAR BIOLOGY AND BIOTECHNOLOGY



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This is to certify that the thesis entitled **"Bioprospecting of thermotolerant bacteria from hot water springs of Himachal Pradesh for production of thermostable pullulanase enzyme"** submitted in partial fulfilment of the requirements for the award of the degree of **MASTER OF SCIENCE MOLECULAR BIOLOGY & BIOTECHNOLOGY** to Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.) is a bonafide research work carried out by **Mr Anshul Sharma Manjul (H-2012-29-M)** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation has been fully acknowledged.

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ACKNOWLEDGEMENT

Humbly, I would like to thank ***Goddess Kushmanda*** *for giving me a gift of life and providing me everything that I would hope for. A loving family, friends who care and a road which leads to the attainment of my dreams. I would like to express my heartful regards towards Nani ji, Mumma, Pappa and my younger brother* ***Dhruv****. Their selfless persuasion, sacrifices, blessings and firm faith has made me achieve this goal. Emotions of my heart should find new boundaries to contain my love for my God gifted angel (sis)* ***Poonam****.*

I feel a great contentment to express my heartiest venerations and heartful gratitude to my esteemed teacher and chairperson of my Advisory Committee, ***Dr (Mrs.) Pooanm Shirkot****, Professor, Department of Biotechnology for her benevolent and everlasting guidance, unstinted help, painstaking efforts, constant inspiration and encouragement with her scientific acumen during the entire course of the present investigations. She always emphasized on hard work, total dedication, sincerity, efficiency and think positive attitude always builds up new hopes which was a learning experience for me.*

'Thanks' is too small a word to express my deep sense of gratitude to ***Dr D K Srivastava and Dr (Mrs.) Anjali Chauhan*** *for their sincere, selfless and invaluable help as and when required.*

Nevertheless, my successful accomplishment of the tasks at hand also owes much to the cooperation, personal inspiration, unfailing encouragement and moral support of ***Ruchika mam, Ambika mam and Ankita mam.*** *Words are inadequate to divulge my illimitable veneration towards my friends* ***Himani, Ishani****, and* ***Rajnikant****. Their constant support and help during the entire course of the present investigations will always be acknowledged.*

Facilities and cooperation provided by Verma ji, Manohar ji and office staff members of Department of Biotechnology is thankfully acknowledged.

I shall always remember the jovial company of my juniors, Ankita, Shalini, Abhishek, Bhuvnesh and Vishal for boosting up my spirits.

Needless to say, errors and omissions are solely mine.

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LIST OF ABBREVIATIONS

BLAST	-	Basic Local Alignment Search Tool
$CaCl_2$	-	Calcium chloride
$CoSO_4$	-	Cobalt sulphate
$CuSO_4$	-	Copper sulphate
dNTP	-	Deoxy nucleotide 5' triphosphate
DNS	-	Dinitrosalicylic acid
EBI	-	European Bioinformatics Institute
EDTA	-	Ethylenediamine tetraacetate
EtBr	-	Ethidium bromide
l	-	litres
EMBL	-	European Molecular Biology Laboratory
et al.	-	Co-workers
etc.	-	et cetera
$FeSO_4$	-	Ferrous sulphate
g/l	-	Gram per liter
hrs	-	hours
HCl	-	Hydrogen chloride
i.e.	-	id est (that is)
KCl	-	Potassium chloride
KH_2PO_4	-	Potassium dihydrogen phosphate
L	-	Litre
m	-	molar
mg	-	milligram
min	-	minutes
ml	-	millilitre
mM	-	millimolar
$MnSO_4$	-	Manganese sulphate
N	-	Normal
Na_2CO_3	-	Sodium carbonate
Na_2HPO_4	-	Disodium hydrogen phosphate
Na_2SO_4	-	Disodium sulphate
NaCl	-	Sodium chloride
$NaNO_3$	-	Sodium nitrate

NaOH	-	Sodium hydroxide
NCBI	-	National Centre for Biotechnology Information
ng	-	nanogram
NH_4Cl	-	Ammonium chloride
NJ	-	Neighbor Joining
No.	-	number
nt	-	Nucleotide
^oC	-	degree centigrade
bp	-	base pair
cm	-	centimeter
DNA	-	Deoxyribonucleic acid
OD	-	Optical density
PCR	-	Polymerase chain reaction
pmoles	-	picomoles
RAPD	-	Random amplified polymorphic DNA
RFLP	-	Restriction fragment length polymorphism
rpm	-	revolutions per minute
SDS-PAGE	-	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
Sp.	-	Species
Taq	-	*Thermus aquaticus*
U	-	units
UPGMA	-	Unweighted Pair Group Method of Arithmetic Averages
URL	-	Uniform Resource Locator
µl	-	microlitre
µg	-	microgram
µM	-	Micromolar

Chapter-1

INTRODUCTION

The microbial world is the largest unexplored reservoir of biodiversity on the earth, and is an important front in biology under investigation. Microbial diversity involves investigation of microorganisms in relation to their biotic and abiotic environments. The vast array of microbial activities and their importance to the biosphere and to human economics provide strong rationale for understanding their diversity, conservation and exploitation. This is done with a variety of approaches and tools, including microscopy, culturing, biochemistry and molecular biology. The microorganisms are adapted to a variety of habitats all round the biosphere. The opportunities of new industrial applications from microorganisms is as large as the variety of environments, they confront. Microbial diversity also describes the complexity and genetic variability of the communities. The total genomic complexity denotes the confines of diversity in terms of genetic information present and provides information about the overall taxonomic and functional variability at the community level.

The microorganisms are the living entities which have ability to survive under extreme environmental conditions in which other life forms are unable to survive. Microorganisms, specifically bacteria, continue to be discovered living under extreme environmental conditions such as high temperature, low pH, high salinity and low temperature etc. Thermophilic microorganisms are a group of extremophiles whose optimal growth temperature lies between 45-80°C, while extremophiles whose optimum temperature is above 80°C are called hyperthermophiles. Thus, thermophiles and hyperthermophiles are the microorganisms which live at very high temperature conditions such as hot water springs, volcanic eruption areas etc. Springs are concentrated discharge of groundwater that appears at the surface as a current of flowing water. Springs that discharge water which has a temperature above that of the normal local ground water are called thermal springs (Todd, 1980). Most of the springs are the result of long cracks in sedimentary rock. Hot springs contained the life even long

before they reach the surface, and the warm water of the springs allows an abundance of algae and bacteria to survive which are called as thermophilic microorganisms. The thermophiles may be among the first living entities on the earth, developing and evolving during the primordial days of earth when surface temperature were quite hot, and thus been called the "Universal Ancestor" (Sen *et al.,* 2010). The ability of the enzymes and proteins which control the specific metabolic and biological functions to work at such high temperature makes survival of these microorganisms under adverse conditions. Scientists are taking great interest in the study of thermophiles ever since the discovery of *Thermus aquaticus* from the Great Fountain region of Yellow Stone National Park (Brock and Freeze, 1969).

Thermotolerant microorganisms are potential sources of suitable enzymes that are more thermostable, as well as more resistant than their mesophilic counterparts to organic solvents, detergents, low and high pH, and other denaturing agents. The guaranteed properties of the thermostable enzyme isolated from thermophilic microorganisms are thermostability, stability against denaturing substances like detergents, organic solvents, stability against high alkalinity and acidity and suitable for fermentation processes. These extremophiles perform metabolic functions producing highly thermostable enzymes such as DNA polymerases, pullulanases, amylases, lipases, laccases, proteases etc and proteins capable of significant geochemical functions *in situ* and potentially adaptable to industrial, biotechnological and bioremediation applications.

The enzyme pullulanase belongs to α-amylase class of enzymes and is an extracellular carbohydrase which was first reported by Bender and Wallenfels in 1961 from bacteria *Klebsiella pneumoniae* (Wallenfels *et al.,* 1966). Pullulanase is also called de-branching enzyme as it can hydrolyze starch, amylopectin, pullulan and related oligosaccharides. Pullulanase belongs to class EC 3.2.1.41 and are classified as type I (true pullulanases) or type II (amylopullulanase) depending on their abilities to degrade α-1,4 glycosidic linkages in starch, amylopectin, and related oligosaccharides. Type I pullulanase is unable to attack α-1,4 glycosidic linkages whereas both type I and type II pullulanases attack α-

1,6-glycosidic linkages in pullulan or branched oligosaccharides, forming maltotriose or linear oligomers, respectively. Pullulanases from thermophiles are interesting, both from an enzyme stability perspective and because novel forms of these enzymes can potentially be applied in the industrial starch hydrolysis process. Thermostable pullulanases that are active above 60°C and specifically attack the branching points of amylopectin are of special interest, because they would allow more efficient and rapid conversion reactions. Hence, a number of pullulanases have been isolated, particularly from thermophilic microorganisms (Niehaus *et al.,* 1999).

Now a day, thermostable enzymes are gaining wide industrial and biotechnological interest due to the fact that they are better suited for harsh industrial processes. Thermostable pullulanase enzyme has many applications in detergent, textiles, food, pharmaceuticals, leather, bioremediation of waste material etc. The commercial applications of pullulanase is manufacturing of low caloric beer, and as a dental plaque control agent. In addition to this it is also used in starch processing industry for the production of maltose syrups and high purity glucose and fructose. Pullulanases are used in sugar syrup industries to complete the hydrolysis of starch initiated by α-amylases. The combined application of pullulanase with other amylolytic enzymes increases the quality of sugar syrups. When starch is treated with amylase and pullulanase simultaneously and consequently increases the efficiency of a saccharification reaction. Pullulanase play an important role in the enzymatic anti-staling treatment (Modderma and Fol, 1995). Further it has a great potential to convert agro-waste into biofuel as it ferments starch to ethanol (Malviya *et al*., 2010). Resistant starch and substitute of natural gums are also produced by action of pullulanase; these products have various potential pharmaceutical and neutraceutical applications (Prakash *et al.,* 2012).

Bioprospecting is an umbrella term describing the process of discovery and commercialization of new products based on biological resources. Bioprospecting often draws an indigenous knowledge about uses and characteristics of plants, animals and microbes. In this way, bioprospecting involves the exploitative appropriation of indigenous forms of knowledge by

commercial representatives, as well as the search for previously unknown compounds in organisms that have never been used traditionally. Thus it involves the discovery of new products which are beneficial for humans on earth.

The local biodiversity of Himachal Pradesh has got great possibilities of hitting upon a source which is much more effective in addition to being economical. The aim of this research was to study the presence and diversity of thermophilic and hyperthermophilic microorganisms producing thermostable pullulanases from the hot water springs from Himachal Pradesh. Because of both, industrial and environmental interests in these unique ecosystems, it is important to get a better understanding of the microbial diversity. Useful bacteria producing thermostable enzymes are nature's gifts to man and Himachal Pradesh has been bestowed with hot water springs. So it is worth exploring the microbial diversity of these hot water springs of the state to assess the status of microbial wealth in Himachal Pradesh w.r.t. thermostable pullulanase producing bacterial species. Himachal Pradesh is a mountainous state having an altitude ranging from 350 to 7000 meters above the sea level. Nature has blessed Himachal Pradesh with a number of hot water springs and these are located at Manikaran, Vashist, Kasol, Kalath, Khirganga and Tattapani which includes the Kullu and Mandi districts. Keeping this in view, it was proposed to isolate thermostable pullulanase producing bacteria from these hot water springs of Himachal Pradesh followed by production of thermostable pullulanases with significant applications.

The present study was, therefore, proposed to accomplish the following objectives-

OBJECTIVES

i) Survey of sites for the presence of hot water springs in Himachal Pradesh and collection of samples.

ii) Isolation of thermostable pullulanase producing thermotolerant bacteria.

iii) Morphological, biochemical and molecular characterization of thermotolerant bacterial isolates.

iv) Production and partial purification of thermostable pullulanase enzyme.

Chapter-2

REVIEW OF LITERATURE

Pullulanase (pullulan α-glucanohydrolase; EC 3.2.1.41) is an extracellular carbohydrase, debranching enzyme which hydrolyses the extracellular yeast, polysaccharide and pullulan. Pullulanase also termed as α-dextrin 6-glucanohydrolase or true pullulanase or limit dextrinase (Bender and Wallenfels, 1966). It consists of repeating units of α-maltotriose joined "head to tail" by 1, 6 bonds. Pullulanase specifically attack on α-1,6-glycosidic linkage in pullulan to produce maltotriose and isopullulanase which hydrolyzes α-(1,4)-linkage to produce isopanose and neopullulanase which acts on α-(1,4)-linkage to produce panose (Doman-Pytka and Bardowski 2004). These properties have made pullulanase a useful agent in structural studies of oligosaccharide and polysaccharide (Ling *et al.,* 2009; Drummond *et al.,* 1969; Saha *et al.,* 1988 and Enevoldsen, 1977). Although in nature the pullulanases are ubiquitous and produced from various sources including plants, fungi, yeast, animals and microorganisms but pullulanases of microbial origin are the most versatile enzymes and are known to bring about a range of bioconversions (including alcoholysis, aminolysis, hydrolysis and esterification). Pullulanases of microbial origin was first discovered in *Klebsiella* sp. (Bender and Wallenfels, 1966). Besides, microbes can be easily cultivated and pullulanase production is extracellular in nature mostly.

Starch is a major industrial raw material and is chemically and/or enzymatically processed into variety of products for subsequent use in various industries, ranging from food (especially high-fructose and glucose syrups) to washing detergent industries. Pullulanases are used at 60°C or above for saccarification process. One of the general prerequisites for an enzyme to be applicable in industrial processes is thermotolerance and thermostability (Vieille *et al.,* 1996; Vielle and Zeikus, 2001). In order to facilitate novel and more efficient biocatalytic process applications, there is a need for with improved biochemical properties, such as thermostability and thermotolerance. It is not only

their thermostability but also their greater stability under other extreme conditions such as high pH or lower water concentration. Thermozymes are much more useful in the processing of lower viscosity fluids, as at high temperature viscosity is usually reduced heat and mass transfer rates are also improved at high temperature. Proteins from thermophiles show higher thermostability as well as more stability towards organic solvents and higher activity at elevated temperatures (Jaeger and Eggert, 2002). Natural environments for thermophilic microorganisms are widespread on Earth's surface (Barns *et al.,* 1994), in form of hot water springs, volcanic eruption areas and peat bogs, etc. Hot water springs are found to be the potential source of a large microbial diversity producing thermostable enzymes. The isolation of different thermophilic microbes from hot water springs producing different thermostable enzymes have showed that there is a lot of possibilities in this area to isolate different products of economic value.

2.1 THERMAL SPRINGS

A thermal spring is a hot water body produced by the emergence of geothermally heated groundwater from the earth's crust. There are geothermal hot springs in many locations all over the crust of the earth. Thermal springs are high temperature aquatic ecosystems and usually maintain a constant temperature and flow of water thus can be considered as 'laboratories in the natural environment. The thermal springs are widely distributed in the world but are most numerous in volcanically active areas. The chemical properties of the springs have been related with medicinal properties of certain springs (Waring, 1965). Many hot water springs have significant amount of hydrogen sulphide. The concentration of elements such as fluoride, arsenic, rare earths and gold varies very much from spring to spring. Many springs are highly radioactive and some springs precipitate silica, others deposit travertine ($CaCO_3$) and still others form elemental sulphur.

In India, thermal springs are scattered throughout the country and occur either as solitary or in groups from sea levels up to nearly three thousand meters above the sea level. Early studies were mainly concerned with the medicinal properties of the springs (Newbold, 1848; Buist, 1851; Macpherson, 1855;

Schlagintweit, 1862). Oldham & Oldham (1882) prepared an exhaustive catalogue of Indian hot water springs and reported more than three hundred thermal springs in the country. Some springs in Bombay have been recorded as being permanently radioactive (Sierp and Steichen, 1911) containing radium salts in solutions. Nag (1933) and Chatterjee (1936) studied the radioactive properties of Rajgir thermal spring. Ghosh (1948) reported hot water springs at Jarom and studied 112 springs and recognized 4 main broad belts in which majority of the thermal springs occur in India (Fig-1) *viz*., Bihar, along the west coast of India, the Himalayan belt, East India. Further on the basis of chemical nature, the thermal springs are classified into four main groups *viz*., simple or indifferent waters, having low mineral contents; alkaline waters; sulphur waters; chloride or saline waters. In recent years a number of studies concerning primary productivity, nutrient cycle, trophodynamics and succession of communities have been undertaken in hot springs which has been helpful in understanding the hot spring ecosystem (Brock, 1967; 1970 and 1973; Brock and Brock, 1966; 1968; Castenholz, 1967; 1970 and 1976). Stockner (1968) and Naiman (1976) extensively studied the different aspects of thermal spring ecosystem and reviewed extensively the environmental requirements and biology of thermophilic microbes.

2.2 THERMOPHILES

Thermophiles are the organisms which are adapted to live at high temperatures and these high temperature environments are widely distributed on the earth. The availability of thermophiles to grow at temperatures above 60^{o}C is associated with extremely thermostable macromolecules present in them. Thermophilic bacteria have been reported in most boiling water environments, where they reproduce normally (Brock, 1967). Thermophilic microorganisms have been found to be potential and good alternative sources of thermostable enzymes. The investigation and application of thermostable enzymes from thermophilic microorganisms have received a lot of attention in industries ranging from food to waste management industries.

A great bacterial diversity has been reported in various solar heated environments ranging from rod shaped spore forming genera *viz*., *Bacillus, Clostridium, Thermoactinomyces* to rod shaped non-spore forming genera *viz*., *Thermus anaerobium* (Zeikus, 1979). The major nutritional categories include phototrophic species *viz*., *Chloroflexus auratiacus*, chemoautotrophic species *viz*., *Methanobacterium, Thermoautotrophicum,* chemoheterotrophic species *viz*., *Thermoanareobium brockii,* chemosynthetic mixotrophic species that grow autotrophically or heterotrophically *viz*., *Sulfolobus acidocaldarius* (Transey and Brock, 1978).

Thermophilic bacteria may have tremendous potential in future microbial and enzyme technology because of their unique property to work at high temperature and enables development of improved or new biotechnology industry (Zeikus, 1979).

2.3 THERMOSTABLE ENZYMES

Thermostable enzymes were the result of discovery of thermophilic microorganisms from hot water springs in 1960s. It was discovered that many bacteria in hot springs not only survived but also thrive at higher temperatures. The first thermostable enzyme Taq polymerase was obtained from *Thermus aquaticus* in 1974 and it was followed by more thermostable enzymes listed in Table-1.

Although the importance of enzymes as catalysts in industrial processes has been widely recognized, very few have high market value for industrial and household applications (Jarnagin and Ferrari, 1992). However, thermophilic bacteria, having growth at high temperature and specific macromolecular properties, possess high metabolic rates, physically and chemically stable enzymes. Thermophilic processes appear to be more stable, rapid and less expensive and facilitate reactant activity and product recovery and replaced the traditional methods to some extent (Zeikus, 1979). In addition the ability to clone and express virtually any desired gene has removed this obstacle and increased the number of enzymes conceivably available for industrial experimentation from

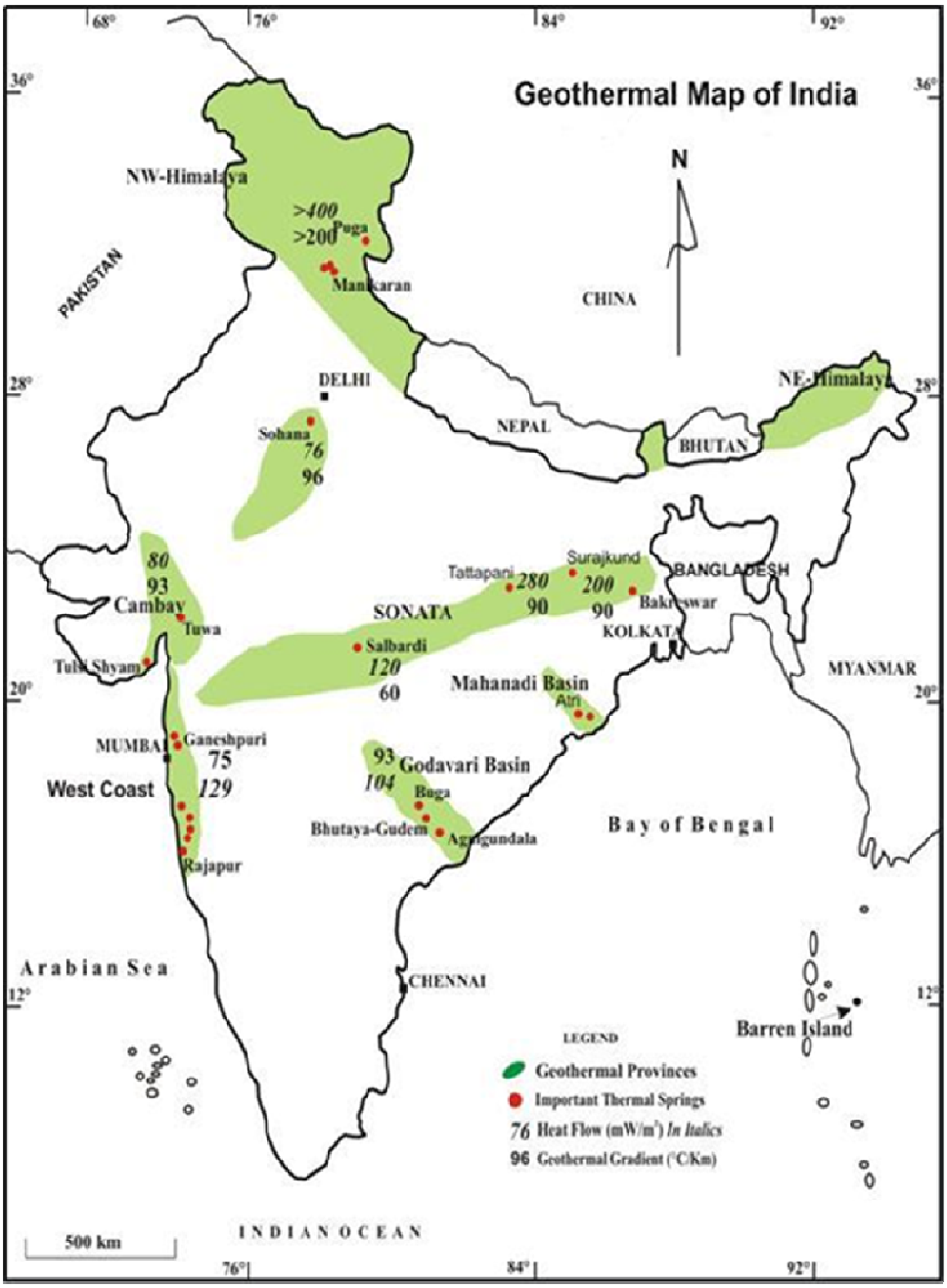


Fig. 1 Distribution of hot water springs in India

a few hundred to several thousand (MacQuitty, 1988). Expression of newly available neutral enzymes or novel ones created by protein engineering require the availability of reliable production hosts that are easy to transform. In present era, thermophiles have become the subject of special interest for structural investigations and biotechnological applications (Herbert, 1992).

Table 1: Summary table depicting reported thermophilic microorganisms and thermostable enzymes

Enzymes	Organisms	Optimal temperature (^{o}C)	References
α-Amylases	*Bacillus stearothermophilus*	70-80	Vihinen and Mantsala, (1990)
	Bacillus stearothermophilus	70	Jeayoung *et al*., (1989)
	Myceliophthora thermophila	100	Ramkrishna *et al*., (1993)
	Pyrococcus furiosus	100	Laderman *et al,.* (1993a,b)
	Thermococcus aggreganes	100	Canganella *et al*., (1994)
β-Amylase	*Clostridium thermosulphurogenes*	75	Shen *et al.,* (1988)
Pullulanase	*Pyrococcus furiosus*	98	Brown and Kelly, (1993)
	Thermococcus aggregans	100	Canganella *et al.,* (1994)
	Thermococcus hydrothermalis	95	Gantlet and Duchiron, (1998)
Xylanases	*Pyrococcus furiosus*	100	Bauer *et al.,* (1999)
	Scytalidium thermophilum	65	Nazan and Zumrut, (2000)
	Sulfolobuss olfataricus	105	Nucci *et al*., (1993)
	Thermotoga thermarum	80	Sunna *et al.,* (1996a)
Cellulases	*Pyrococcus furiosus*	102-105	Kengen *et al*., (1993)
	Rhodothermus marinus	95	Hreggvidsson *et al*., (1996)
	Thermotoga neapoltana	95	Bok *et al.,* (1998)
	Thermotoga neapoltana	106	Bok *et al*., (1998)
Proteases	*Bacillus stearothermophilus*	85	Rahman *et al.,* (1994)
	Bacillus thermoruber	45	Manchini *et al*., (1988)
	Thermococcus celer	95	Klingberg *et al.,* (1991)
Lipases	*Bacillus stearothermophilusa*	68	Gupta *et al*., (1999)
	Pseudomonas sp.	90	Rathi *et al*., (2000)
	Pyrococcus horikoshii	95	Yan *et al.,* (2000)
	Bacillus sp. RSJ-1	50	Sharma *et al*., (2002)
	*Bacillus thermoleovorans*ID-1	75	Lee *et al.,* (2006)
	Acinetobacter sp.	94	Bisht and Panda, (2011)
	Geobacillus sp.	94	Zuridah *et al*., (2011)

2.4 DISTRIBUTION OF PULLULANASE/THERMOPULLULANASES

Pullulanase is an amylolytic enzyme that is capable of cleaving α-1,6-glycosidic bonds in polysaccharides such as starch, pullulan, amylopectin and related oligosaccharides. This enzyme became of interest due to its debranching capability. However, for industrial purposes, more stable enzymes are in high demand, leading to various researches done on producing thermostable pullulanase from thermophilic and hyperthermophilic organisms. The enzyme pullulanase can be isolated from various sources like bacteria, fungi, plant and yeast.

The enzyme pullulanase in plants readily cleaves the α-1,6-glycosidic linkages in pullulan β-limit dextrin and α-limit dextrin while debranching amylopectin to a lesser extent than polyglucans. Potato and broad beans are common plant sources of pullulanase. Pullulanase from these sources produced maltose and maltotetraose, accompanied by traces of maltohexose, from waxy maize β-dextrin with an overall yield of 12.8%. In contrast to pullulanase from potato, the one isolated from corn produced a high yield of maltohexose. Pullulanase isolated from malted barley had only low activity until it had been thoroughly purified. Rice can also serve as a source of pullulanase.

Fungi and yeasts also produce pullulanase. The fungus *Streptomyces* strain NCIB 12235 is a source of pullulanase. The enzyme performs best at pH: 4-6 and 55-65°C. Pullulanase secreted from *Aspergillus oryzae* and *Aspergillus usamii* could not be separated from amylases. Pullulanase was also extracted from brewery yeast.

Pullulanase is produced by a variety of microorganisms, such as mesophiles- *Bacillus macerans* (Adams and Priest, 1977) and *Bacillus circulans* F-2 (Sata *et al.,* 1989); thermophiles- *Thermoactinomyces thalpophilus* (Odibo and Obi, 1990) and *Thermus* sp. AMD33 (Nakamura *et al.,* 1989); and hyperthermophiles- *Pyrococcus woesei* (Rudiger *et al.,* 1995), *Thermotoga maritima* (Kriegshauser and Liebl, 2000) and *Rhodothermus marinus* (Gomes *et al,* 2003). *Bacillus sp*. US149 (Roy *et al.,* 2003) and *Thermoanaerobacter* strain

B6A (Saha *et al.,* 1990) produced acidic pullulanase while *Bacillus* sp. S-1 (Lee *et al.,* 1997) and *Bacillus* sp. KSM-1378 (Ara *et al.,* 1995) secreted alkaline pullulanase.

2.5 STRUCTURE OF PULLULANASE

Pullulanase is divided into two groups, pullulanase type I (pullulanase I) containing classical α-1,6-specific pullulanase, and pullulanase type II (pullulanase II, amylopullulanase), which can act on both α-1,6 and α-1,4-glycosidic linkages of pullulan (Doman-Pytka and Bardowski 2004; Bertoldo and Antranikian 2002). Other types of enzyme that can hydrolyse pullulan are pullulan hydrolase type I (neopullulanase), pullulan hydrolase type II (isopullulanase), and pullulan hydrolase type III (Doman-Pytka and Bardowski, 2004; Bertoldo and Antranikian, 2002). According to the recommendation of the International Union of Biochemistry and Molecular Biology (IUBMB) enzyme classification relies on two biochemical features: the catalytic reaction and substrate specificity (Kuriki and Imanaka, 1999; Webb, 1992). Enzymes that hydrolyze pullulan have been classified into four types (Kuriki and Imanaka 1999; Plant *et al*., 1987): 1) glycoamylases (EC 3.2.1.3), which hydrolyze pullulan from non-reducing ends to produce glucose; 2) pullulanases or α-dextrin 6-glucanohydrolase (EC 3.2.1.41), which hydrolyze α-(1,6)-glucosidic linkage of pullulan to produce maltotriose: 3) isopullulanase (EC 3.2.1.57), which hydrolyze α-(1,4)-glucosidic linkage of pullulan to produce isopanose: 4) neopullulanases (EC 3.2.1.135), which hydrolyze α-(1,4)-glucosidic linkage of pullulan to produce panose (Fig 2).

The pullulanase is also known as limit dextranase (EC 3.2.1.42), previously called R-enzyme (Manners 1997). The structure of pullulanase comprises five domains: N1 from residues 39–172, N2 from 32–38 and 173–287, N3 from 288-395, A from 396–966, and C from 967–1083. The N1 domain, having the highest average B-factor, could be refined only in the complexes with G2, G3, and G4. The average deviation of the C^{α} position in the N1 domain between the G4 and G3 complexes reached 4.1Åafter the whole C^{α} was superimposed (r.m.s.=0.33 Å for 946 C^{α} atoms within 2Å), indicating the

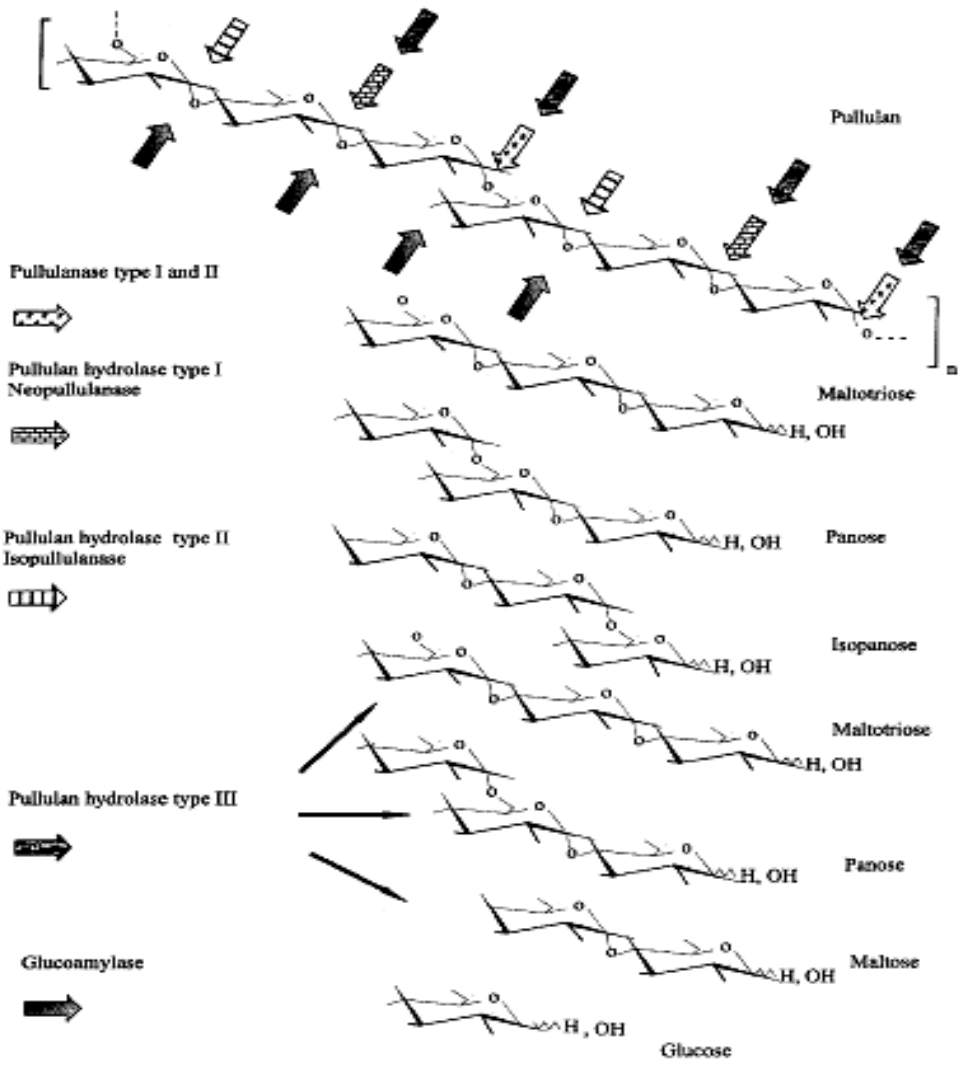


Fig 2: Enzymatic degradation of pullulan by pullulanase

mobility of the N1 domain. The average B-factors of the five domains were: N1 54.0Å^2, N2 36.7Å^2, N321.4Å^2, A 18.5Å^{2}and C 26.1Å^2 in the structure of the G4 complex. Figure 3 shows the whole structure of pullulanase complexed with G4. The enzyme has dimensions of 102Å×65Å×71Å, and has a hole of 25Å diameter surrounded by the N1, N2 and A domains. This hole is faced by the active cleft of the A domain and the possible carbohydrate binding site of the N1 domain. The three N-terminal domains, N1, N2 and N3, and the C-terminal domain C are composed of a motif of two-sheeted β-sandwich structures each composed of 5β/3β, 4β/4β, 3β/4β, and 5β/3β, respectively, while the catalytic domain A has a $(\beta/\alpha)_8$-barrelstructure. Domains N1, N2 and C have α-helices, mostly flanking

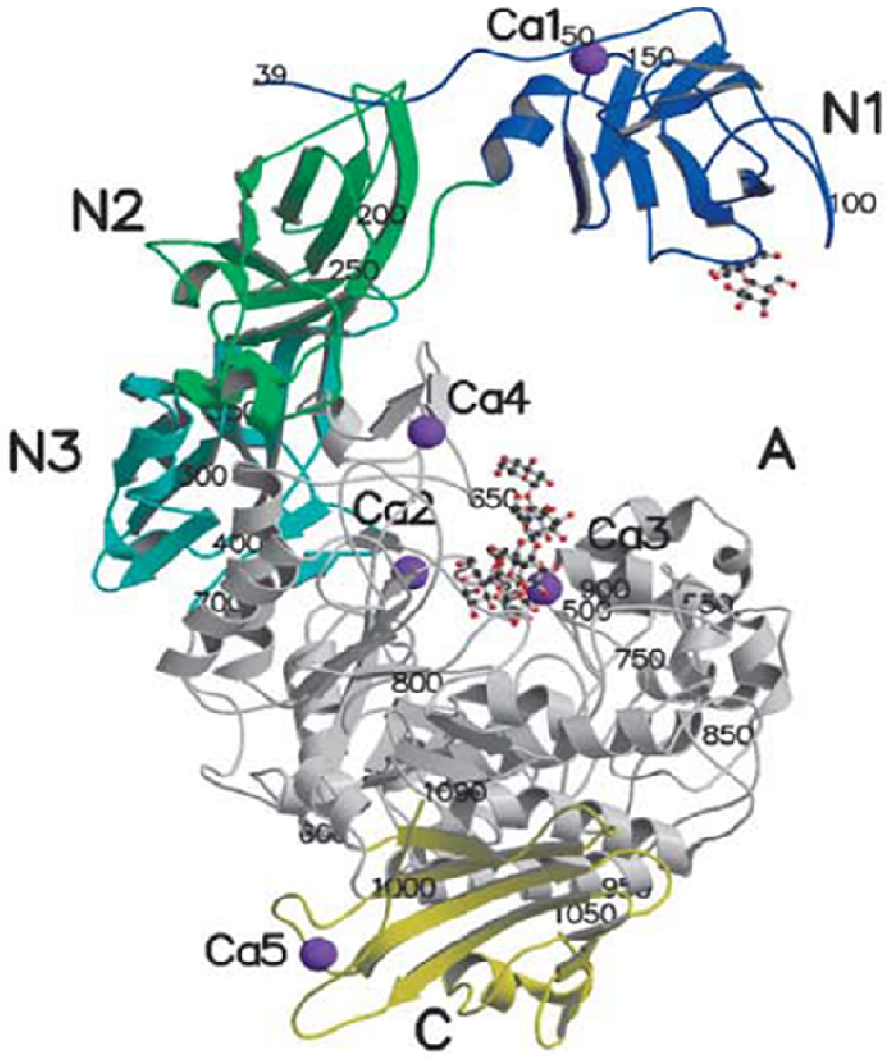


Fig 3: **Overall structure of the pullulanase with G4 (ribbon stereo diagram). The Figure shows N1 (blue), N2(green), N3 (cyan), A (white) and C' (yellow) domains, together with five calcium ions (purple sphere) and two G4 (ball and-stick) in the active site.**

their C-terminal sides. The domains of N2, N3, A and C are bound tightly with hydrogen bonds and van der Waals interactions. The numbers of hydrogen bonds are ten N2–N3, eight N2–A, six N3–A and fifteen A–C. In contrast, the N1 domain, having the highest average B-factor, is rather isolated, with no hydrogen bond to another domain.

2.6 ISOLATION OF PULLULANASE/ THERMOSTABLE PULLULANASE PRODUCING MICROBES

The isolation of independent microbes from various environmental sources has been important in all research work related to microbes, since bacteria are ubiquitous and live in microbial communities of mixed populations. Population in microbial communities or ecosystems may interact and co-operate in their efforts to obtain nutrients from the environment and with the waste products from one group of microbes serving as nutrients for another. The wastes/metabolic wastes from various industries and fermentation processes also provide a favourable ecological niche for bacteria. Pullulanase producing microbes can be isolated from various sources like rice husk, soil, waste water, hot water springs etc.

The first report of bacterial pullulanase was reported from *Aerobacter aerogenes* by Bender *et al.,* (1961) using Czapeck medium. This was followed by various reports of bacterial pullulanase produced by *Aerobactcr aerogenes* (Enevoldson and Nielson, 1974), *Bacillus stearothermophilus* strain TRS128 (Kuriki *et al.,* 1988) and *Bacillus polymyxa* (Castro *et al.*, 1992).

Koch *et al.,* (1997) reported isolation of thermostable pullulanase from *Fervidobacterium pennavorans* Ven5 from water and mud samples of Abano terme spa of North Italy. They used a complex medium given by Balch and Wolfe (1976). The cells were gram negative motile rods occurring single and in pairs. This new isolate of *Fervidobacterium pennavorans* was found similar to *Fervidobacterium islandicum* and *Fervidobacterium nodosum* and was identified as *Fervidobacterium pennavorans* Ven5.

Roy *et al.,* (2003) isolated and purified an acidic pullulanase type II from *Bacillus* sp. US149. They collected soil samples from Tunisian hot spring zone. The strain US149 was routinely grown in M9 minimal medium with pH 5.0 and starch as the sole carbon source. Based on the area of pullulan degradation, the strain designated US149 was selected as it exhibited a comparatively high activity. The strain could grow between 30-55°C on solid medium with 45°C as optimum temperature. Microscopic studies revealed single or oligo-chain of the cells in culture. Physiological test showed the strain was gram positive, catalase positive, oxygenase negative, non-motile, and non-sporulating.

Ling *et al*., (2009) isolated pullulanase type II producing *Bacillus* H1.5. They isolated *Bacillus* H1.5 from various sources and locations (soils samples from cassava, corn, paddy farm fields, decayed samples from different types of fruits, vegetables and tubers and water samples from hot springs and sea water) of Malaysia. They used basal medium for isolation (Katsutoshi *et al.,* 1995). Based on the area of pullulan degradation, the strain designated H1.5 was selected as it exhibited a comparatively high activity (0.05–0.1 U/mL). Microscopic studies revealed rods, single cell or pairs or chains of 3-5 cells in culture. Physiological test showed the strain was Gram positive, non-motile, and sporulating. From the identification using API kits, it was found that the isolate H1.5 belongs to *Bacillus* sp.

Chai *et al.,* (2011) isolated and characterized pullulan-degrading *Anoxybacillus* sp. isolated from Malaysian hot springs using modified *Thermus* medium (Atlas, 2004). Pullulan degrading bacterial colonies produced clear halozones on solid media containing pullulan/starch and these were white, cream to dull yellow in colour. These pullulan degrading bacteria were found positive for catalase and oxidase tests and negative for motility and nitrate reduction tests. Identification was done by 16S *rrna* gene sequencing.

Ahmad and Milad (2012) isolated a new glucoamylopullulanase from thermotolerant alkalophilic *Bacillus subtilis* DR8806 from a hot mineral spring in Iran. Samples were grown on the super Luria-Bertani medium at 60°C and 180

rpm on a rotary shaker incubator for 48 hrs and subsequently subcultured on the Luria-Bertani agar plate to isolate single colonies.

Asha *et al.,* (2013) isolated pullulanase producing bacterium from potato grown fields of Bangalore on modified pullulan agar medium and identified as *Bacillus halodurans* based on the microscopic examination and biochemical tests. Maximum pullulanase production occurred in the presence of soluble pullulan at 1.5% concentration and in the presence of 0.5% peptone at pH 10.0 and 37°C.

Khalaf and Aldeen (2013) isolated pullulanase producing *Bacillus licheniforms* (BS18) from different sources of soil in Iraq. Sample was spread over nutrient agar plates and incubated aerobically at 37°C for 24 hrs. Bacterial isolates were identified as *Bacillus* sp. according to the morphological and microscopic examinations. Pullulan agar medium containing 1% pullulan as a carbon source was used for screening the pullulanase production. Five isolates (BS18, BS13, BS19, BS11, and BS8) which have largest pullulan hydrolysis zones were selected for quantitative screening of pullulanase production. Results indicated that *Bacillus* BS18 showed the highest specific activity for the production of pullulanase (18 U/mg protein) with largest pullulan hydrolysis ability.

2.7 MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF BACTERIAL ISOLATES

The term “colony morphology” refers to the visible characteristics of a colony. Colonies that differ in appearance are typically different bacterial strains, species, or genera. However, colony morphology is not a reliable way to identify bacteria, as many different types of bacteria have similar colony morphology. Still, recognizing differences in colony morphology is useful when trying to isolate bacteria. Although classification based on genetic divergence highlights the evolutionary relationships of bacteria, classification based on the morphological and biochemical features of bacteria remains the most practical way to identify these organisms. A definitive identification scheme for bacteria was first presented in 1984 in Bergey’s Manual of Systematic Bacteriology. In this scheme, bacteria are classified on the basis of many characteristics. Cell

shape, nature of multi cell aggregates, motility, formation of spores, and reaction to the Gram stain are important. Morphological features are important in microbial taxonomy for many reasons. Morphology is easy to study and analyse, particularly in eukaryotic microorganisms and the more complex prokaryotes. In addition, morphological comparisons are valuable because structural features depend on the expression of many genes, are usually genetically stable, and normally do not vary greatly with environmental changes. Thus, morphological similarity often is a good indication of phylogenetic relatedness. The different morphological features employed in the classification and identification of microorganisms are: cell shape, cell size, colonial morphology, ultrastructural characteristics, staining behaviour, mechanism of motility, endospore shape and location, spore morphology and location, cellular inclusions and colour.

Biochemical/metabolic characteristics are also very useful because they are directly related to the nature and activity of microbial enzymes and transport proteins. Since proteins are gene products, analysis of these characteristics provides an indirect comparison of microbial genomes. Important in the identification of a genus and species of bacteria are biochemical tests, including the determination of the kinds of nutrients a cell can use, the products of its metabolism, the response to specific chemicals, and the presence of particular characteristic enzymes. Other criteria used for the identification of some types of bacteria might be their antigenic composition, habitat, disease production, and requirement for specific nutrients. Some tests are based on the ultrastructure of the bacteria revealed under the electron microscope by negative staining and preparation of thin sections.

Both morphological as well as biochemical characterization technologies have been immensely useful and have been applied in carrying out characterization of most of the microorganisms.

Kuriki *et al.,* (1988) carried out morphological and biochemical characterization of pullulanase producing bacterial strain which was identified as

Bacillus stearothermophilus strain TRS128 and found it to be strictly aerobic, gram negative, catalase positive and endospore forming Bacillus.

Tomimura *et al.,* (1990) carried out morphological and biochemical characterization of pullulanase producing *Bacillus naganoensis* strain D39 which was found to be gram positive and the colony was found to be opaque, smooth, glistering, convex and circular with entire margins. It tested positive for catalase, starch hydrolysis, growth in the presence of 2% NaCl and growth at pH 6.

Koch *et al.,* (1997) carried out morphological and biochemical characterization of pullulanase producing thermophilic anaerobic bacterium *Fervidobacterium pennavorans* Ven5 and found it to be gram negative, straight rod with pointed ends, occurring singly or in pairs or sometimes in short chains. Sulphate and nitrate reduction were negative.

Roy *et al.,* (2003) carried out morphological and biochemical characterization of *Bacillus* sp. US149 capable of producing acidic pullulanase type II. The cells of this strain were found to be rod shaped, gram positive, non-motile and could grow only in acidic pH (4.4-5.0) between 37°C and 55°C. Tests showed that the strain was catalase positive, oxygenase negative and no spore formation.

Ling *et al.,* (2004) reported pullulanase type II from a local *Bacillus* isolate H1.5. Microscopic studies revealed rod shaped, single cell or pairs or chains of 3-5 cells in culture. The strain was gram positive, non-motile and spore formation.

Chai *et al.,* (2011) reported isolation of pullulan degrading *Anoxybacillus* sp. from Malaysian hot springs. Two strains which were able to degrade starch and pullulan were SK3-4 and DT3-1. The colonies of SK3-4 were glistering and white to cream coloured and the colonies of DT3-1 were dull and yellow coloured. The cells of both strains were straight/slightly curved rods in single arrangements. Both cells strained gram positive and formed endospores. Both strains were positive in the catalase and oxidase tests and negative in the motility and nitrate reduction tests. Both strains hydrolysed starch.

Khalaf and Aldeen (2013) reported 30 isolates of *Bacillus* sp. Colonies on the nutrient agar medium showed opaque with dull to rough surface mounds and lobes consisting largely of slime often accumulate on colony. Among these isolates BS18 showed maximum pullulanase activity. The strain BS18 was rod shaped gram positive, motile and spore forming. BS18 was positive in catalase, oxidase and citrate test and negative in indole test. It also showed hydrolysis of glucose, maltose, sucrose and fructose.

2.8 MOLECULAR CHARACTERIZATION AND PHYLOGENETIC STUDIES

Molecular characterization technology is now widely used for wide range of bacterial species. The analysis of DNA has been used in a large number of studies on bacterial taxonomy and bacterial typing, and as well to further our understanding of the basic mechanisms of evolution. The availability of DNA sequences for the complete genome of a number of bacteria has paved the way for the identification of orthologous families of genes within genomes based on DNA sequence and gene function (Eisen, 1998). The characterization of orthologous gene families has been termed phylogenomics (Eisen, 1998). Related to phylogenomics is the concept of exaptation defined as the recruitment of DNA or RNA sequences into variant or novel function: RNAs from all categories have been identified that may be accepted as novel genes or regulatory elements (Brosius, 1999; Brosius and Gould, 1992). Similarly, bacterial morphology provides significant evolutionary insights when mapped onto rRNA phylogeny since bacterial morphology has been shown to be remarkably consistent with rRNA phylogeny (Siefert and Fox, 1998). A phylogenomic approach that includes information about the function of genes, exaptation and bacterial genetics of morphology and or biochemistry in the phylogenetic analysis may in the future be of value in inter-genomic as well as intra-genomic analyses of isolates.

The evolution of bacteria has long been viewed primarily through the perspective of rRNA sequences (Woese, 1987). The gene content of the genome (Snel *et al*., 1999), the evolutionary rates of different classes of genes (Jain *et al.,*

1999), genome signatures (defined as the ratios between the observed dinucleotide frequencies and the frequencies expected if neighbors were chosen at random). G+C content (Sueoka, 1999) and the occurrence and location of gene duplications, rearrangements, insertions, gene capture (Szpirer *et al.,* 1999) and horizontal gene transfer (Lawrence and Hartl, 1992) are all indices of genome organization that vary between species. A much more complete analysis of the taxonomy and evolution of bacteria may be possible with the current rapid DNA analysis techniques if these parameters are used as a framework.

The analysis of DNA sequences has significantly increased further knowledge about the relatedness of different isolates in at least three categories including random whole-genome analysis, specific gene variation and mobile genetic elements. The numerous studies in these areas has provided considerably more information than traditional phenotypic methods and with this, more questions have been raised that were previously not evident.

The analysis of whole genomes by DNA band pattern analysis has been facilitated by the discovery of repetitive elements and their analysis by a number of PCR based techniques (including RAPD, ERIC, BOX and REP) and the analysis of restriction enzyme sites on bacterial chromosomes by PFGE and AFLP. These two advances led to a plethora of studies using these techniques in a wide range of bacterial species. All of these techniques recognize random sites on the genome that cannot be predicted without the whole genome sequence. These random whole-genome analysis techniques have been applied mainly in strain differentiation (RAPD, PFGE) and to a smaller extent in taxonomy (AFLP). But technically, RAPD suffers from non-reproducibility and non-portability.

The recent techniques for molecular typing and taxonomy and evolution of bacterial isolates include both single-locus and multi-locus approaches. The single-locus approach uses highly variable genes. In contrast, multi-locus approaches include multi-locus sequence typing (MLST) and the analysis of multigene families such as RRN operons and tRNA genes. The latter includes 16S rRNA gene variations: intergenic (16S-23S rRNA gene) spacer regions (ISR)

typing; ISR concerted evolution; ISR tRNA genes and tRNA genes not associated with RNA operons.

2.8.1 16S rDNA technology

The analysis of small *rrna* gene sequences is another important landmark in the study of evolution and classification of living organisms. Traditionally, living organisms were classified, according to similarities and differences in their phenotypic characteristics, into prokaryotes and eukaryotes, and these were in turn further classified into various kingdoms, phyla, classes, orders, families, genera and species. However, objective taxonomic classification by these methods can be difficult because of variations in phenotypic characteristics. Three decades ago, Carl Woese and others started to analyse and sequence the 16S *rrna* genes of various bacteria, using DNA/RNA sequencing, and used the sequences for phylogenetic studies. The invention of PCR and automated DNA sequencing two decades ago, and subsequent work on 16S *rrna* gene sequencing of bacteria, as well as 18S *rrna* gene sequencing of eukaryotes, has led to the accumulation of a vast amount of sequence data on the *rrna* /rDNA genes of the smaller subunit of the ribosomes in a large number of living organisms. Comparison of these sequences has shown that the *rrna* gene sequences are highly conserved within living organisms of the same genus and species, but that they differ between organisms of other genera and species.

In addition to the calculation of similarity coefficients among organisms, a detailed analysis of 16S *rrna* oligonucleotide catalogues or complete sequences reveals that there are both quite variable and highly conserved regions of the molecule, presumably reflecting different degrees of functional constraint on different parts of the molecule. Separate comparison of the variable and conserved regions allows determination of both close and distant relationships. In addition, careful inspection of the conserved regions has revealed that there are sequences that are characteristic of major groups of bacteria, termed signature sequences. At great phylogenetic distances these signature sequences are important in allowing the determination of relationships.

Using these *rrna* gene sequences for phylogenetic studies, three domains of life, Archaea, Bacteria and Eukarya, as opposed to the traditional classification of living organisms into prokaryotes and eukaryotes only, were described. Using 16S *rrna* gene sequences, numerous bacterial genera and species have been reclassified and renamed, classification of uncultivable bacteria has been made possible, phylogenetic relationships have been determined, and the discovery and classification of novel bacterial species has been facilitated. In the last decade, sequencing of various bacterial genomes and comparison between genome and 16S *rrna* gene phylogeny has confirmed the representativeness of the 16S *rrna* gene in bacterial phylogeny.

To get in depth information about the sequence of various microorganisms and to look for its relationship with other related species, sequence analysis and phylogenetic studies conducted by different scientists were reviewed critically. Some of the available literature has been presented as follows:

Koch *et al.,* (1997) reported molecular characterization of thermostable pullulanase producing thermophilic anaerobic bacterium. Almost the complete 16S rDNA sequence of strain Ven5 was determined. The phylogenetic dendrogram indicates that strain Ven5 belongs to the order *Thermotogales* and shows highest 16S rDNA sequence similarity to *Fervidobacterium pennavorans*, *F. islandicum*, and *F. nodosum*, with similarities of 99.0, 98.6, and 96.0%, respectively.

Chai *et al.,* (2011) carried out 16S *rrna* gene sequencing of two strains of pullulan-degrading *Anoxybacillus* sp. from Malaysian hot springs. They found almost full-length 16S *rrna* gene sequence was 1522 bp for strain SK3-4 and 1518 bp for strain DT3-1. Both gene sequences were submitted to the GenBank nucleotide sequence database with accession numbers GQ184213 and GU129931, respectively. The 16S *rrna* gene sequences of the two strains were 99% similar to each other. Subsequent construction of a phylogenetic tree revealed that both strains clustered with representatives of the genus

Anoxybacillus and were separate from other genera, such as *Bacillus*. The closest relative of the SK3-4 was *Anoxybacillus ayderensis* with 99.65% similarity, while the DT3-1 was most closely related to *Anoxybacillus gonensis* (99.27%).

Ahmad and Milad (2012) carried out 16S *rrna* gene sequencing of glucoamylopullulanase producing thermotolerant alkaliphilic *Bacilllus subtilis* DR8806. Searches in databases against the sequenced segment of the 16S *rrna* gene, resulted to categorize the isolate in *B. subtilis* that it was in accordance to biochemical tests. The sequence was submitted to GenBank which could be identified with an accession number; JF309277 and under the name of *Bacillus subtilis* DR8806.

2.9 BIOINFORMATICS APPLICATIONS AND MULTIPLE SEQUENCE ALIGNMENT

Advances in computational biology and bioinformatics has been remarkable in the last few decades, that established large scale sequencing, structure and function determination, gene prediction, and specific landmarks on the genome as well as proteome analysis on strong foundations. Several services emerged as the need for access to bioinformatics increased considerably and the most two significant were the European Molecular Biology Laboratory (EMBL) and the United States National Centre for Biotechnology Information (NCBI). There are various tools in bioinformatics and they are needed whenever sets of homologous sequences are compared and are essential precursors to numerous further analyses. Use of these tools in sequence alignment, phylogenetic studies and proteomics is being reviewed in the following sections:

2.9.1 Multiple sequence alignment

One of the cornerstones of modern bioinformatics is the comparison or alignment of nucleic acid/protein sequences. With the aid of multiple sequence alignments, biologists are able to study sequence patterns conserved through evolution and the ancestral relationships between different organisms. The most widely used programs for global multiple sequence alignment are from the Clustal series of programs. The first Clustal program was written by Des Higgins

and Sharp in 1998. This program constructed guide tree using the UPGMA (Unweighted Pair Group Method of Arithmetic Averages) method. In 1992, Higgins *et al.,* released ClustalW which generated trees from the multiple alignments using Neighbour-joining (NJ) method. ClustalW was released in 1994 by Thompson *et al.,* and it included sequence weighting, position-specific gap penalties and the automatic choice of a suitable residue comparison matrix at each stage in the multiple alignments. In this version the dendrogram construction by UPGMA was replaced by NJ.

2.9.2 Phylogenetic tree construction

Phylogenetic tree is a construction which describes the ancestor-descendants relationships of a set of entities. Usually the leaves of the trees represent the present-day entities and the internal nodes represent common ancestors which existed in the past. Thus, a phylogenetic tree is a model of evolutionary divergence events. These trees contain two types of information, the tree topology, which illustrates the order of divergence events and the branch lengths, which indicate the extent of sequence divergence. It is now well known that in genomics, the phylogeny of the sequences is of more interest than the phylogeny of the organisms.

2.10 ENZYME PRODUCTION

Cultivation of thermophiles at high temperature is technically and economically interesting as it reduces the risk of contamination reduces viscosity, thus making mixing easier, and leads to a high degree of production purity. Bringing an enzyme into the solution from its source is known as production. For this the cell wall or cell membrane has to be ruptured. The extraction medium generally used is a suitable buffer which may contain additives such as EDTA, Mg^{2+}, DTT (dithiothreitol), glycerol and protease inhibitors etc. However some of the enzymes are secreted extracellularly. Therefore the cell constituents must be separated from the production medium in order to get the crude enzyme by centrifugation. Production medium is formulated in such a way that should provide all nutrients supporting for enzyme production in high amount but not for

microbial growth. For this purpose, an ideal medium must have a cheap source of carbon, nitrogen, amino acids, growth promoters, trace elements and little amount of salts. Care must be taken to maintain pH during fermentation. For a specific microbe pH, temperature and formulation of culture medium was optimized prior to inoculation. Production of enzymes increases with the concentration of culture medium. This may help in obtaining stable enzyme extract. Buffer helps to maintain the pH as on rupturing of tissue. EDTA is included for chelating heavy metals which may be present in traces in water and other reagents etc. Many enzymes require Mg^{2+} for their activity so Mg^{2+} was quite often added in the extraction medium. DTT stabilizes the enzyme by keeping thiol groups in their reduced state. Upon disintegration of cellular structure, the proteolytic enzymes present in the tissue come in contact with the enzymes and may degrade it. To avoid this proteolytic cleavage, use of protease inhibitors was recommended. An ideal extraction medium must, therefore, contain all the components which contribute towards stabilization or protection of the enzyme.

Gantelet and Duchiron (1998) studied the effect of various carbohydrates on pullulanase production and found that only maltose strongly increased pullulanase production. Addition of maltose (4 g/l) to the medium was found to increase the production of pullulanase activity between 4-7 folds. Extracellular pullulanase synthesis was mainly associated with the exponential growth phase and with the late exponential growth phase. The maximum level of extracellular pullulanase was reached after 22 hrs of fermentation i.e. 33 U/l.

Gomes *et al.,* (2003) optimized highly thermostable pullulanase from bacterium *Rhodothermus marinus*. Low maltose concentrations of 0.5–2.0 g/l showed a pronounced stimulatory effect on pullulanase syntheses by the bacterium. Addition of maltose at 1.5 g/l yielded 45 nkat m/l pullulanase, which were about 19-24 folds higher than those obtained without maltose. Increase of maltose concentrations above 2 g/l reduced the enzyme production remarkably.

Noorwez *et al.,* (2006) reported that a extremophilic bacterium *Geobacillus thermoleovorans* secreted high titres of amylopullulanase in the

basal medium within 20 hrs in shake flasks when the organism was cultivated at 70°C, 200 rpm, pH 7.0 with 2% of 12 hrs old inoculum. Among different starches, 2% soluble starch supported high enzyme production.

Ling *et al.,* (2009) obtained extracellular pullulanase from *Bacillus cereus* H1.5. The culture medium consisted of 1% gelatinized sago starch, 0.5% yeast extract, 0.03% K_2HPO_4, 0.02% $MgSO_4.7H_2O$, 0.1% $(NH4)_2SO_4$, 0.02% $CaCl_2.2H_2O$, 0.001% $FeSO_4.7H_2O$ and 0.0001% $MnCl_2.4H_2O$. The pH of all media was adjusted to 5.5 with 1 M HCl prior to sterilization at 121°C for 20 min. After incubation of the cultures at 30°C for 24 hrs, the cells were removed from the culture medium by centrifugation at 10,000 rpm for 15 mins. The supernatant was used for further purification.

Mrudula *et al.,* (2011) reported production of thermostable amylopullulanase by *Clostridium thermosulfurogenes*. PYE medium containing 0.5% soluble starch as carbon source was used for enzyme production. An incubation temperature of 60°C was found to be optimum for the growth and enzyme production by *C. thermosulfurogenes.* The optimum pH for growth and enzyme production was 7.5.

Asha *et al.* (2013) reported production of crude pullulanase from *Bacillus halodurans* by submerged fermentation by inoculating pure culture of the isolate into the fermentation medium i.e. modified pullulan medium followed incubation at 37°C for 3 days. The broth was centrifuged (REMI C-30 BL centrifuge, India) at 10,000 rpm for 10 mins and the supernatant was used as crude enzyme.

2.11 ENZYME PURIFICATION AND CHARACTERIZATION

Purification is the first step in the study of physio-chemical, kinetic and biological properties of the enzyme. Purification generally involves a series of fractionations by which the enzyme protein was separated from the other proteins. The basic aim in enzyme purification is to isolate pullulanase enzyme from other contaminating proteins so that its structure and other properties can be studied. Once a suitable cellular source of the protein has been identified, the

protein was liberated into solution and then separated from contaminating materials by sequential use of a series of different fractionation techniques or separations. These separations exploit one or more of the following basic properties of the protein such as its solubility, its size, its charge or its specific binding affinity. These separations may be ammonium sulphate presipitation technique. Other electrophoretic procedures mainly SDS-PAGE was used to monitor the extent of purification and to determine the molecular mass and subunit composition of the purified protein E.

2.11.1 Ammonium Sulphate Precipitation Technique

Ammonium sulphate precipitation is a technique used to precipitate proteins from solution by increasing the ionic strength of the solution. The technique is reliant on the hydrophobic nature of proteins, since they contain hydrophilic and hydrophobic groups. When the proteins are dissolved, water is forced into contact with the protein's hydrophobic groups and in the process becomes ordered around the proteins. Increasing the concentration of salt ions by the addition of ammonium sulphate causes the water to be removed from around the protein exposing the hydrophobic portions of the proteins. Precipitation of the proteins will then occur due to the aggregation of proteins via the exposed hydrophobic portions. This technique is used to fractionate the proteins from solution since proteins with larger or more hydrophobic portions will aggregate and precipitate before those with smaller or fewer proteins of hydrophobic groups.

The solubility of protein depends on the salt concentration in the solution. At low concentrations, the presence of salt stabilizes the various charged groups on a protein molecule, thus attracting protein into the solution and enhancing the solubility of protein. This is commonly known as salting-in. However, as the salt concentration is increased, a point of maximum protein solubility is usually reached. Further increase in the salt concentration implies that there is less and less water available to solubilise protein. Finally, protein starts to precipitate when there are not sufficient water molecules to interact with protein molecules.

This phenomenon of protein precipitation in the presence of excess salt is known as salting-out.

Many types of salts have been employed to effect protein separation and purification through salting-out. Of these salts, ammonium sulphate has been the most widely used chemical because it has high solubility and is relatively inexpensive. Because enzymes are proteins, enzyme purification can be carried out by following the same set of procedures as those for proteins, except that some attention must be paid to the consideration of permanent loss of activity due to denaturation under adverse conditions. In ammonium sulphate precipitation, chart of Gomori (1955) as mentioned by Dixon and Webb (1964) has been applied to calculate the solid ammonium sulphate to be added to achieve any given concentration of the cell free filtrate under investigation.

2.11.2 SDS Polyacrylamide gel electrophoresis (SDS-PAGE)

In this technique, protein is dissociated into their constituent subunits using anionic detergent such as sodium dodecyl sulphate (SDS) or sodium lauryl sulphate. The protein mixtures are denatured by heating the sample at 100^{o}C in the presence of excess SDS. Under these conditions most polypeptides bind SDS in a constant weight ratio (1.4 gm SDS per gm of polypeptide).The intrinsic charges of the polypeptides are insignificant compared to negative charges provided by the bound detergent. Consequently SDS polypeptide complexes have essentially identical charge densities and these migrate in polyacrylamide gels of an appropriate porosity strictly according to polypeptide size or their molecular mass. Thus in addition to analysis of polypeptide composition of the sample, the molecular weight of the polypeptides of the sample protein can also be determined by using standard molecular weight markers.

As a prerequisite for the survival of thermophiles, they contain enzymes that can function at high temperature. The enzymes isolated from some extremophiles have proven to be of great use in the biotechnology industry, because they are able to function under conditions that would denature enzymes taken from most “normal” organisms.

Rudiger *et al.,* (1995) characterized heat-stable pullulanase from hyperthermophilic archaeon *Pyrococcus woesei*. After incubation of the homogeneous enzyme with 1% SDS and 1.5% β-mercaptoethanol at temperatures of 40°C to 60°C for 10 mins, a single protein band with a molecular mass of 45 kDa was observed. Incubation at temperatures above 60°C caused the appearance of a second protein band with a molecular mass of 90 kDa.

Duffner *et al.,* (2000) reported purification and characterization of thermoactive pullulanase from *Desulfurococcus mucosus*. After anion-exchange chromatography, hydrophobic interaction, and gel filtration, pullulanase enzyme preparation was purified 76-folds, with a specific activity of 26 U/mg and a final yield of 8.2%. Proteins from the purification steps were separated using SDS-PAGE (12%).The sample after gel filtration revealed two protein bands with apparent masses of 66 and 50 kDa.

Roy *et al.,* (2003) purified an acidic pullulanase type II from newly isolated *Bacillus* sp. US149. The protein was purified with a recovery yield of 13.3% and the homogeneity was verified by HPLC size exclusion column, which showed a single peak of 200 kDa, approximately. Also, SDS–PAGE revealed a single band of 95 kDa.

Ling *et al.,* (2009) purified pullulanase type II from *Bacillus cereus* H1.5 to 23.6-folds with 8.5% yield and a final specific activity of 14.250 U/mg proteins. The homogeneity of the purified pullulanase was ascertained by SDS-PAGE. A single band of protein was obtained with the molecular weight 93 kDa.

2.12 APPLICATIONS OF THERMOSTABLE PULLULANASES

The pullulanase enzyme is of two types, pullulanase type-I and pullulanase type-II. Pullulanase type-I hydrolyses only α-1,6-glycocidic linkages but pullulanase type –II specifically attack on α-1,4-glycocidic linkage and α-1,6-glycocidic linkage. Various bacteria and plants act as the source of pullulanase enzyme. The enzyme is produced by several bacteria also like *Klebsiella pneumoniae* and *Bacillus* species. The highly thermostable pullulanase type-II

enzyme is the most interesting from industrial point of view which is obtained from hyperthermophilic archaea, for example members of genera *Thermotoga maritime* and *Pyrococcus* etc. Pullulanase shows maximum enzymatic activity at pH 6.0 and temperature 55°C. The α-1,6-glycosidic bonds are easily broken by the pullulanase enzyme and this property is widely used in the industries for the productions of various important products.

Production of bioethanol

Duckweeds, or water lentils, are small aquatic plants which are found floating on or just beneath the surface of still or slow-moving fresh water bodies. It is also known as "bayroot". It can grow in all seasons in the areas of warm climate. Also its biomass gets double within two days under the optimal conditions. The total starch content of duckweed can vary from 3-75% of the dry weight depending on strains and species of the duckweed. Some species like *Spirodela polyrrhiza* when grown on swine wastewater and grown anaerobically contains a starch content of almost 59%. Hence they act as a good source for the production of bioethanol. The duckweed biomass is hydrolysed by the action of pullulanase enzyme and amyloglucoside to produce reducing sugars. These sugars are then converted into ethanol. The overall starch conversion rate by the action of pullulanase is very high; hence it is economical and easy method for the production of bioethanol from the high-starch duckweed.

Food processing industry

Pullulanase obtained from *Bacillus stearothermophilus* is highly thermostable and acidophilic. This kind of pullulanase is used in sugar syrup industries for the complete hydrolysis of starch which is started by α-amylases. The combination of amylase and pullulanase simultaneously increases the efficiency of the starch saccharification reaction which in turn increases the quality of sugar syrups. This method is highly advantageous for the generation of higher yields of a desired end product from starch. Pullulanase enzyme is also in use as a processing aid in the food industries, like in the production of certain corn sweeteners by debranching of corn starch. Pullulanase is also use to improve

the glucose content and shorten saccharification time in the manufacture of monosodium glutamate, alcohol, vinegar and soy sauce.

Baking industry

Starch and starch–modifying enzymes are used by baking industries in large amounts around the world. One of the major problems that are faced by baking industry is the staling effect. The staling effect leads to increase in the firmness of crumb, loss of crispness of the crust, reduction of moisture content of the crumb and flavour of the bread is changed. This greatly reduces the quality of bread and other products. This problem is mainly solved by the chemical treatments. But now a days the consumers prefer the enzymatic treatment as chemical possess certain health hazards. On the other hand enzymes are produced naturally and are highly accepted by the consumers. Pullulanase plays a major role in the enzymatic anti-staling treatment.

Production of cyclodextrins (CDs)

Cyclodextrins are the compounds which are made up of sugar molecules joined together in a ring hence are the members of cyclic oligosaccharides family. CDs are mainly composed of 5 or more α-D-glucopyranoside units joined together by α-1, 4-glycosidic linkages. The production of cyclodextrins is quite simple and involves treatment of ordinary starch with a set of starch modifying enzymes. Commonly cyclodextrin glycosyltransferase (CGTase) is used with α-amylase. But the α-1, 6-glycosidicbond of the amylopectin blocks the action of the CGTase. These bonds are broken by the pullulanase enzyme hence increasing the percentage yield of the cyclodextrin production. Also pullulanase is used for the production of branched cyclodextrins (CDs) like maltosyl-CDs and glucosyl-CDs. The, CDs and branched CDs are widely used as stabilizers for labile materials, masking odours, and as a solvent for insoluble or poorly soluble drugs. Also these are used for the production of cholesterol free products in the food industry.

Brewing industry

Beer is an alcoholic beverage which is part of our social life for thousands of years. It is produced form barley by the yeast fermentation process of sugars.

Barley contains starch which is first broken down to fermentable sugars on which yeast acts to produce alcohol. The beer is produced by mixing hot water and crushed barley malt in a mash. This process is known as mashing. During mashing the starch is broken down into dextrin and fermentable sugars. After mashing, the mash is filtered to form a liquid known as sweet wort. Hops are boiled with the sweet wort to produce hopped wort. The yeasts then act on the hopped wort to ultimately produce beer. Pullulanase is added during the mashing step of beer production. Pullulanase enzyme causes the hydrolysis of the α-1,6 branch points of starch which leads to the maximum fermentability of the wort. Hence increases the percentage yield of the low caloric beer production. Also pullulanase reduces the amount of dextrin during the manufacture of beer. This causes improvement in the quality of beer.

Production of food gums

Natural gums are polysaccharides obtained from natural sources. These are used as thickening agents, gelling agents, emulsifying agents, and stabilizers in the food industries. Also they are used in other industries as adhesives binding agents, clarifying agents, encapsulating agents, flocculating agents, swelling agents and foam stabilizers. One of the natural gums is Locust bean gum. Locust bean gum (LBG, also known as carob gum) is a galactomannan vegetable gum which is extracted from the seeds of the carob tree. This tree is mostly found in the Mediterranean region. But the main problem associated with LGB is that it is very costly and difficult to extract. This problem is solved by the use of pullulanase enzyme on gaur galactomannan. Pullulanase enzymes remove galactose residues from the gaur galactomannan to produce modified gaur galactomannans. These modified gaur galactomannans have all the functional properties of the locust bean gum and also have better rheological properties like viscosity and elasticity. Pullulanase is relatively inexpensive, easily available and the modified guar galactomannan which is produced by its action has enhanced rheological properties along with the functional properties of LGB .Hence these modified galactomannan can be utilized for various food and non-food applications of the locust bean gum.

Production of resistant starch

Resistant starch (RS) is that portion of the starch which is partially or wholly fermented in the large intestines. These cannot be broken down by the enzymes present in the human small intestine. It is generally considered to be one of the most important components of the total dietary fibre (TDF). Resistant starch is produced by the hydrolysis of maize starch by pullulanase enzyme. Resistant starch is produced by the amylose retrogradation process. Amylopectin interferes with amylose retrogradation. Retrograded amylose yield is increased by the removal of amylopectin with the pullulanase. Resistant starch acts as a replacement for wheat products in the foods that are required to be gluten-free. Resistant starch is also called as "prebiotic fiber" because it increases the growth of the healthy bacteria and also reduces the growth of the potentially harmful bacteria in the bowel. The fermentation of the resistant starch in the intestines results in the reduction of intestinal pH, secondary resistant acids, ammonia and phenols. Resistant starch also leads to the formation of butyrate. Butyrate acts as the primary source of energy for the colonic cells .also butyrate has anti-carcinogenic and anti–inflammatory properties hence it keeps colon tissue healthy.

Chapter-3

MATERIALS AND METHODS

The present investigation on **"Bioprospecting of thermotolerant bacteria from hot water springs of Himachal Pradesh for production of thermostable pullulanase enzyme**" was carried out in the department of Biotechnology, Dr Y S Parmar, UHF, Nauni, Solan (HP). The experimental details with respect to materials and methodology adopted for the study are described here in:

3.1 Survey and selection of sites
3.2 Sample collection
3.3 Physico-Chemical analysis
3.4 Statistical data analysis of physico-chemical descriptors
3.5 Isolation of thermophilic bacteria
3.6 Enumeration of thermophilic bacterial population
3.7 Morphological characterization of thermophilic bacterial isolates
3.8 Screening of thermophilic bacteria producing thermostable pullulanase enzyme
3.9 Biochemical characterization
3.10 Quantitative screening for thermostable pullulanase activity
3.11 Molecular characterization of a selected bacterial isolate
3.12 Optimization of culture conditions for maximum growth and maximum enzyme production
3.13 Partial purification of thermostable pullulanase enzyme
3.14 Characterization of thermostable pullulanase enzyme

3.1 SURVEY AND SELECTION OF SITES

The study was conducted for the selection of various hot water springs of Himachal Pradesh. Various hot water springs *viz*., Manikaran, Vashist, Khirganga, Kasol, Kalath and Tattapani located in Kullu and Mandi districts of

Himachal Pradesh were surveyed and selected (Plate-1). The geographical locations of these sites along with their altitudes (m) and code numbers of samples are presented in Table 2 (Fig. 4).

Table 2: Geographical location of selected sites and sample codes:-

Sr. No.	Sites	Districts	Subsite code	Sample code	Altitude (m)
1.	MANIKARAN	KULLU	MS1	MS1W	1735
				MS1S	
				MS1R	
				MS1P	
			MS2	MS2W	
				MS2S	
				MS2R	
				MS2P	
			MS3	MS3W	
				MS3S	
				MS3P	
				MS3R	
2.	VASHISHT	KULLU	VS1	VS1W	2050
				VS1S	
				VS1P	
				VS1R	
			VS2	VS2W	
				VS2S	
				VS2P	
				VS2R	
			VS3	VS3W	
				VS3S	
				VS3P	
				VS3R	
3.	KASOL	KULLU	KS1	KS1W	1640
				KS1S	
				KS1P	
				KS1R	
			KS2	KS2W	
				KS2S	
				KS2P	
				KS2R	
			KS3	KS3W	
				KS3S	
				KS3P	
				KS3R	
4.	KALATH	KULLU	KaS1	KaS1W	680
				KaS1S	
				KaS1P	
				KaS1R	
			KaS2	KaS2W	
				KaS2S	
				KaS2P	
				KaS2R	
			KaS3	KaS3W	
				KaS3S	
				KaS3P	
				KaS3R	



Manikaran



Vashisht



Khirganga



Kasol



Kalath



Tattapani

Plate 1: Selected hot water springs of Himachal Pradesh



Fig 4: Map showing six selected sites of hot water springs in Himachal Pradesh.

Sr. No.	Sites	Districts	Subsite code	Sample code	Altitude (m)
5.	KHIRGANGA	KULLU	KhS1	KhS1W	**2960**
				KhS1S	
				KhS1P	
				KhS1R	
			KhS2	KhS2W	
				KhS2S	
				KhS2P	
				KhS2R	
			KhS3	KhS3W	
				KhS3S	
				KhS3P	
				KhS3R	
6.	TATTAPANI	MANDI	TS1	TS1W	**680**
				TS1S	
				TS1P	
				TS1R	
			TS2	TS2W	
				TS2S	
				TS2P	
				TS2R	
			TS3	TS3W	
				TS3S	
				TS3P	
				TS3R	

W-Water sample **S**-Soil sample **P**- Pebbles sample **R**- Rock matting sample

These samples were brought to the laboratory and kept at 4^{o}C in refrigerator till further processing.

3.2 SAMPLE COLLECTION

Seventy two samples in the form of water, soil, pebbles and rock matting from different sites of Manikaran, Vashist, Khirganga, Kasol, Kalath and Tattapani were collected in sterilized screw capped vials and jars (Plate- 2).

3.3 PHYSICO-CHEMICAL ANALYSIS

Water samples from eighteen subsites were studied for various physico-chemical characters. The following parameters were evaluated during the survey:

3.3.1 pH

The pH of the water samples was recorded using pH meter (ELICO, India).

3.3.2 Temperature

The temperature of all the water samples was recorded at the site using temperature probe.

3.3.3 Chloride

Chloride content of all the water samples was determined using the standard technique (APHA, 1976).

- Silver nitrate
- Potassium nitrate indicator (100.0 g/l)

Procedure

Water samples were assayed titrametrically. 10 ml of the sample was taken in the flask and 5 ml of chromate reagent was added to it. The yellow coloured solution was then titrated against standard solution of silver nitrate till the appearance of brick red colour. A blank titration was also done.

Calculations

$$\text{Chloride mg/l} = \frac{\text{ml of titrant} \times N \times 35.46 \times 10^3}{\text{ml of sample}}$$

3.3.4 Sulphate

Sulphate content of all the water samples was determined using the standard technique (APHA, 1976).

- Barium chloride (20-30 crystals)
- NaCl-HCl (100.0 g NaCl added to 800 ml distilled water and then the volume was made up to 1 litre).
- Conditioning reagent (Glycerol-ethanol solution mixed 50 ml glycerol in 100 ml ethanol).
- 0.147 g anhydrous Na_2SO_4 in 1 litre of distilled water: 1 ml of solid contains 100.0 µg SO_4.



Plate 2: Samples collected from hot water springs of H.P. in the form of water, soil, pebbles and rock mattings.

Procedure

Diluted 10 ml of the sample to 100 ml. 5ml of the conditioning reagent was added to the flask with constant stirring using a magnetic stirrer. A few crystals of barium chloride were added and mixed for 1 minute. Immediately absorbance was read at 420 nm against a blank. Estimated sulphate concentration of the sample by comparing the turbidity reading with a calibration curve prepared by carrying the sulphate standard through entire procedure. Sulphate was expressed then in mg/l.

Calculations

$$SO_4 \quad mg/l = \frac{MgSO_4 \times 1.0}{\text{ml of sample}}$$

3.3.5 Total hardness

Total hardness of all the water samples was determined using the standard technique (APHA, 1976).

- Standard EDTA titrant (0.01M)
- Ammonium buffer solution, 114 ml concentrated NaOH was added to 13.5g NH_4Cl and made the final volume 200 ml.
- Erichrome Black-T indicator: Dip 5.0 g in 100 ml of 80% ethyl alcohol.

Procedure

To 50 ml of water sample, 1 ml of ammonium buffer and 5 drops of Erichrome Black-T indicator were added. The wine red coloured solution was then titrated against the standard EDTA solution (0.01M) till the blue colour appeared.

Calculations

$$\text{Total hardness as mg/l as } CaCO_3 = \frac{A \times 1.05 \times 1000}{\text{ml of sample}}$$

Where, A= ml of titrant used

3.3.6 Calcium hardness

Calcium hardness of all the water samples was determined using the standard technique (APHA, 1976).

- Standard EDTA titrant (0.01M)
- Sodium hydroxide solution (8.0%)
- Murexide indicator mixture.

Procedure

To 50 ml of the sample, added 1 ml NaOH solution and a pinch of murexide indicator till the pink coloured solution changed to purple. Checked the end point by adding 1 to 2 drops of titrant in excess till no further change in colour occurred.

Calculations

$$\text{Calcium hardness as mg/l } CaCO_3 = \frac{A \times B \times 1000}{\text{ml of sample}}$$

$$\text{Calcium hardness as mg/l } Ca^{++} = \frac{A \times B \times 400.8}{\text{ml of sample}}$$

Where, A= ml of titrant

B= mg $CaCO_3$ equivalent to 1.0 ml EDTA titrant at calcium indicator end point B=1.05.

3.3.7 Magnesium content

Magnesium content of all the water samples was determined using the standard technique (APHA, 1976).

Procedure

If the calcium hardness as calcium carbonate is known, the magnesium content can be calculated as follows:

Magnesium in mg/l= (Total hardness – Calcium hardness) × 0.243.

3.4 STATISTICAL DATA ANALYSIS OF PHYSICO-CHEMICAL CHARACTERS

3.4.1 Simple Correlation Studies

Simple Correlation coefficient (Karl Pearson's coefficient) between characters was worked out by using the following formula (Panse and Sukhatme, 1978).

$$r(xy) = \frac{Cov(xy)}{\sqrt{V(x), V(y)}}$$

Where:

r (xy) = Simple correlation between x and y

V (x) = Variance of x character

V (y) = Variance of y character

For testing of simple correlation the 't' value was calculated by the following formula given by Gosset (1908), also known as student-t-test.

$$t = \frac{r \times \sqrt{n-2}}{\sqrt{1-r}}$$

Where:

r = Simple correlation

n = Number of coefficients in the sample

This value was compared with the tabulated 't' value at (n-8) degrees of freedom.

3.4.2 Variability analysis

In order to compare the consistency, the coefficient of variation has been worked out for each of the observed characteristics under each site as given below:

$$CV = (\sigma / \overline{x}) \times 100$$

Where,

$$\overline{X} = (\text{Arithmetic mean}) = n^{-1}\sum_{i=1} xi$$

$$\sigma \text{ (standard deviation)} = \left(n^{-1}\sum_{i=1}^{N} (x_i - \overline{x})^2 \right)^{1/2}$$

Analysis of Variance Technique (ANOVA)

Analysis of variance technique (one way classification of data) was applied to know the average performance of various characters *viz.*, pH, temperature, chloride, sulphate, total hardness, calcium hardness, calcium carbonate and magnesium content w.r.t. sites.

3.5 ISOLATION OF THERMOSTABLE BACTERIA

3.5.1 Medium for isolation

Isolation of bacterial isolates from the hot water springs was done using nutrient agar medium (pH 6.8).

Table 3: Composition of nutrient agar medium

Component	Composition (g/l)
Peptone	5.0
NaCl	5.0
Beef extract	3.0
Agar	15

pH 6.8 ± 0.2 at 60^{o}C

3.5.2 Inoculation of samples on medium

1g soil/rock matting/pebbles/1ml water sample was taken and transferred to 150 ml flask containing 100 ml nutrient broth and incubated for 24-48 hrs at 60^{o}C in water bath incubator shaker at 150 rpm. Turbid cultures were streaked on plates of solidified nutrient agar medium and these were incubated for 24-48 hrs in autoclavable polythene bags to prevent drying out. Each of the colonies were transferred to nutrient broth and after incubation each culture was re-streaked on

nutrient agar plates, and the pure colonies obtained were stored at $4^{o}C$ in refrigerator till further processing.

3.6 ENUMERATION OF THERMOPHILIC BACTERIAL POPULATION IN VARIOUS SAMPLES FROM HOT WATER SPRINGS

Enumeration of thermophilic bacterial population in various samples from hot water springs was done by serial dilution method. Serial dilution is a process of diluting a sample several times. 9 ml of distilled water is transferred into each of 5 tubes using sterile pipettes (10 ml) with aseptic technique. By using sterile pipette, 1ml of water sample/1g soil sample is transferred into 10^{-1} tube. Mix the test tube properly. Continue dilution with aseptic technique for test tubes 10^{-2} until 10^{-5}. After that each of serial dilution is transferred into nutrient agar plate by using spread plate method. 0.1ml of an appropriately diluted culture is spread over the surface of nutrient agar plate using sterile glass spreader. The plate is then incubated until the colonies appear.

3.7 MORPHOLOGICAL CHARACTERIZATION OF BACTERIAL ISOLATES (Kristjansson *et al*., 1986)

All the thermophilic bacterial isolates were further studied for various morphological characters which include the following colony and microscopic characters.

3.7.1 Colony characters of bacterial isolates

a) Colour

1. White
2. Cream
3. Yellow

b) Size

1. Pinpointed (Punctiform)
2. Small
3. Medium
4. Large

c) Texture

1. Smooth
2. Rough

d) Elevation

1. Flat
2. Raised
3. Sunken

e) Margin

1. Entire
2. Irregular
3. Rhizoid

3.7.2 Microscopic Characters of bacterial isolates

a) Gram's reaction

- Crystal violet
- Gram's iodine solution
- 95% ethyl alcohol (Decolourizer)
- Safranin

Procedure

Thin smears of each bacterial culture were prepared and then heat fixed. Few drops of crystal violet were added on the smear for 60 secs. Slides were washed with distilled water and each smear was then covered with gram's iodine solution for 60 secs. Then the slides were washed with alcohol and safranin was applied to the smears for 30 secs. Washed the slides with distilled water and observed under low power magnification and oil immersion objective of microscope to observe the colour, shape and arrangement of the bacterial cells.

b) Shape of the cells

Shape of the cells was observed under microscope using oil immersion objective (1000x magnification).

c) Spore formation

- Malachite green (5% aqueous)
- Safranin (0.5% aqueous)

Procedure

Bacterial smears were heat fixed and malachite green was applied to the smears. The slides were then heated to steaming for 5 mins and more stain was kept on adding from time to time. After that the slides were washed under running tap water and counterstained with safranin for 60 secs. Then washed the slides with distilled water and observed under microscope.

3.7.3 Pellicle in liquid culture

Pellicle formation was observed directly by seeing the suspension cultures.

3.7.4 Analysis of microscopic data

Results obtained from morphological observations of all the bacterial isolates were recorded and the data matrices were analysed by the SAHN module of NTSYS-pc version 2.20 and similarities between isolates were estimated using the Jaccard's coefficient, calculated as J= A/(N-D), where A is the number of positive matches, D is the number of negative matches and N is the total sample size including both the number of matches and unmatches (Rohlf & Milligan, 1994).

3.8 SCREENING OF THERMOPHILIC BACTERIA PRODUCING THERMOSTABLE PULLULANASE ENZYME

3.8.1 Medium selection for screening of thermostable pullulanase producing bacteria

Three different culture media were investigated for screening of thermostable pullulanase producing bacterial isolates *viz.*, Pullulan agar medium containing 10 g/l pullulan (Asha *et al.*, 2013); Basal medium containing pullulan 10 g/l (Nair *et al.,* 2006) and KWF medium containing 10 g/l pullulan (Chai *et*

al., 2011). Medium producing maximum growth OD along with best proliferation of thermostable pullulanase producing bacterial colonies were selected for isolation of thermostable pullulanase producing bacterial isolates.

Table 4: Composition of different isolation media (A1-A3)

Media code	Composition	References
A1	Soluble pullulan 10 g, NaCl 2 g, $MgSO_4.7H_2O$ 0.1 g, K_2HPO_4 0.17 g, $KH_2PO_4.7H_2O$ 0.12 g and Agar 15 g at pH 7 to make final volume 1 litre.	Asha *et al.*, 2013
A2.	Tryptone 2 g, yeast extract 2 g and NaCl 2 g, soluble pullulan 10 g and Agar 15 g at pH 6.5 to make final volume of 1 litre.	Nair *et al.,* 2006
A3	Peptone 17.5 g, Yeast extract 0.5 g, $MgSO_4$ 0.4 g, K_2HPO_4 2 g, Agar 15 g, soluble pullulan 10 g at the pH 7.5±2 to make final volume 1 litre.	Chai *et al*., 2011

A1: Pullulan agar medium

A2: Basal medium

A3: KWF medium

3.9 BIOCHEMICAL CHARACTERIZATION OF THERMOPHILIC BACTERIA PRODUCING THERMOSTABLE PULLULANASE ENZYME

3.9.1 The selected thermophilic bacterial isolates were further examined for the following biochemical reactions:

a) Catalase test (Harrigan, 1998)

I. Hydrogen peroxide (3%)

Procedure

Add few drops of hydrogen peroxide over the surface of an agar culture and observed the effervescence if any, caused by the liberation of free oxygen as gas bubbles.

b) Oxidase test (Aneja, 2003)

I. Oxidase reagent

Procedure

Autoclaved filter paper strips were dipped in wurster reagent and bacterial cultures were inoculated on it with the help of loop. Production of blue or purple colouration was taken as positive test.

c) Urease test (Aneja, 2003)

Procedure

Urea agar slants were inoculated with bacterial isolates and incubated at 60ºC for 48 hrs. Change in colour from yellow to red or deep pink was taken as positive reaction for the degradation of urea by means of the production of an enzyme urease.

d) Citrate utilization test (Aneja, 2003)

Procedure

Simmon's citrate agar slants were inoculated with bacterial isolates and incubated at 60ºC for 48 hrs, change in colour of the medium from green to blue was taken as positive test.

e) Methyl Red & Voges-Proskauer (MR-VP) test (Case and Johnson, 1984)

I. MR-VP broth (1% glucose supplemented in nutrient broth).
II. Methyl red (0.1g methyl red dissolved in 300ml of 95% ethanol).
III. VP reagent I (6g α-napthol dissolved in 100ml of 95% ethanol)
IV VP reagent II (40% KOH)

Procedure

The tubes containing MR-VP broth were inoculated with bacterial isolate and incubated at 60ºC for 12 hrs and 24 hrs. Five drops of methyl red were added to one of the tubes and change in colour (brick red) if any was recorded. To the other tube, 0.6 ml of VP reagent I and 0.2 ml of VP reagent II were added. After shaking the tubes gently with their caps off to expose them to air, these were allowed to stand for 30 mins and the change in the colour from pink to red was

taken as positive and no change in colour or black colour formation was taken as negative.

f) Indole production test (Aneja, 2003)

I. Kovac's reagent

II. 1% trypton broth

Procedure

Tubes containing 1% trypton broth were inoculated with bacterial isolates and incubated at 60ºC for 48 hrs. 1 ml of Kovac's reagent was added to the tubes. After shaking the tubes gently, these were allowed to stand to permit the reagent to come to the top and development of a cherry (deep) red colour in the top layer of the tube. This is taken as positive test for indole production.

g) Fermentation of sugars (glucose, sucrose, lactose, mannitol) (Aneja, 2003)

I. Glucose broth

II. Sucrose broth

III. Lactose broth

IV. Mannitol broth

Procedure

Tubes containing glucose, sucrose, mannitol and lactose broth were inoculated with bacterial isolates and incubated at 60ºC for 48 to 72 hrs, change in colour to yellow from the colour of broth in all the type of fermentation broths indicates that fermentation occurred and the test was taken as positive for fermentation of sugars.

h) Triple Sugar Iron Agar Test (TSI)

Procedure

TSI agar slants were inoculated with bacterial isolates and incubated at 60ºC for 48 hrs. Change in colour of slants from red to pink and butt from red to yellow was taken as positive to ferment glucose and not to ferment sucrose or lactose whereas if both slant and butt were pink in colour means none of the

sugar was fermented and H_2S gas formation was showed by black colouration in the butt.

3.9.2 Analysis of biochemical data

Biochemical observations of selected thermostable pullulanase producing bacterial isolates were studied and the data matrices were analysed by the SAHN module of NTSYS-pc version 2.20 and similarities between isolates were estimated using the Jaccard's coefficient, calculated as J= A/(N-D), where A is the number of positive matches, D is the number of negative matches and N is the total sample size including both the number of matches and unmatches (Rohlf & Milligan, 1994).

3.10 QUANTITATIVE SCREENING FOR THERMOSTABLE PULLULANASE ACTIVITY

Selected bacterial isolates further screened for pullulanase activity by quantitative methods. 1% inoculums was inoculated in 50 ml KWF broth at pH 9.0 and incubated at 60ºC for 24-48 hrs. The culture supernatant was obtained by centrifugation of overnight cultures of selected bacterial isolates at 10000 rpm at 4ºC for 10 mins and used as crude extracellular enzyme.

3.10.1 Quantitative determination of thermostable pullulanase activity

Thermostable pullulanase activity was determined spectrophotometrically at 540 nm via dinitrosalicylic (DNS) method as proposed by Miller (1959) with slight modification. The reaction mixture contained 0.3 ml crude enzyme extract and 3 ml of 0.1M sodium phosphate buffer (pH: 9.0). The reaction was held at 60ºC for 15 mins and transferred to cooling ice bath for 1 min followed by addition of 1.5 ml of DNS acid and 150 µl 0.1N NaOH. The reaction tubes were again incubated for 5 mins at 100ºC. The reaction was stopped by immersing the reaction tubes in ice bath. One unit of thermostable pullulanase activity was defined as the amount of enzyme which liberates 1 µmol of reducing sugar in 1 min under standard assay conditions. On the basis of quantitative screening, the thermophilic bacterial isolate depicting maximum thermostable pullulanase activity was selected for further studies.

3.11 MOLECULAR CHARACTERIZATION OF SELECTED BACTERIAL ISOLATE

3.11.1 Extraction of genomic DNA

Total genomic DNA of selected isolate was extracted using Genomic DNA extraction kit-Mini (Real Genomics) using the following protocol:

Lysozyme buffer for Gram positive bacteria: 20 mg/ml lysozyme, 20mM TrisHcl, 2mM EDTA (pH 8.0) and 1% Triton X-100.

Preparation of bacterial suspension

Twenty five ml of KWF medium of pH 9.0 was prepared and inoculated with two loop full culture of the selected isolate was taken in 150 ml flask and incubated at 60^{o}C in a water bath incubator shaker (MAC) for 24 hrs.

Cell harvesting for Gram negative bacteria

Overnight grown bacterial culture (approx.10^{9} cells/ml) was transferred to 1.5 ml microfuge tubes and centrifuged at 13,000 rpm for 1 min in a microcentrifuge (REMI). Supernatant obtained was discarded and to each tube 200 µl of GT buffer (Genomic DNA extraction kit, Real Genomics) was added. There after the cell pellet was re-suspended by vortexing which was followed by incubation at room temperature for 5 mins.

Cell harvesting for Gram positive bacteria

Overnight grown bacterial culture (approx.10^{9} cells/ml) was transferred to 1.5 ml microfuge tubes and centrifuged at 13,000 rpm for 1 min in a microcentrifuge (REMI). Supernatant obtained was discarded and to each tube 200 µl of lysozyme buffer (freshly prepared) was added. There after the cell pellet was re-suspended by vortexing, followed by incubation at room temperature for 10 mins.

Cell lysis

Two hundred µl of GB buffer (lysis buffer) was added to sample and mixed for 5 secs by vortexing followed by incubation at 70^{o}C for 10 mins until

the sample lysate was clear. During incubation the tubes were inverted every 3 mins and cells were eluted using 200 µl per sample of elution buffer, at 70^{o}C.

RNA degradation

Two µl of 50 mg/ml RNase A solution was added to the sample lysate and mixed by vortexing.

DNA binding

Two hundred µl of 95% ethanol was added to the sample lysate and mixed for 10 secs by vortexing. During mixing precipitation was avoided by pipetting. Then placed GB column (Genomic DNA extraction kit, Real Genomics) in a 2.0 ml collection tube and sample was applied. After closing the caps, centrifugation was done at 13,000 rpm for 2 mins.

Washing

Four hundred µl of W1 (Wash buffer) buffer was added in the GB column (Genomic DNA extraction kit, Real Genomics) and centrifuged at 13,000 rpm for 30 secs. The flow-through was discarded and the GB columns were placed back in a 2 ml collection tube followed by centrifugation at 13,000 rpm for 3 mins to dry the column matrix.

DNA Elution

Dried GB column was transferred into a clean 1.5 ml microfuge tube and 100 µl of preheated elution buffer was added into the center of the column matrix. The tube was allowed to stand for 3-5 mins until elution buffer got absorbed by the matrix followed by centrifugation at 13,000 rpm for 30 secs to elute the purified DNA. The eluted purified DNA was stored at -20^{o}C for further use.

3.11.2 Characterization of selected bacterial isolate by universal primers

The above extracted genomic DNA of selected bacterial isolate was amplified by using universal primers for 16S *rrna* gene. The PCR amplification was carried out in 0.2 ml PCR tubes with 20 µl reaction volume with composition

as depicted in Table-5. Amplifications were performed using thermal cycler with different temperature transitions for each primer set (Table-6).

B27F 5`-AGAGTTTGATCCTGGCTCAG-3'

U1492R 5`-GGTTACCTTGTTACGACTT-3'

Table 5: Composition of PCR reaction mixture

S. No.	Reaction mixture	Quantity (µl)
1	PCR buffer (10x) with $MgCl_2$ (1.5 mM)	2.0
3	dNTPs mixture (0.5 mM each)	2.0
4	Taq DNA polymerase (5U/µl)	0.5
5	Primer F (10 nM)	1.0
6	Primer R (10 nM)	1.0
7	Template DNA	1.0
8	Distilled water	12.5
Total volume		**20.0**

Table 6: PCR reactions followed in amplification studies

Stage of PCR		Time (mins or secs)	Temp (^oC)	No. of cycle
Pre-PCR	Hot start	5 mins	94	1
PCR	Denaturation	1 mins	94	30
	Annealing	1 mins	48	
	Extension	1.5 mins	72	
Post-PCR	Final extension	8 mins	72	1

3.11.3 Agarose gel electrophoresis of PCR products

The amplified PCR product of test isolate was resolved by electrophoresis using 1.0 percent agarose gel in tris acetate EDTA buffer (2M tris base, 57.10 ml acetic acid and 0.5 M EDTA, pH 8.0, 50X) containing ethidium bromide (0.5 µg/ml). DNA ladder of high range was used for comparison. The gel was run at 80 V for 2-3 hrs using Bangalore Genei power pore system. The gel was viewed and image was captured using gel documentation system (Alphalmager 2200, Alpha Infotech Corporation, USA).

3.11.4 Gel extraction protocol

Gel Dissociation

The agarose gel slice containing the relevant DNA fragment was excised and extra agarose was removed to minimize the size of the gel slice followed by transferring the gel slice into a microfuge tube. 500 µl of DF buffer (Genomic DNA extraction kit, Real Genomics) was added to the sample followed by vortexing and incubated at 55^{o}C for 10-15 mins until the gel slice was completely dissolved. The dissolved sample mixture was cooled down to room temperature.

DNA Binding

The DF column (Genomic DNA extraction kit, Real Genomics) was placed in a 2 ml collection tube and 800 µl of the cooled sample mixture was applied into it followed by centrifugation at 13,000 rpm for 30 secs. After centrifugation, the flow-through was discarded and the DF column was placed back in the 2 ml collection tube.

Wash

Then added 600 µl of wash buffer (ethanol added) into the DF column and centrifuged at 13,000 rpm for 30 secs. The flow-through was discarded and column was placed back in the 2 ml collection tube. The column was dried by centrifugation at 13,000 rpm.

DNA Elution

The dried column was transferred in a new microfuge tube and added 20 µl of elution buffer into it. The microfuge tube was allowed to stand for 2 mins until elution buffer got absorbed by the matrix followed by centrifugation for 2 mins at 13,000 rpm to elute the purified DNA.

3.11.5 16S *rrna* gene sequencing

Sequencing is the process of determining the nucleotide (A, T, G and C) order of a given DNA fragment. DNA sequencing has been performed using the chain termination method developed by Sanger and his coworkers in 1977. The

PCR products obtained through amplification with universal primers targeting 16S *rrna* gene were sent for sequencing using same upstream and downstream primers to Merk lab, India.

3.11.6 *In silico* analysis of sequences

In silico is an expression used to mean "performed on computer or via computer simulation". Raw sequence data were obtained as a set of two sequences for each sample one with forward and other with reverse universal primers of sequencing reaction. Analysis of raw sequences data was done using Chromas Lite (version 2.1) of Technelysium Pvt. Ltd. Sequence of the selected bacterial isolate was analyzed using different approaches which have been discussed below briefly.

3.11.6.1 BLASTn

Basic Local Alignment Search Tool (BLAST) uses an algorithm of Altshul *et al*., (1997) for searching similarities above certain threshold between a query sequence and all other sequences present in a database. 16S *rrna* gene sequences of selected bacterial isolate was analyzed using BLASTn to align it with corresponding sequences of 16S *rrna* from the database.

Reference URL

http://www.ncbi.nlm.nih.gov/blast

3.11.6.2 Multiple Sequence Alignment

Sequence alignment is done for comparing sequences and this comparison of sequences provides a foundation for usage of bioinformatics tools and hence it becomes easier to study the sequence at genomic and proteomic level.

Multiple sequence alignment is the sequence alignment of three or more biological sequences. During present investigation, multiple sequence alignment of nucleotide sequence of the selected isolate and other isolates retrieved from NCBI database was generated. Multiple sequence alignment was performed using

CLUSTAL W program (Thompson *et al*., 1994). From such alignment studies, inference of sequence homology and phylogenetic analysis has been drawn.

1.11.6.3 Phylogenetic analysis

Phylogenetic analysis has been used in comparative genomics and proteomics to depict evolutionary relationships. The analysis began with aligning of sequences using tools like Clustal W and after alignment, phylogenetic trees are constructed.

During present investigations entire set of nucleotide sequences (nucleotide sequences of selected isolate and rest all sequences from NCBI) were placed in a single notepad in their Fasta format, and these were aligned through EBI tool, Clustal W program, integerated with MEGA (Molecular Evolutionary Genetics Analysis; Tamura *et al*., 2007) version 5.0 and the tree was generated using MEGA version 5.0 and analyzed. The evolutionary history/phylogenetic analysis were inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 100 replicates was taken to represent the evolutionary history of the taxa analysed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) has been shown above the branch (Felsenstein, 1985).

3.12 OPTIMIZATION OF CULTURE CONDITIONS FOR GROWTH AND MAXIMUM PRODUCTION OF THERMOSTABLE PULLULANASE

The culture conditions for industrially useful microorganisms are generally optimized to obtain higher yields of their industrially important products including enzymes. The thermophilic bacterial isolate selected by quantitative screening depicting maximum thermostable pullulanase activity was further investigated to study effect of different factors such as medium composition, incubation time, pH and incubation temperature on pullulanase production as well as growth of the bacterial isolate.

3.12.1 Effect of different medium

Thermostable pullulanase production by selected bacterial isolate in three different media (A1-A3) was investigated. The medium that induced the maximum bacterial growth and thermostable pullulanase activity was selected for further studies of enzyme production.

3.12.2 Effect of incubation time

Effect of different incubation time for maximum bacterial growth and maximum thermostable pullulanase activity were investigated ranging from 24, 48, 72, 96 and 120 hrs using selected medium. The optimum incubation time leading to maximum production of pullulanase enzyme as well as maximum growth of the bacterial isolate was selected for further studies.

3.12.3 Effect of pH

To find optimum pH for the maximum bacterial growth and maximum thermostable pullulanase activity by selected bacterial isolate, a pH range of 4.0-12.0 was examined using selected medium at an optimum temperature and optimum time. The best pH was selected and used in further experiments.

3.12.4 Effect of incubation temperature

Effect of incubation temperature on the maximum bacterial growth and maximum thermostable pullulanase activity was examined. The bacteria was grown at a temperature range of $40^{o}C$-$90^{o}C$ using selected medium and optimum conditions of incubation time, pH and then optimum temperature were selected for further experiments.

3.13 ENZYME PRODUCTION AND PARTIAL PURIFICATION OF PULLULANASE ENZYME

3.13.1 Enzyme production

3.13.1.1 Preparation of inoculum

An axenic culture of selected bacterial isolate was inoculated into 10 or 20 ml of standardized medium for thermostable pullulanase production followed

by incubation at optimum temperature for optimum period of time with the shaking speed of 150 rpm.

3.13.1.2 Preparation of bacterial culture for pullulanase production

1% inoculum was inoculated in 50 ml KWF broth and incubated at 60ºC for 48 hrs at 150 rpm**.** The cell free supernatant was collected by centrifugation 10,000 rpm at 4ºC for 10 mins and extracellular pullulanase activity was tested.

3.13.1.3 Protein estimation of crude enzyme extract by Lowry's method (Lowry *et al*., 1951)

Protein concentration estimated in crude enzyme extract by standard Lowry's method.

Reagents

a) 1 percent copper sulphate

b) 2 percent sodium carbonate in 0.1N sodium hydroxide

c) 2 percent sodium potassium tartarate

d) To 50 ml of reagent b, 0.5 ml of reagent a and 0.5 ml of reagent c were added. This solution was prepared fresh.

e) 1 N Folin's Ciocalteau reagent (prepared fresh)

Procedure

5 ml of Reagent d was added to 1 ml of suitably diluted protein solution and mixed immediately. 1N Folin's reagent (0.5 ml) was added after 10 mins and the test tube was shaken vigorously to ensure proper mixing. After 30 mins, the intensity of color developed was measured at 660 nm against the reagent blank. The amount of protein was calculated from standard curve using Bovine Serum Albumin (0-100 µg per ml).

3.13.1.4 Enzyme Assay

Thermostable pullulanase activity was determined spectrophotometrically at 540 nm via dinitrosalicylic (DNS) method as proposed by Miller (1959) with slight modification. The reaction mixture contained 0.3 ml crude enzyme extract

and 3 ml of 0.1M sodium phosphate buffer (pH: 9.0). The reaction was held at 60ºC for 15 mins and transferred to cooling ice bath for 1 min followed by addition of 1.5 ml of DNS acid and 150 µl 0.1N NaOH. The reaction tubes were again incubated for 5 mins at 100ºC. The reaction was stopped by immersing the reaction tubes in ice bath. One unit of thermostable pullulanase activity was defined as the amount of enzyme which liberates 1 µmol of reducing sugar in 1 min under standard assay conditions.

Determination of pullulanase activity (U/ml)

Enzyme activity = (A x V)/(t x e x v)

A	=	Absorbance
V	=	Total volume of reaction mixture (ml)
v	=	Enzyme volume (ml)
t	=	Incubation time (min)
e	=	Extinction coefficient

3.13.2 Partial purification of thermostable pullulanase enzyme

All steps of purification were performed at a temperature of 4ºC using 0.1 M sodium phosphate buffer, pH: 9.0. Techniques used for the purification of thermostable pullulanase enzyme were ammonium sulphate precipitation, gel filtration and ion exchange chromatography. The enzyme preparation at each step of thermostable pullulanase enzyme purification was analyzed for protein concentration and enzyme activity.

3.13.2.1 Ammonium sulphate precipitation

The cell free extract was subjected to various saturation/concentration of ammonium sulphate (0-80%). The ammonium sulphate precipitation table (Scopes, 1982) was followed to calculate the required amount of ammonium sulphate to be added in cell free extract. Ammonium sulphate was added with continuous stirring and stored at 4ºC for 45 mins. The precipitated proteins were recovered by centrifugation at 20,000 rpm for 20 mins and the pellet was dissolved in minimum quantity of 0.1 M sodium phosphate buffer pH: 9.0. The

dialysis was carried out for 24 hrs against three successive changes of dialysis buffer in dialysis bag. The dialysis bag was rinsed in distilled water and then boiled in excess of distilled water containing 50 µM EDTA to remove the impurities and eliminate the release of ultraviolet light absorbing material during dialysis. The dialysis bag was washed thrice with distilled water. One end of the dialysis bag was tied with thread and the crude extract was poured into the bag. The other end of the dialysis bag was also tied and was kept in dialysis buffer at 4ºC for concentration till the volume was reduced considerably. Protein concentration and thermostable pullulanase enzyme activity were estimated in protein sample using Lowry's method and enzyme assay respectively. The fraction or percentage cut exhibiting maximum activity of thermostable pullulanase was termed as ASF (ammonium sulphate fraction) and was taken further for purification.

3.13.2.2 Gel filtration column chromatography

Four grams of Sephadex G-100 was suspended in 200 ml of sodium phosphate buffer pH: 9.0 for 24-48 hrs. It was packed into glass column having dimensions of (50x1.5 cm) avoiding entrapment of any air bubble in the gel bed. It was then washed with sufficient amount of sodium phosphate buffer of pH: 9.0. Then the 4 mg/ml blue dextran solution was loaded on the column and 3 ml fractions were collected. The absorbance was recorded at 660 nm. This was done to check the uniformity of packed column and for calculating the void volume.

2 ml of concentrated ASF fractions from dialysis was loaded on the Sephadex G-100 column. This column was eluted with sodium phosphate buffer of pH: 9.0 and 3ml fractions were collected. The absorbance was recorded at 280 nm. The collected fractions were analyzed for protein content and for enzyme assay. The most active fractions were pooled and stored at 4^{o}C till further purification step.

3.13.2.3 Ion exchange chromatography

Two grams of DEAE-Sephadex was suspended in 200 ml of sodium phosphate buffer of pH: 9.0 for 48-72 hrs. It was packed in a glass column (8x2.5

cm). The column was washed with sufficient amount of sodium phosphate buffer of pH: 9.0. Then the active pooled fractions obtained from G-100 gel filtration were loaded on ion exchange column. The column was first eluted with sodium phosphate buffer to wash out unbound proteins. The bound proteins were eluted with linear salt gradient using four bed volumes of 0.1M NaCl, 0.2M NaCl, 0.3M NaCl, 0.4M NaCl and 0.5M NaCl in sodium phosphate buffer of pH: 9.0. Fractions of 2 ml each were collected and absorbance was recorded at 280 nm. The collected fractions were analyzed for protein content and for enzyme assay. The active fractions were pooled together and concentrated by dialysis against solid sucrose. The concentrated sample was used for carrying out further studies.

3.13.2.4 Protein estimation of partially purified enzyme extract by Lowry's method (Lowry *et al*., 1951)

Protein concentration estimated in crude enzyme extract by standard Lowry's method.

Reagents

a) 1 percent copper sulphate

b) 2 percent sodium carbonate in 0.1N sodium hydroxide

c) 2 percent sodium potassium tartarate

d) To 50 ml of reagent b, 0.5 ml of reagent a and 0.5 ml of reagent c were added. This solution was prepared fresh.

e) 1 N Folin's Ciocalteau reagent (prepared fresh)

Procedure

5 ml of Reagent d was added to 1 ml of suitably diluted protein solution and mixed immediately. 1N Folin's reagent (0.5 ml) was added after 10 mins and the test tube was shaken vigorously to ensure proper mixing. After 30 mins, the intensity of color developed was measured at 660 nm against the reagent blank. The amount of protein was calculated from standard curve using Bovine Serum Albumin (0-100 µg per ml).

3.13.2.5 Measurement of activity of the thermostable pullulanase enzyme

The activity of the partially purified thermostable pullulanase enzyme after each step of purification was measured. The activity was determined

spectrophotometrically at 540 nm via dinitrosalicylic (DNS) method as proposed by Miller (1959) with slight modification. The reaction mixture contained 0.3 ml crude enzyme extract and 3 ml of 0.1M sodium phosphate buffer (pH: 9.0). The reaction was held at 60ºC for 15 mins and transferred to cooling ice bath for 1 min followed by addition of 1.5 ml of DNS acid and 150 µl 0.1N NaOH. The reaction tubes were again incubated for 5 mins at 100ºC. The reaction was stopped by immersing the reaction tubes in ice bath. One unit of thermostable pullulanase activity was defined as the amount of enzyme which liberates 1 µmol of reducing sugar in 1 min under standard assay conditions.

3.13.3 Native PAGE (Polyacrylamide gel electrophoreses)

The purity of the pullulanase enzyme obtained after the course of purification was detected by native polyacrylamide gel electrophoresis using 7.5 percent acrylamide gel and anionic system of Davis, 1964 on horizontal slab gel electrophoresis system.

3.13.3.1 Reagents

(A) Acrylamide bisacrylamide stock solution

Acrylamide - 30.00 g

Bisacrylamide - 0.8 g

Dissolved in distilled water and final volume was made to 100 ml with distilled water and stored in brown bottle at 4ºC, till use.

(B) 1.5 percent (w/v) ammonium persulphate (APS) solution.

It was prepared by dissolving 0.15 g of APS in 10 ml of distilled water. This reagent was prepared fresh just before use.

(C) Bromophenol blue solution (0.01 percent)

Ten mg of bromophenol blue was dissolved in 100 ml of distilled water.

(D) Staining solution for protein

Coomassie brilliant blue R-250	-	1.25 g
Methanol	-	200 ml
Glacial acetic acid	-	35 ml

The above components were mixed together and final volume was made to 500 ml with distilled water. It was then filtered to remove undissolved materials and was stored at room temperature.

(E) Destaining solution

Glacial acetic acid	-	75 ml
Methanol	-	50 ml

Above components were mixed and the final volume was made to 1 litre.

(F) Resolving gel buffer stock (Tris-HCl, pH 8.8)

Tris	-	36.3 g
1M HCl	-	48.0 ml

Adjusted its pH to 8.8 and then the final volume was made to 100 ml with distilled water and stored at 4^{o}C.

(G) Reservoir buffer (Tris glycine, pH 8.3)

Tris	-	3.0 g
Glycine	-	14.4 g

Adjusted its pH to 8.3 and the final volume was made to 1 litre with distilled water.

(H) Stacking gel buffer stock (Tris-HCl, pH 6.8)

Tris	-	6.0 g
1M HCl	-	48.0 ml

Adjusted its pH to 6.8 and final volume was made to 100 ml with distilled water and stored at 4^{o}C.

(I) 40 percent (w/v) sucrose

40 g of sucrose was dissolved in 100 ml of distilled water

3.13.3.2 Sample preparation

To 0.3 ml of purified sample 0.1 ml of 40 per cent sucrose was added prior to loading.

3.13.3.3 Gel preparation and electrophoresis

Glass plates were washed with distilled water and oven dried. The plates were placed on gel casting assembly. The plates were clamped properly avoiding any leakage and air bubble entrapment. Various components of resolving gel were mixed as specified in the Table 7 and were poured into glass plate assembly after addition of TEMED. The gel was allowed to polymerize for 30 mins. After the polymerization of gel, the whole assembly was placed in reservoir buffer tank. Buffer tank was filled with prechilled reservoir buffer and 30 µl of protein samples were loaded in each well with a few drops of marker dye (bromophenol blue) loaded in one of the well as tracking dye.

Table 7: Composition of stacking and resolving gel for native PAGE

Reagents	Stacking gel (2.5 %)	Resolving gel (7.5 %)
Acrylamide- bisacrylamide stock solution	2.500	7.50
Stacking gel buffer stock solution (Tris HCl, pH 6.8)	5.000	-
Resolving gel buffer stock solution (Tris HCl, pH 8.8)	-	3.75
1.5 per cent APS	1.500	1.50
Water	11.000	17.25
TEMED	0.015	0.15
Total volume	20	30

Electrophoresis was carried out at a constant current of 3 mA/well. The current was applied till the tracking dye reached at the other end of the gel. Gel was removed from the electrophoresis apparatus and stained for 3 hrs in coomassie brilliant blue R-250 staining solution for proteins. It was then destained by changing the destaining solution frequently.

3.13.4 SDS- polyacrylamide slab gel electrophoresis

3.13.4.1 Reagents

(A) Acrylamide – bis acrylamide stock solution

As described in section 3.7.7.1

(B) Stacking gel buffer stock (Tris HCl, pH 6.8)

As described in section 3.7.7.1

(C) Resolving gel buffer stock (Tris HCl, pH 8.8)

As described in section 3.7.7.1

(D) 1.5 percent (w/v) ammonium per sulphate (APS) solution

As described in section 3.7.7.1

(E) Staining solution

As described in section 3.7.7.1

(F) Destaining solution

As described in section 3.7.7.1

(G) Reservoir buffer (Tris glycine, pH 8.3)

Tris	-	3.0 g
Glycine	-	14.4 g
SDS	-	1.0 g

Dissolved in 900 ml of distilled water and adjusted its pH to 8.3. The final volume was made to 1 litre with distilled water.

(H) SDS (10 percent, w/v)

One gram of SDS was dissolved in 10 ml of distilled water. This reagent was stored at room temperature.

(I) Sample buffer - 2

1M Tris HCl pH 6.8	-	12.5 ml
SDS	-	4.0 g
β- mercaptoethanol	-	10.0 ml
Glycerol	-	20.0 ml
Bromophenol blue (1 per cent)	-	4.0 ml

Distilled water was added to make the final volume to 100 ml.

3.13.4.2 Sample preparation

The purified enzyme was mixed with equal volume of sample buffer. The mixture was boiled for 3 mins in a boiling water bath and cooled at room temperature.

3.13.4.3 Preparation of slab gel and electrophoresis

Gel was prepared in the same way as described for native PAGE. Various components of resolving gel mixed has been specified in Table 8. About 20 µl of protein sample was loaded in the sample wells and standard molecular weight protein markers in one of the well. Electrophoresis was carried out at a constant current of 3 mA/well. The current was applied till the tracking dye (bromophenol blue) reached at the other end of the slab gel. After the electrophoresis was complete, the gel was removed carefully and placed for 3-4 hrs in protein staining solution and destained by frequently changing the destaining solution.

Table 8: Composition of stacking and resolving gel for SDS-PAGE

Stock solution	Stacking gel (2.5 %)	Resolving gel (12.5 %)
Acrylamide – bis acrylamide stock solution	2.50	12.50
Stacking gel buffer (Tris HCl, pH 6.8)	5.00	-
Resolving gel buffer (Tris HCl, pH 8.8)	-	3.75
10 per cent SDS	0.20	0.30
1.5 per cent APS	1.00	1.50
Water	11.30	11.95
TEMED	0.015	0.015
Total volume	20.00	30.00

3.14 CHARACTERISATION OF THERMOSTABLE PULLULANASE ENZYME

3.14.1 Molecular weight determination

Molecular weight of the purified pullulanase enzyme was determined by SDS polyacrylamide gel electrophoresis (as described under section 3.13.4). The standard protein markers consisted of five precisely sized recombinant proteins of molecular weight 205,000; 97,400; 66,000; 43,000 and 29,000 daltons.

3.14.2 Effect of pH

Different buffers *viz.,* citrate (pH: 6.0 to 7.0), Tris buffer (pH: 7.0 to 8.0) and phosphate buffer (pH: 8.0 to 9.5) were prepared and used in the reaction mixture. The activity of the pullulanase enzyme was monitored.

3.14.3 Effect of temperature on the stability of pullulanase enzyme

The pullulanase enzyme was incubated at different temperatures *viz.*, 20, 30, 40, 50, 60, 70, 80 and 90ºC for 10 mins. After incubation, the test tubes were immediately cooled in ice bath and enzyme activities were measured.

Chapter-4

EXPERIMENTAL RESULTS

The major objectives of the present study were the isolation and identification of thermostable pullulanase producing thermotolerant bacteria from the hot springs of Himachal Pradesh and production of thermostable pullulanase enzyme from these indigenous strains. To achieve the objectives, a number of experiments were conducted and the results obtained during the course of this study have been presented in this chapter under the following sections:

4.1 Survey and selection of sites
4.2 Sample collection
4.3 Physico-Chemical analysis
4.4 Statistical data analysis of physico-chemical descriptors
4.5 Isolation of thermophilic bacteria
4.6 Enumeration of thermophilic bacterial population in various samples from hot water springs
4.7 Morphological characterization of thermophilic bacterial isolates
4.8 Screening of thermophilic bacteria producing thermostable pullulanase enzyme
4.9 Biochemical characterization of thermophilic bacteria producing thermostable pullulanase enzyme
4.10 Quantitative screening for thermostable pullulanase activity
4.11 Molecular characterization of selected bacterial isolate
4.12 Optimization of culture conditions for maximum growth and maximum enzyme production
4.13 Partial purification of thermostable pullulanase enzyme
4.14 Characterization of thermostable pullulanase enzyme

4.1 SURVEY AND SELECTION OF SITES

Six hot water springs *viz*., Manikaran, Vashist, Khirganga, Kasol, Kalath and Tattapani located in two districts of Himachal Pradesh, were surveyed and

selected for sample collection. Manikaran thermal spring has been found to be one of the hottest water springs of H.P. with a temperature of water 106^{o}C. It is located at a distance of 46 km from Kullu in a village Manikaran at an altitude of 1735 m along the banks of the river Parvati. Vashisht hot spring is located in the village Vashisht located at a distance of 5 km from Manali and 44 km from Kullu, at an altitude of 2050 m from sea level. Khirganga is also located in Kullu district of H.P. and situated at a distance of 46 km from Kullu and 12 km from village Bershani at an altitude of 2960 m. Kasol is situated in Parvati valley on the banks of Parvati River on the way between Bhuntar to Manikaran. It is located 42 km east of Kullu at the height of 1640 m. Kalath is 6.5 km from Manali whereas Tattapani is located in Mandi district at an altitude of 680 m and it is 51 km from Shimla and 29 km from Naldhera (Figure-4, Plate-1). From each selected site three subsites were selected for the collection of the samples and appropriate codes have been given (Table-2).

4.2 SAMPLE COLLECTION

A total of seventy two samples were collected by using a 50 ml syringe with a 1.0 cm wide opening in sterile 30 ml screw capped vials. Samples consisted of water, soil, pebbles and rock matting (Plate-2). All these samples were kept at 4°C in refrigerator in laboratory till further experimentation. Out of these seventy two samples, only eighteen water samples were subjected to physico-chemical analysis.

4.3 PHYSICO-CHEMICAL ANALYSIS

4.3.1 pH

Variability in pH was studied for the eighteen water samples, rest of the samples were in the form of pebbles, soil and rock matting. It was found to range from a minimum of 5.7 to a maximum of 9.5. Water sample KhS1W has been found to possess the minimum pH value of 5.7 whereas maximum pH of 9.5 has been exhibited by sample MS3W (Table-9, Fig-5).

Table 9: Variability in pH amongst eighteen water samples of six hot water springs in H.P.

Sites	pH		
	S1	**S2**	**S3**
Manikaran	8.0	8.4	**9.5**
Vashisht	8.0	7.8	8.0
Khirganga	**5.7**	6.0	5.8
Kasol	6.0	6.0	5.9
Kalath	7.5	7.3	7.0
Tattapani	6.4	6.0	5.8

4.3.2 Temperature

Temperature was also investigated for only eighteen water samples, rest of the samples were in the form of pebbles, soil and rock matting. It was found to range from a minimum of 35^{o}C to a maximum of 106^{o}C. Variability in temperature among eighteen water samples of six hot springs has been depicted in Table-10 and it was found that sample KaS2W of Kalath showed a minimum temperature of 35^{o}C whereas sample MS3W of Manikaran showed a maximum temperature of 106^{o}C (Table-10, Fig-6).

Table 10: Variability in temperature amongst eighteen water samples of six hot water springs in H.P.

Sites	Temperature (^{o}C)		
	S1	**S2**	**S3**
Manikaran	97	81	**106**
Vashisht	46	48	52
Khirganga	58	56	55
Kasol	62	65	60
Kalath	38	**35**	37
Tattapani (S6)	56	58	53

4.3.3 Chloride

Variability in chloride content was also explored for eighteen water samples, rest of the samples were in the form of pebbles, soil and rock matting. The chloride content has been observed to range from 578-1978 mg/l.

Persual of Table-11 showed that the minimum chloride content of 578 mg/l was observed in VS1W sample of Vashisht site whereas maximum chloride

content of 1978 mg/l was recorded in MS3W sample of Manikaran site (Table-11, Fig-7).

Table 11: **Variability in chloride content amongst eighteen water samples of six hot water springs in H.P.**

Sites	Chloride (mg/l)		
	S1	**S2**	**S3**
Manikaran	1823	1772	**1978**
Vashisht	**578**	659	592
Khirganga	1043	998	1121
Kasol	786	884	865
Kalath	1231	1278	1351
Tattapani	978	1112	1076

4.3.4 Sulphate

Sulphate content has been tested in eighteen water samples, rest of the samples were in the form of pebbles, soil and rock matting. It has been observed that the values of sulphate content ranged from a minimum of 9 mg/l to a maximum of 54 mg/l.

Variability in sulphate content amongst eighteen water samples of hot water from six sites has been presented in Table 12 (Fig- 8). It has been found that the minimum value of sulphate content (9 mg/l) was recorded in VS1W sample of Vashisht site whereas maximum value of sulphate content (54 mg/l) was observed in sample MS3W of Manikaran site.

Table 12: Variability in sulphate content amongst eighteen water samples of six hot water springs in H.P.

Sites	Sulphate (mg/l)		
	S1	**S2**	**S3**
Manikaran	32	48	**54**
Vashisht	**09**	16	13
Khirganga	12	18	19
Kasol	24	27	29
Kalath	17	23	29
Tattapani	19	33	38

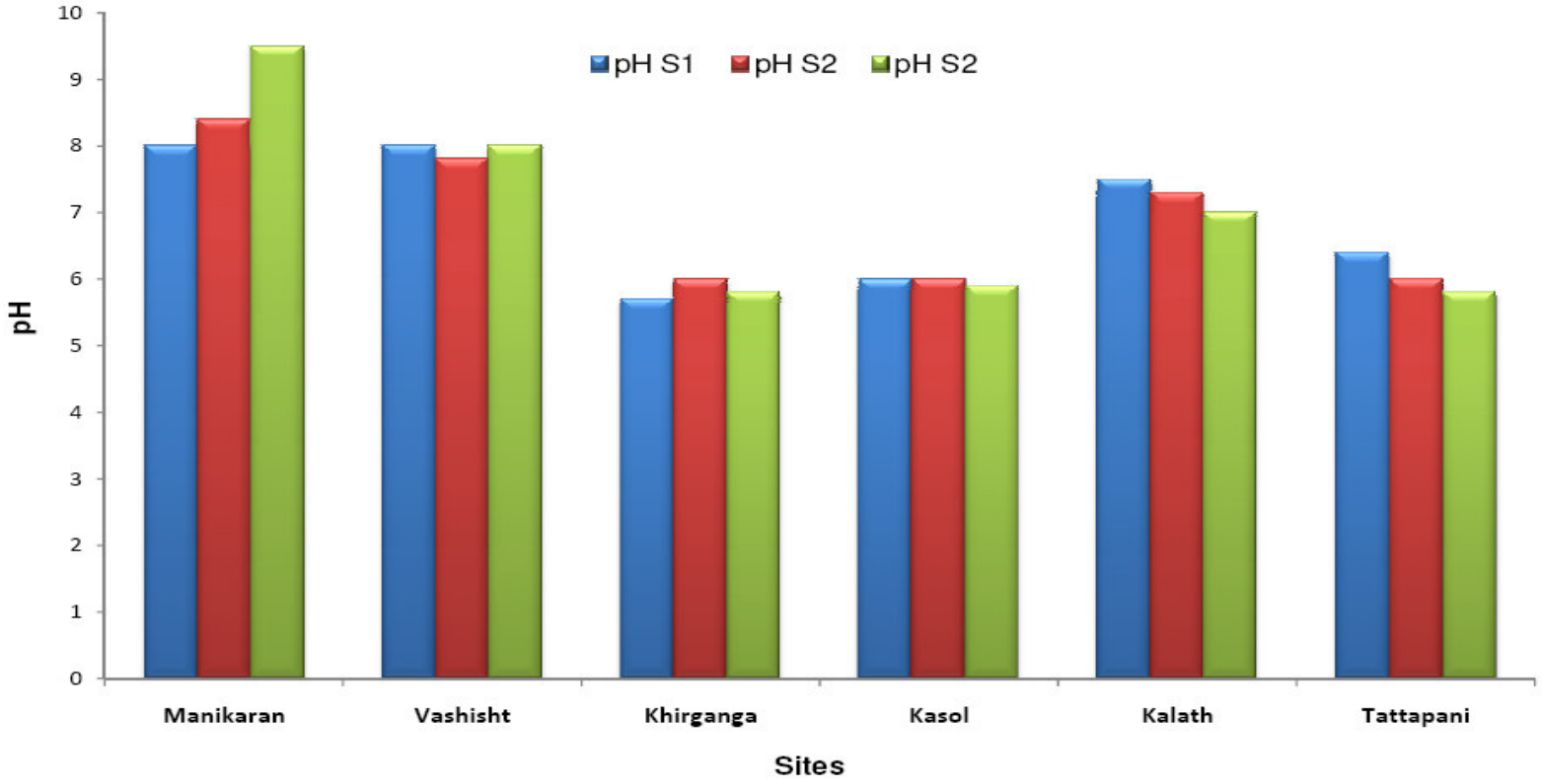


Fig 5: Variability in pH among eighteen water samples of six hot water springs in H.P.

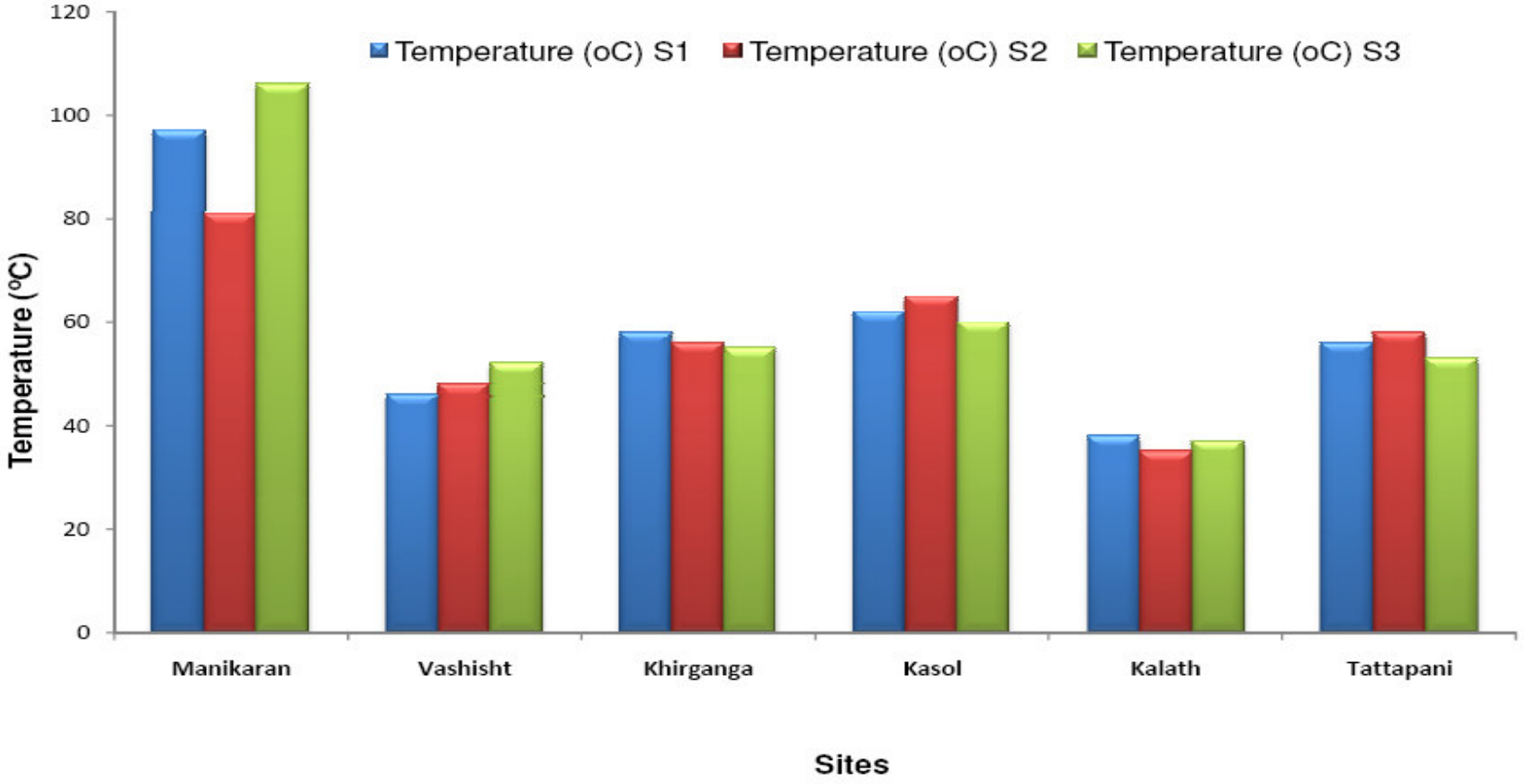


Fig 6: Variability in temperature among eighteen water samples of six hot water springs in H.P.

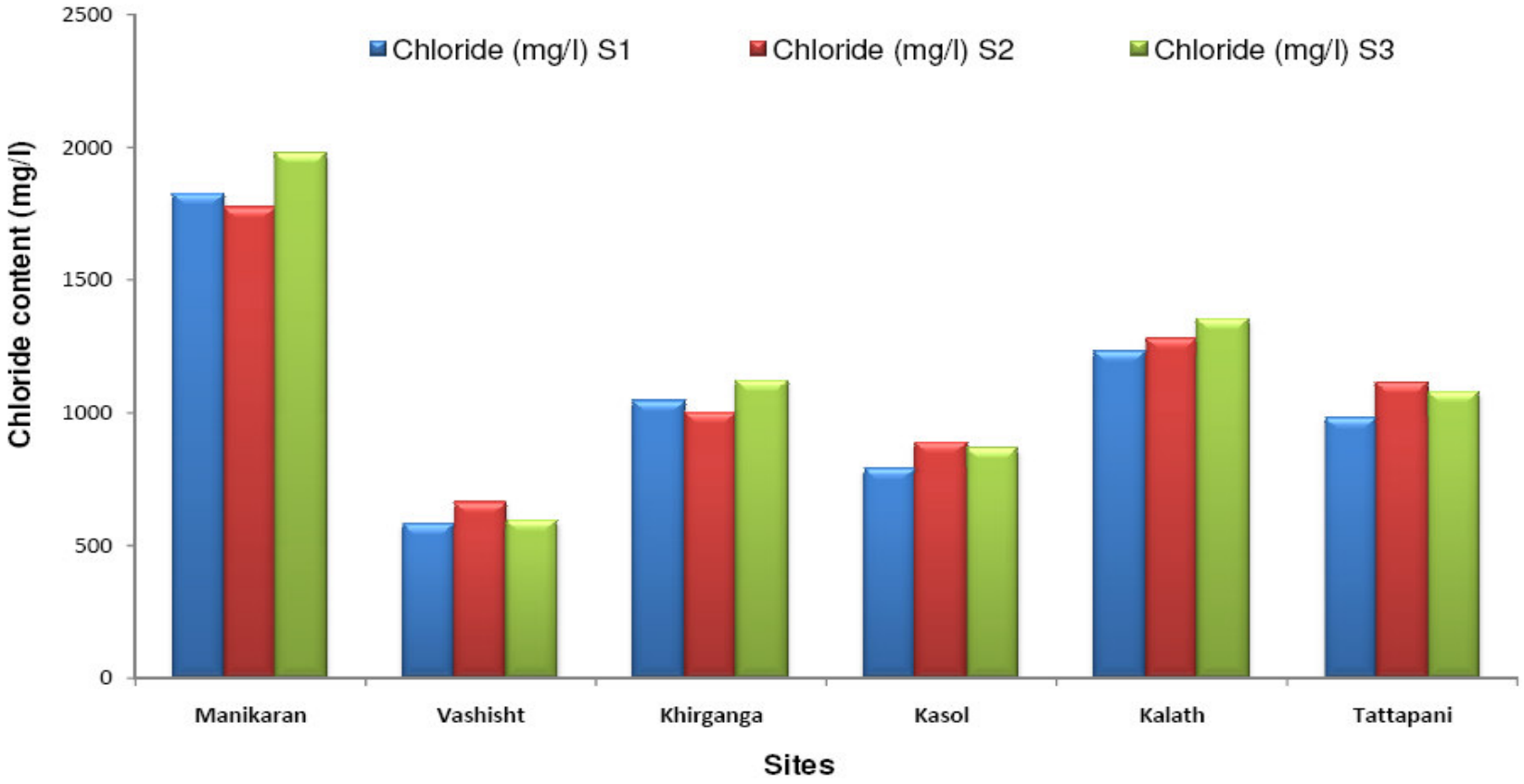


Fig 7: Variability in chloride content among eighteen water samples of six hot water springs in H.P.

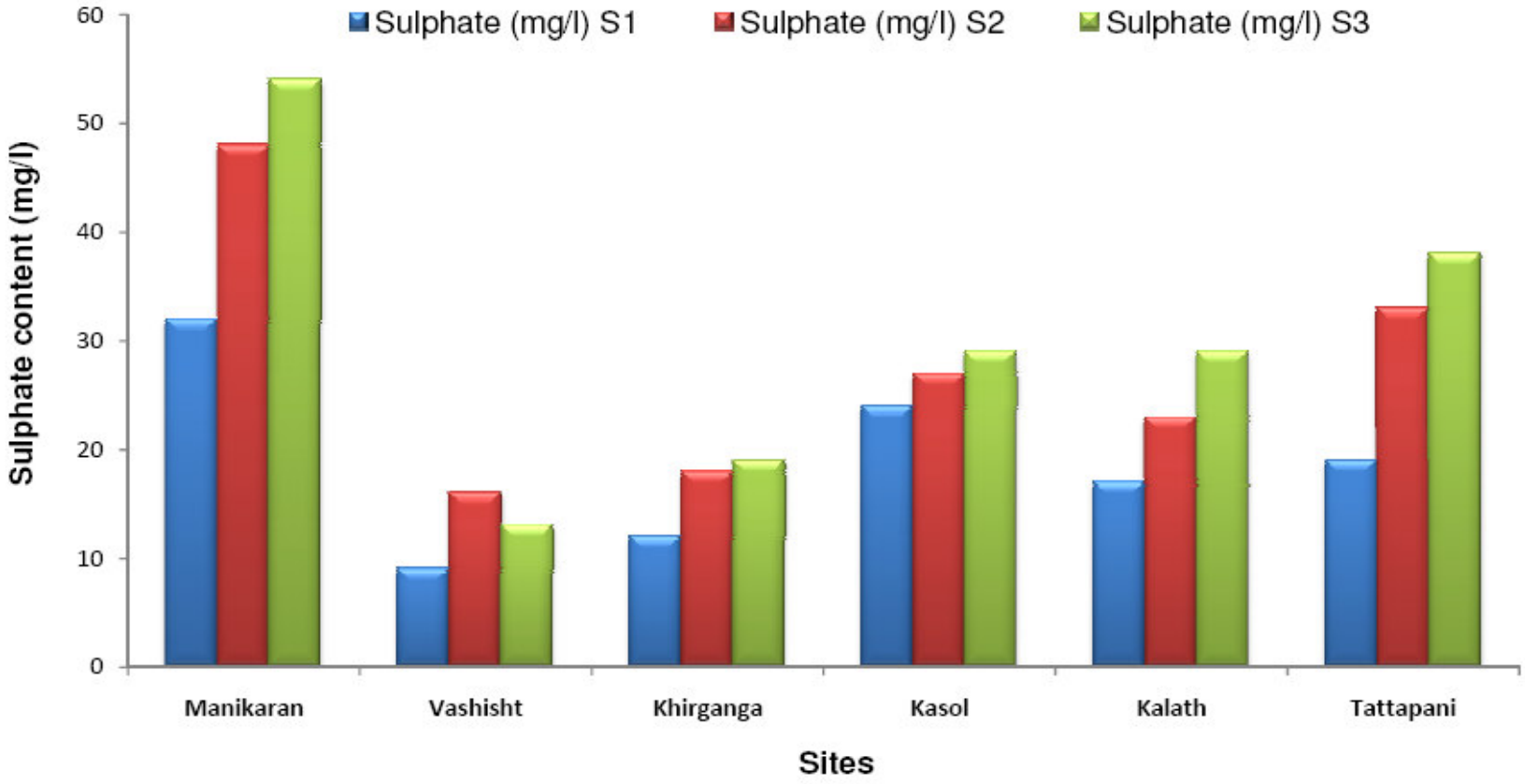


Fig 8: Variability in sulphate content among eighteen water samples of six hot water springs in H.P.

4.3.5 Total Hardness

The eighteen water samples were analyzed for total hardness whereas rests of the samples were in the form of pebbles, soil and rock matting. The total hardness values have been found to range from 32.9-141.3 mg/l.

Table-13 (Fig-9) depicts the variability in total hardness among eighteen water samples of six sites and the maximum value of 141.3 mg/l was found to be exhibited by MS2W sample of Manikaran site whereas minimum value of 32.9 mg/l of total hardness was observed in sample VS3W of Vashisht site.

Table 13: Variability in total hardness content amongst eighteen water samples of six hot water springs in H.P.

Sites	Total hardness (mg/l)		
	S1	**S2**	**S3**
Manikaran	134.7	**141.3**	118.8
Vashisht	42.2	43.8	**32.9**
Khirganga	69.2	73.7	63.5
Kasol	35.3	36.1	33.1
Kalath	49.2	41.8	50.5
Tattapani	101.2	89.8	110.8

4.3.6 Calcium Hardness

Variability in calcium hardness in the form of (Ca^{++}) has also been studied in eighteen water samples. The values have been found to range from a minimum of 22 mg/l to a maximum of 134 mg/l.

Table 14: Variability in calcium hardness amongst eighteen water samples of six hot water springs in H.P.

Sites	Calcium hardness (mg/l)		
	S1	**S2**	**S3**
Manikaran	129	**134**	112
Vashisht	32	33	**22**
Khirganga	43	47	38
Kasol	31	35	29
Kalath	43	36	45
Tattapani	97	86	106

Variability in calcium hardness among eighteen water samples of six sites has been shown in Table-14 (Fig- 10) and it has been observed that the minimum calcium hardness of 22 mg/l was found in sample VS3W of Vashisht while maximum calcium hardness value of 134 mg/l was observed for MS2W sample of Manikaran site.

4.3.7 Calcium Carbonate Hardness ($CaCO_3$)

The eighteen water samples were also analyzed for calcium carbonate hardness ($CaCO_3$). The values have been found to range from 52 -283 mg/l.

It is clear from Table-15 (Fig-11) that minimum (52 mg/l) value of calcium carbonate hardness ($CaCO_3$) has been recorded in VS3W sample of Vashisht site whereas the maximum value (283 mg/l) has been observed in TS2W sample of Tattapani site.

Table 15: Variability in calcium carbonate hardness amongst eighteen water samples of six hot water springs in H.P.

Sites	Calcium Carbonate hardness (mg/l)		
	S1	**S2**	**S3**
Manikaran	85	92	98
Vashisht	63	59	**52**
Khirganga	68	75	79
Kasol	178	165	152
Kalath	109	122	98
Tattapani	256	**283**	278

4.3.8 Magnesium hardness

Magnesium content has also been investigated in eighteen water samples of six hot water springs. The values ranged from a minimum of 3.15 mg/l to maximum of 26.75 mg/l.

Variability in magnesium content among eighteen water samples from hot water spring has been shown in Table-16 (Fig-12). The minimum value of 3.15mg/l has been observed in KS2W sample of Kasol whereas the maximum (26.75 mg/l) has been recorded in the KhS2W sample of Khirganga site.

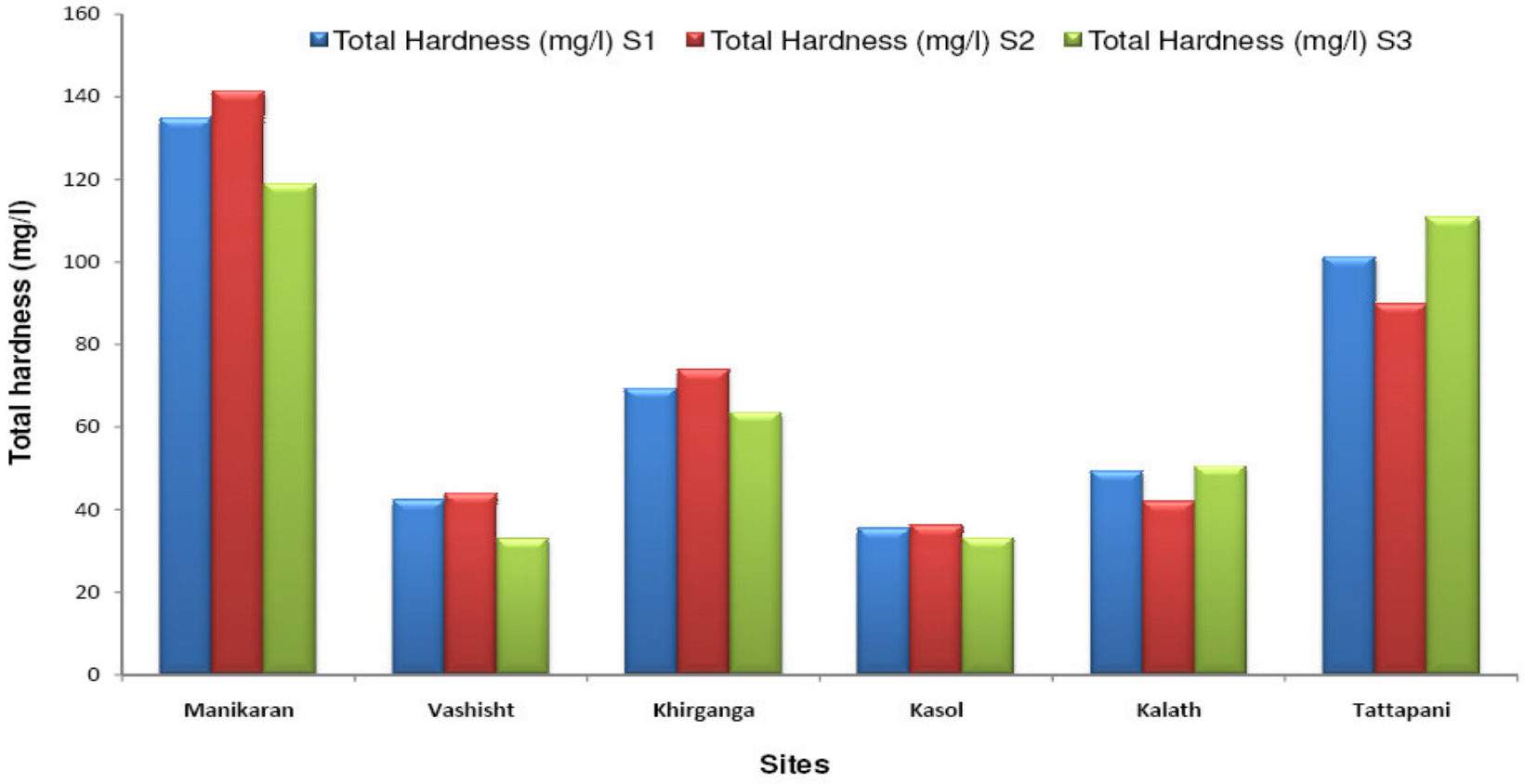


Fig 9: Variability in total hardness among eighteen water samples of six hot water springs in H.P.

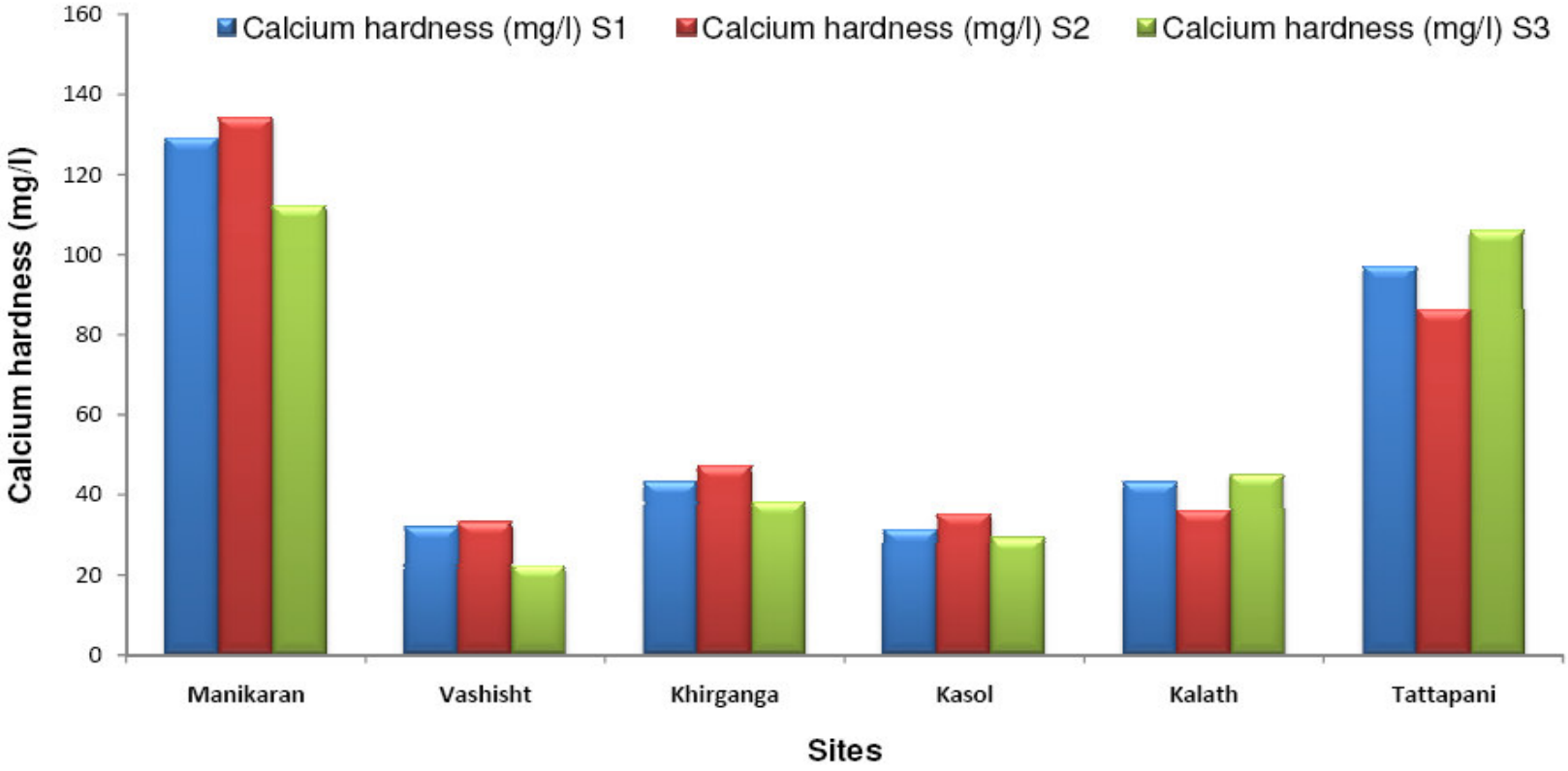


Fig 10: Variability in calcium hardness among eighteen water samples of six hot water springs in H.P.

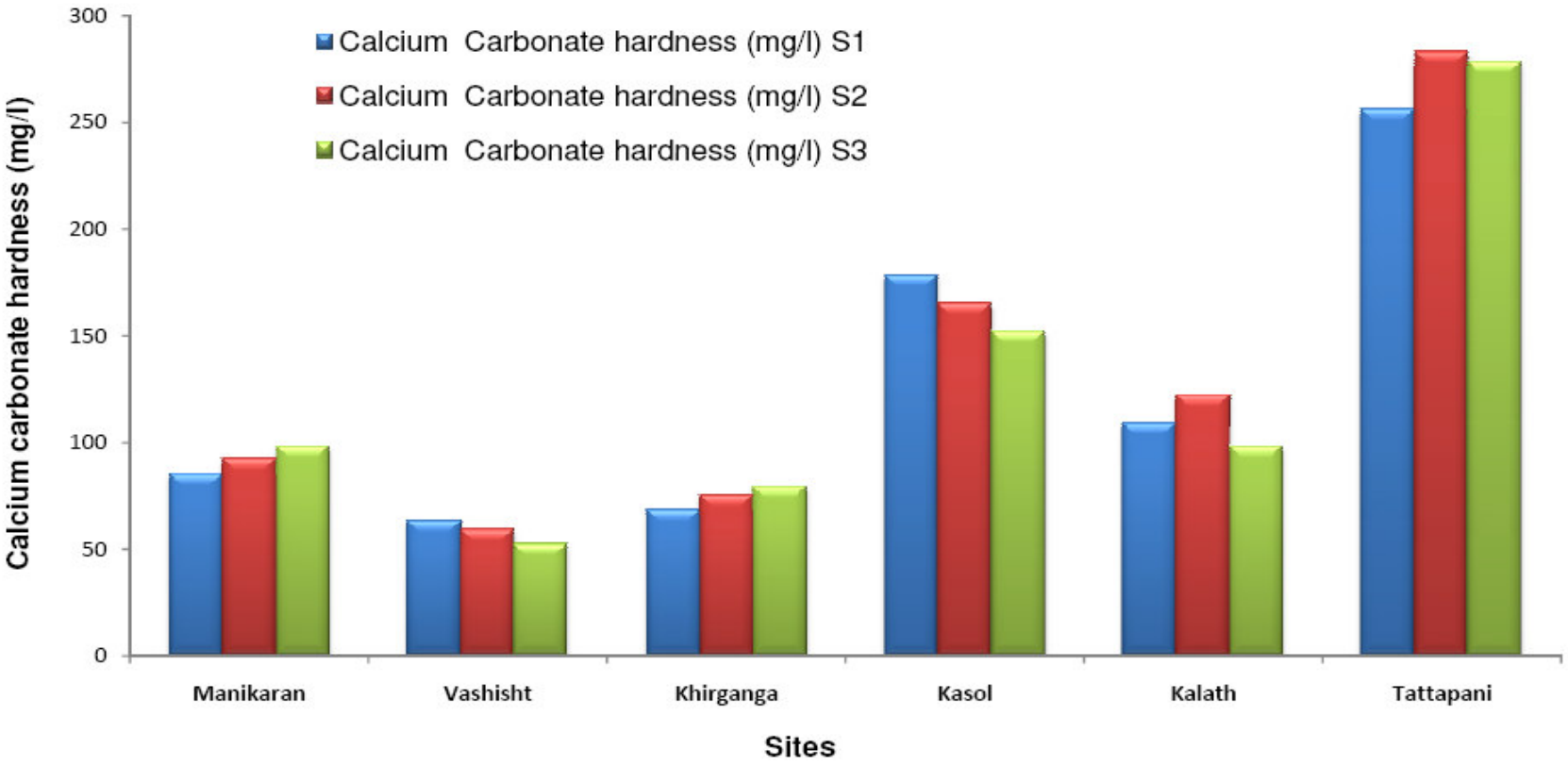


Fig 11: Variability in calcium carbonate hardness among eighteen water samples of six hot water springs in H.P.

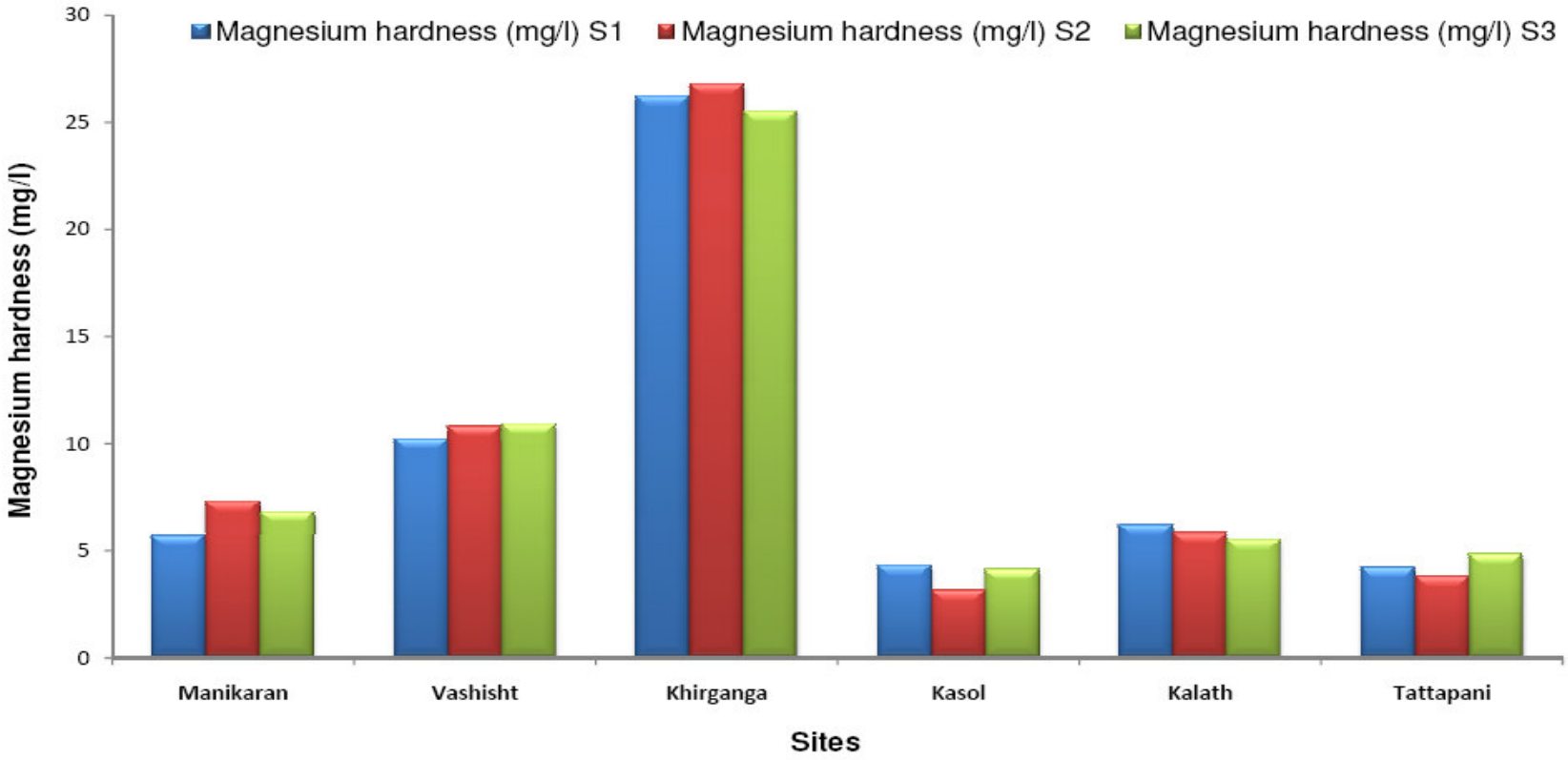


Fig 12: Variability in magnesium hardness among eighteen water samples of six hot water springs in H.P.

Table 16: Variability in Magnesium hardness amongst eighteen water samples of six hot water springs in H.P.

Sites	Magnesium hardness (mg/l)		
	S1	**S2**	**S3**
Manikaran	5.70	7.30	6.80
Vashisht	10.20	10.80	10.90
Khirganga	26.20	**26.75**	25.50
Kasol	4.30	**3.15**	4.10
Kalath	6.20	5.80	5.50
Tattapani	4.20	3.80	4.80

4.4 STATISTICAL DATA ANALYSIS OF PHYSICO-CHEMICAL CHARACTERS

4.4.1 Correlation studies

To study the correlation amongst 8 variables obtained from physico-chemical characters, has been subjected to standard statistical analysis and a correlation matrix was obtained.

Correlation matrix showing pairwise correlation coefficients has been presented in Table-17. The significance was tested at 5% level of significance and it has been observed that all variables are significantly correlated except the pairs X1 and X5; X1 and X7; X1 and X8; X2 and X7; X2 and X8; X3 and X7; X3 and X8; X4 and X8; X5 and X8; X6 and X8; X7 and X8.

Table 17: Correlation studies

	X1	X2	X3	X4	X5	X6	X7	X8
X1	1							
X2	.108	1						
X3	.227	.654	1					
X4	.092	.799	.834*	1				
X5	-.223	.387	.439	.720	1			
X6	.130	.741	.788	.875*	.806	1		
X7	-.545	-.049	-.059	.318	.824*	.333	1	
X8	-.263	-.095	-.140	-.506	-.528	-.280	-.525	1

.*Correlation is significant at the 0.05 level (2-tailed).

X1: pH
X2: Temperature
X3: Chloride content
X4: Sulphate content
X5: Total Hardness
X6: Calcium hardness (Ca^{++})
X7: Calcium carbonate Hardness
X8: Magnesium hardness (Mg^{++})

4.4.2 Variability analysis

Coefficient of variability and other related parameters with respect to various physico-chemical characters under study for different sites were further analyzed by standard statistical methods. The minimum value of 0.968% for coefficient of variation has been observed for pH at Kasol site and a maximum of 32.83 % for sulphate at Tattapani site.

Comparison of all the sites amongst themselves depicted that for pH maximum value of coefficient of variation of 8.997% was found for Manikaran site and minimum value of 0.968% was found for Kasol site. Similarly, for temperature, coefficient of variation was maximum 13.38% for site Manikaran and minimum 2.71% for site Khirganga. In case of chloride, maximum value of coefficient of variation 7.10% was for Vashisht site and minimum 4.70% for Kalath site and for total hardness maximum value of coefficient of variation 14.85% was for Vashisht site and minimum 4.46% for Kasol site. Also for calcium hardness when noticed as Ca^{++} maximum value of coefficient of variation 20.98% was for Vashisht site and minimum 9.23% for Manikaran site and for calcim carbonate hardness as $CaCO_3$ maximum value of coefficient of variation 10.95% was for Kalath site and minimum 5.27% for Tattapani. Maximum value of coefficient of variation 15.96% has been recorded for Kasol site and minimum 2.40% for Khirganga site and for sulphate, coefficient of variation was maximum 32.83% for Tattapani site and minimum 9.44% for Kasol site (Table-18).

4.4.3 Analysis of Variance Technique (ANOVA)

Average performance of various characters was studied using Analysis of Variance Technique (ANOVA) and it has been observed that in case of pH, maximum mean pH of 8.633 was observed for site Manikaran however, the minimum value of 5.833 was found for site Khirganga.

In case of temperature, the maximum mean temperature of 94.667ºC was observed for Manikaran site whereas minimum of 36.667ºC was observed for Kalath. For chloride content, maximum mean of 1857.667 mg/l was observed for

Table 18: Site wise coefficient of variability and other related parameters

Treatment	pH		Temperature		Chloride		Sulphate		Total Hardness		Calcium Hardness		CaCO3 Hardness		Magnesium Hardness	
	σ	CV	σ	CV	σ	CV	σ	CV	σ	CV	σ	CV	σ	CV	σ	CV
Manikaran	0.777	8.997	12.66	13.38	107.29	5.78	11.37	25.46	11.57	8.79	11.53	9.23	6.51	7.10	0.82	12.40
Vashisht	0.115	1.456	3.06	6.28	43.29	7.10	3.51	27.73	5.89	14.85	6.08	20.98	5.57	9.60	0.38	3.56
Khirganga	0.153	2.619	1.53	2.71	62.23	5.90	3.79	23.18	5.11	7.43	4.51	10.57	5.57	7.52	0.63	2.40
Kasol	0.058	0.968	2.52	4.04	51.97	6.15	2.52	9.44	1.55	4.46	3.06	9.65	13.00	7.88	0.61	15.96
Kalath	0.252	3.463	1.53	4.17	60.47	4.70	6.00	26.09	4.69	9.95	4.73	11.43	12.01	10.95	0.35	6.02
Tattapani	0.306	5.036	2.52	4.52	69.35	6.57	9.85	32.83	10.51	10.45	10.02	10.40	14.36	5.27	0.50	11.80

σ = Standard Deviation
CV (%) = Coefficient of variation
N=8 (No. of variables)

Table 19: Mean Value Table

Variables / Sites	pH	Temperature	Chloride	Sulphate	Total hardness	Ca^{++} hardness	$CaCO_3$	Mg^{++} hardness
Manikaran	8.633	94.667	1,857.667	44.667	131.600	125.00	91.667	6.600
Vashisht	7.933	48.667	609.667	12.667	39.633	29.000	58.000	10.633
Khirganga	5.833	56.333	1,054.000	16.333	68.800	42.667	74.000	26.150
Kasol	5.967	62.333	845.000	26.667	34.833	31.667	165.000	3.850
Kalath	7.267	36.667	1,286.667	23.000	47.167	41.333	109.667	5.833
Tattapani	6.067	55.667	1,055.333	30.000	100.600	96.333	272.333	4.267

Manikaran where as minimum of 609.667 mg/l was observed in Vashisht. For sulphate content, maximum mean value of 44.667 mg/l was observed in Manikaran followed by Tattapani (30.00 mg/l) and minimum mean value of 12.667 mg/l was observed for site Vashisht. But for total hardness maximum value of 131.600 mg/l was observed in Manikaran followed by Tattapani (100.600 mg/l) however, the minimum of 34.833 mg/l was observed in site Kasol. For Ca^{++} maximum mean value of 125.00 mg/l was observed in Manikaran whereas minimum of 29.00 mg/l was observed in Vashisht. Also, for $CaCO_3$ maximum mean performance of 272.333 mg/l was seen in Tattapani followed by Kalath (109.667 mg/l). For magnesium content, Khirganga gave maximum mean performance of 26.150 mg/l followed by Vashisht (10.633 mg/l), whereas Kasol site was found to give minimum mean performance of 3.850 mg/l (Table-19).

4.5 ISOLATION OF THERMOPHILIC BACTERIA

Nutrient agar/broth was used for isolation of thermophilic bacterial isolates from seventy two samples of hot water springs of H.P. Each sample of water, soil, pebbles and rock matting was inoculated in 150 ml flask containing 100 ml nutrient broth, incubated at 60^oC at 150 rpm for 48 hrs leading to isolation of fifty eight bacterial isolates (Plates 3a, b, c, d).

4.6 ENUMERATION OF THERMOPHILIC BACTERIAL POPULATION IN VARIOUS SAMPLES FROM HOT WATER SPRINGS

Bacterial population colonizing water and soil samples from the hot water springs were observed after 48 hrs on nutrient agar (NA) as presented in Table 20 (Fig-13). Bacterial population in water was highest (3.45×10^5 cfu/ml) at S1 site of Tattapani and minimum (0.76×10^5 cfu/ml) bacterial population was noted at S2 site of Kalath. Similarly Bacterial population in soil was highest (3.54×10^5 cfu/g) at S3 site of Vashisht and minimum (0.54×10^5 cfu/g) at S3 site of Kalath.

4.7 MORPHOLOGICAL CHARACTERIZATION OF THERMOPHILIC BACTERIAL ISOLATES

4.7.1 Morphological characterization

All the fifty eight thermophilic bacterial isolates were further studied for various morphological characteristics (Table 21).

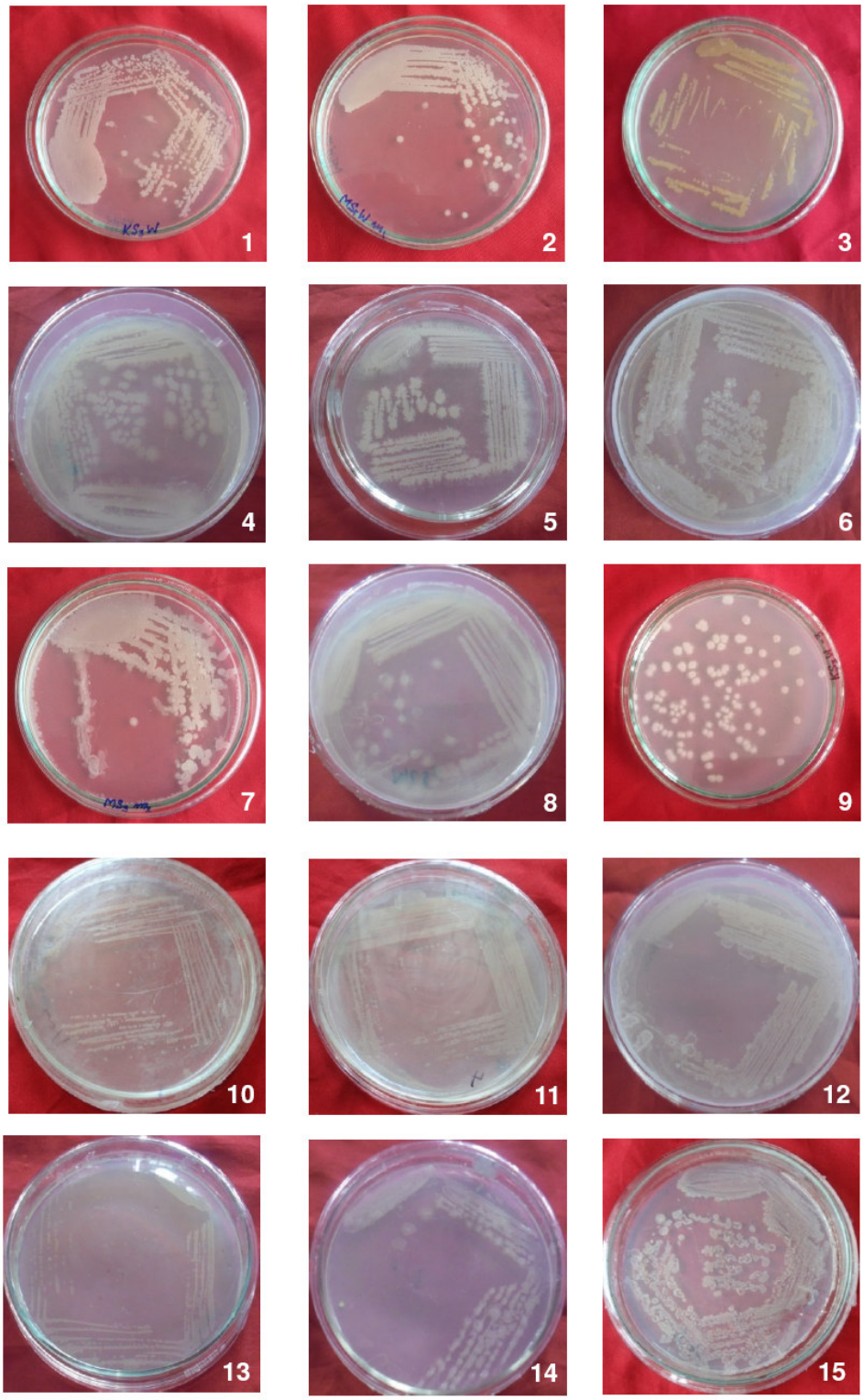


Plate 3 (a): Different colony morphotypes obtained from samples of hot springs (H.P.)

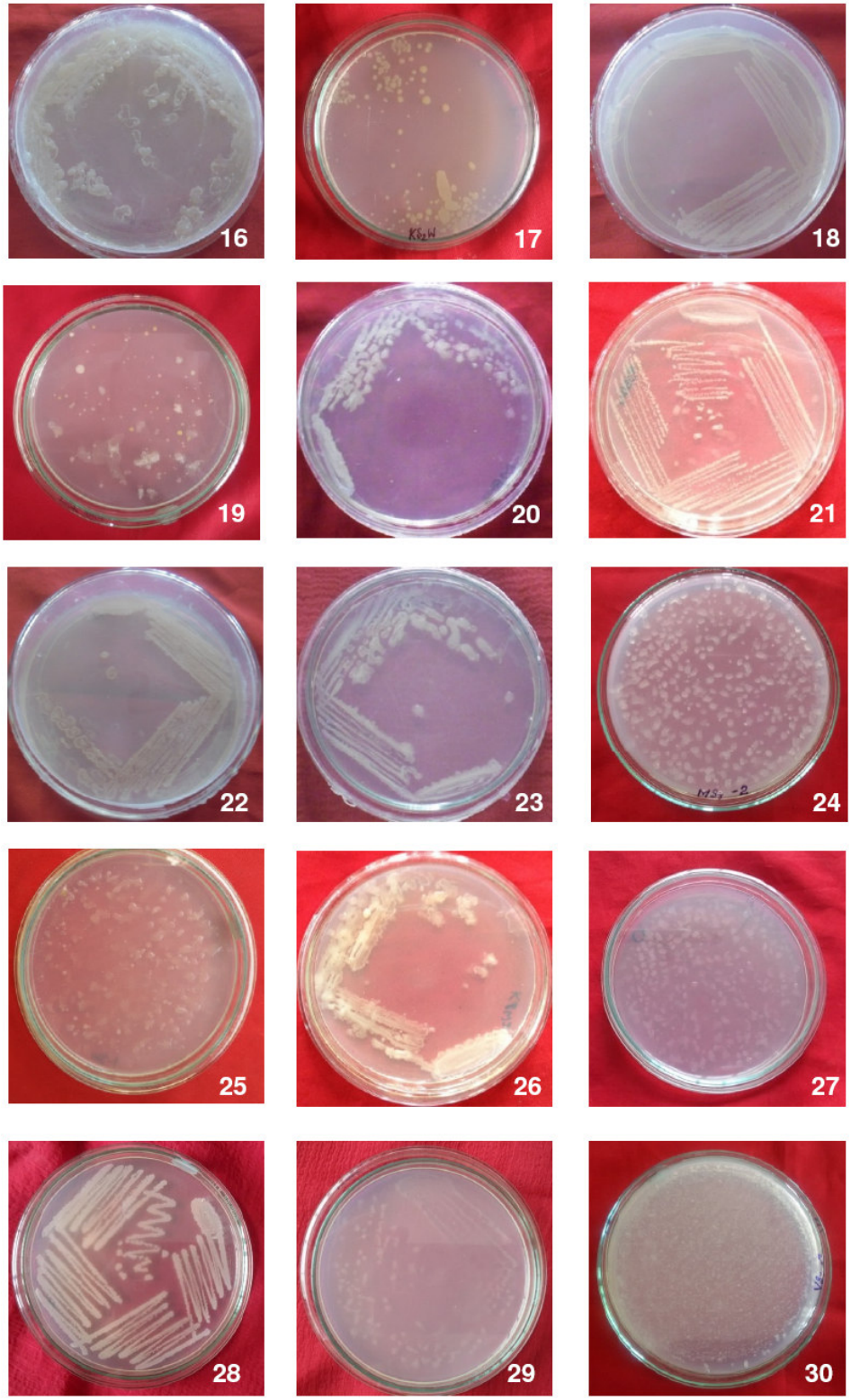


Plate 3 (b): **Different colony morphotypes obtained from samples of hot springs (H.P.)**

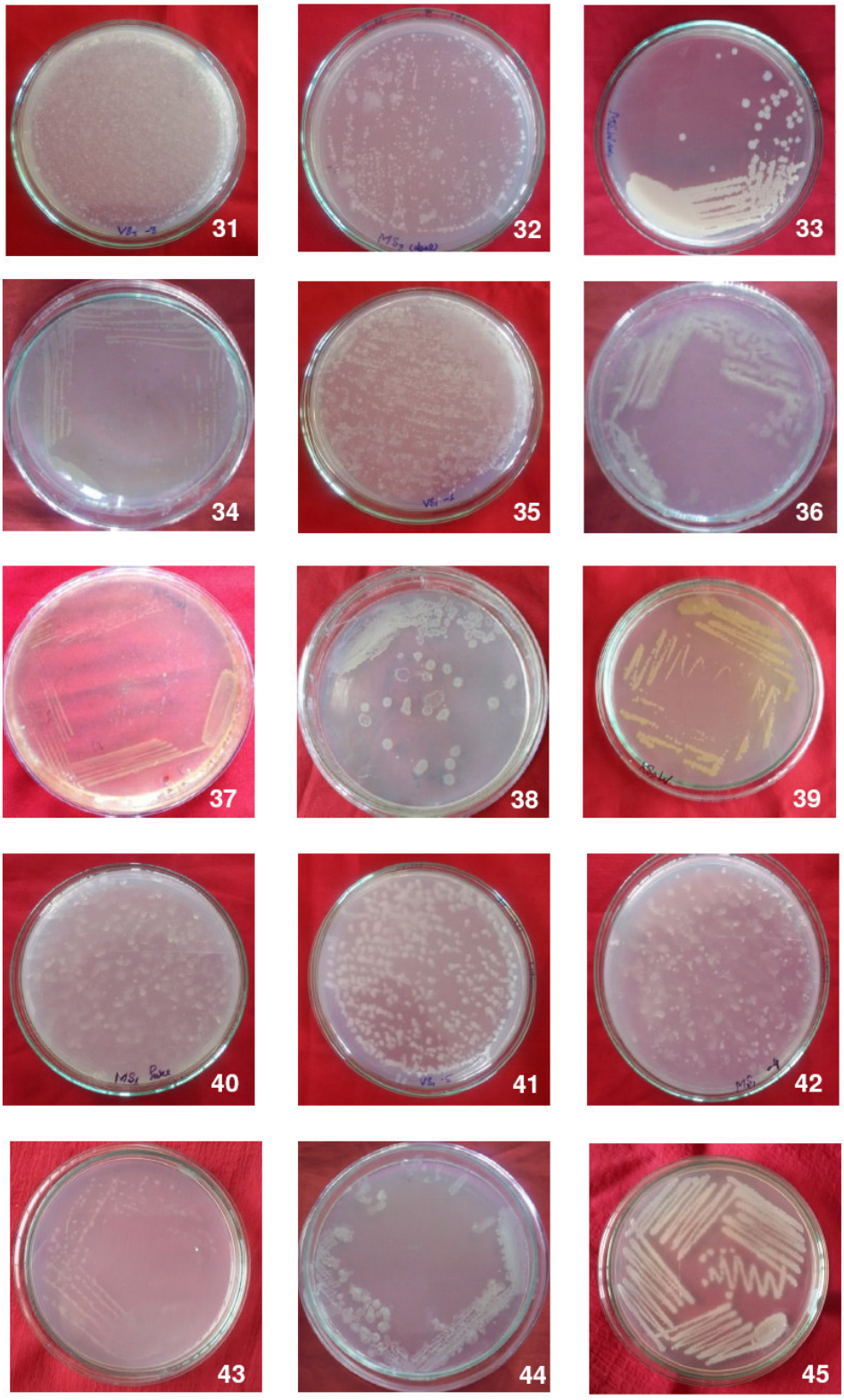


Plate 3 (c): Different colony morphotypes obtained from samples of hot springs (H.P.)

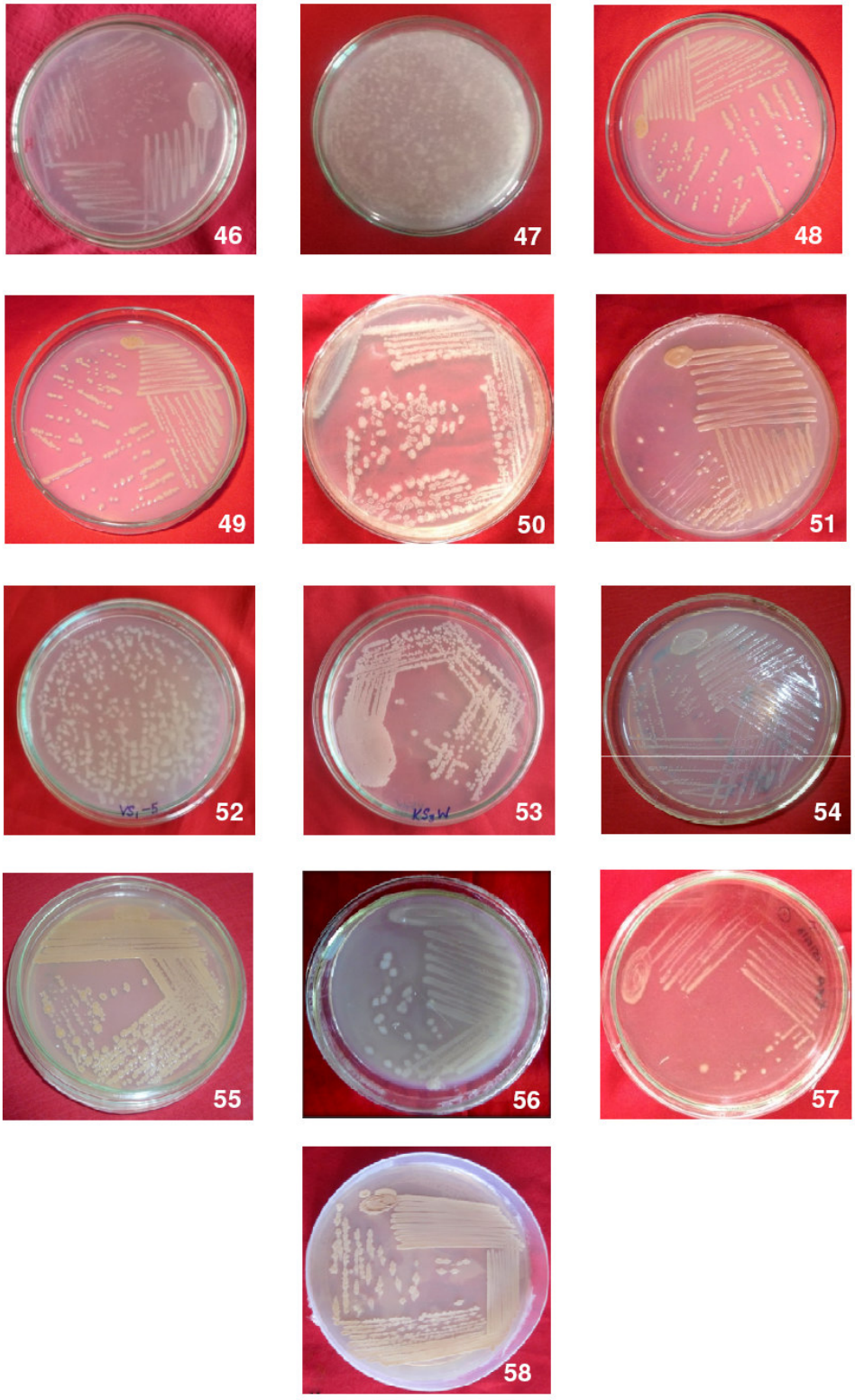


Plate 3 (d): **Different colony morphotypes obtained from samples of hot springs (H.P.)**

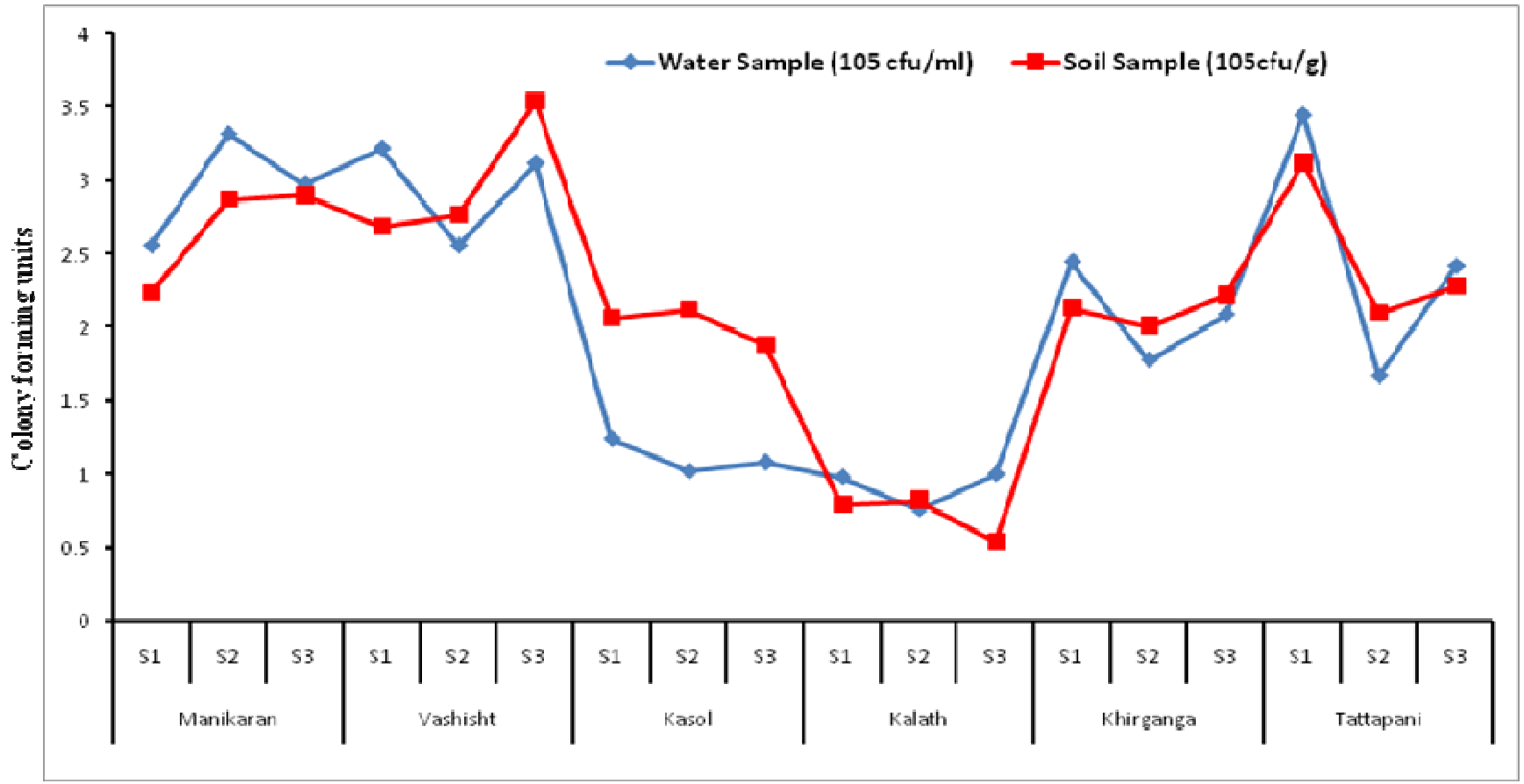


Fig 13: Variability in bacterial population among eighteen water samples of six hot water springs in H.P.

Table 20: Enumeration of thermophilic bacteria from six hot water springs of H.P.

	Bacterial population on nutrient agar after 48 hours of incubation at 60ºC																	
	Manikaran			**Vashisht**			**Kasol**			**Kalath**			**Khirganga**			**Tattapani**		
	S1	**S2**	**S3**	**S1**	**S2**	**S3**	**S1**	**S2**	**S3**	**S1**	**S2**	**S3**	**S1**	**S2**	**S3**	**S1**	**S2**	**S3**
Water Sample (10^5 cfu/ml)	2.56	3.32	2.98	3.22	2.56	3.12	1.24	1.02	1.08	0.98	0.76	1.00	2.45	1.78	2.09	**3.45**	1.67	2.42
Soil Sample (10^5cfu/g)	2.24	2.87	2.90	2.69	2.77	**3.54**	2.06	2.12	1.88	0.79	0.82	0.54	2.13	2.01	2.22	3.12	2.10	2.28

S1, S3 and **S3** are the subsites of six hot water springs of H.P.

Table 21: **Colony characterization of 58 isolates**

Sr.No.	Isolate	Colour	Size	Texture	Elevation	Margin
1.	MS1W1	W	P	R	F	I
2.	MS1W2	T	M	S	R	E
3.	MS1W3	T	L	S	R	I
4.	MS1S1	W	P	S	F	E
5.	MS1S2	W	M	R	R	I
6.	MS1P	W	P	S	F	E
7.	MS1R	W	M	S	F	E
8.	MS2W1	W	P	R	F	I
9.	MS2W2	T	M	S	R	E
10.	MS2W3	T	L	S	R	I
11.	MS2S1	W	P	R	F	I
12.	MS2S2	W	S	R	F	E
13.	MS2P	W	S	R	F	E
14.	MS3W1	W	P	R	F	I
15.	MS3W2	T	M	S	R	E
16.	MS3W3	T	L	S	R	I
17.	MS3W4	C	S	S	S	E
18.	MS3S	W	M	S	R	E
19.	VS1W1	W	P	S	F	E
20.	VS1W2	C	S	S	F	I
21.	VS1S	W	S	S	F	E
22.	VS1R	W	S	S	F	E
23.	KS1W	T	P	S	F	E
24.	KS1S	T	S	S	F	E
25.	KS1R	T	P	S	F	E
26.	KS2W	Y	S	S	R	E
27.	KS2S1	W	P	S	F	E
28.	KS2S2	W	S	S	F	I
29.	KS3W1	W	S	S	R	R
30.	KS3W2	W	L	S	R	R
31.	KS3S	T	S	S	R	R
32.	KS3R	W	P	S	R	E
33.	KaS1W1	W	S	S	F	E
34.	KaS1W2	T	P	S	F	E
35.	KaS1S	W	P	S	F	E
36.	KhS1W1	C	S	R	F	E
37.	KhS1W2	W	P	S	F	E
38.	KhS1S1	W	P	S	F	E

39.	KhS1S2	W	S	S	R	I
40.	KhS1R	W	P	S	F	E
41.	KhS2W	W	S	S	R	R
42.	KhS2S	W	L	S	R	I
43.	KhS2R	T	S	S	R	R
44.	KhS3W	W	M	R	F	I
45.	KhS3S	W	S	R	F	I
46.	KhS3R	W	S	R	F	I
47.	TS1W1	C	S	R	F	E
48.	TS1W2	W	M	S	R	I
49.	TS1S1	W	S	R	F	I
50.	TS1S2	C	S	R	F	E
51.	TS1R	W	S	R	F	I
52.	TS2W1	T	S	S	F	I
53.	TS2W2	W	L	S	F	E
54.	TS2S1	T	P	R	R	I
55.	TS2S2	T	M	S	R	E
56.	TS3W	T	M	S	R	E
57.	TS3S	W	P	R	F	E
58.	TS3R	W	P	R	F	E

S- Soil
W-Water
P- Pebbles
R- Rock matings

VS1- Sample from first subsite of Vashisht
VS2- Sample from second subsite of Vashisht
VS3- Sample from third subsite of Vashisht

KaS1- Sample from first subsite of Kalath
KaS2- Sample from second subsite of Kalath
KaS3- Sample from third subsite of Kalath

TS1- Sample from first subsite of Tattapani
TS2- Sample from second subsite of Tattapani
TS3- Sample from third subsite of Tattapani

MS1- Sample from first subsite of Manikaran
MS2- Sample from second subsite of Manikaran
MS3- Sample from third subsite of Manikaran

KS1- Sample from first subsite of Kasol
KS2- Sample from second subsite of Kasol
KS3- Sample from third subsite of Kasol

SIZE
Punctiform-**P**
Small-**S**
Medium-**M**
Large-**L**

ELEVATION
Flat-**F**
Raised-**R**
Sunken-**S**

COLOUR
White-**W**
Translucent-**T**
Crimish-**C**
Yellow-**Y**

TEXTUTE
Smooth-**S**
Rough-**R**

MARGIN
Irregular-**I**
Entire-**E**
Rhizoid-**R**

KhS1- Sample from first subsite of Khirganga
KhS2- Sample from second subsite of Khirganga
KhS3- Sample from third subsite of Khirganga

4.7.1.1 Colony characterization

Colony characteristics of all the fifty eight bacterial isolates showed significant variation for various morphological descriptors of colour, size, texture, margin and elevation of the colonies.

a) Colour

Colour of colonies exhibited by fifty eight bacterial isolates was found to vary from white, translucent and creamish to yellow. Thirty six colonies were found to be white, sixteen were translucent, five were creamish whereas only one colony possessed yellow colour (Table-21).

b) Size

Size of the colonies varied from pinpointed to large (Table-21).The maximum sized (large) colonies were produced by six bacterial isolates, medium sized colonies were ten while small sized colonies were produced by twenty three isolates whereas pinpointed colony size was possessed by nineteen bacterial isolate.

c) Texture

Colonies of fifty eight bacterial isolates exhibited smooth or rough texture (Table-21). Among these isolates forty colonies were smooth while eighteen were rough textured.

d) Margin

Colonies of thirty three bacterial isolates were found to possess entire margins, twenty isolates showed irregular margins and only five bacterial colonies *viz*., KS3W1, KS3W2, KS3S, KhS2W and KhS2R were found to possess rhizoid margins (Table 21).

e) Elevation

Colonies of thirty six isolates were flat, twenty one were found to have raised elevation whereas only one i.e. MS3W4 isolate was having sunken elevation (Table 21).

4.7.2 Microscopic characterization

The fifty eight bacterial isolates obtained in primary screening were further characterized for various microscopic characters (Table 22). Gram's reaction showed that forty six bacterial isolates were found to be blue in colour and thus gram positive in nature while twelve isolates were found to be gram negative. Fifty two isolates were found to be rod shaped and rest of six isolates were coccus in shape (Table 22). Arrangement of cells showed variation ranging from singles, clustered to chains, 39 were found to be singles and paired whereas 19 were found to possess singles as well as clusters like arrangement. Out of fifty eight isolates 14 isolates were found to be non-sporulating while rest of the isolates were able to form spores (Table 22).

On the basis of all the morphological observations of 58 bacterial isolates a dendrogram was constructed. The first major bifurcation divides the dendrogram into 2 clusters. Cluster A consists of 7 bacterial isolates and rest of the 51 were grouped in cluster B which is further divided into subclusters, sub-subclusters and so on forming 15 groups. The first cluster A formed after first major bifurcation excluded seven strains *viz*., MS2S2, MS2P, KhS1W1, TS1W1, TS1S2, TS3S and TS3R from rest of the strains (Fig-14).

4.7.3 Pellicle formation

All the 58 bacterial isolates were tested for their ability to form pellicle in the suspension culture and it was found that all the 58 bacterial isolates were positive for the morphological descriptor of pellicle formation (Plate-4).

4.8 SCREENING OF THERMOPHILIC BACTERIA PRODUCING THERMOSTABLE PULLULANASE ENZYME

Selective screening of thermostable pullulanase producing bacteria from fifty eight isolates was done on selective medium i.e. KWF medium of pH: 7.5. Seven bacterial isolates were isolated from water samples only, while no bacterial growth has been observed from soil, pebbles and rock matting samples on KWF (selective) medium. Turbid cultures were streaked on plates of solidified KWF agar medium pH: 7.5. Within 48 hrs of incubation at 60°C temperature spreading

Table 22: Microscopic characterization of fifty eight isolates

Sr. No.	Isolate	Grams Reaction	Shape	Arrangement	Spore formation
1.	MS1W1	+	Rods	Singles & Paired	+
2.	MS1W2	+	Rods	Singles & Paired	+
3.	MS1W3	+	Rods	Singles & Clusters	+
4.	MS1S1	-	Rods	Singles & Paired	+
5.	MS1S2	-	Rods	Singles & Paired	+
6.	MS1P	+	Rods	Singles & Clusters	-
7.	MS1R	+	Rods	Singles & Paired	+
8.	MS2W1	+	Rods	Singles & Paired	-
9.	MS2W2	+	Rods	Singles & Clusters	-
10.	MS2W3	+	Rods	Singles & Clusters	-
11.	MS2S1	-	Rods	Singles & Clusters	-
12.	MS2S2	-	Rods	Singles & Paired	+
13.	MS2P	+	Cocci	Singles & Clusters	+
14.	MS3W1	-	Rods	Singles & Clusters	+
15.	MS3W2	+	Rods	Singles & Clusters	+
16.	MS3W3	+	Rods	Singles & Clusters	+
17.	MS3W4	+	Rods	Singles & Clusters	+
18.	MS3S	+	Rods	Singles & Clusters	-
19.	VS1W1	+	Rods	Singles & Paired	+
20.	VS1W2	+	Rods	Singles & Paired	-
21.	VS1S	-	Rods	Singles & Clusters	-
22.	VS1R	-	Rods	Singles & Paired	+
23.	KS1W	+	Rods	Singles & Paired	+
24.	KS1S	-	Rods	Singles & Clusters	+
25.	KS1R	-	Rods	Singles & Clusters	+
26.	KS2W	+	Cocci	Singles & Clusters	+
27.	KS2S1	+	Rods	Singles & Paired	+
28.	KS2S2	+	Rods	Singles & Paired	+
29.	KS3W1	+	Rods	Singles & Paired	+
30.	KS3W2	+	Rods	Singles & Paired	+
31.	KS3S	+	Rods	Singles & Clusters	-
32.	KS3R	+	Rods	Singles & Paired	-
33.	KaS1W1	+	Rods	Singles & Paired	-
34.	KaS1W2	+	Rods	Singles & Paired	-
35.	KaS1S	+	Rods	Singles & Paired	-
36.	KhS1W1	+	Rods	Singles & Paired	+
37.	KhS1W2	+	Rods	Singles & Paired	+
38.	KhS1S1	+	Cocci	Singles & Clusters	+
39.	KhS1S2	+	Rods	Singles & Paired	+
40.	KhS1R	+	Rods	Singles & Paired	+
41.	KhS2W	+	Rods	Singles & Paired	+
42.	KhS2S	+	Rods	Singles & Paired	+
43.	KhS2R	+	Cocci	Singles & Clusters	+
44.	KhS3W	+	Rods	Singles & Paired	+
45.	KhS3S	+	Cocci	Singles & Paired	+
46.	KhS3R	+	Rods	Singles & Paired	+
47.	TS1W1	+	Rods	Singles & Paired	+
48.	TS1W2	+	Rods	Singles & Paired	-
49.	TS1S1	-	Rods	Singles & Paired	-
50.	TS1S2	+	Rods	Singles & Paired	-
51.	TS1R	+	Rods	Singles & Paired	+
52.	TS2W1	+	Rods	Singles & Paired	+
53.	TS2W2	-	Rods	Singles & Paired	+
54.	TS2S1	-	Rods	Singles & Paired	+
55.	TS2S2	+	Rods	Singles & Paired	-
56.	TS3W	+	Cocci	Singles & Clusters	+
57.	TS3S	+	Rods	Singles & Paired	+
58.	TS3R	+	Rods	Singles & Paired	+

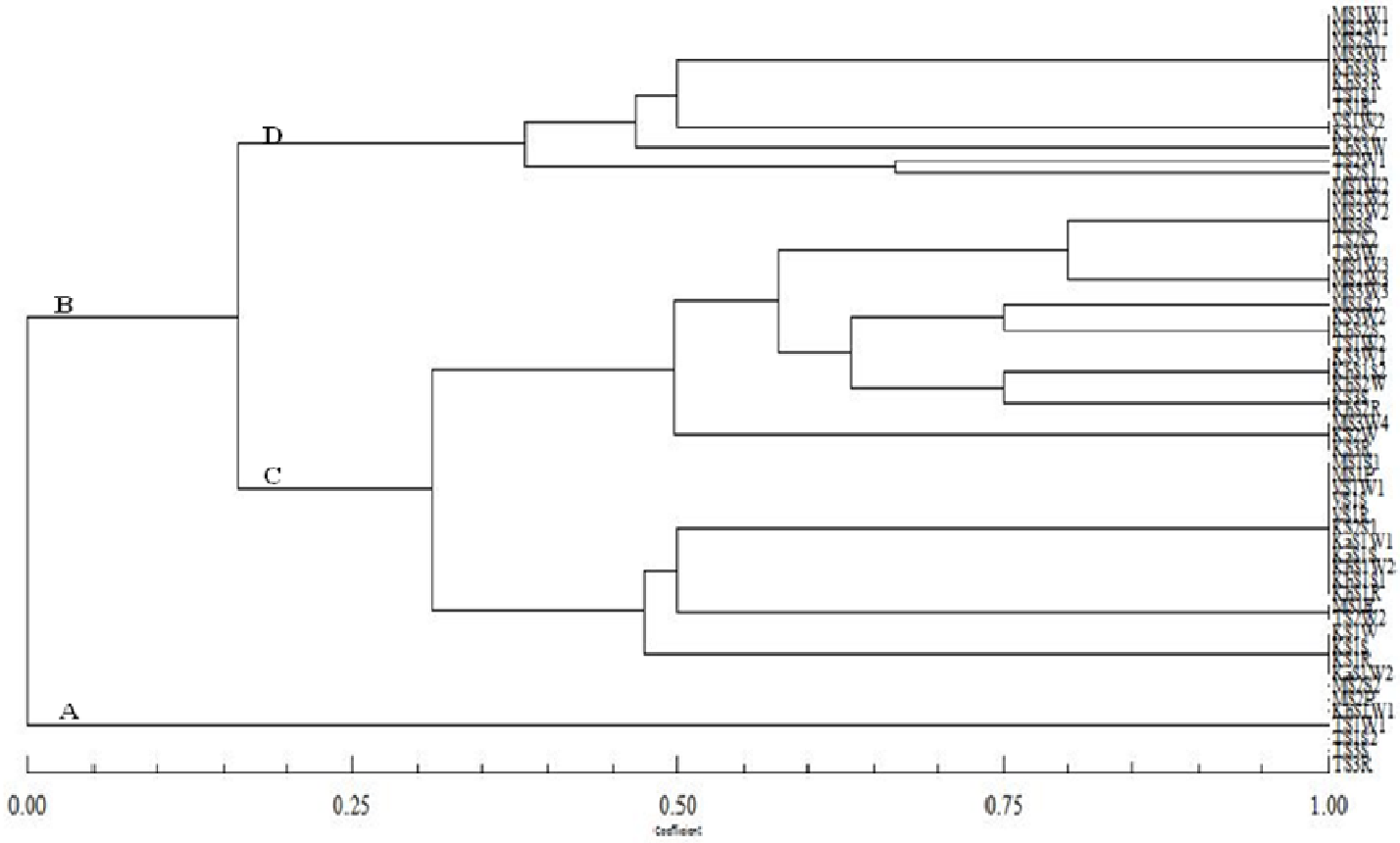


Fig 14: Dendrogram showing relatedness among 58 bacterial isolates based on morphological characters.

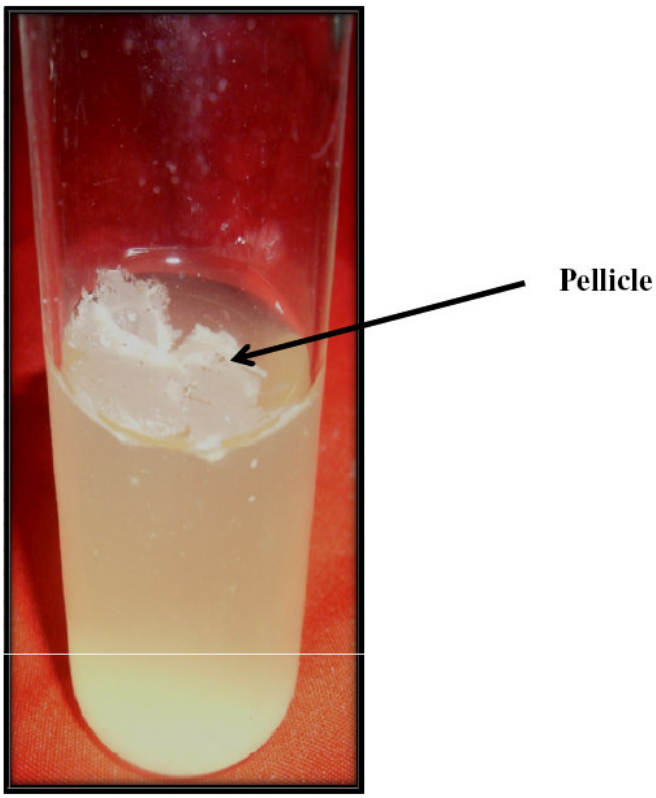


Plate 4: Pellicle formation in suspension culture

colonies were obtained. Individual colonies were restreaked repeatedly on the same KWF medium to get the axenic cultures of the selected seven isolates (Plates-5). These axenic cultures were stored at 4^{o}C in refrigerator till further processing.

4.9 BIOCHEMICAL CHARACTERIZATION OF SELECTED BACTERIAL ISOLATES

Out of a total of 58 bacterial isolates, seven were selected on their ability to produce colonies on KWF medium, for biochemical characterization. Thus bacterial isolates were examined for various biochemical tests *viz.*, catalase, oxidase, urease, indole, methyl red, Voges-Proskauer, citrate, triple sugar iron agar and fermentation of sugars i.e. glucose, sucrose, lactose and mannitol.

a) Catalase test

All the seven bacterial isolates *viz.,* MS1W1, KS3W1, KS2W, KS3W2, MS1W2, MS3W4 and MS3W2 tested positive for catalase (Table-23).

b) Oxidase test

This test depicted variation within the 7 bacterial isolates. Only one thermostable pullulanase producing bacterial isolate i.e. KS3W2 tested negative and rest of the isolates were tested positive for oxidase enzyme activity (Table-23).

c) Urea production

The selected seven bacterial isolates were studied for the urease production. Only one bacterial isolate *viz.*, KS2W was found negative for the urease production while rest of the bacterial isolates were found positive for the urease production (Table-23).

d) IMViC test

IMViC test also showed variation within the 7 tested thermostable pullulanase producing bacterial isolates. In case of indole production test, all isolates were tested negative for indole production except MS3W2. Methyl red

test was found positive for 2 isolates *viz*., KS3W2 and MS3W2 whereas five isolates were tested negative and for VP test six bacterial isolates were found to be positive and one isolates *viz*., KS3W2 was found to be negative (Table-23).

e) Citrate test

All the seven thermophilic bacterial isolates were found positive for citrate test (Table-23).

f) Fermentation of sugars (glucose, lactose, sucrose and mannitol)

Bacterial isolates showed significant variation to ferment different sugars *viz*., glucose, lactose, sucrose and mannitol. It was depicted that all the seven bacterial isolates were able to ferment glucose. Sucrose sugar was fermented by only 5 isolates whereas rest of two isolates *viz*., KS2W and KS3W2 were found unable to ferment sucrose. Lactose and mannitol was fermented by 4 bacterial isolates whereas 3 bacterial isolates were found unable to ferment lactose and mannitol (Table-23).

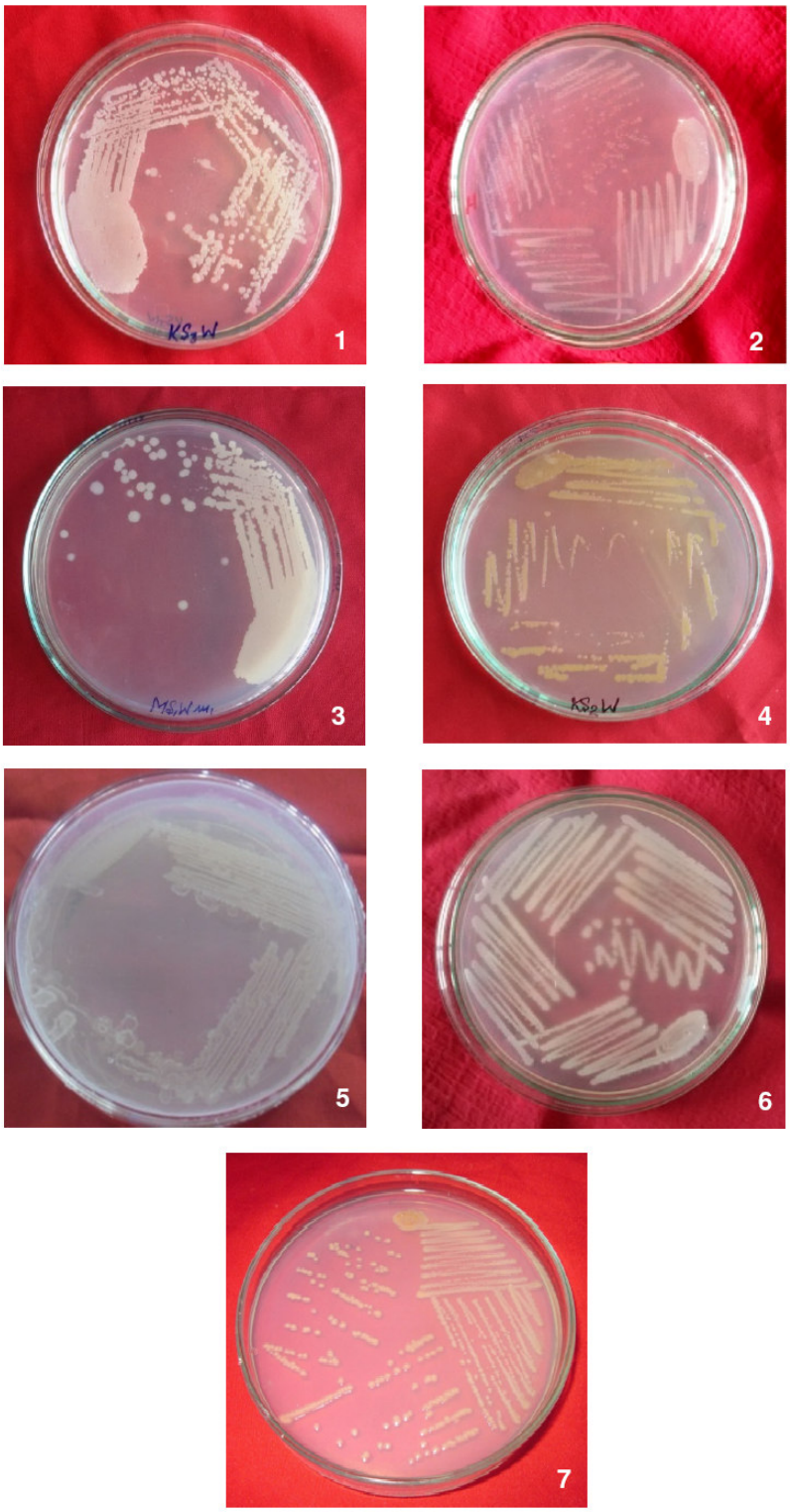
g) Triple sugar iron agar test

In triple sugar iron agar test only one bacterial isolate *viz*., KS2W was tested negative and rest of the six bacterial isolates showed positive results.

Results of all the 7 bacterial isolates were analysed by the SAHN module of NTSYS-Pc version 2.20 and dendrogram was constructed using UPGMA. The dendrogram constructed based on results of biochemical characters showed that all the bacterial isolates possess 65% similarity. The cluster B formed after first major bifurcation clearly separates out the KS3W2 bacterial isolate, isolated from Kasol from rest of the six bacterial strains. The cluster A is further divided into subclusters C and D which were further divided into sub subclusters (Fig-15).

4.10 QUANTITATIVE SCREENING FOR THERMOSTABLE PULLULANASE ACTIVITY

Extracellular thermostable pullulanase activity of the crude enzyme preparations of selected seven bacterial isolates was screened quantitatively by



Plates 5: Plates of selected pullulanase producing bacterial isolates on KWF medium

Table 23: Biochemical characterization of selected thermophilic pullulanase producing bacterial isolated

Sr. No.	Isolate	Catalase	Oxidase	Urea	IMViC test				Fermentation of sugars				TSI
					Indole	MR	VP	Citrate	Glucose	Sucrose	Lactose	Manitol	
1	MS1W1	+	+	+	-	-	+	+	+	+	+	+	+
2	KS3W1	+	+	+	-	-	+	+	+	+	-	-	+
3	KS2W	+	+	-	-	-	+	+	+	-	+	+	-
4	KS3W2	+	-	+	-	+	-	+	+	-	-	-	+
5	MS1W2	+	+	+	-	-	+	+	+	+	-	-	+
6	MS3W4	+	+	+	-	-	+	+	+	+	+	+	+
7	MS3W2	+	+	+	+	+	+	+	+	+	+	+	+

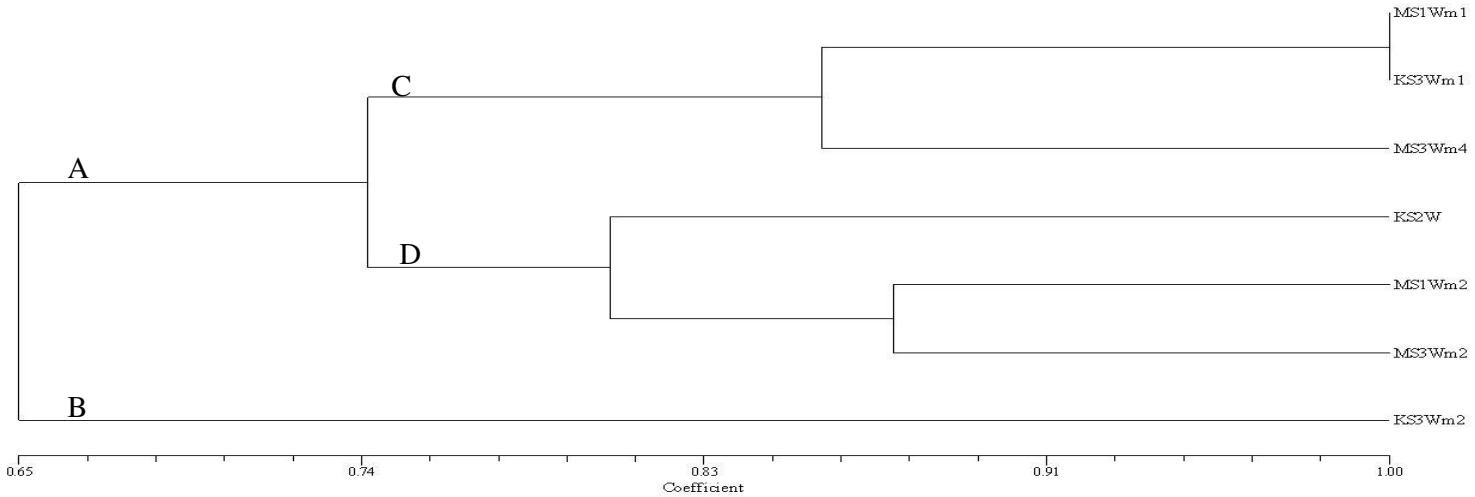


Fig 15: Dendrogram showing relatedness among 7 bacterial isolates based on biochemical characters

pullulanase enzyme activity assay. Thermostable pullulanase activity was determined after 24 and 48 hrs of incubation for all selected seven bacterial isolates and all the bacterial isolates were found to produce thermostable pullulanase extracellularly.

Table 24: Thermostable pullulanase activity of selected 7 bacterial isolates

Sr. No.	Isolate	Enzyme activity after 24 hrs(U/ml)	Enzyme activity after 48 hrs (U/ml)
1.	**MS1W1**	0.93	1.73
2.	**KS3W1**	0.69	1.67
3.	**KS2W**	**1.43**	**3.96**
4.	**KS3W2**	1.25	2.48
5.	**MS1W2**	0.43	2.10
6.	**MS3W4**	0.21	1.27
7.	**MS3W2**	0.68	2.12

Bacterial isolate KS2W showed maximum extracellular thermostable pullulanase activity of 1.43 U/ml after 24 hrs of incubation and 3.96 U/ml after 48 hrs of incubation followed by KS3W2, MS1W1, KS3W1, MS3W2, MS1W2, MS3W4 and MS3W2 depicting thermostable pullulanase activity of 1.25, 0.93, 0.69, 0.68, 0.43, 0.21 and 0.68 U/ml respectively after 24 hrs of incubation and 2.48, 1.73, 1.67, 2.12, 2.10, 1.72 and 2.12 U/ml respectively after 48 hrs of incubation (Table-24). KS2W strain which was found to produce maximum extracellular thermostable pullulanase activity was selected for further molecular characterization and enzyme investigation.

4.11 MOLECULAR CHARACTERIZATION OF SELECTED KS2W BACTERIAL ISOLATE

The selected thermostable pullulanase producing bacterial isolate KS2W was subjected to molecular characterization using 16S *rrna* gene technology.

4.11.1 Extraction of genomic DNA

Total genomic DNA of the selected bacterial isolate was extracted successfully using Genomic DNA extraction Mini kit (Real Genomics) and presence of DNA and its quality was checked using 1.0% agarose gel. Sharp

DNA band obtained in the lane clearly revealed absence of contamination of proteins and RNA (Plate-6).

4.11.2 16S *rrna* gene characterization of selected thermophilic bacterial isolate KS2W

The DNA sample extracted from thermophilic bacterial isolate KS2W was selectively amplified using PCR technology/16S rDNA technology. Universal primers for 16S *rrna* gene was used for the amplification. After 30 cycles of PCR amplification, (as described under section 3.7.2), universal primers for 16S *rrna* gene were able to amplify 16S *rrna* gene of thermophilic bacterial isolate KS2W and produced an amplicon of expected size i.e. 1500 bp (Plate-7). The emplified product was eluted and purified DNA of selected bacterial isolate KS2W was sequenced.

4.11.3 Sequencing

To determine the nucleotide sequence of 16S *rrna* gene of the selected isolate KS2W, sequencing was carried out using same upstream and downstream universal primers which were used for amplification and as a result, the size of the 16S *rrna* gene sequence of the selected bacterial isolate KS2W was found to be approximately 556 base pairs. On the basis of results obtained from 16S *rrna* gene analysis in addition to G+C content analysis (Table-25), the selected bacterial isolate KS2W producing thermostable pullulanase was found to belong to genus *Bacillus.* The nucleotide sequence of the selected thermostable pullulanase producing bacterial isolate KS2W, obtained after sequencing of 16S *rrna* gene has been depicted here as under:

16S *rrna* partial sequence of KS2W isolate

Total Bases: 556

The sequence obtained from KS2W isolate is as under:

AGGACCCCCTTTCGGCGGCTGGCTCAAAAAGGTTACCTCACCGACTTC
GGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCC
GGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCC
AGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGA
TTTGTGGGATTGGCTTAGCCTCGCGGCTTCGCTGCCCTTTGTTCTGCCC
ATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGAC

GTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTG
CCCAACTGAATGCTGGCAACTAAAATCAAGGGTTGCGCTCGTTGAGG
GACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGAACC
ACCTGTCACTCTGCCCCCGAAGGGGAAGCACTATCTCAAAGGATGTC
ATATGATTGGGGACCTGGTAAGGCTCTTCGCGATGCTTTCAACCATAC
ACTTGACCTATCGCTTGTATCCTCCCCCAACA

Table 25: Nucleotide base composition in the query sequence (KS2W isolate)

Nitrogenous Base	Nucleotide Count	
	Total	**Percentage (%)**
Adenine (A)	123	22.12
Thymine (T)	132	23.74
Cytosine (C)	161	28.95
Guanine (G)	140	25.17
G+C	255	45.86
A+T	301	54.13

4.11.4 *In silico* analysis of the sequences

Further *in silico* analysis pertaining to the sequence, so obtained, was carried out using various bioinformatic tools available online. Analysis of 16S *rrna* gene of the selected thermostable pullulanase producing bacterial isolate KS2W revealed its homology with various other 16S *rrna* gene sequences. Various online approaches were followed to assess and predict relationship of the selected isolate KS2W with other 16S *rrna* gene sequences.

4.11.4.1 BLASTn

BLASTn search of this nucleotide sequence with the most similar 16S *rrna* gene sequences of the GenBank database (http:// www. ncbi.nlm.nih. gov/blast) revealed the closest sequence identities from the sequence database. All the similarities have been summarized in Table-26. In BLASTn analysis, nucleotide test sequence of strain KS2W showed 96% homology with *Bacillus licheniformis* strain BCRC 11702, 16S ribosomal RNA, partial sequence (NR_116023.1) and *Bacillus licheniformis* strain NBRC 12200 16S ribosomal RNA gene, partial sequence (NR_113588.1) and 95% homology with *Bacillus aerius* strain 24K 16S ribosomal RNA gene, partial sequence (NR_042338) whereas 94% homology was observed with *Bacillus sonorensis* strain NBRC

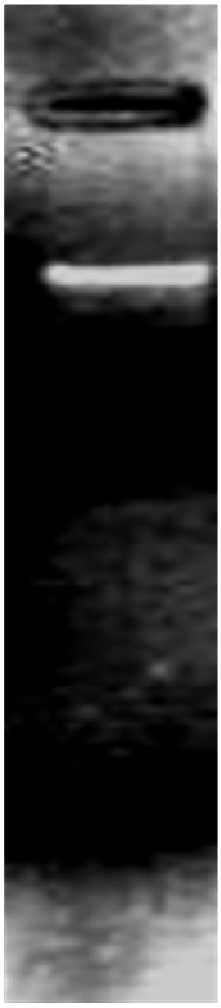


Plate 6: Gel image showing genomic DNA extracted from selected thermostable pullulanase producing bacterial isolate (KS2W).

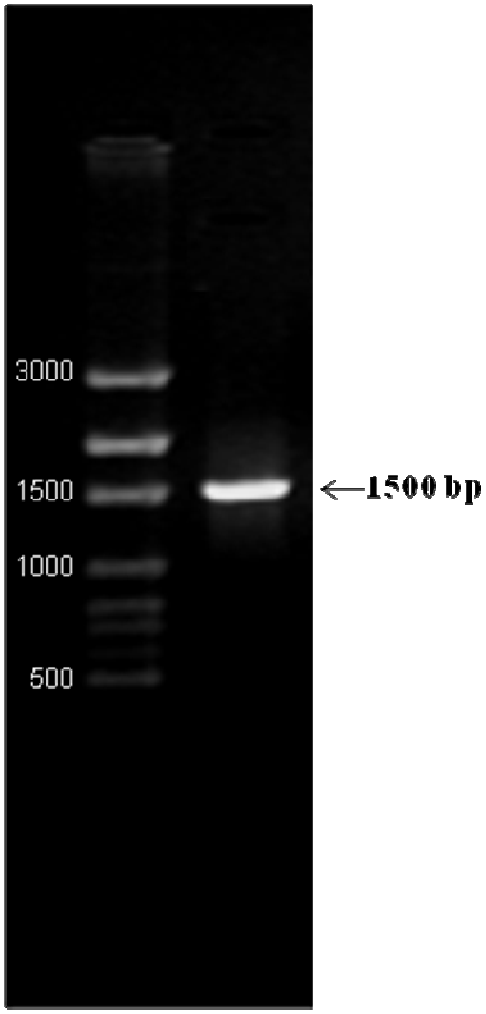


Plate 7: Amplicon of 16S *rrna* gene of selected thermostable pullulanase producing bacterial isolate KS2W

101234 16S ribosomal RNA gene, partial sequence (NR_113993.1), *Bacillus sonorensis* strain NRRL B-23154 16S ribosomal RNA gene, partial sequence (NR_025130.1) and many more present in the database. Sequence analysis revealed that KS2W is identical to *Bacillus licheniformis* as it showed maximum homology (96%) with *Bacillus licheniformis* strain BCRC 11702 (Fig 16). Thus the bacterial isolate KS2W was submitted to NCBI as *Bacillus licheniformis* strain ASM3.

Table 26: Percent homology of nucleotide query sequence of KS2W thermostable pullulanase producing bacterial isolate from hot water springs of H.P. with other nucleotide sequences present in the database using BLASTn analysis

Accession No.	Description	Percent Homology
NR_116023.1	*Bacillus licheniformis* strain BCRC 11702	96%
NR_113588.1	*Bacillus licheniformis* strain NBRC 12200	96%
NR_042338.1	*Bacillus aerius* strain 24K	95%
NR_113993.1	*Bacillus sonorensis* strain NBRC 101234	94%
NR_025130.1	*Bacillus sonorensis* strain NRRL B-23154	94%
NR_118290.1	*Bacillus mojavensis* strain ifo 15718	92%
NR_113994.1	*Bacillus vallismortis* strain NBRC 101236	91%
NR_104873.1	*Bacillus subtilis* subsp. *inaquosorum* strain BGSC 3A28	91%
NR_117946.1	*Bacillus amyloliquefaciens* strain MPA 1034	91%
NR_112725.1	*Bacillus mojavensis* strain NBRC 15718	91%
NR_112723.1	*Bacillus atrophaeus* strain NBRC 15539	91%
NR_112686.1	*Bacillus subtilis* subsp. *spizizenii* strain NBRC 101239	91%
NR_112685.1	*Bacillus amyloliquefaciens* strain NBRC 15535	91%
NR_116022.1	*Bacillus amyloliquefaciens* strain BCRC 11601	91%
NR_115930.1	*Bacillus malacitensis* strain CECT 5687	91%

4.11.4.2 Multiple sequence alignment

Multiple sequence alignment of test nucleotide sequences of *Bacillus licheniformis* strain ASM3 with that of the selected nucleotide sequences was performed using Clustal W program (Higgins *et al*., 1992) available online at European Bioinformatics Institute (EBI) (http:// www. ebi. ac. uk/) and the Clustal W output was then used in (MEGA 5.0 software) bioinformatic tool for constructing phylogenetic tree.

Pairwise percentage similarity score of 15 nucleotide sequences with test isolate KS2W from Kasol, Himachal Pradesh (India) were arranged in tabular form. The pairwise similarity score of 15 nucleotide sequences with test sequence KS2W elucidates that sequence-1 (*Bacillus licheniformis* strain ASM3) showed maximum similarity score of 74.4% (Table-27).

Table 27: Pairwise percentage similarity score table of *Bacillus licheniformis* ASM3

Seq A	Name	Length (bp)	Seq B	Name	Length (bp)	Score
1	*Bacillus*	556	2	gi|636559963|ref|NR_116023.1|	1468 bp	74.4
1	*Bacillus*	556	3	gi|126653866|gb|EF433411.1|	1468 bp	73.6
1	*Bacillus*	556	4	gi|115333962|gb|DQ993679.1|	1549 bp	73.6
1	*Bacillus*	556	5	gi|256265123|gb|GQ375247.1|	1448 bp	73.0
1	*Bacillus*	556	6	gi|291088018|emb|FN652005.1|	845 bp	72.8
1	*Bacillus*	556	7	gi|636559957|ref|NR_116017.1|	1468 bp	72.0
1	*Bacillus*	556	8	gi|126567887|gb|EF423603.1|	1538 bp	72.0
1	*Bacillus*	556	9	gi|291088017|emb|FN652004.1|	929 bp	70.4
1	*Bacillus*	556	10	gi|636559466|ref|NR_115526.1|	1486 bp	68.0
1	*Bacillus*	556	11	gi|343202909|ref|NR_043403.1|	1486 bp	67.2
1	*Bacillus*	556	12	gi|295027210|emb|FN667913.1|	1355 bp	67.2
1	*Bacillus*	556	13	gi|7321091|emb|AJ288157.1|	1340 bp	67.2
1	*Bacillus*	556	14	gi|343202907|ref|NR_043401.1|	1486 bp	61.6
1	*Bacillus*	556	15	gi|457650|dbj|D16273.1|	1486 bp	61.6

4.11.4.3 Phylogenetic analysis

To gain insight of the evolutionary pattern, phylogenetic tree was constructed using MEGA 5.0. Neighbour-Joining (NJ) technique of mathematical averages (UPGMA) was used. The results have been presented in the form of phylogenetic tree (Fig-17).

Phylogenetic tree analysis

To trace out the evolutionary pattern of the selected KS2W bacterial isolate, and assess its relationship with other selected sequences at NCBI, phylogenetic tree was constructed using Neighbour-Joining (NJ) method of mathematical averages (UPGMA) among 16S *rrna* gene sequence and corresponding gene sequences. The bacterial isolates selected were united with quite high statistical support by the bootstrap method estimates for 100

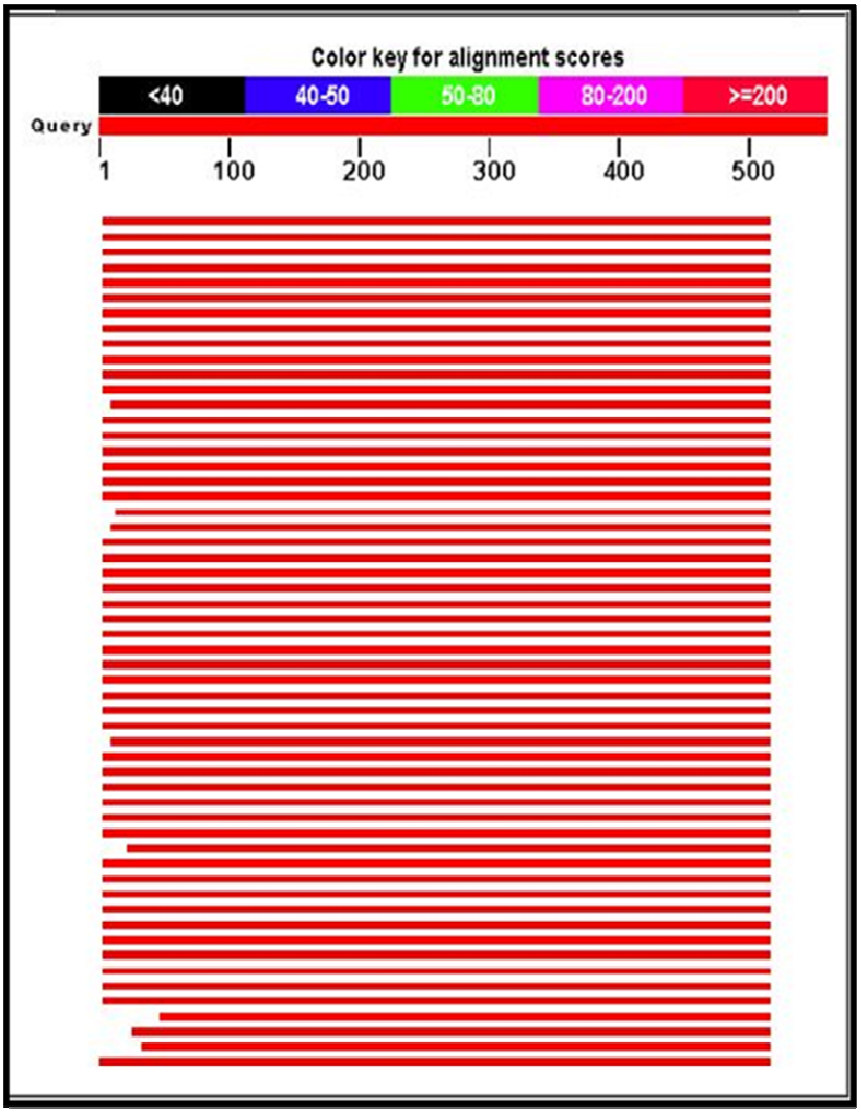


Fig 16: Colour key for alignment score of nucleotide query sequence of KS2W isolate with other nucleotide sequence present in the database using BLAST analysis (Red lines show the similarity of the query sequence is more than 200).

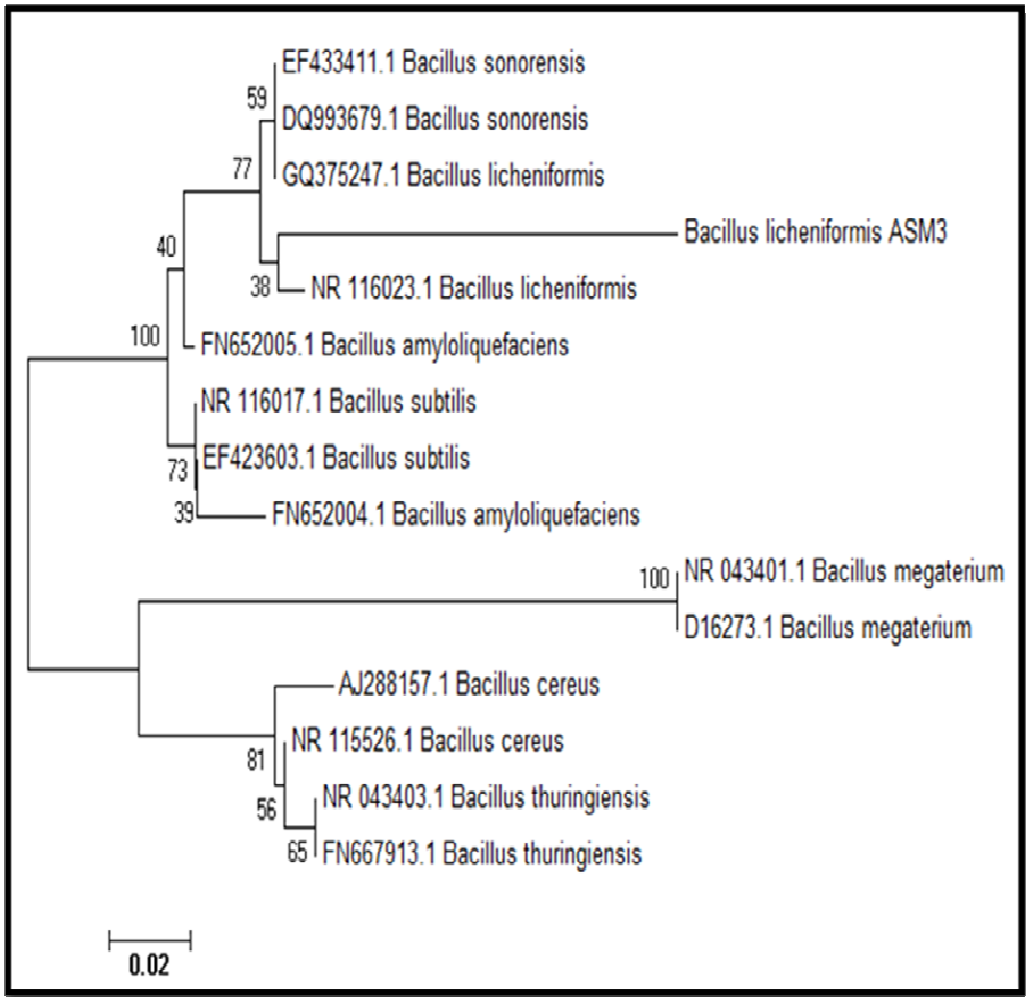


Fig 1: Phylogenetic tree of genus Bacillus 16S rDNA. The GenBank accession numbers of the analyzed sequences are shown. A total of 100 bootstrap replicates were performed, and the bootstrap values are indicated at the branching points.

replications and values inferred greater than 38 percent, only were presented (Fig-17).

By Neighbour-Joining algorithm, the phylogenetic tree (Fig-17) has been divided and subdivided into clusters and sub clusters with bootstrap values ranging from 38 to 100. These clusters were subclustered further and verified the bacterial isolate KS2W as *Bacillus licheniformis*, as the isolate KS2W clustered closely with *Bacillus licheniformis* strain BCRC 11702 (NR_116023.1) with boot strap value of 38.

4.12 OPTIMIZATION OF CULTURE CONDITIONS FOR MAXIMUM GROWTH AND MAXIMUM ENZYME PRODUCTION

The maximum thermostable pullulanase producing isolate KS2W was identified as *Bacillus licheniformis* in the previous step was selected for further enzyme production. Optimization of various culture conditions such as different media, incubation time, pH of the medium and incubation temperature were carried out for maximum thermostable pullulanase enzyme activity as well as for best growth of the selected *Bacillus licheniformis* strain ASM3.

4.12.1 Effect of different media on growth and thermostable pullulanase enzyme activity

Effect of different media (Table-28) was studied on growth and thermostable pullulanase enzyme activity. Growth of *Bacillus licheniformis* strain ASM3 was observed in different liquid media. The selected bacteria showed a maximum growth OD of 4.26 at a wavelength of 540 nm in KWF broth where as growth in other two media *viz*., Pullulan agar broth and basal medium was observed to be negligible (Table-28, Fig-18).

Table 28: Effect of different media on growth and thermostable pullulanase enzyme activity of selected *Bacillus licheniformis* strain ASM3

Sr. No.	Media	Growth (OD at 540nm)	Enzyme activity (U/ml)
1.	**Pullulan broth**	0.81	0.52
2.	**KWF broth**	**4.26**	**2.76**
3.	**Basal medium**	0.57	0.36

In case of enzyme activity, maximum enzyme activity of 2.76 U/ml was observed using KWF broth, whereas much less enzyme activity was observed using pullulan broth and Basal medium (Table-28, Fig- 18). Thus KWF medium was selected for further experiments.

4.12.2 Effect of time of incubation on the growth and thermostable pullulanase enzyme activity

Growth of *Bacillus licheniformis* strain ASM3 was monitored up to 120 hrs using KWF broth. It has been observed that bacterial growth increased exponentially depicting an OD of 2.110 at a wavelength of 540 nm after first 24 hrs and then it enhanced to 3.380 after 48 hrs, thereafter it declined up to 120 hrs (Fig-19). The isolate exhibited maximum growth OD of 3.380 at wavelength of 540 nm after 48 hrs, thus 48 hrs was selected as optimum incubation time for best growth of *Bacillus licheniformis* ASM3.

Table 29: **Effect of time of incubation (hrs) on the growth and thermostable pullulanase enzyme activity of selected *Bacillus licheniformis* strain ASM3**

Sr. No.	Time (hrs)	Growth (OD at 540nm)	Activity (U/ml)
1.	0	0.001	0
2.	24	2.110	0.707
3.	**48**	**3.380**	**2.187**
4.	72	1.820	0.938
5.	96	0.430	0.071
6.	120	0.019	0.007

Effect of incubation time on enzyme activity of thermostable pullulanase enzyme produced by *Bacillus licheniformis* strain ASM3 was also determined. It was found that maximum enzyme activity of 2.187 U/ml was produced after 48 hrs of incubation followed by a sharp decline up to 120 hrs, thus for production of extracellular thermostable pullulanase, 48 hrs incubation time has been found to be optimum (Table-29, Fig-19). On the basis of these results incubation time of 48 hrs was selected for further experiments.

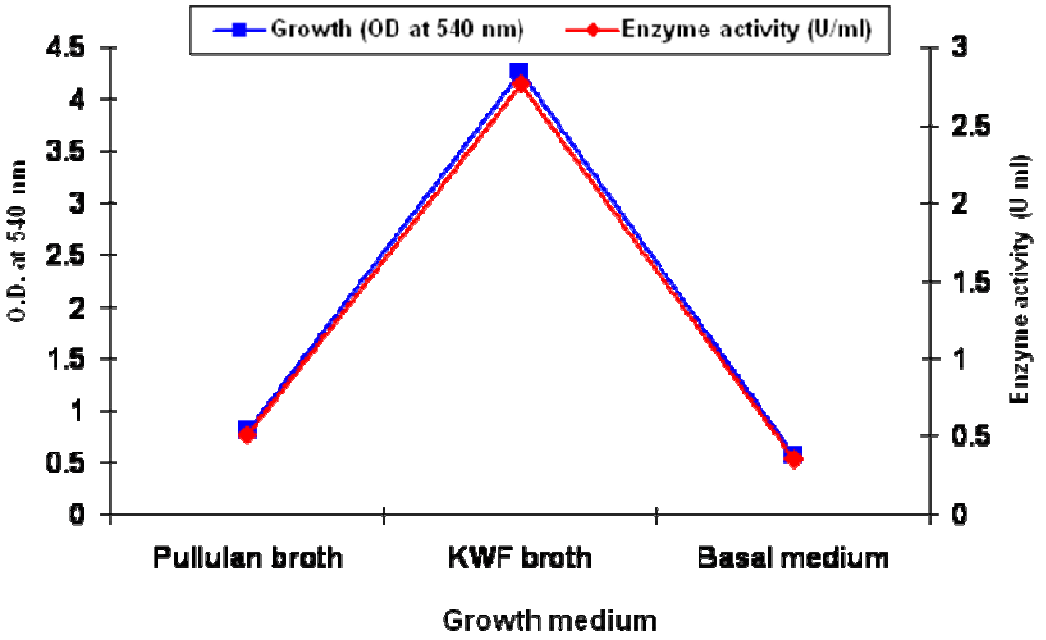


Fig 18: Effect of different media on growth and thermostable pullulanase enzyme activity of selected *Bacillus licheniformis* strain ASM3

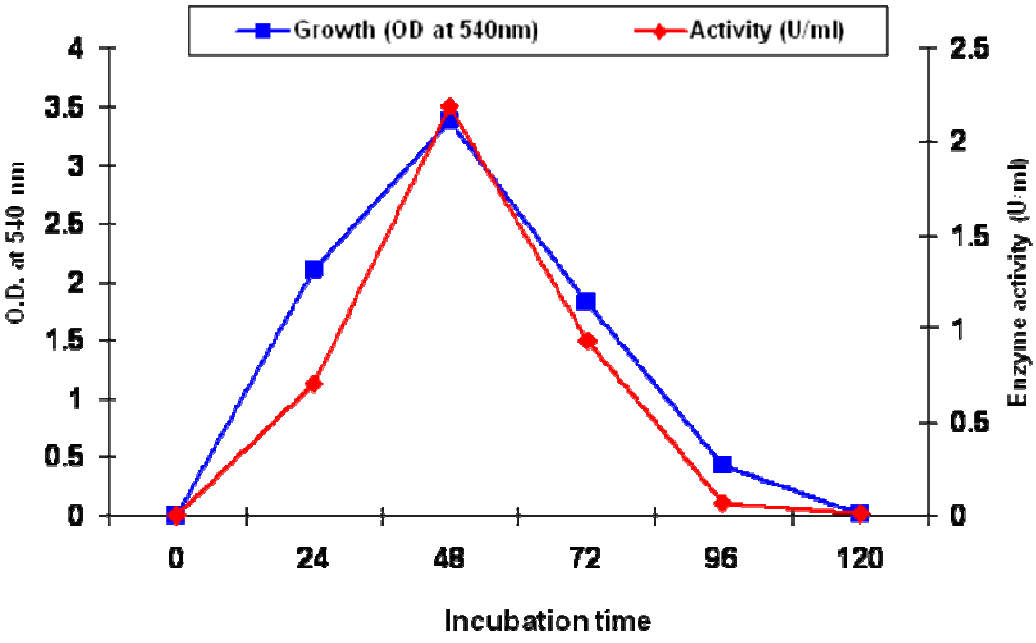


Fig 19: Effect of incubation time on growth and thermostable pullulanase enzyme activity of selected *Bacillus licheniformis* strain ASM3

4.12.3 Effect of pH on growth and thermostable pullulanase enzyme activity

Effect of pH in the range of 4.0-12.0 of the KWF broth was examined and it was found that growth of the bacterial isolate increased with increase in pH of the medium up to pH 9.0 and thereafter it declined (Fig-20). Maximum growth OD of 3.341 was observed at 540 nm wavelength. Thus pH 9.0 was selected as optimum pH for best growth of *Bacillus licheniformis* strain ASM3.

Table 30: Effect of pH on growth and thermostable pullulanase enzyme activity of selected *Bacillus licheniformis* strain ASM3

Sr. No.	pH	Growth (OD at 540nm)	Activity (U/ml)
1.	4.0	0.143	0.027
2.	5.0	0.702	0.260
3.	6.0	1.122	0.531
4.	7.0	1.842	0.868
5.	8.0	2.296	1.22
6.	9.0	**3.341**	**2.161**
7.	10.0	1.527	1.117
8.	11.0	0.713	0.396
9.	12.0	0.193	0.124

The enzyme activity was also found to increase gradually from pH 4.0-9.0 thereafter it showed gradual decrease till pH 12.0. Maximum enzyme activity of 2.161 U/ml was observed at a alkaline pH of 9.0. Thus optimization studies indicated that pH 9.0 was optimum pH for the growth as well as for maximum thermostable pullulanase activity by *Bacillus licheniformis* strain ASM3 (Table-30, Fig-20). On the basis of these results 9.0 pH of the medium was selected for further experiments.

4.12.4 Effect of incubation temperature on growth and thermostable pullulanase enzyme activity

A study on growth and thermostable pullulanase activity was carried out at different temperature using KWF broth at a pH 9.0. Growth of *Bacillus licheniformis* strain ASM3 was observed at temperature range of 40-90^{o}C for 48 hrs and it was found to increase with increase in temperature up to 60^{o}C and then

a steady decrease was observed till 90°C. *Bacillus licheniformis* ASM3 showed a maximum growth OD of 3.131 at an incubation temperature of 60°C and thus it was selected as optimum incubation temperature for optimum growth of KS2W strain (Fig-21).

Table 31: **Effect of incubation temperature on growth and thermostable pullulanase enzyme activity of selected *Bacillus licheniformis* strain ASM3**

Sr. No.	Incubation temperature (^{0}C)	Growth (OD at 540nm)	Activity (U/ml)
1.	40	0.875	0.566
2.	50	1.529	1.118
3.	**60**	**3.131**	**1.767**
4.	70	2.589	1.015
5.	80	1.984	0.636
6.	90	0.583	0.377

The enzyme activity of thermostable pullulanase enzyme produced by *Bacillus licheniformis* ASM3 has been studied in the temperature range 40-90°C. There was very low enzyme activity detected at 40°C however it was observed to increase with increasing temperature from 50°-60°C showing maximum enzyme activity of 1.767 U/ml at 60°C temperature and enzyme activity gradually decreased at 90°C. Thus the optimum incubation temperature selected for maximum growth and thermostable pullulanase activity was 60°C (Table-31, Fig-21). On the basis of these results incubation temperature of 60°C was selected for further experiments.

4.13 PURIFICATION OF THERMOSTABLE PULLULANASE ENZYME

4.13.1 Preparation of bacterial culture of *Bacillus licheniformis* ASM3

The selected thermophilic bacterial isolate, *Bacillus licheniformis* ASM3 was cultured in KWF broth, at a pH of 9.0 for 48 hrs for the production of extracellular pullulanase enzyme. The bacterial culture was centrifuged at 20,000 rpm for 20 mins at 4°C. The cell free supernatant was used as crude enzyme.

Crude extracellular enzyme extract was further purified using different purification techniques such as ammonium sulphate precipitation, gel filtration and ion exchange chromatography.

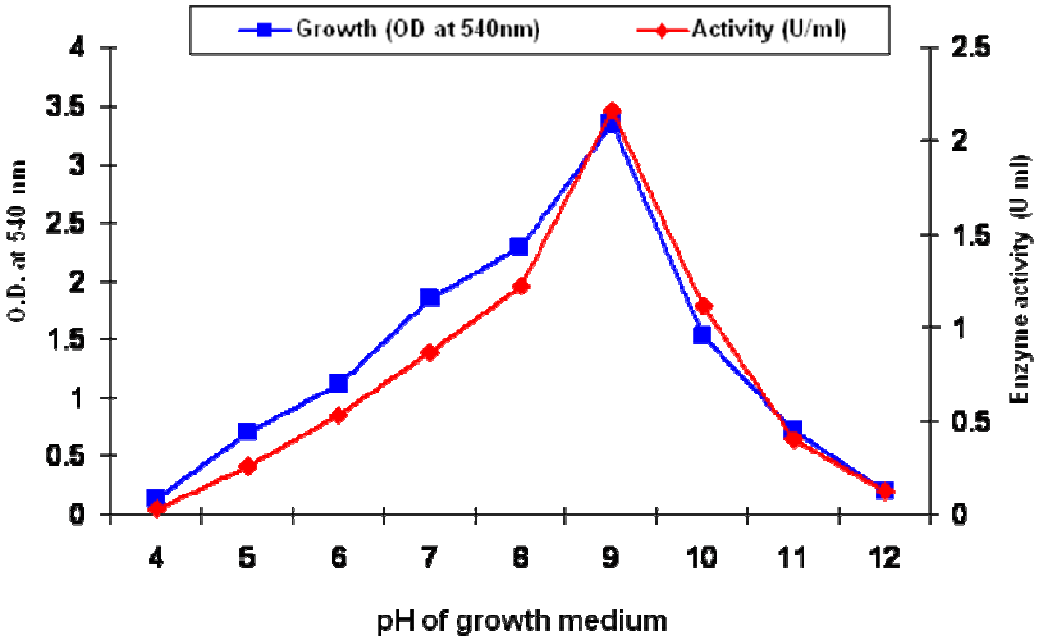


Fig 20: Effect of different pH on growth and thermostable pullulanase enzyme activity of selected *Bacillus licheniformis* strain ASM3

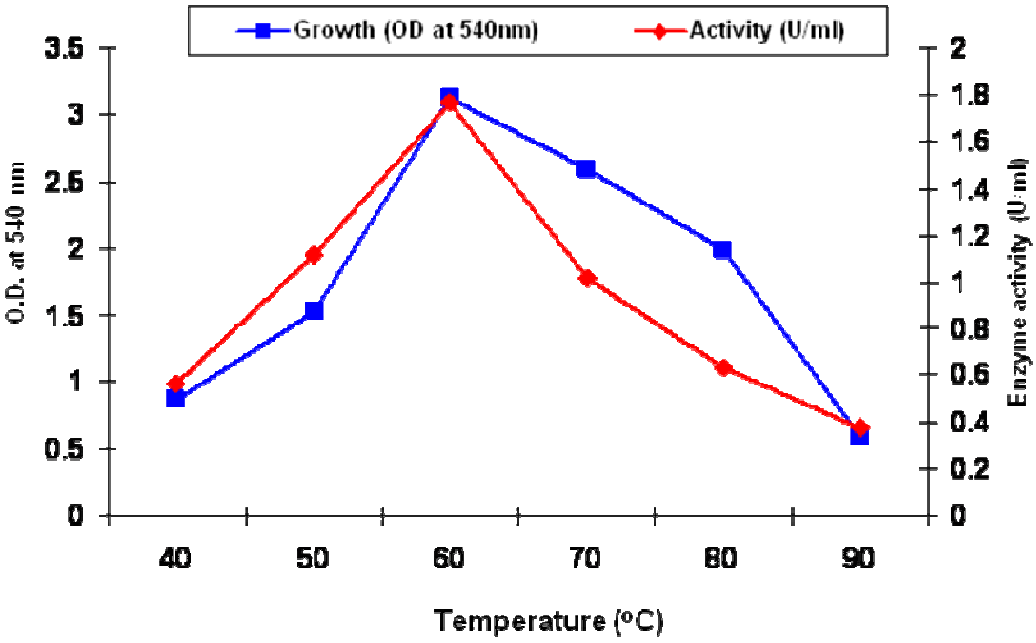


Fig 21: Effect of incubation temperature on growth and thermostable pullulanase enzyme activity of selected *Bacillus licheniformis* strain ASM3

4.13.2 Ammonium sulphate precipitation

Crude extracellular enzyme extract was subjected to ammonium sulphate (0-80%) saturation and maximum pullulanase enzyme activity was detected in 20-80 percent level of saturation. The enzyme preparation at this stage was treated as ammonium sulphate fraction (ASF). The dialysis was carried out for 24 hrs against three successive changes of dialysis buffer in dialysis bag.

4.13.3 Gel filtration column chromatography

The elution profile of blue dextran on Sephadex G-100 column is shown in Fig-22. The void volume (V_o) of Sephadex G-100 was calculated as 30 ml by loading 4mg/ml blue dextran on Sephadex G-100 column.

The ASF (proteins precipitated after the overnight dialysis of 80% ammonium sulphate cut) was applied on Sephadex G-100 column equilibrated with sodium phosphate buffer (pH: 9.0) used as start buffer. Maximum absorbance at 280 nm was detected in the fractions numbering 15-26 and the elution profile obtained on Sephadex G-100 column has been shown in Fig-23. Pullulanase activity for each fraction was calculated using 3,5-dinitrosalicylic acid (DNS) assay and it was found that maximum pullulanase activity was obtained in the fraction numbering 15-26 (Fig- 23). These most active fractions were pooled. The dialysis was carried out for 24 hrs against three successive changes of sucrose in dialysis bag.

4.13.4 Ion exchange chromatography

The active pooled fractions prepared previously were loaded on ion exchange column equilibrated with sodium phosphate buffer pH 9.0 as a start buffer. The proteins were eluted with linear salt gradient of 0.1M–0.5M NaCl and maximum absorbance at 280 nm was detected in the fractions numbering 36-38. It was found that pullulanase fractions were eluted with 0.3 M NaCl gradient and the elution profile obtained on DEAE sephadex has been depicted in Fig- 24.

The data of total pullulanase activity (U), protein content, specific activity, fold purification and yield at different stages of purification has been presented in Table-32. The pullulanase enzyme was partially purified up to 29.04

fold with 15.48 percent recovery. The specific activity increased significantly from 3.88 U/mg in crude extract to 112.66 U/mg in ion exchange chromatography fraction.

Table 32: Partial purification of pullulanase from *Bacillus licheniformis* strain ASM3

Steps	Total Enzyme Activity (U/ml)	Total soluble protein (mg)	Specific activity (U/mg protein)	Fold purification	Percent yield
Crude extract	2642.42	680	3.88	1.0	100
Ammonium sulfate precipitation	1692	253	6.69	1.72	64.03
Gel filtration chromatography	583.2	11	53.01	13.66	22.07
Ion exchange chromatography	408.96	3.63	112.66	29.04	15.48

4.13.5 Native PAGE (Polyacrylamide gel electrophoresis)

The purity as well activity after each step of purification was checked by native-PAGE technology, using 7.5 per cent acrylamide gel and anionic system of Davis (1964) on vertical slab gel electrophoresis. Native PAGE of fraction after gel filtration chromatography followed by ion exchange chromatography revealed a single band of purified protein (Plate-8).

4.14 CHARACTERIZATION OF PARTIALLY PURIFIED THERMOSTABLE PULLULANASE ENZYME

Purified enzyme was further characterized biochemically to determine its molecular weight, optimum temperature and pH.

4.4.1 Determination of molecular weight of pullulanase of *Bacillus licheniformis* strain ASM3

SDS polyacrylamide gel electrophoresis was carried out to determine the purity as well as molecular weight of pullulanase enzyme. The purified fractions of ion exchange chromatography showed the presence of a distinct protein band (Plate-9), which confirmed the purity of enzyme. Standard protein molecular weight markers were used for determining molecular weight of the enzyme and it was found that molecular weight of the partially purified pullulanase preparation was ~96 kDa (Plate-9).

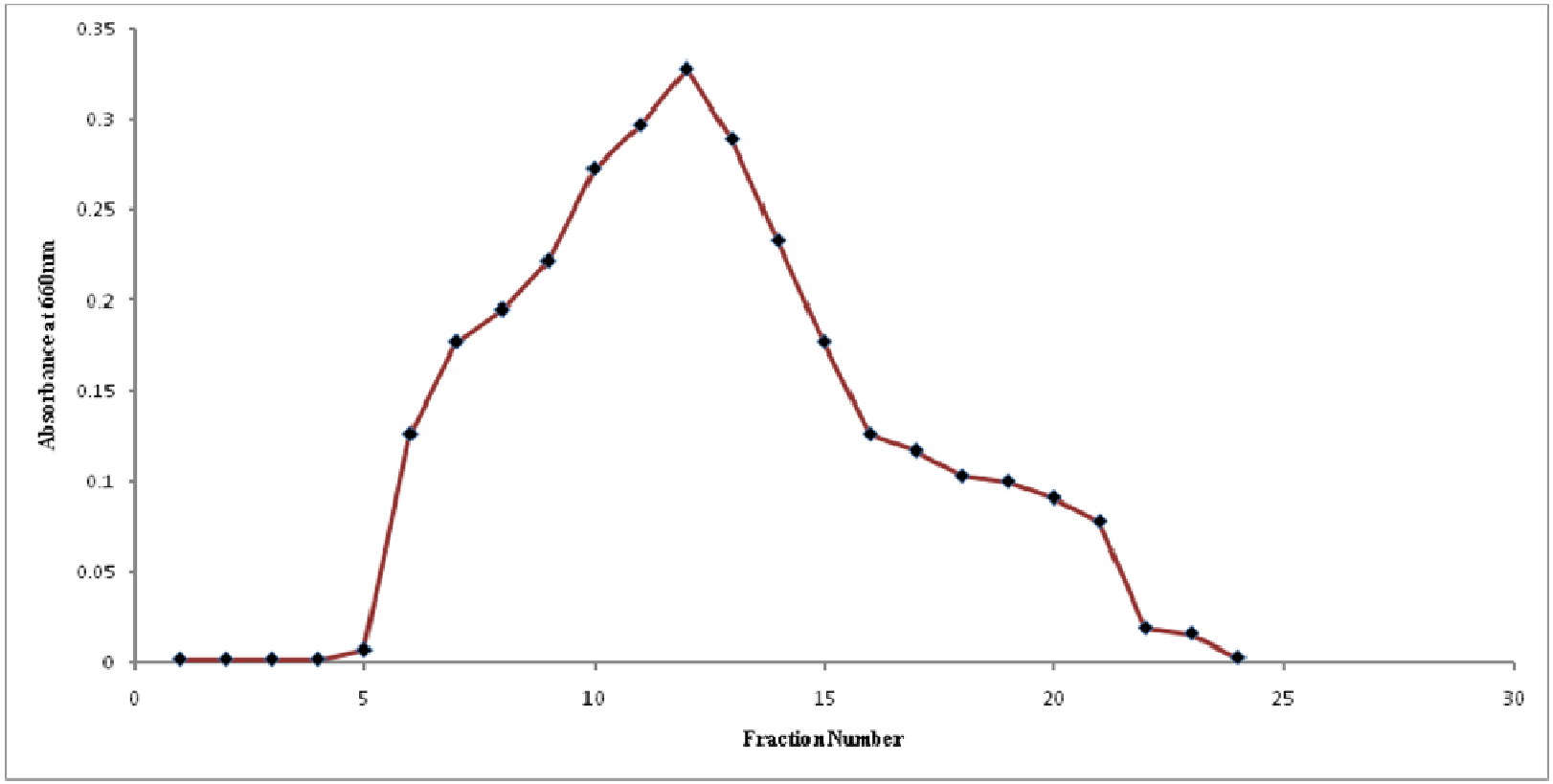


Fig. 22: Elution profile of blue dextran on Sephadex G-100 column.

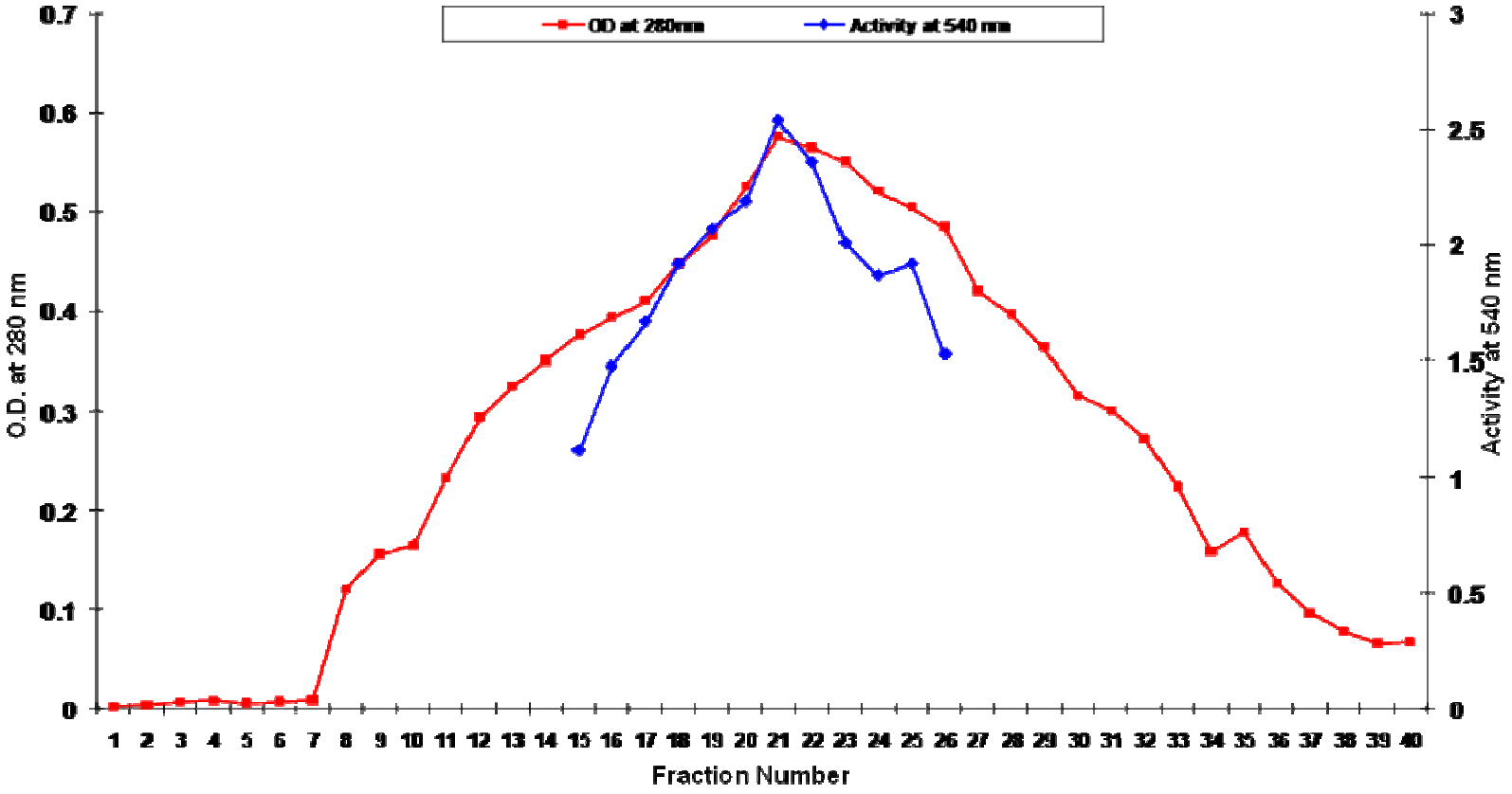


Fig. 23:- Gel filteration column chromatography of dialysed ammonium sulphate fraction on Sephadex G-100.

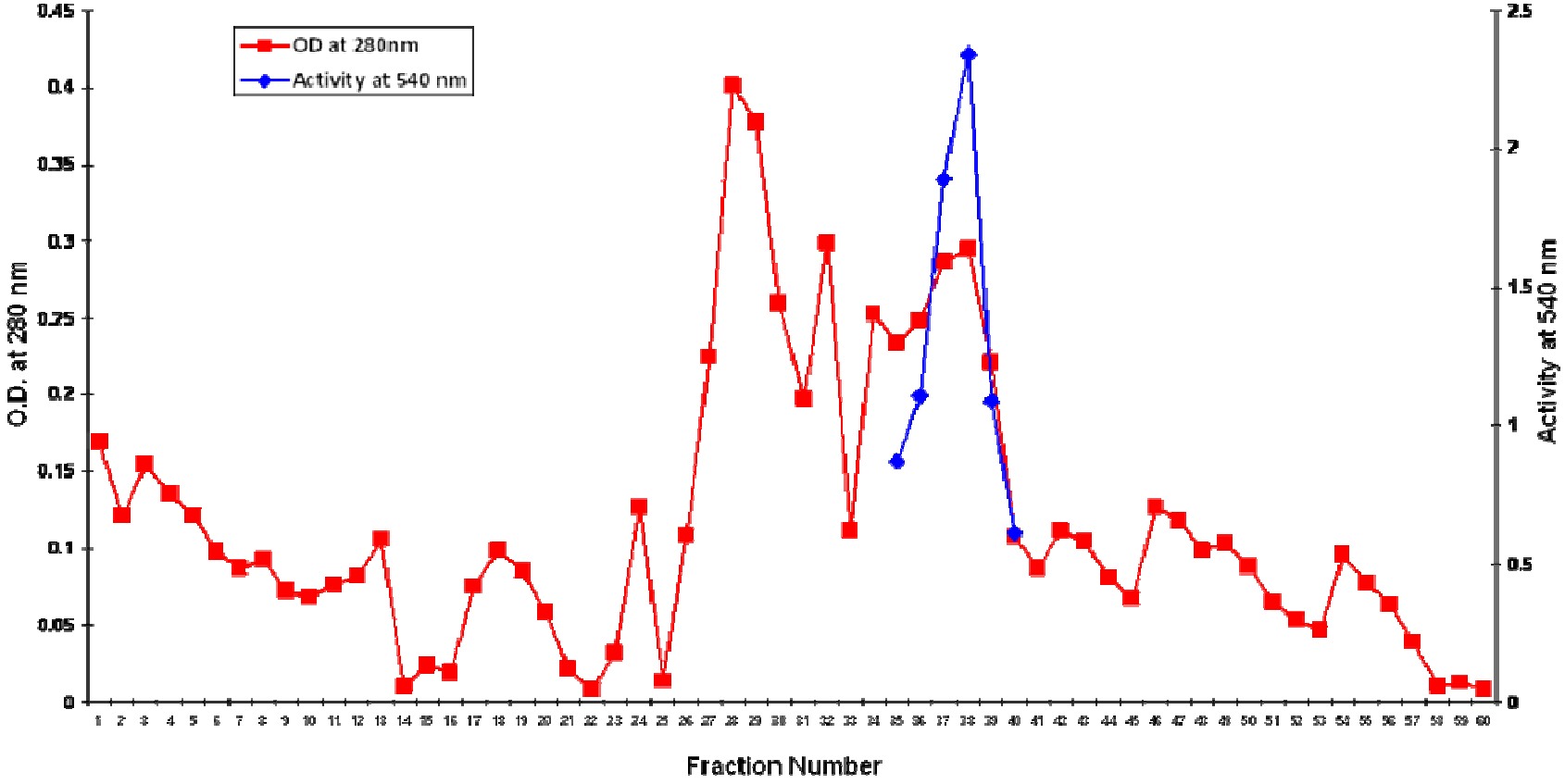


Fig. 24. Ion exchange chromatography of active pooled fractions obtained from G-100 gel filteration chromatography on DEAE sephadex column.



Plate 8:- Native polyacrylamide gel electrophoresis of pullulanase enzyme *Bacillus licheniformis* strain ASMB.

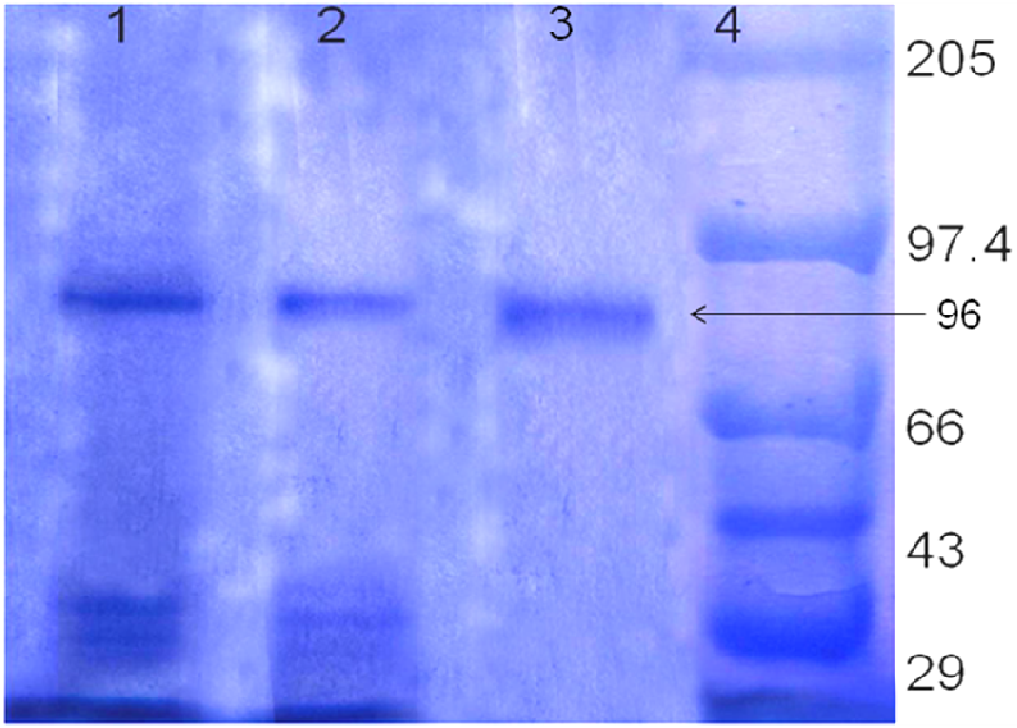


Plate 9: SDS polyacrylamide gel electrophoresis of pullulanase enzyme *Bacillus licheniformis* strain ASMB.

Lane 1: Extracellular crude extract

Lane 2: Protein fraction after ammonium sulphate fraction.

Lane3:Partially purified protein eluted from ion exchange chromatography

Lane 4: Protein molecular weight marker (kDa)

4.4.2 Effect of pH on thermostable pullulanase enzyme activity

An optimum pH was found to be 9.0 for maximum pullulanase enzyme activity. It has been depicted in Fig-29 that the enzyme activity of the purified fraction of ion exchange chromatography, increased with increase of pH. A maximum value was achieved at pH 9.0 and after that the activity decreased up to the pH 9.5 (Table-33, Fig-25).

Table 33: Effect of pH on thermostable pullulanase enzyme activity

Buffer	pH	Activity (U/ml)
Citrate	6.0	0.39
	6.5	0.82
	7.0	0.98
Tris	7.0	1.27
	7.5	1.03
	8.0	0.68
Phosphate	8.0	1.63
	8.5	2.12
	9.0	**2.73**
	9.5	1.34

4.14.3 Effect of temperature on thermostable pullulanase enzyme activity

Optimum temperature of the purified fraction of ion exchange chromatography of pullulanase was determined at optimum pH of 9.0 and maximum pullulanase activity of 1.962 U/ml was observed at high temperature of 60°C. It has been observed that the enzyme activity increased with increase of temperature up to 60°C and after that gradual decrease was observed in the enzyme activity up to 90°C (Table-34, Fig-26)**.**

Table 34: Effect of temperature on thermostable pullulanase enzyme activity

Temperature (°C)	Activity (U/ml)
40	0.861
50	1.437
60	**1.962**
70	1.379
80	0.934
90	0.593

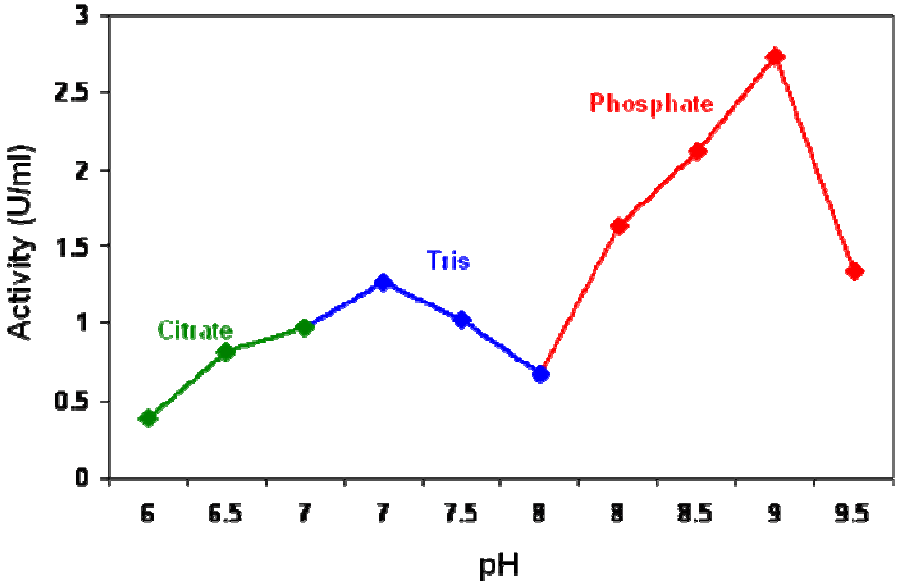


Fig. 25. Effect of different pH on activity of thermostable pullulanase enzyme

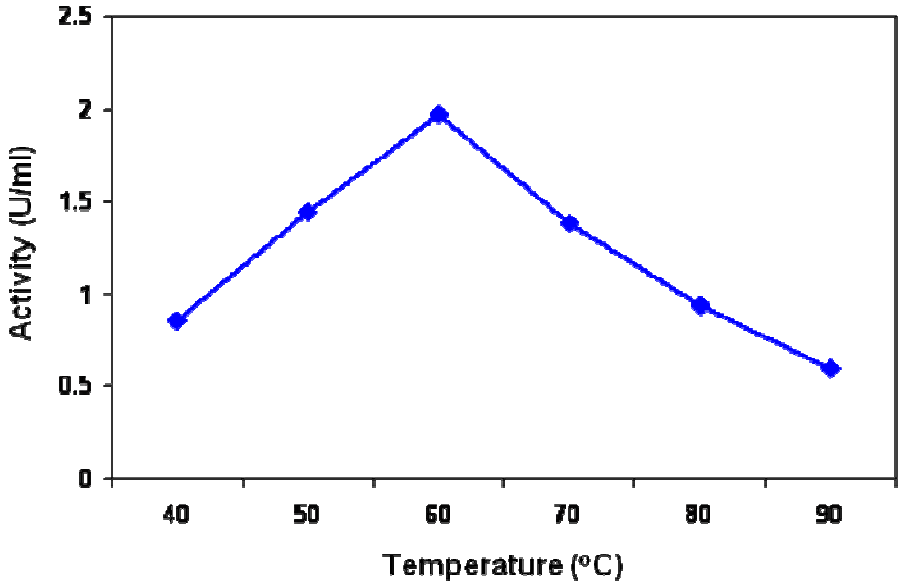


Fig. 26. Effect of different temperature (°C) on activity of thermostable pullulanase enzyme

Chapter-5

DISCUSSION

Advances in biotechnology and associated areas have enhanced the value of biodiversity and indigenous biological wealth and knowledge. It has been reported that global markets for some products derived from genetic resources and biodiversity lies in the range of US $ 500-800 billion (Kate *et al*., 2000). Over the past few decades the development of new approaches in the field of biology, genomics, chemistry and information technology has given impetus to the pace of change in the industry, setting new targets for development. This in turn has created greater demands for adequate supply of bioresources, further encouraging bioprospecting-the exploration of biodiversity for commercially valuable biological and genetic resources. This activity involves the applications of advanced technologies to develop new industrial enzymes in addition to many more products from biodiversity. Such advances in laboratory based biotechnology have increased the value of genetic resources and indigenous microbial wealth, providing important leads to commercially exploitable properties of resources. Bioprospecting and commercialization of bioresources by indigenous biological communities in India is moving towards the new paradigm. Bioprospecting of microbial diversity of extremophiles including thermophiles have been explored with considerable success with positive results. The opportunity of new industrial applications from microorganisms is as large as the variety of environments they confront.

Thermophiles like all other living things have adapted to the conditions in which they have to live, grow and survive. They are reported to contain specialized proteins known as ‘chaperonins’, which help after their denaturation to refold the proteins to their native form and restore their functions. The cell membrane of thermophiles is made up of saturated fatty acids which provides a hydrophobic environment for the cell and keeps the cell rigid enough to live at elevated temperatures. The DNA of thermophiles contains a reverse DNA gyrase which produces positive supercoils in the DNA. This raises the melting point of the DNA to at least as high as the organism’s maximum temperature for growth.

Thermophiles also tolerate high temperature by using increased interactions that non-thermotolerant organisms use, namely, electrostatic, disulphide bridges and hydrophobic interactions. In addition, the study of proteins from thermophilic organisms has provided important insight into the mechanism of protein folding because these proteins must be stable at temperatures that would denature typical proteins. The genome and proteome composition of thermophiles are characterized by over representation of purine bases in protein coding sequences, higher G+C content of the structural RNAs, distinct synonymous codon usage, enhanced usage of positively charged residues and aromatic residues and decrease in polar uncharged residues in the encoded protein (Das *et al*., 2006). Thermophiles are found in various geothermally heated regions of the earth such as hot water springs and deep sea hydrothermal vents, as well as decaying plant matter such as peat bogs and compost. As a prerequisite for their survival, thermophiles contain enzymes that can function at high temperatures and mankind has successfully exploited these microbial enzymes for the benefit of mankind. Over about 70% of the world use of thermophilic enzymes is to be found in a number of industries. It is certainly worthwhile to intensify screening programmes to explore and identify rare genera and species of microorganisms, culture and conserve them *in vitro* and intensively investigate them for enzymes with novel properties. Indeed such screening programmes in combination with advances in molecular biology have already been yielding spectacular results in the developed world. It is essential that similar studies are intensified in other developing countries of the world including India to remain competent and competitive in biotechnology in the future decades.

In India also thermal springs have been found scattered throughout the country and occur either as solitarily or in groups upto 3000 m above sea level. In state of Himachal Pradesh, which is located in northern India, between 30"22' and 30"12' north latitude and between 75"47' and 79"4' east longitude, thermal springs occur as terrestrial biotopes of thermophiles and have been found located in the districts of Kullu and Mandi at places known as Manikaran, Vashisht, Khirganga, Kasol, Kalath and Tattapani. The mountainous state has altitudes ranging from 350-7000 m above the sea level. These are mainly sulphur containing solfataric fields with a temperature range of 35-106°C and pH range

of 5.7-9.5 and sulphate content varies from 9-54 mg/l. Physico-chemical analysis of water samples of these thermal springs revealed that highest mean values of chloride content (1857.667 mg/l), sulphate content (44.667 mg/l), calcium hardness (125.00 mg/l) and total hardness (131.600 mg/l) were determined in Manikaran water samples only, though maximum calcium carbonate hardness (272.333 mg/l) was observed in Tattapani samples and highest value of 26.150 mg/l of magnesium hardness was observed in Khirganga water samples.

In addition enumeration of thermophilic bacteria of both water and soil samples of six hot water springs was carried out by the most dependable serial dilution technique and to the best of our knowledge this is the first report. The results obtained revealed that cfu/ml of water samples and cfu/g of soil samples were found to be 10^5 in both the cases. Though the maximum number $3.45x10^5$ cfu/ml was determined from water sample of S1 site of Tattapani and $3.54x10^5$ cfu/g were observed in soil sample of S3 site of Vashisht.

Isolation and screening of thermostable pullulanase producing bacteria

With the discovery of *Thermus aquaticus* and success of Taq DNA polymerase at the commercial level, importance of thermostable enzymes has been realized and research is being undertaken all over the world for the isolation of more and more thermostable enzymes producing bacteria from diverse niches. For many years, biotechnologists and microbiologists have enriched thermophilic bacteria by incubation at 55^{o}C. However thermophilic bacteria do not grow optimally at 55^{o}C; they represent a continuum of organisms, from those with optima near mesophilic range to those with optima of 70^{o}C or above and are called hyperthermophiles (Brock, 1967; Brock and Brock, 1968). In the present investigation of bioprospecting of thermostable pullulanase bacteria, initially fifty eight thermophilic bacterial isolates were obtained using very common nutrient agar medium, pH: 6.8, with the optimum temperature of incubation being 60^{o}C and optimum time of 48 hrs, from six hot springs of Himachal Pradesh *viz*., Manikaran, Vashist, Khirganga, Kasol, Kalath and Tattapani. Screening of thermostable pullulanase producers was carried out on KWF medium enriched with 1% pullulan substrate. Only seven isolates were selected on their ability to

use pullulan as substrate. Similarly KWF medium was also used for successful isolation of thermostable pullulanase producing bacteria from hot springs of Malaysia have been reported (Chai *et al.,* 2011). However other authors have reported Pullulan agar medium and Basal medium for the isolation of thermostable pullulanase producing thermophilic bacteria (Asha *et al.,* 2013 and Nair *et al.,* 2006).

Characterization of bacterial isolates

Both morphological as well as biochemical characterization technologies have been immensely useful and have been applied in carrying out characterization of most of the bacteria. In the present study, 58 thermophilic bacterial isolates of six hot water springs were characterized initially on the basis of different morphological characters *viz*., colour, size, texture, elevation and margin of the colonies (Table-20) along with microscopic characters such as gram reaction, shape, spore formation and pellicle formation and it was observed that 20.7% of thermostable bacterial isolates were gram negative and 79.3% were gram positive whereas 89.6% were rod shaped and only 10.4% cocci. It was interesting to note that 75.8% were found to be spore formers (Table-21) giving us an idea that most of the thermophilic isolates belongs to genus *Bacillus*. In literature also variations in gram character, shape, arrangement and spore formation have been reported. Chai *et al.,* (2011) reported that the colonies of SK3-4 were glistening white to cream coloured, and the colonies of DT3-1 were dull yellow coloured and cells of both strains were straight or slightly curved rods in single arrangements respectively. Roy *et al.,* (2003) observed pullulanase producing strain US149 as gram positive, catalase positive, oxygenase negative, non motile and non spore forming. Biochemical characterization of selected seven pullulanase producers revealed that all the seven bacterial isolates tested positive for catalase, citrate and glucose whereas variation was observed in utilization of carbon sources sucrose, lactose and mannitol, in addition to urease, indole and MR/VP tests. However two biochemical tests *viz*., sucrose and TSI showed negative results in case of KS2W isolate, which also produced maximum thermopullulanase enzyme activity.

Quantitative screening for thermostable pullulanase activity

The selected seven putative bacterial thermostable pullulanase producers, when quantitatively screened showed considerable variation in the activity of the enzyme. The isolate KS2W produced maximum enzyme activity of 1.43 U/ml and 3.96 U/ml after 24 and 48 hrs respectively and this isolate of Kasol site was selected for further thermostable pullulanase studies.

***In silico* analysis of nucleotide sequence**

The term *in silico* was coined by Antoine Danchin in 1991 and first expression was used by Miramontes in 1992 and has been applied in many research areas including sequence analysis, protein structure prediction and computational phylogeny.

In the present study, *in silico* analysis was applied to determine nucleotide sequence of selected thermostable pullulanase producing bacterial isolate i.e. KS2W on the basis of results of sequencing and BLASTn was found to belong to genera *Bacillus* (http//www.ncbi.nlm.gov/BLAST/). BLASTn anlysis of KS2W nucleotide sequence was found to possess 96% homology with *Bacillus licheniformis* strain BCRC 11702, 16S ribosomal RNA, partial sequence (NR_116023.1). Finally the phylogenetic relationship of the test isolate with 15 sequences retrieved from NCBI database using MEGA 5.0 software generated a Neighbour-Joining tree which was divided into many branches and 16S *rrna* gene of KS2W bacterial isolate was placed in cluster B supported by a bootstrap value of 36. In the present study, a thermotolerant bacteria producing thermostable pullulanase identified as *Bacillus licheniformis* strain ASM3 was successfully isolated from hot water springs of Himachal Pradesh, India using 16S *rrna* gene technology.

Optimization of culture conditions

The various culture conditions such as different media, incubation time, pH of media and incubation temperature were optimized for the maximum bacterial growth and thermostable pullulanase activity of selected *Bacillus licheniformis* strain ASM3. On the basis of experimental results it has been

established that KWF medium was found to be optimum for both, growth and thermostable pullulanase enzyme activity. Both maximum growth of 3.38 OD at 540 nm and maximum thermostable pullulanase activity (2.18 U/ml) was achieved after 48 hrs of incubation. An alkaline pH of 9.0 and incubation temperature of 60°C were also established as optimum for growth as well as maximum thermostable pullulanase activity. A maximum growth OD of 3.34 and 3.13 respectively for pH 9.0 and temperature 60°C at 540 nm and maximum thermostable pullulanase enzyme activity of 2.16 U/ml & 1.76 U/ml was observed at pH 9.0 and 60°C temperature respectively. The selected optimal parameters were used for the production of thermostable pullulanase enzyme. However in other studies, optimum pH of 10.0 has been reported (Asha *et al*., 2013) for maximum pullulanase activity of *Bacillus cereus* strain FDTA-13. However Hyun and Zeikus (1995) reported that pH of 6.0 and temperature of 85^{o}C was found optimum for thermostable pullulanase activity of *Clostridium thermohydrosulfuricum* 39E.

Partial Purification of the thermostable pullulanase enzyme

Purification of an enzyme is an essential step for the determination of accurate kinetic parameters due to the possible presence of compounds from the host that may act as natural mediators, or the presence of similar enzymes that may exhibit significantly different reaction kinetics (Rittstieg *et al*., 2003).

In the present endeavour thermostable pullulanase was partially purified by ion exchange chromatography to 29.04 folds with 15.48% of yield from selected *Bacillus licheniformis* strain ASM3 (Table-31).

SDS-PAGE revealed that this partially purified thermostable pullulanase possessed a molecular weight of approximately 96 kDa (Plate 9). In addition to 48 hrs of incubation time an alkaline pH and high temperature of 9.0 and 60^{o}C respectively were established as optimum conditions for maximum thermostable pullulanase activity. However Koch *et al.,* (1997) reported pullulanase enzyme of 77.6 kDa which showed maximum activity at pH 6.0 and at 85^{o}C. Roy *et al.,* (2003) reported pullulanase of 95 kDa. Ling *et al.,* (2009) reported pullulanase enzyme with molecular weight of 93 kDa which showed maximum activity at pH 6.0 and temperature 55^{o}C.

Chapter-6

SUMMARY AND CONCLUSION

The results of the present investigation so obtained are summarized below:

- Six thermal springs *viz.,* Manikaran, Vashisht, Khirganga, Kasol, Kalath and Tattapani were purposely selected for the present studies. Seventy two samples were collected in sterile 30 ml screw capped vials. Samples consisted of water, soil, pebbles and rock matting.
- The pH and temperature of the six thermal springs were found to range from 5.7-9.5 and 35-106^{o}C respectively. The chloride, sulphate, total hardness, calcium hardness, $CaCO_3$ and magnesium content ranged from 578-1978, 09-54, 32.9-141.3, 22-134, 52-283 and 3.15-26.75 mg/l respectively.
- Bacterial population colonizing water and soil samples from the hot water springs were observed after 48 hrs on nutrient agar (NA). Bacterial population in water was highest (3.45×10^5 cfu/ml) at S1 site of Tattapani and minimum (0.76×10^5 cfu/ml) bacterial population was noted at S2 site of Kalath. Similarly Bacterial population in soil was highest (3.54×10^5 cfu/g) at S3 site of Vashisht and minimum (0.54×10^5 cfu/g) at S3 site of Kalath.
- Total 58 thermophilic bacterial isolates were isolated from the samples of selected six hot water springs using nutrient medium. All the bacterial isolates were studied for various morphological characters *viz.,* Gram reaction (+/-), shape (rods/cocci), spore formation (+/-) and pellicle formation (+/-), colour, size, elevation, margin and texture of the colonies and all 58 thermophilic bacterial isolates showed variation with respect to all morphological characters.
- On the basis of ability to grow on KWF medium, 7 bacterial isolates were screened out for having ability to produce thermostable pullulanase enzyme.

- The selected 7 bacterial isolates were further investigated for biochemical characters *viz.,* catalase, oxidase, urea, indole, methyl red & Voges-Proskauer (IMViC), citrate and fermentation of sugars (glucose, sucrose, mannitol and lactose). All 7 thermophilic bacterial isolates showed variation with respect to all biochemical characters.
- Quantitative screening was done to select one bacterial isolate showing maximum thermostable pullulanase activity and KS2W thermostable bacterial isolate was found to show maximum thermostable pullulanase activity of 3.96 U/ml.
- Genomic DNA was isolated from the thermostable bacterial isolate KS2W. PCR of the isolated DNA was carried out using universal primers for 16S *rrna* gene and an amplicon of expected size i.e. 1500 bp was produced.
- Sequencing of the PCR product was done using same primers and nucleotide sequence obtained after sequencing were analyzed using online NCBI BLAST program (http://www.ncbi.nlm.nih.gov/).
- BLASTn analysis showed 96% homology of the query sequence with *Bacillus licheniformis* strain BCRC 11702, 16S *rrna* gene, partial sequence (NR_116023.1).
- Multiple sequence alignment of 16S *rrna* gene sequence of the test bacterial isolate KS2W was performed with 16S *rrna* gene sequences of *Bacillus* sp. collected from GenBank, using ClustalW software of EBI (http://www.ebi.ac.uk/).
- Alignment score was highest for *Bacillus licheniformis* strain BCRC 11702, 16S *rrna*, partial sequence (NR_116023.1). Thus the bacterial isolate KS2W was identified as *Bacillus licheniformis* ASM3.
- Phylogenetic analysis based on nucleotide sequences using NJ method was achieved *via.* MEGA 5.0 and *Bacillus licheniformis* ASM3 was closely matched with *Bacillus licheniformis* strain BCRC 11702, 16S *rrna*, partial sequence (NR_116023.1) with bootstrap value of 38.
- Optimization of culture conditions for maximum growth and maximum thermostable pullulanase activity of isolate *Bacillus licheniformis* strain ASM3 was carried out.

- KWF medium was found to be the best medium for maximum growth (4.26 OD at 540 nm) and maximum thermostable pullulanase activity (2.76 U/ml).
- The optimum incubation time for maximum growth and maximum thermostable pullulanase enzyme activity was recorded to be 48 hrs.
- The optimum pH for the maximum growth and thermostable pullulanase activity was found to be 9.0.
- The optimum temperature for the maximum growth and thermostable pullulanase activity was observed to be 60ºC.

❖ The crude extracellular thermostable pullulanase was produced using optimized conditions and partially purified using ammonium sulphate precipitation technique, gel filtration chromatography and ion exchange chromatography. The procedure yielded with 29.04 fold purification with a percent yield of 15.48% after ion exchange chromatography.

❖ Molecular weight of the partially purified enzyme was found to be 96 kDa using SDS-PAGE.

In the present study, indigenous thermophilic bacteria producing thermostable pullulanase has been isolated from Kasol thermal spring, where water was at a temperature of 65ºC. This bacterium has been identified as *Bacillus licheniformis* strain ASM3 after morphological, biochemical and molecular characterization by 16S *rrna* gene technology. Extracellular thermostable pullulanase enzyme activity of 3.96 U/ml was found to be significant and after partial purification its molecular weight was determined to be ~96 kDa.

Chapter-7

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Title of Thesis	**:**	**"Bioprospecting of themotolerant bacteria from hot water springs of H.P. for production of thermostable pullulanase enzyme"**
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Admission Number	**:**	H-2012-29-M
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Minor Field(s)	**:**	Microbiology
Degree Awarded	**:**	M.Sc. (Molecular Biology and Biotechnology)
Year of Award of Degree	**:**	2014
No. of Pages in Thesis	**:**	115+II
No. of words in Abstract	**:**	545

ABSTRACT

Thermophiles are the organisms which are adapted to live at high temperatures. The survival of these organisms at high temperature is possible due to the thermostability of their enzymes. Many thermostable enzymes such as Taq DNA polymerase, pullulanase, amylase, lipase, protease, cellulase, RNA polymerase etc. find a number of commercial applications because of their thermostability. Therefore, the isolation of thermophilic bacteria from natural sources and their identification are very important in terms of discovering new industrial enzymes. Keeping in view, hot water springs of Himachal Pradesh could serve as a good source for new thermophilic microorganisms with novel industrially important properties. Therefore aim of the present study was the isolation and characterization of thermotolerant bacteria from hot water springs of Himachal Pradesh for production of thermostable pullulanase enzyme. Six hot water springs *viz.,* Manikaran, Vashisht, Khirganga, Kasol, Kalath and Tattapani were purposely selected for the present studies. Samples consisted soil, water, rock matting and pebbles were collected from each hot water spring. The pH and temperature of the six thermal springs ranged from 5.7-9.5 and 35-106^{o}C respectively. The chloride, sulphate, total hardness, calcium hardness, $CaCO_3$ and magnesium content ranged from 578-1978, 09-54, 32.9-141.3, 22-134, 52-283 and 3.15-26.75 mg/l respectively. Fifty eight bacterial isolates were isolated from the samples using nutrient agar medium. Bacterial population in water was highest (3.45×10^5 cfu/ml) at S1 site of Tattapani and minimum (0.76×10^5 cfu/ml) bacterial population was noted at S2 site of Kalath. Similarly Bacterial population in soil was highest (3.54×10^5 cfu/g) at S3 site of Vashisht and minimum (0.54×10^5 cfu/g) at S3 site of Kalath. All the bacterial isolates were studied for various morphological characters and on the basis of ability to grow on KWF medium seven bacterial isolates were selected, which were further investigated for biochemical characters. Quantitative screening was done to select one bacterial isolate showing maximum thermostable pullulanase activity. KS2W bacterial isolate was found to show maximum thermostable pullulanase activity of 3.96 U/l and was selected further for molecular characterization. Genomic DNA was isolated from the selected bacterial isolate KS2W. PCR amplification of the isolated DNA was carried out using universal primers for 16S *rrna* gene and an amplicon of 1500 bp was obtained. Sequencing of the PCR product was done using same primers. Sequence of the KS2W bacterial isolate so obtained was found to be 556 bp. *In silico* analysis using BLASTn showed 96% homology of the query sequence with *Bacillus licheniformis* strain BCRC 11702. A total of 20 sequences were mined and these sequences were used to compare the 16S *rrna* gene sequence of the test isolate KS2W. Alignment score was highest for *Bacillus licheniformis* strain BCRC 11702, 16S ribosomal RNA, partial sequence (NR_116023.1). Phylogenetic analysis based on nucleotide sequences using NJ method was achieved via MEGA 5.0. *Bacillus licheniformis* strain ASM3 was studied further for enzymatic studies. Optimization of culture conditions for growth and thermostable pullulanase activity of isolate *Bacillus licheniformis* strain ASM3 was carried out. The crude extracellular extract was produced using optimized conditions and further partially purified using ammonium sulphate precipitation, gel chromatography and ion exchange chromatography. The procedure yielded 3.96 g protein with 29.04 folds of purification with a percent yield 15.48%. Molecular weight of the partially purified enzyme was found to be ~96 kDa using SDS-PAGE gel.

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ANNEXURE-I

A. Solutions, Chemicals and Reagents used for DNA extraction

1. Real Genomics, Genomic DNA isolation kit (Mini)

B. Solvent for DNA

10X TNE Buffer:

S. No.	Reagent	Quantity added (1000 ml of 10X TNE)	Final concentration (100 ml 10X TNE)
1.	Tris	12.11 g	100 mM
2.	EDTA	3.72 g	10 mM
3.	NaCl	116.89 g	2 M

C. Gel Electrophoresis

1. Agarose gel (1 per cent)

2.0 g agarose was added to 200ml of 1x TAE buffer and molten down. The molten gel was cooled down to 40^{o}C and 18 µl of ethidium bromide solution was added and mixed well. The molten gel was cast in a gel tray with a comb containing 51 teeth to produce wells.

2. Ethidium bromide 10 mg/ml

10 mg of ethidium bromide was dissolved in sterile water and volume made upto 1 ml the solution was stored in an amber coloured bottle at 4^{o}C.

3. Loading dye (6 x solution) (MBI fermentas)

Tris-HCl (pH 7.6)	:	10mM
Bromophenol blue	:	0.03%
Xylene cyanol FF	:	0.03%
Glycerol	:	60%
EDTA	:	60mM

The volume was made up to 20 ml with sterile water.

D. PCR cocktail

1. *Thermus aquaticus* DNA polymerase

A stock solution 3 units μl^{-1} was provided by the manufacture that was stored at-20^{o}C.

Dexyribonucleotide Tri-phosphate

dATP (10 mM)	:	100 µl
dCTP (10 mM)	:	100 µl
dGTP (10 mM)	:	100 µl
dTTP (10 mM)	:	100 µl

Sterile water

The solution was mixed well and stored at -20^{o}C.

2. Primer

The manufacturer in a lyophilized form provided the primer. Based on the molecular weight of given primer, a solution of 10 pmoles/l was prepared by adding the required amount of TE buffer. Storage was done at - 20^{o}C.

ANNEXURE-II

The sources of instruments

i) Instruments used in DNA extraction

Microfuge	:	Eppendorf
Waterbath	:	Neolab
Deep freezer (-20^{o}C)	:	Sanyo Ultra Low
Refrigerator	:	Godrej
Water purification system	:	Millipore
Vortex	:	Vortex Genie-2
Pipetteman	:	Eppendorf
Eppendorf tubes	:	Axygen

ii) Instruments used in molecular analysis

PCR machine	:	MJ Research Inc., USA, model PTC-100
Laminar flow	:	Stericlean
Weighing balance	:	Sartorius
Gel electrophoresis unit	:	Biorad
UV transilluminator	:	Hoefer
Camera	:	Kodak
Power Pack 300	:	Biorad
Microwave Oven	:	LG
Pipetteman	:	Finipipette
Thinwalled PCR tubes	:	Axygen
Gel dryer	:	Biorad
Gel plate	:	Hoefer

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Whether sponsored by some state/ Central Govt./Univ./SAARC : NA

Scholarship/Stipend/Fellowship, any other financial assistance received during the study period : NA,

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