ISOLATION AND CHARACTERIZATION OF CELLULOSE DEGRADING BACTERIA FROM HOT SPRINGS

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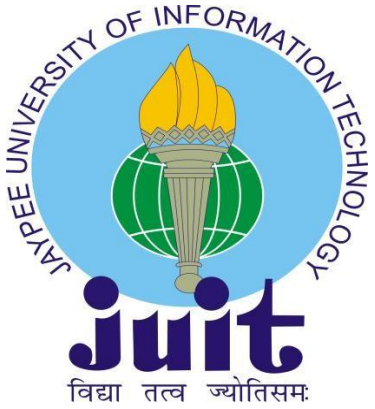

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WAKNAGHAT

Chapter 1

Chapter 2

REVIEW OF LITRETURE

Summary

In summary the eight isolates were isolated from the Tattapani hot springs. No isolate was shown good activity for cellulase enzyme. The different isolated bacteria were able to grow at different range of temperature starting from 40°C to 70°C. The bacteria isolated from Base soil sample (B2, B3,B4) were able to grow from 40 °C to 70°C but the bacteria isolated from Right side soil sample (R1&R2) were unable to grow at the temperature of 70°C the reason cloud be as the temperature of the right side location fluctuate according to the surrounding climate so the temperature always goes less. The bacteria isolated from the top outlet was able to grow at the temperature range of 70°C-50°C and unable to grow at 40 C the reason could be that the temperature at the top is near almost constant so the bacteria were unable to grow at 40°C. On the basis of the structural identifition of bacteria we can assume that they may belong to Micrococcus species for proper conformation 16S rRNA DNA amplification was done and the samples were send for sequencing.

Signature of the Student

Signature of the Supervisor

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Any assignment puts to litmus test an individual's knowledge, credibility and experience and thus, sole efforts of an individual are not sufficient to accomplish the desired work. Successful completion of a project involves interest and efforts of many people and so this becomes obligatory on my part to record thanks to them.

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CHAPTER 1

1.0 INTRODUCTION

Cellulose is the most abundant natural organic substance and the predominant component throughout the plant kingdom; because of its large abundance we could manipulate it for the benefit of mankind. Thus travelling back to many years in 1910, the first plant was constructed by Ewen and co-workers for the production of ethanol from sawmill dust, several improvements have been developed for the proper utilization of this natural cellulolytic material (Ramamurthy *et al.*, 1992).

For many years, researchers have been engaged in the process of improving technological methods and equipment for hydrolysing wood and other cellulose-containing substances for various industrial purposes (Julian *et al.*, 1990, Saxena *et al.*, 1992; Chen & Wayman, 1992; Teunissen, 1992; Wayman *et al.*, 1992; Doran & Ingram, 1993; Chaudhuri & Sahai, 1993; Sakata *et al.*, 1985; Tewari *et al.*, 1987; Tewari *et al.*, 1988; Malek *et al.*, 1988; Schafener & Toledo, 1991; Shiang *et al.*, 1991). The degradation of the highly crystalline cellulose biomass, in both efficient and cheaply is still a challenge for the researcher for the current cost sensitive markets. Many attempts have been done in using mixed microbial cultures in order to improve the hydrolysis of cellulose. Attempts included co-culturing of microorganisms to complement each other on cellulases enzyme production. The need for these mixed cultures is to improve on the production of biomass, and cellulolytic enzymes for the effective digestion of cellulose (Bailey & Viikari, 1993; Gutierrez-Correa & Tengerdy, 1997 Manonmani & Sreekantiah, 1987; Soundar & Chandra, 1987; Pavlostathis *et al.*, 1990).

Dunning and Lathrop (1945) had designed a system, which could hydrolyze agricultural wastes such like corncobs, cottonseed hulls and sugarcane bagasse to produce sugars that could be fermented to ethanol. The method of hydrolysing cellulose involved the use of concentrated hydrochloric acid. The products yielded by this method had other toxic materials because of the chemical complexity of the natural cellulose. The disposing of waste generated from this method after the glucose extraction causes problems of pollution to the already strained environment.

The biological conversion of abundant cellulosic biomass to man-manufactured products which include single cell protein (SCP), ethanol, methanol, butenediol, acetic acid, sugars, biogas, chemical feedstock and liquid fuels by employing microbial fermentation. (Tsao *et al.,* 1999; Ho *et al.,* 1999; Gong *et al.,* 1999; Sun & Cheng, 2002 Leisola & Linko, 1976, Manonmani & Sreenkantiah, 1987, Julian *et al.,* 1990; Dolan & Ingram, 1993; Bhat & Bhat, 1997). Attempts were made in In the late 19th century to use acid or alkali pretreatment of cellulose followed by enzymatic hydrolysis in order to improve the yield of the glucose (Mandels *et al.,* 1976; Tewari *et al.,* 1987; Taherzaeh *et al.,* 1998; Meyer *et al.,* 1992; Tenborg *et al.,* 1998Lee *et al.,* 1999' Millet & Baker, 1975). Under current scenario there is a need from to substitute fossil fuels with environmentally friendly renewable sources of energy. This need has motivated scientists in order to look for treatment of solid or solubilised organic cellulose wastes into bioconvertable fuels and other products (Saxena *et al.,* 1992; Taherzadeh *et al.,* 1998 Rogers *et al.,* 1972; Wayman *et al.,* 1992). The production of energy from wastes like cellulose material is affected by the total potential energy that can be extracted from these sources is the measure of the efficiency of converting the raw material source to the energy carrier of interest. Other parameters that can affect the production of fuels are the cost of producing, harvesting and converting the organic sources (Miller & Churchill, 1986; Horikoshi, 1999). Cellulose as energy source can be of higher efficiency of conversion to fuels than current sources because there is room for technical improvements and higher rate of processing.

Natural sources of cellulolytic enzymes which are majorly available includes bacteria, fungi and plants (Gilkes *et al*., 1991; Pearson *et al.,* 1991; Horikoshi, 1999). Production of cellulases by bacteria and fungi has attracted interests by scientists. Scientists aretrying to isolate and study the organisms that can produce cellulases that are thermostable, have temperature optima, broader pH optima and have application potential (Ito, 1997; Bhat & Bhat, 1997; Ito *et al.,* 1998). Organisms isolated from natural environments such as hot springs, alkaline lakes and sea waters can be able to produce enzymes with these properties. There are a number of advantages for isolating enzymes from thermophilic microbes.

The advantages are:

a) Enzymes from these organisms have potential economic value,

b) Selective growth of certain microbes at high temperatures because of low oxygen solubility,

c) Their enzymes can be stable in extreme conditions under which the microorganism thrive,

d) Fermentation techniques using microbes have high yields, and recovery of fermentation products downstream is easy and

e) Microorganisms can be genetically manipulated to improve protein production and quality (Miller & Churchill, 1986; Mosier *et al.,* 1999).

Although fungal cellulase enzyme studies have been extensively (Schimdhatter & Canevascini, 1992 Harmova *et al.,* 1986; Ali &Akhand, 1992; Yazdi *et al.,* 1990; Meyer *et al.,* 1992) the bacterial cellulases has recently received much attention at molecular level (Horikoshi, 1999; Takami & Horikoshi 2000 Nakamura *et al.,* 1987, Blanco *et al.,* 1988) with the aim of improving the production output and characteristic or qualities of the enzyme. Cellulase regulation and synthesis from various wild and cloned sources like eukaryotes and prokaryotes are ongoing research activities (Baird *et al.,* 1990; Lindhl *et al.,* 1994; Miyatake & Imada 1987; Dei *et al.,* 2000; Hakamada *et al.,* 2001; 2001; Ozaki *et al.,* 1990 and 1991; Shima *et al.,* 1991; Park *et al.,* 1991; Hansen-Sonne *et al.,* 1993 Wicher *et al*).

The production of enzymes by microbes is not independent of each other. The plethora of enzymes produced tend to interact which leads to the process of proteolytic posttranslational modifications of other proteins turning these prepro proteins into functional proteins (Creighton, 1993). Microbial proteases are produced as gene products (Pero & Sloma, 1993) or as a control response to environmental stimuli. In certain classes of proteins the proteolytic cleavage of polypeptide chains occurs after synthesis, primarily for those proteins destined for extracellular excretion in addition to the removal of signal peptides. The proteolytic process modification for the pro-enzyme will have an extracellular function that under goes outside the cell system (Creighton, 1993). Some bacterial proteases have been shown to undergo auto proteolysis when they reach the extracellular region of the cell. The extracellular conditions are sometimes suitable for induction of autocatalysis e.g.

Bacillus subtilisin protease (Ikemura & Inouye, 1988). This proteolytic enzyme production is triggered by depletion in either carbon or nitrogen nutrient sources which leads to the formation of spores (Pero & Sloma, 1993). In the present study extra cellulase production was studied and the temperature tolerance of the isolates was studied.

CHAPTER 2

2 REVIEW OF LITERATURE

2.1 Natural occurrence of cellulose

Cellulose has become the important point in commercial and industrial applications. It has many applications in food, clothing, paper and wood industries. It is also being used as a natural fertiliser.

Naturally, cellulose occurs in abundance in wood as lignified cellulose, fungi, tunicates and bacteria (Colvin, 1980). The cellulose from various raw materials can be processed into papers, fibres and textiles. Many agricultural wastes like stalks, stems and husks of cereal grains have cellulose as the predominant component and can be recycled as bio fertilizers. The plant polysaccharides have notable features of certain types of sugar residues as main polysaccharide backbone component. Other different sugar units can be attached to the main chain. Sometimes the hydroxyl groups are substituted with O-acetyl, O-methyl derivatives or carboxylic acids of methyl esters. (Aspinall, 1980; Bacic, 1988; Morohoshi, 1991).

2.2 NATURAL CELLULOSE PHYSIOCHEMICAL PROPERTIES

Naturally occurred cellulosic materials predominantly consists of two different sections, namely microfibrillar and matrix components. These two sections have different sugar units and differ in their residues bond linkages.

2.2.1 The Microfibrillar component of cellulose

The microfibril component is made up of long, thin and microfibril like structures of β(1,4) linked glucose units. The linear homopolymer of D-glucopyranosyl residues per native cellulose molecule vary from 15 to 15 000 conferring a varying degree of polymerisation (Morohoshi, 1991). Intra-molecular and inter-molecular hydrogen bonds cellulose chains made the structure in paracrystalline lattice. The hydrogen bonds arise between intra-chain and inter-chain hydroxyl groups of microfibril cellulose chains (figure 1). This structure confers a certain order of inflexibility hence the cellulose's considerable tensile strength. Cellulose can occur in a number of different forms. Designations of cellulose by Millet and

Baker (1975) were after reacting cellulose with 20 % (w/v) sodium hydroxide, which macerate the cellulose polymer. The products that were obtained from such treatment gave rise to two forms of cellulose designated cellulose I and II. However, initial studies postulated the existence of possible parallel and antiparallel configurations of the cellulose chains thereby giving rise to different forms of cellulose designated as cellulose I, cellulose II, cellulose III and cellulose IV. Gardner and Blackwell (1974) resolved that cellulose microfibril have same polarity and are parallel oriented.

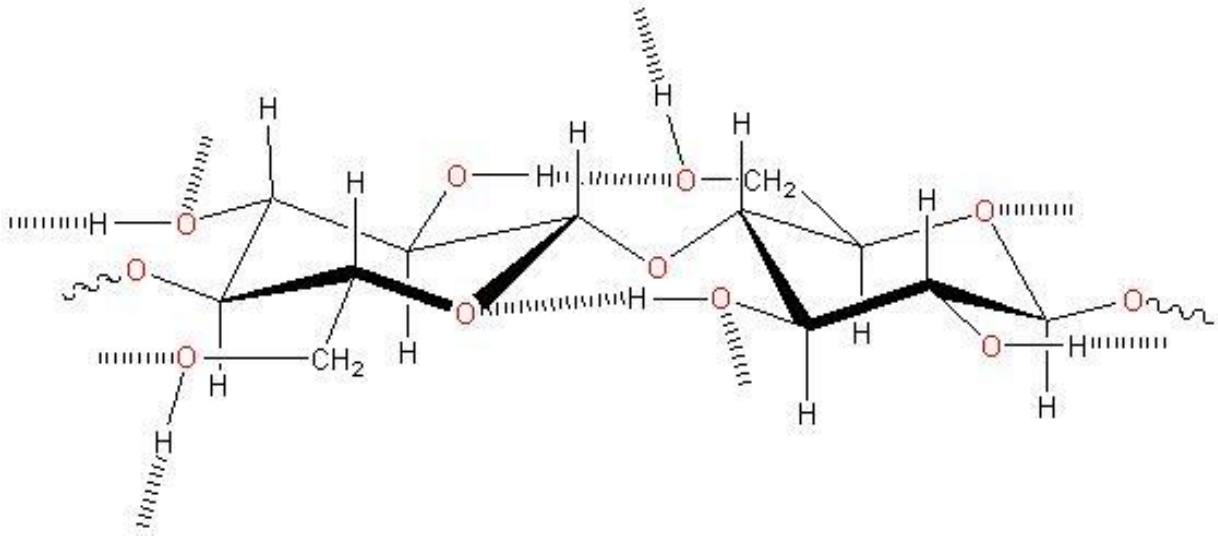


Figure 1. Conformation of β(1,4)-glucan linkages in microfibril component showing sticking out hydroxyl groups involved in intramolecular hydrogen bonding between adjacent glucose residues. (adapted from Aspinall, 1980)

Currently, native cellulose is known as cellulose I and other forms such as cellulose II, cellulose III and cellulose IV are obtained after subjecting the native cellulose to mechanical or thermal treatment (Langan *et al.*, 1999).

2.2.2 The Matrix component of cellulose

The matrix component of native cellulose is made up of different parts which were identified after solubilization, separation and analysis of these components from the microfibrillar component. The components of matrix were grouped into hemicellulose, lignin, pectin, protein and phenolic compounds. The compositions of these materials are extensively complex and vary from species to species, cells, and stage of cell development.

The matrix component further complicates cellulose's natural composition by having variations in proportions of its heteropolymer units (Table 1).

Cellulose is made up of only β(1,4) linked glucose sugar units where as hemicellulose is a heteropolymer that is made up of several sugar units with several bond links. Hemicellulose can easily be hydrolysed by acids to their monomer components that are glucose, mannose, galactose, xylose, arabinose, rhamnose, glucuronic acid, methyl glucuronic acid and galacturonic acid (Dekker & Richards, 1976). The sugar composition gives rise to the differentiation of wood into softwood or hardwood hemicellulose. The major difference between these two types is depending on the concentration of xylan. If the concentration of xylan is more it is considered as hardwood. The lignins are polymers of phenylpropanoid residues that are almost exclusively derived from p-coumaryl, (p-hydroxyphenyl) coniferyl (guaicyl) and sinapyl (syringyl) alcohols. Lignin and hemicellulose are linked together by ester, ether and glycosidic chemical bonds.

Component	Constituents	Glucan(s) types
Microfibril	cellulose β(1,4)	glucan
Matrix	i) pectins	R hamnogalacturonan, homogalacturonan, ararabinan, galactan, ar rhamnogalacturonan II
	ii) hemicelluloses	xylan, glucomannan, mannan, xyloglucan, galactomannan, glucuronomannan, callose β(1,3) glucan, β(1,3)-β1,4) glucan, arabinogalactan, galactoglucomannan
	iii) proteins	extensin, arabinogalacta protein, others including enzymes
	iv) phenolics	lignin (coniferyl and sinapyl), ferulic acid and others (coumaric acid and traullic acid)

(adapted from Aspinall, 1980 and Morohoshi, 1991)

Table 1. Microfibril and matrix heteropolymers constituents in nature.

These linkages are quite strong and are extremely resistant to chemical and enzymatic hydrolysis. The other naturally occurring extractives from cellulose are terpenoids and steroids, fats and waxes and inorganic components like carbonates, silicates, oxalates and phosphates (Kai, 1991; Morohoshi, 1991). Based on the sugar involved in the main backbone chain plant polysaccharide nomenclature has done. The bonds that are also identified in hemicellulose components are α(1,4); α(1,3); α(1,5); β(1,3); β(1,5); β(1,6) or a combination of these bonds giving rise to β(1,3-1,4); β(1,3-1,5) or α(1,3-1,5) branch linkages (Aspinall, 1980). Major residues that are found in the matrix component of plants are α-L-rhaminose, α-L-fucose, α-L-arabinose, α-D-galacturonic acid, β-D-mannose, β-D-galactose, β-D-glucose, β-D-apiose, β-L-aceric acid and ketodeoxyoctulosonic acid.

Therefore we can summarise and conclude that naturally occurring cellulose comprises of microfibril of β(1,4) glycosides linked polysaccharides which are impregnated with plasticing matrix resin of heteropolymers.

2.3 USES OF CELLULOSE AND CELLULOSE DERIVATIVES

Besides the biotechnological by-products from cellulose produced by microbial fermentations they are some commercial products currently produced from cellulose and cellulose derivatives are being used for plastics, textiles, packaging, films, lacquers, explosives and pharmaceuticals. For instance, food and pharmaceutical industries require polysaccharides and cellulose as stabilisers or thickening agents to improve on the viscosity of their food, pastes, ointments and creams. These thickening agents vary in monosaccharides composition and properties (Scaman, 2000).

Also paint and oil industries use cellulose derivatives that have high degree of polymerisation in order to protect their colloidal products, as emulsifiers or pigments suspending and dispersing agents (Tothill & Seal, 1993). The chemically modified cellulose or cellulose derivatives that are used in paint and oil industries include hydroxyethyl cellulose (HEC), ethylhydroxyethyl cellulose (EHEC), hydroxypropylmethyl cellulose (HPMC) and carboxymethyl cellulose (CMC). The clothing industry is no exception in the use of microfibril cellulose for making cotton thread and fibres. Commercially available cellulose derivatives used for cellulase assays have little resemblance to natural cellulose considering that some of these cellulose forms lack the cellulose matrix component. The

accessibility and extent of cellulose derivative hydrolysis becomes much important when a microorganism produces a single cellulase enzyme component. Currently marketed cellulosic products that are substrates for cellulase enzymes include filter paper, Avicel, Sigmacel, Solfa floc, cellulose azure, sodium salts of CMC and HEC.

Recent studies have shown that cellulose derivatives like cellulose acetate, cellulose-propionate and cellulose-acetate-butyrate can be used as membrane supports that can covalently immobilise enzymes. Such immobilised enzymes have shown better storage stabilities, and could give more enzymatic activity. (Murtinho *et al.,* 1998).

2.4 METHODS OF HYDROLYSING CELLULOSE

There are various methods of hydrolysing cellulose like physical, chemical and enzymatic methods. To make cellulose more accessible to enzymatic hydrolysis physical or chemical methods of hydrolysing are sometimes incorporated as preliminary pretreatment stages (Tewari *et al.,* 1987; Meyer *et al.,* 1992). The physical and chemical pretreatment methods alter the fine structure and disrupt or open up lignin-carbohydrate association and weaken the crystalline complex of cellulose. By-products from natural cellulose inhibit microbial hydrolysis or fermentation of cellulose. Strategies have been developed to minimise production of cellulosic hydrolysates that inhibit fermentation of cellulose (Taherzadeh *et al.,* 1998; Tengborg *et al.,* 1998).

2.4.1 Physical and mechanical methods of hydrolysing cellulose

2.4.1.1 Grinding method

Grinding method is usually incorporated as an initial stage to ease cellulose hydrolysis by cellulase enzymes. After the grinding pretreatment, the bioavailability of the cellulose substrate for both enzymes and microorganisms is significantly increased. Vibrator balling, roll and hammer milling methods are used to produce fine sand dust from wood. Grinding of wood generates fine particles that can be easily hydrolysed by cellulase enzymes, fungi and bacteria. Studies on compression milling or pressing and drying of cellulose showed an

increase in moisture content retention by the cellulose thus improving the properties of the cellulose for enzymatic and bacterial hydrolysis (Ryu & Mandels, 1980; Haggkvist & Odberg, 1998).

2.4.1.2 Use of high and low temperatures

Temperatures of 200°C in solvents like kerosene, dry air, nitrogen or oxygen enhances hydrolysis of cellulose but this requires specialised equipment. The degree of polymerisation of cellulose enhances its strength, can be greatly reduced if the temperature is rapidly dropped to –75°C. Repeated freezing and thawing process can further augment the hydrolysis of cellulose with this method. This process results in reducing the compactness of cellulose due to the weakened microfibril intra- and intermolecular hydrogen bonds (Millet & Baker, 1975; McMillan, 1994).

2.4.2 Chemical methods of hydrolysing cellulose

2.4.2.1 Alkali and acid hydrolysis

Sodium hydroxide is the most widely used alkali agent. The main problem with Sulphuric, hydrochloric and phosphoric acids is esterification reactions that facilitate hydrolysis of the matrix and microfibril components of cellulose. It was observed that alkali treated straw that was fed to ruminants showed an improved nutritional value to the host ruminants. Sugarcane bagasse digestibility by *Cellulomonas* bacteria was shown to increase by about 40 % after sodium hydroxide pretreatment (Han & Callihan, 1974), 80 % with *Trichoderma viridie*, 90 % with a mixed culture of *Aspergillus* and *Trichoderma* strains (Manonmani & Sreekantiah, 1987). Alkali treatment improved the dissolution of hemicelluloses that occur in nature resulting in the conversion of crystalline cellulose form I to cellulose form II that is readily hydrolysed by *Trichoderma reseei* cellulases (Rahkamo *et al.,* 1998).

2.4.2.2 Ammonia treatment of cellulose

In this method of hydrolysis either aqueous or gaseous ammonia at 130°C is udes for cellulose hydrolysis. The ammonia hydrolysis method has been employed to ligninocellulosic materials fed on ruminants, and there was improved digestibility by the

animals. The ammonia was realised to improve the swelling of wood. The ruminants gut flora will then produce enzymes that in turn will digest the cellulose that the ruminants feed on (Holtzapple *et al.,* 1991; McMillan, 1994). Ammonia pretreatment method is more effective with agricultural residues like corn straws than with woody materials (Dunning & Lathrop, 1945; Mandels *et al.,* 1976; McMillan, 1994).

2.4.2.3 Delignification pretreatment

This process of hydrolysis use of agents that cause pulping is done, such as the treatment of cellulose with sodium chlorite. In the presence of acetic acid, lignin solubilization is enhanced when pulping agents like sodium chlorite are used. Another pulping agent that can be used is ammonium bisulphate. Most of the processes are selective in removing lignin. Pulping agents like ammonium bisulphate allow the usage of the end product after the delignification pretreatment (Royer & Nakas, 1987).

2.4.2.4 Steaming of cellulose

Steam treatment of aspen chips fed to sheep resulted in a remarkable live weight gain. Steam treatment helps in the reduction of degree of polymerisation of cellulose. Steam method is the mostly used method for pretreatment of ligninocellulosic materials. In this method, chipped material is treated with high pressure saturated with steam and then pressure is swiftly reduced. The reduction in pressure causes the material to undergo an explosion decomposition (McMillan, 1994).

2.4.3 Enzymatic hydrolysis of cellulose

Chemical and physical agents used in pretreatment of cellulose helps in breaking down the bonds of the matrix component of natural cellulose thereby reducing their crystalline region which leads to a little resistance to enzymatic attack. Due to the presence of multiple bonds in naturally occurring cellulose, a single cellulase enzyme is not capable for the total hydrolysis. Besides cellulases, other enzymes like endo- and exo-β-1,4-xylanases, hemicellulases, and lichenases, β(1,3)(1,4) glucanases and pectinases pectin methyl esterases, polygalacturonases, and poly-α-1,4-D-galacturonide lyase are required for a

complete hydrolysis of native cellulose. For an efficient hydrolysis of cellulose a system of enzyme entities collectively called cellulases are required.

The cellulases enzyme system is a complex of three different enzymes. This further complicates the assays of cellulase activity. The cellulase enzymes breakdown cellulose to glucose, cellobiose and different length sized oligosaccharides. The following is a schematic summary of the cellulase enzymes involved in cellulose hydrolysis (Kluepfel, 1988; Gilkes, 1991; Schmidhalter & Canevascini, 1993; Ito, 1997).

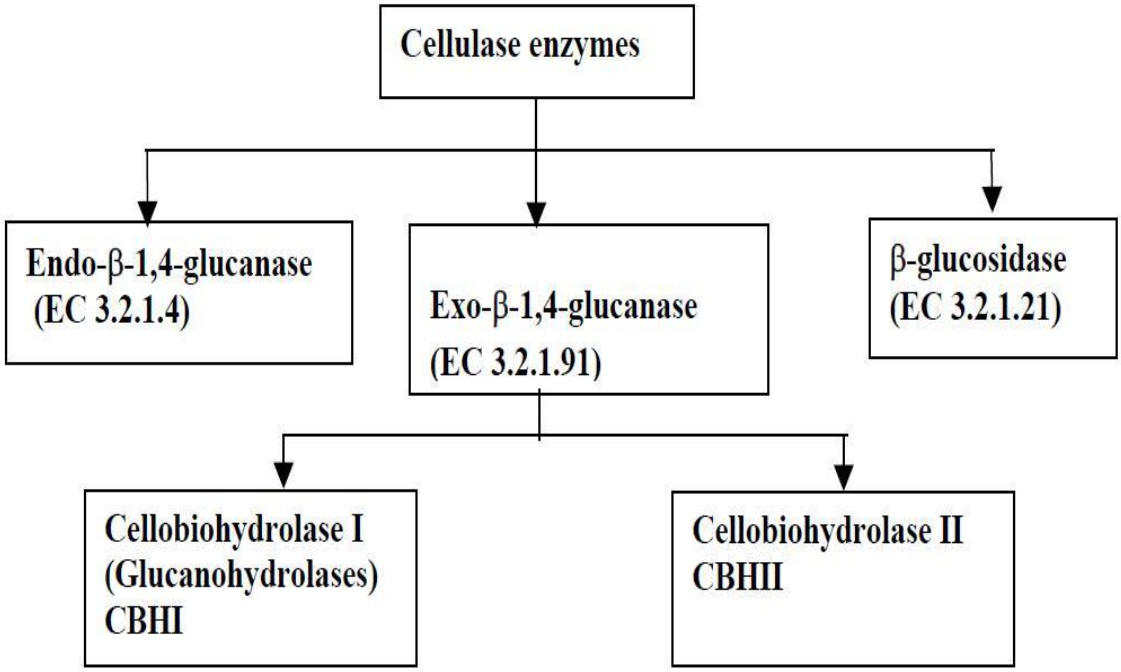


Figure 2. Cellulase enzymes involved in cellulose hydrolysis (Kluepfel, 1988).

The cellulase enzyme complex comprises of three different enzyme activities that act synergistically (i.e. endo-β-1,4-glucanase (EC 3.2.1.4), exo-β-1,4-glucanase (3.2.1.91), and β-glucosidase (3.2.1.21)) to efficiently decrystallise and hydrolyse native cellulose. These cellulase enzymes have different substrate specificities. Endo-β-1,4-glucanase activity is involved in random hydrolysis of internal β-1,4 glycosidic bonds of amorphous cellulose microfibril chains producing glucose, cellobiose and different sized celloligosaccharides.

The exo-β-1,4-glucanase or 1,4-β-glucan cellobiohydrolase has two types of cellobiohydrolase activity namely CBHI and CBHII (Schmidhalter & Canevascini, 1993).

Exo-β-1,4-glucanases that include cellobiohydrolase preferentially liberate cellobiose (glucose dimers) from either end of cellulose chain and glucanohydrolases liberate glucose monomers. The exo-β-1,4-glucanase hydrolyses microfibril cellulose chains from the non-reducing ends and gives more sites for endo-β-1,4-glucanase enzyme. The cellobiose disaccharides are then split into glucose moieties by the activity of β–glucosidase or β–D-glucoside glucohydrolase. This enzyme is sometimes regarded as non-cellulolytic enzyme because it is not specific to β-1,4 linkages although it participates in the complete hydrolysis of cellulose. This enzymatic mode of cellulose hydrolysis was however postulated based on fungal cellulases and can be different with bacterial cellulases because most bacterial cellulases do not have all enzyme activities as illustrated in figure 2 (Person *et al.*, 1991; Coutinho *et al.*, 1993). They are some bacterial cellulases that have been shown to possess endoglucanase activity only or both endoglucanase and exoglucanase activity (Béguin, 1990).

All cellulases have modular structures with one catalytic domain (CD) linked to one or several non-catalytic modules called cellulose binding domains (CBD). These two domains are structurally and functionally independent (figure 3). The catalytic domain is responsible for cellulose hydrolysis of β-1,4-glycosidic bonds while CBD mediates the attachment of cellulases to cellulose. The non-catalytic module is involved in protein-carbohydrate (ligand binding) and protein-protein (cellulosomes) interaction.

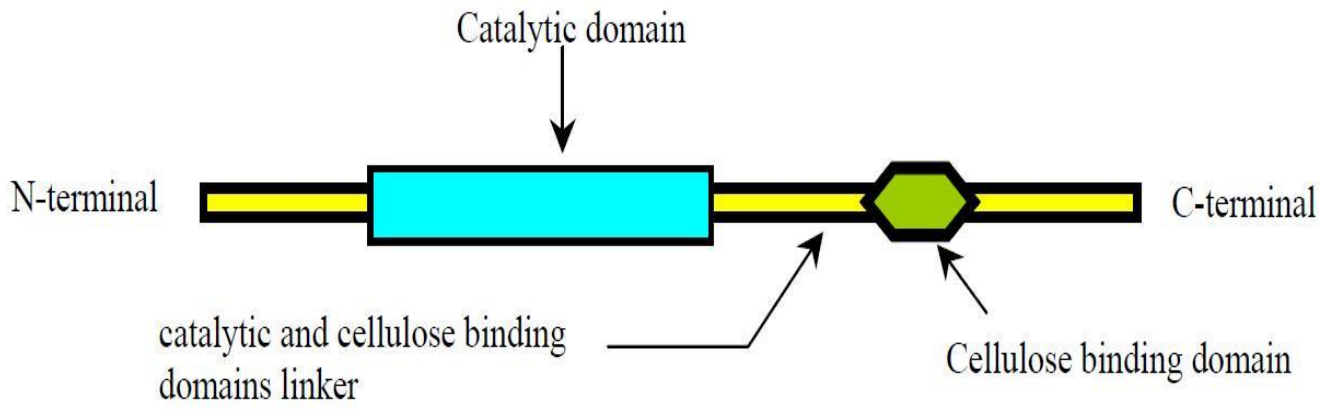


Figure 3: Architectural primary sequence organisation of cellulase domains of glycosyl hydrolase family-5A.

CBD domains are ubiquitous and occur in glycosidic hydrolase enzymes. CBD domains show a wide spectrum of substrate binding properties that include cellulose, xylan, chitin, and cellulose derivatives. There are differences within CBD domains from different sources regarding affinities to substrates. All cellulase enzymes are structurally either all α-helix domains or β-sheet domains or a combination of α/β barrels. Endoglucanase family-5A glycosyl hydrolases, the class of the *Bacillus subtilis* CHZ1 endoglucanase which is under investigation, have $(\alpha/\beta)_8$ barrel folds and have two highly conserved Glu active site amino acid residues (figure 4). Enzymatic hydrolysis of cellulose microfibrillar structures by bacterial endo-β-1,4-glucanase enzyme is generally thought to be an acid/base catalysis mechanism promoted by Asp and/or Glu residues. There are 8 invariant amino acid sequences that are highly conserved on the active sites of bacterial endo-β-1,4-glucanases. These are 2 tryptophan, lysine, serine, 2 tyrosine, glutamate and histidine residues, see figure 4A.

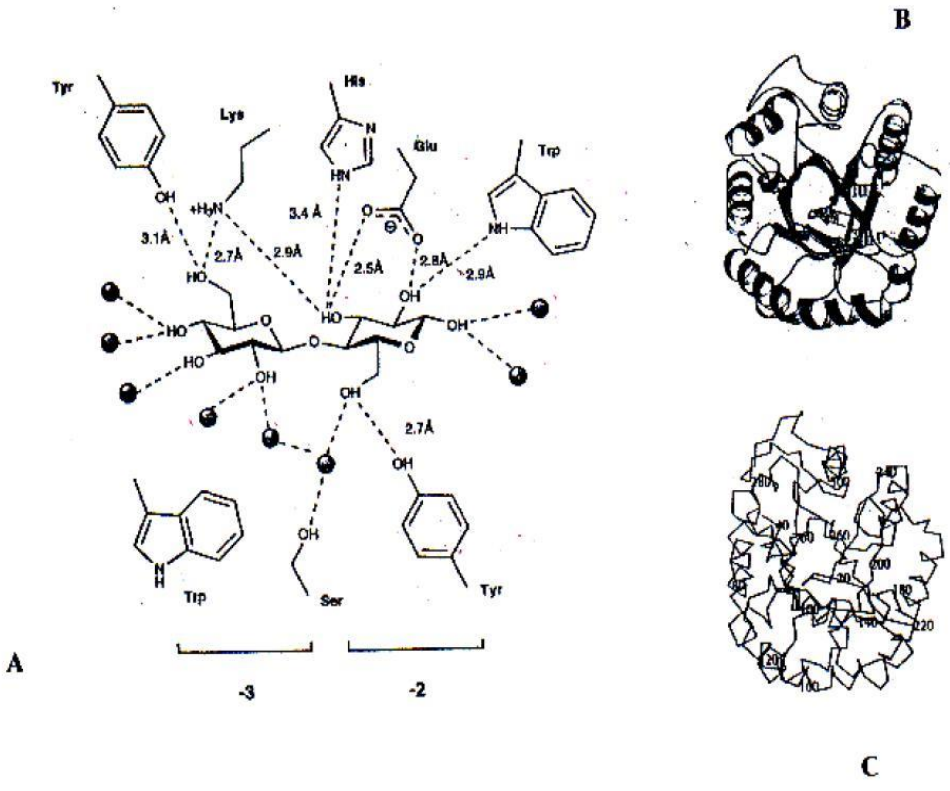


Figure 4: (**A**) Schematic representation of an endoglucanase enzyme interacting with cellobiose moiety of cellulose microfibril, (**B**) ribbon diagram of the enzyme showing the catalytic acid/base and nucleophile Glu residues positions and (**C**) its stereo C_α-trace. (adapted from Davies *et al.*, 1998)

The mode of stereochemical hydrolysis of β-glycosidic bonds of cellulose by cellulases proceeds by either a double-displacement catalytic mechanism (retention of configuration) or by a single-displacement catalytic mechanism (inversion) (figure 5). Protonic assistance will be provided by inverting enzymes (from an Asp residue) to a glycosidic hydroxyl group and then a catalytic base deprotonates a water molecule for nucleophilic substitution at the anionic centre of the leaving glycoside group. The inversion hydrolysis mechanism involves formation of a covalent glycosyl-enzyme intermediate. This reaction proceeds with the aid of an enzymatic nucleophile and a catalytic base/acid residue that facilitates formation of oxocarbonium ion-like transition states (Gilkes *et al.*, 1991; Varrot *et al.*, 2000; Schülein, 2000).

The cellulase enzymes are now grouped into 13 families (5-9, 12, 45 and 48) based on their CD and CBD amino acid sequence similarities and hydrophobic clusters (Henrissat & Bairoch, 1993; Linder & Teeri, 1997; Schülein, 2000). The families are also grouped according to either retention or inversion mechanism of cellulose hydrolysis.

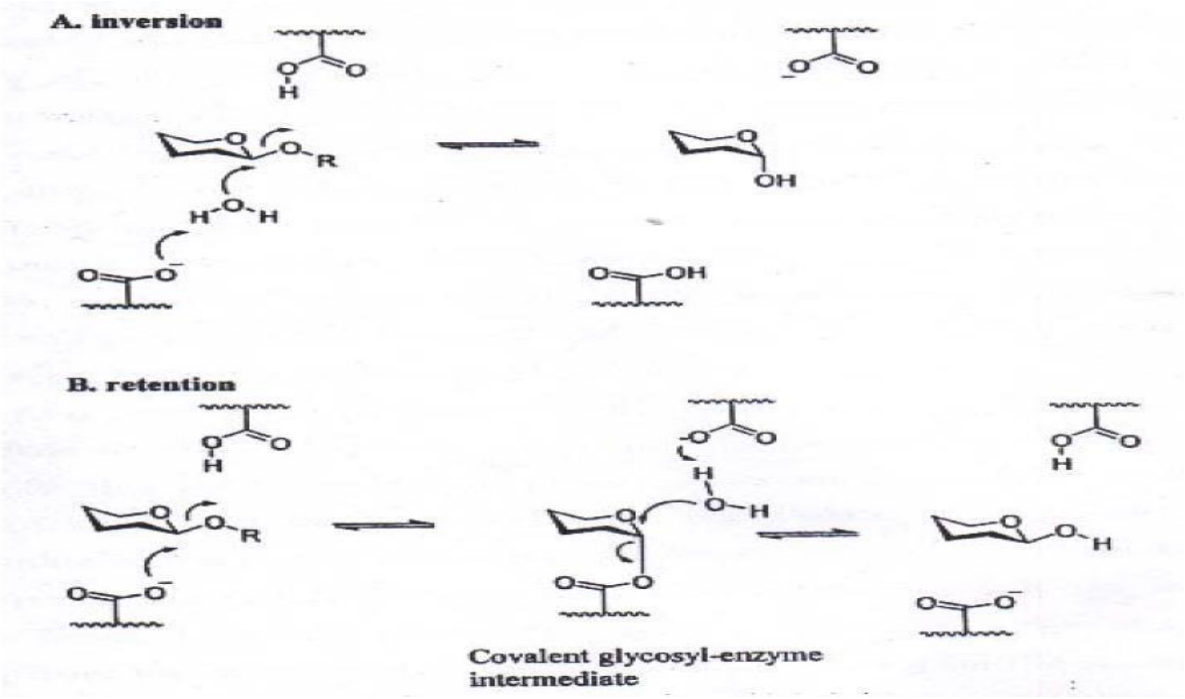


Figure 5. The proposed mechanisms of cellulose hydrolysis by glycoside hydrolases. (adapted from Schülein, 2000)

2.5 Cellulases in the industry

In the early 1970s the commercial production of cellulase enzymes by submerged fermentation, known to be has began with cellulose made by Trichoderma sold for use in research and pilot studies. The mid 1980s saw the first large industrial uses of cellulases for stonewashing denim and as an additive for animal feeds. This was accompanied by the introduction of commercial cellulases made by fungi of the genera Aspergillus, Penicillium, and most importantly Humicola.

2.5.1 Current industrial application of cellulases

2.5.1.1 Paper Industry

In paper production cellulases are used for various purposes like

(i) To decrease the viscosity of the processed material during the pulping process (bio-mechanical pulping) thus saving energy during refining.
(ii) To improve drainage of recycled fibers thus increasing the runnability of paper mills.
(iii) To release ink from fiber surface found in used paper material by partial hydrolysis (Bio-De-Inking).
(iv) To improve sheet-strength properties of the end-product, and
(v) To characterize fibers by selective solubilization of pulp carbohydrates.

2.5.1.2 Textile Industry

In textile industry they are extensively used in the bio-stoning of denim, as a sustainable alternative for stone-washing to gain faded, worn, aged appearance, by the careful removal of excess dye from the fabric. This is also used in softening the textile without fiber damage thereby minimizing the production of low-quality garments. Cellulases are extensively used in the production of environmentally friendly washing powders (Galbe, M.; Zacchi, G. 2002. 20, 151-159).

2.5.1.3 Beverage Industry

In beer brewing cellulase use is restricted to improve the brewing process starting form a poor quality barely. The main ole oc cellulases in this process is to avoid gel formation which cause poor filtration, slow run-off times, and low extract yields. In wine production cellulases are applied to obtain better skin degradation, improved color extraction, easier must clarification, better extraction, and improved quality and stability of the end product.

2.5.1.4 Animal Industry

Cellulases are widely used to supplement monogastric and ruminant feed. Their role is

(i) To eliminate anti nutritional factors present in grains and vegetables,
(ii) To degrade certain cereal compounds to improve the nutritional value of feed,
(iii) To improve feed conversion rate, and
(iv) To enable the utilization of less expensive feed components.

2.5.1.5 Fuel Industry

Ethanol Fuel Production: **A**pproaches developed in parallel for conversion of lignocellulose to ethanol are "acid based" and "enzyme based". Biomass hydrolysis, i.e. the depolymerization of the polysaccharides of the biomass to fermentable sugars is performed via environmentally friendly and economically feasible technologies. The enzyme based ethanol production gives an advantage over chemical procedure, because of its higher conversion efficiency, and the loss of substrate which occur during chemical modifications is less or nearly negligible and the use of more moderate and noncorrosive physical-chemical operating conditions. Atsushi *et al.* directly produced ethanol from barley β-glucan by shake yeast using *Aspergillus oryzae* β-glucosidase and endoglucanase.

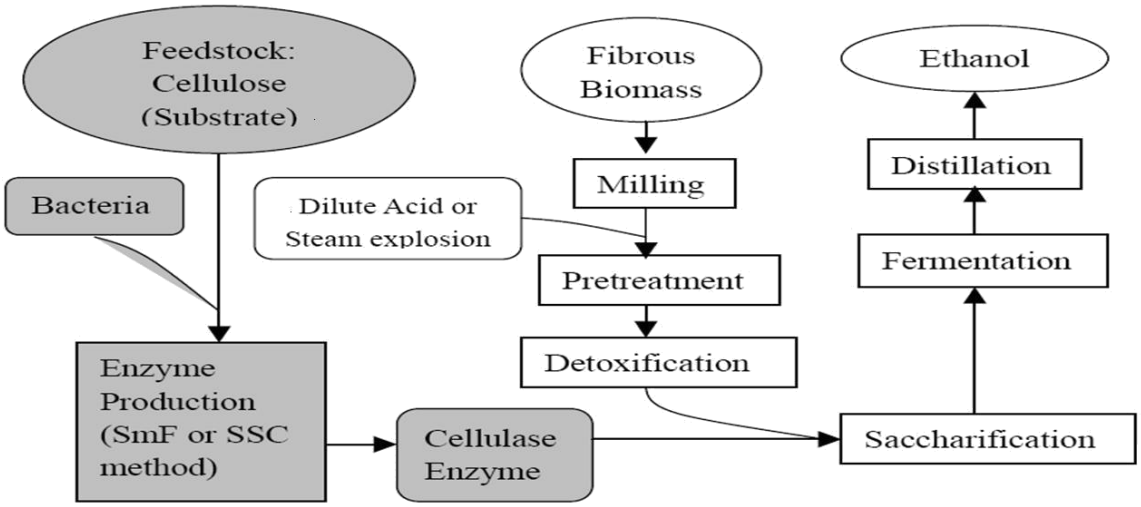


Figure 6: Enzyme production component within the ethanol production.

Enzyme	**Temperature range (°C)**	**Bioconversions**	**Applications**
α-amylase (bacterial)	90-100	Starch to dextrose syrups	Starch hydrolysis, brewing, baking, detergents
α-amylase (fungal)	50-60	Starch to dextrose syrups	Production of maltose
Pullulanase	50-60	Starch to dextrose syrups	Production of glucose syrups
Xylanase	45–65, 105a	Craft pulp to xylan+lignin	Pulp and paper industry
DNA polymerase	90–95	DNA amplification	Genetic engineering/PCR
Protease	65–85	Protein to amino acids and peptides	Baking, brewing, detergents, leather industry
Lipase	30–70	Fat removal, hydrolysis, interesterification, alcholysis, aminolysis	Dairy, oleo chemical, detergent, pulp, pharmaceuticals, cosmetics and leather industry

Table 2: Bioconversion reactions and applications of thermostable enzymes

2.6 THERMOPHILIC BACTERIA AND THEIR ENVIRONMENTS

The work of thermopiles, as we know it today, was established by Thomas Brock through his extensive and pioneering studies in Yellowstone National Park (Wyoming, USA) from 1968 to 1978. It is more than 30 years until now since he discovered *Thermus aquaticus* which was the first isolated organism that had shown grow above 70 °C. Ever since, thermophiles and extremophiles, in general, have attracted a constantly increasing level of interest. Before 1970, several thermophiles had been isolated that could grow up to 70 °C, and much research had been done on strains of *Bacillus stearothermophilus*, including the isolation and study of thermostable enzymes.

Brock also isolated may other bacteria and archaea like *Sulfolobus acidocaldarius* and *Thermoplasma acidophilum*. The discovery of archaea as a third domain of life, in addition to eukarya and bacteria, was a major scientific event. The next big wave in thermophile research started in 1981 when Karl Stetter and Wolfram Zillig isolated *Thermoproteus*, the first anaerobic, extreme thermoacidophile, from Icelandic hot springs. Since then, several new species and genera have been isolated, including the landmark discoveries by Karl Stetter and his colleagues of *Pyrodictium*, the first organism to grow optimally at 105 °C, and *Pyrolobus*, which has a maximum growth temperature of 113 °C—the highest growth temperature yet recorded for any living organism.

The research topics of thermophily are still expanding into ever more diverse fields, but it is molecular-biology methods, including whole genome sequencing and bioinformatics, that have had the most profound impact on our current understanding of thermophily.

2.6.1. Thermophily and Geochemical History

Evolution has since led to extensive diversification in microorganisms. The present-day microbial diversity is so great that it is now generally accepted that we have yet only explored a small fraction of it, and much discovery work still remains ahead.

Nowadays we can put together a set of strong arguments for the hypothesis that the first living organisms on earth were thermophiles. There is no geological evidence that argues against this. In fact, some geologists now feel that even 3 billion years ago, it was much hotter than it is now. Furthermore, we can state that hot springs have always existed on

earth, and they may have been much more widespread in the past than they are now. It is, therefore, also conceivable that life originated in a hot spring. The reduced chemical conditions in the hot soils of present solfatara fields probably correspond well with those of the early earth .

In the context of this discussion it is interesting to note that concurrent with increasing biological complexity there is a stepwise lowering of the maximum growth temperature for the organisms (see Table 1). The most thermophilic archaea are fermentative, sulfur-respiring, microaerophilic, or tolerant to extremely low oxygen tension; the most thermophilic bacteria have similar metabolisms (Alfredsson G.A et al;). We first find fully aerobic metabolism among the extremely acidophilic archaea and among the more common bacterial thermophiles, such as *Thermus*. Anoxygenic and oxygenic photosynthesis first occurs in bacteria at 70–73 °C. The first heterotrophic eukaryotes are at 60–62 °C, but the highest temperature for photosynthetic eukaryotes is at 55–58 °C, and, finally, for more complex eukaryotes, at 45–50 °C.

Temperature is certainly the most important variable in the environment. The classification of living organisms based on their relation to temperature has, therefore, always been considered as one of the most basic elements of biological classification and systematics. Organisms that grow optimally at 45–55 °C are ubiquitous, but those with an optimum temperature above 60 °C are, in general, associated with permanently hot places, such as areas of geothermal activity. Many thermophiles, in particular spore-forming organisms, can however be isolated from many nongeothermal areas, including solar-heated soils and manmade environments, such as composts and domestic or industrial heating systems. Microorganisms have traditionally been divided into three main groups in this respect: psychrophiles, mesophiles, and thermophiles. Brock suggested a definition of a thermophile boundary at 55–60 °C, based on two main arguments. First, temperatures below this boundary are common in nature, but higher temperatures are mainly associated with geothermal activity or some very special situations. Second, no eukaryotes are known to grow above this boundary; therefore, it would be an exclusively prokaryotic world. The thermophilic range above the boundary will then be exactly half the currently known temperature span of life (–10 °C to 113 °C).

Organism Group Name	Maximum Growth Temperature (°C)
Animal	
Fish	38
Insects	45-50
Plants	
Vascular plants	45
Bryophytes	50
Eukaryotic Microorganisms	
Protozoa	56
Algae	55-60
Fungi	60-62
Prokaryotes	
Cynaobacteria	70-72
Green bacteria	70-72
Bacteria	95
Archaea	113

Table 3: Maximum growth temperature for main groups of organisms

According to the above discussion we can assumes that life was first created at more extreme environments on the one hand and on the other hand life was created for relatively low temperatures, and evolved simultaneously into to form more complex structures.

2.6.2. Habitats and Ecology

2.6.2.1. Diversity of Thermal Environments

Geothermal areas are extremely varied in terms of geology and chemistry, but they belong mainly to two categories: first category is solfatara type which is characterized with the presence of much sulfur, acidic soils, acidic hot springs, and boiling mud pots; another one is the neutral-alkaline type which is characterized by freshwater hot springs and geysers, which are neutral to a1kaline in pH. These two different classes of geothermal areas differed on the basis of heat source. Type one areas are called as high temperature fields which are primarily located within active volcanic zones and having a magma chamber at a depth of 2–

5 km as a heat source. Here the temperature is between 150–350 °C and emit steam and volcanic gases on the surface.

The type two areas are called as low temperature fields and are located outside the volcanically active zones. They are heated by deep lava flows or by dead magma chambers. The temperature is usually below 150 °C. Groundwater percolates into these hot areas and warms up and returns to the surface containing dissolved minerals like silica, and some dissolved gases, mainly CO_2 and also contains little H_2S. The pH of these areas is nearly neutral. This neutrality is due the presence of more water than sulphide so its surface oxidation has no effect on the pH (Huber R et al; 1995).

2.6.2.2. Energy Sources and Physiology

Since basic primary metabolism is common among all living cells we might assume that all metabolic types (i.e., all energy sources known to be utilized by living organisms) should be found in all thermal biotopes. This is, however, obviously not true since we know that some metabolic types (e.g., photosynthesis) are not found in some biotopes above a certain temperature limit.

If we then analyze the different thermal habitats in terms of the abundance of different energy sources available and what metabolic types of organisms are represented, we will get a picture of the main community structure in each type of environment. In Table 2, some examples are given for the organisms that were thought to be the most important or to be the best representatives of a particular group.

The freshwater hot springs consists of dense growth of phototrophs but only up to 72 °C. If the sulfide concentration in such springs is above about 60 µM it will inhibit growth of many bacterial spices like *Chloroflexus* and only a massive growth of sulfide-oxidizing bacteria, such as the mat-forming *Aquificales* species are seen. Even thay are many redox couples are utilized in the freshwater springs, several are bacterial identifications are still missing like thermophilic methane or ammonia oxidizers (Kristjansson J.K. (1992)).

Primary producers	Name of the organism	T max*	T opt*	pH opt
H2O/Light	Synechococcus lividus	73	65	8.0
H2S/Light	Chloroflexus auranticus	70	56	8.0
H2/ O2	Hydrogenobacter thermophilus	77	72	6.8
H2S /O2	Thermocrinis ruber	89	80	7-8.5
H2/CO2	Methano bacterium	75	65	7.4
H2/SO4	Desulfovibrio thermophilus	85	65	7.5
S/O2	Sulfolobus acidocaldarius	90	75	2.5
H2/S/O2	Acidianus infernus	96	90	2.0
Algae	Cyanidum caldarium	50	40	1-4

Table 4: Community structure in acidic solfatara fields and in freshwater, alkaline hot springs. * Where T_{max} and T_{min} correspond to maximum and minimun temperatures , respectively.

The discussion regarding temperature gradients and leaching of organic material from external phototrophs into the acidic hot springs also applies for acidic springs, although no phototrophs are found growing above 60 °C under acidic conditions. The contribution of photosynthesis in the acidic springs is small but other energy sources like sulfide/sulfur and H_2, are abundant and support good growth of aerobic and facultative anaerobic autotrophs.

2.7 MODE OF THERMOPHILIC ADAPTATIONS

Recently studies have been focussed on enzymology, physiology and molecular genetics of thermophilic microorganisms to elucidate their mechanisms of adaptation to extreme conditions of high or low temperatures and extreme pH values. The adaptation by these microbes to extreme conditions has allowed their enzymes to be exploited for industrial, commercial and pharmaceutical applications.

2.7.1 Morphology and physiology of thermophiles

Spring waters are short of nutrients, the microfloras of these niches have few microbes that can exploit and survive. The bacterial shapes have evolved in such a way that they can cope

with these environments i.e. bacterial cells are either small, cocci, or rods to ease transport of materials across their cell membranes. They have shown adaptations through modifications of their physiological systems, enzymes and cell membranes lipid structures to sustain these extreme habitat conditions (de Mendoza *et al.,* 1993; Horani & Priest, 1994).

2.7.2 Nature of cell membrane fatty acids and lipids of thermophiles

Thermophiles and extremophiles membrane structures should adapt to environmental changes that occur in order to maintain their cellular integrity the cell membrane lipids form simple to complex long chain diglycerol tetraethers in these microorganisms. The nature of fatty acids of various thermophilic microbes changes from temperature to temperature. Due to the structural, quantitative and qualitative nature of their cell membrane lipids and fatty acids biomembranes of the thermophilic microbe resulted in the rigid-to-fluid phase transition of. An increase in temperature resulted in a shift in the fatty acid synthesis of saturated type of fatty acids (Kaneda, 1991; Priest, 1993; Horan & Priest, 1994; Markossian *et al.,* 2000; Yumoto *et al.,* 2000).

Alkalophilic *Bacillus* species have branched, and long chain fatty acids that include pentadecanoic acid, heptadecanoic acid and hexadecanoic acid (iso-$C_{18:0}$) as major membrane fatty acid constituents. The lipid-compositions shift when there are changes in pH or temperature by thermophilic microbes had been observed to occur in *Bacillus* microorganisms (Kaneda, 1991 & 1997; Sturr *et al.,* 1994; Aono *et al.,* 1995; Nicolaus *et al.,* 1995; Markossian *et al.,* 2000; Yumoto *et al.,* 2000). An increase in temperature results in an increase in the amount of high melting point iso- fatty acids and a decrease of the low melting point anteiso- fatty acids particularly iso-$C_{17:0}$. The cell envelope of thermophilic bacteria is generally a composition of cytoplasmic lipid membrane peptidoglycan wall and a proteinaceous surface layer. Additional functional surface inclusions like capsules, slimes, fimbriae and flagella may be present. The cell wall in many other gram-positive bacteria is composed of peptidoglycan and one or more anionic polymers. The synthesis of phospholipids and glycolipids is a membrane bound pathway in *Bacillus* genus. The major lipids are phosphatidylethanolamine, phosphatidylglycerol, lysine esters of phosphatidylglycerol and a small amount of cardiolipin (diphosphatidylglycerol) (Rothman

& Kennedy, 1977). In *B. subtilis* strains diglucosyldiglyceride, monoglucosylglyceride and neutral 1,2-diglycerides are the major lipid components (Bishop *et al.*, 1975).

2.7.3 Extremophiles with novel lipid adaptations

The membrane lipid structure and biosynthetic pathways are quite different from thermophiles producing different lipids. Their glycerolipids lack the usual ester linkages between glycerol backbone and fatty acyl moiety. Instead of ester bonds there are ether linkages. Ether linkages are quite stable and can withstand high temperatures and pH. A notable feature with extremophiles lipids is the presence of the isoprenoids diethers and tetraethers. The tetraether and diether molecules have phytanyl side chains that are covalently bonded to the glycerol-like backbones or substituted four-carbon or branched nine-carbon polyol structures called tetriol and nonitol (Madigan *et al.*, 1997; Priest, 1993). Cell membrane lipids of thermophilic microorganisms have free hydroxyl groups that are sometimes phosphorylated, glycosylated or sulphated. Extreme thermophiles are known to have lipid structures of compounds like squalene, hexaisoprenoids, (C-30), pentaisoprenoids, (C-25) and tetraisoprenoids, (C-20).

2.8 Cellulase Producing Thermophiles

2.8.1 Cellulolytic Fungi

The best source considered for the cellulases is the cellulolytic fungus *Trichoderma* sp., however, the major problem with *Trichoderma* cellulases is that they produce a very low β-glucosidase activity in culture supernatants and the enzyme is also subject to product inhibition. Mandels observed that some species of thermophilic fungi degraded cellulose rapidly but their culture filtrates had low cellulase activity (Fracheboud, D 1989, Rao, U. S 1988 Kawamori, M 1987). This was contradicted by reports that the thermophilic fungi

Sporotrichum thermophile and *Talaromyces emersonii* produced cellulase activity nearly comparable to that of the mesophilic fungus *Hypocrea jecorina* (anamorph *Trichoderma reesei*). Characterization of *Hypocrea jecorina* (anamorph *Trichoderma reesei*) cellulase and xylanase promoters has recently been evaluated by Rahman *et al.* Cellulolytic rates of some thermophilic fungi *Chaetomium thermophile*, *Sporotrichum thermophile* and

Thermoascus aurantiacus has been observed to be two or three times greater than that of *Trichoderma viridae*. Chellapandi and Jani studied on production of endoglucanase by the native strains of *Streptomyces* isolates in submerged fermentation in Brazil.

In general, crystalline cellulose has been found to be a superior carbon source for cellulase production in thermophilic fungi than its amorphous or impure forms except *Thermoascus aurantiacus*, *Humicola insolens* and *H. grisea* var. *thermoidea* which showed high cellulase and xylanase activities even on hemicellulosic substrates without cellulose (Knapp, J.S. (1985), Mandels, M. (1975),et al; 1978). Like the mesophilic fungi, the thermophilic fungi produced multiple forms of the cellulase components. (Knapp, J.S. 1985, Folan, M.A 1978, Rahman, Z 2009, Tansey, M.R. 1971).

2.8.2 Cellulolytic Bacteria

In nature, fungi tend to produce more cellulases than bacteria, however, cellulases produced by bacteria are better catalyst as they encounter less feedback inhibition. Bacteria have the capacity of growing in wide variety of environmental niches like high temperature, alkaline pH, and acidic pH help these strains to produce biocatalysts which are stable under harsh conditions found in increasing the rates of hydrolysis and finally efficient product recovery at the time of the bioconversion processes. The bacterial cellulases have very high activities against crystalline celluloses like cotton or avicel and are also more thermostable. The cellulolytic property has been reported in *Bacillus* strains and in thermophilic anaerobic bacterium *Clostridium thermocellum* (Bon, E.P.S 2002, Macedo 1999, Acharya, S 2011, Mori 1992, Mawadza, C 2000)

Fangdong isolated thermophilic cellulolytic bacteria by using three different kinds of medium and determined its enzyme activity. Acharya and Chaudhary also isolated thermophilic cellulolytic *Bacillus* strains from hot spring, India and reported 60°C for their optimal activity. From *Pyrococcus furiosus* and *Pyrococcus horikoshii*. *Sulfolobus solfataricus* MT4, *Sulfolobus acidocaldarius* and *Sulfolobus shibatae* different thermostable cellulases of archaeal origin showing optimal activity at 102- 105°C have been isolated have also shown to produce significant amount of β-glucosidases . Highly thermostable cellulases acting at 95°C, pH 6.0 and 7.0 has been reported from *Thermotoga maritima* MSB8. The other species of the same organism i.e. *Thermotoga* sp. FjSS3-B1 also produced highly

thermostable cellobiose which was active at 115°C at pH 6.8-7.8. Endocellulase, with the ability to hydrolyze microcrystalline cellulose, was isolated from the extremely thermophilic bacterium *Anaerocellum thermophilum* and maximal activity of this enzyme was observed at pH 5.0-6.0, and at temperature 85-95°C (Zerbini 1999, Zverlov, V et al;1998).

2.9 Cellulase Production Costs and Challenges

The search for the environmental friendly technologies in different industries and green fuel has renewed the interests in cellulases. Even through the enzyme cost is considered as the major impediment in extensive commercialization of it. They are more advantages in production of these enzymes by microorganisms rather than the conventional chemical techniques, because microorganisms can be produced the cellulose enzymes in ample amounts by established fermentation techniques. The yield of cellulase depend on various factors such as inoculums size, carbon source, cellulose quality, pH value, temperature, presence of inducers, medium additives, aeration and incubation period, etc.

Ghosh and Ghosh had studied the relationship between cellulose productions and growth conditions. In order to commercialize an enzyme successful, yield of 1100 FPUL-1h-1(FPU= Filter paper unit) is required and this yield can be obtained from the culture with a growth rate of 70g L-1h-1. This growth rate can be achieved if the microorganisms show any one or both of the following properties:

i) A high enhanced capacity for cellulase production.
ii) An ability to produce enzymes with a high specific activity.

Not only the nature and composition of lignocellulosic substrates but also the sugars present in medium also affect the cellulase biosynthesis. Acharya *et al.* studied the optimization for cellulase production by *Aspergillus niger* using saw dust as substrate and different fungal species producing cellulases were used by Khan *et al.* for bioconversion of rice straw. Ojumu *et al.* reported on cellulase production by *Aspergillus flavus* by using saw dust, bagasse and corn cobs as substrates. The other substrates which also can be used are corn cobs, wheat straw, sugarcane bagasse, aspen wood and waste from newspaper industry.

Cellulase is an inducible enzyme in order to reduce the cost of production of cellulases, cheaper carbon and nitrogen sources are desired. Now a day's agricultural and other waste

residues are used for the production of cellulase, but due to its high cost of the utilization process, the commercialization of this technology has been hampered. The biomass needs pre-treated in order to expose the cellulose fibre to the enzymes and high concentrations of enzymes are required which accounts for more than 50% of the ethanol production cost. The three different approaches proposed to reduce the cost of cellulase:

(i) Improve on-site production of cellulose.

(ii) Optimized reconstitution of cellulase components from different sources into a more effective artificial cellulase system.

(iii) Development of improved ethanologenic biocatalysts which supply a portion of the cellulase needed for the direct microbial conversion of cellulose into ethanol.

These improved enzymes must show different characteristics, such as higher catalytic efficiency, increased stability at high temperatures, and certain pH and higher tolerance to end-product inhibition. For lowering the cost of cellulose production different strategies like development of recombinant organisms, metabolic engineering and native engineering, etc. have been implemented (Figure 7). It is well recognized that the viability of biomass ethanol economically depends on the cost of enzyme contribution.

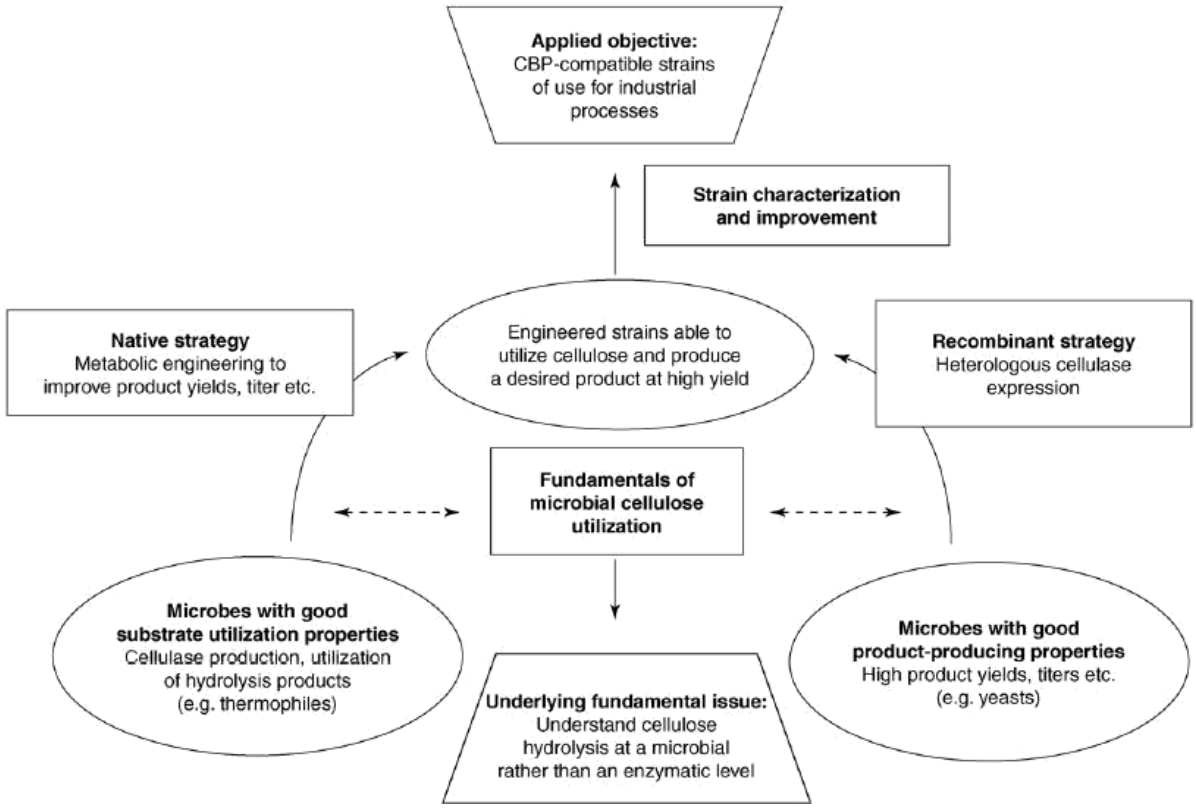


Figure 7: Organism development strategies and related fundamentals Organism development strategies and related fundamentals

2.10 Cellulase Market

A lot of industrial enzymes (60%) are produced in Europe, whereas the remaining 40% are produced by United States and Japan. Presently, the market of the world enzyme is estimated to be worth US$ 4 billion, in which approximately 60% are attributed to industrial enzymes, with a rising tendency of 5.7% per year. In this hydrolases represent 75% of the industrial enzymes and carbohydrases takes the position of second largest group of industrial enzymes. Cellulases demand up to 8% of the world's industrial enzyme market. During the period 2004-2014 a 100% approximate increase in use of cellulases as a special enzyme has been projected. Countries such as China, India, South Korea and Taiwan, which recently emerged as industrialized manufacturing centers with strong national research and development programs, are playing a much larger role in the world market. The largest supplier of enzyme Novozymes has already obtained a 40% reduction in cellulase enzyme costs and now they can supply enzymes at a cost of US$0.5 per gallon of ethanol produced. At this price the conversion processes has started looking attractive. However, for the support of robust biorefinery industry economically a further decrease is still necessary to

US$ 0.10/gallon (or 0.026/L). Data generated from the industries indicate that the present industrial enzymes costing up to US$ 2.24/gallon (US$ 0.59/L). The capital costs can be lowered by different routes of improving the enzyme efficiencies which involves the development of enzymes with more heat tolerance and higher specific activities, and better corresponding enzymes for different plant cell-wall polymers.

AIM OF THE PRESENT STUDY

The aim of our study is to isolate and study the bacterial ecology of hot spring and to isolate those bacteria that are capable to degrade cellulose and lignocellulosic biomass and to characterise those bacteria for the use in industries.

Chapter 3

Materials and Methodology

3.1 Materials

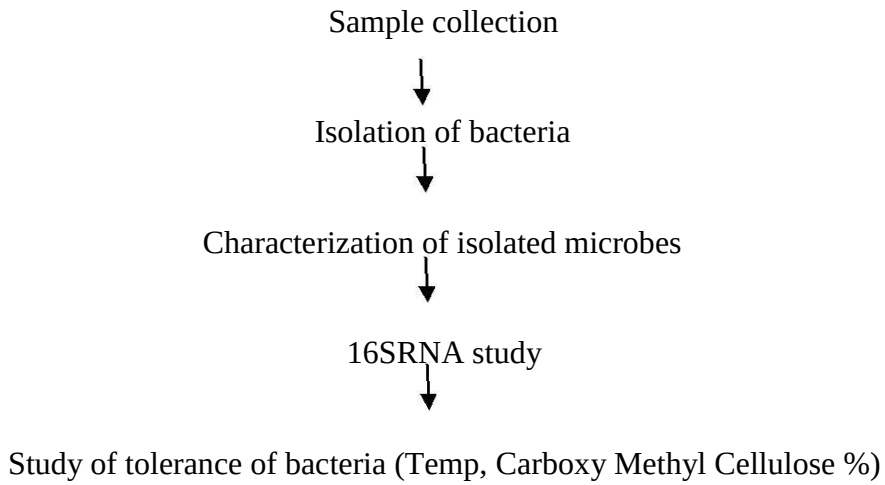
3.1.1 Sampling

Collection of Water and Soil Samples

In Himachal Pradesh of India, - Tattapani (Distt. Mandi), the hot water springs have unique physical location. Tattapani is located 31° 13' 50" to 32° 04' 30" North and 76° 37' 20" to 77° 23' 15" East is one of the popular hot water spring pockets which are scarcely being explored for their microbial diversity. Geographically, Himachal Pradesh is located in the region of western Himalayan. Tattapani hot spring is located near Sunni in district Mandi on the right bank of Sutlej River at an elevation of 625 m above sea level.. The pH of the sample location was taken with the help of pH strip and and the temperature of the sample location was taken with the help of thermometer and were recorded as pH 6.9- 7.1 and temperature is of 60 °C.

Figure 8 showing sample collection region

3.1.2. Overview of the project



3.2 Isolation of Bacteria

Thermophilic microorganisms were isolated directly from Soil and water samples by adding 1grm of soil and 1ml of water in Basic salt medium with Carboxy methyl celloulsoe as a carbon source (ref table 4 below) and incubated at 60 °C for 36 hours. For enrichment of samples, the samples were grown in the same basic salt media and incubated at 60 °C for 24 hours. Plates were constantly observed for the appearance of bacterial colonies. Single colonies with distinct morphology were selected from each of the plates and were purified by sub-culturing on the same selective media. 8 isolates namely B2, B3, B4 (isolated from the sample collected from base) R1, R2 (isolated from the sample collected from right side of hot spring) and cT2 (isolated from the sample collected from the water outlet) were obtained and selected for further studies. The purified bacterial strains were maintained at 4 °C and - 20 °C in Basic salt medium and 60% glycerol, respectively. The medium compositions are given in table number 5.

S.No	Name of chemical	Gram/lit
1.	K2HPO4	1.5 gm
2.	KH2PO4	3 gm
3.	MgSO4.7H2O	0.3 gm
4.	CaCO3 .2H2O	0.05 gm
5.	NaCl	0.5 gm
6.	NaHCO3	0.5 gm
7.	NH4Cl	1gm
8.	FeSO4	1 mg
9.	NH4Fe(III) Citrate	10mg
10.	MnSO4	5mg
11.	CoCl2.6H2O	1mg
12.	ZnSO4.7H2O	1mg
13.	CuSO4.5H2O	0.1mg
14.	H3BO4	0.1mg
15.	Na2MoO4.2H20	0.1mg
16.	Calcium pantothenate	2.5mg
17.	Thiamine-HCl	2.5mg
18.	Riboflavin	2.5mg
19.	Pyridoxine	0.1mg
20.	Yeast extract	5mg
21.	L-cysteine	0.1gm

Table 5 Showing the composition of media

The images of isolated cellulose degrading bacteria were given below

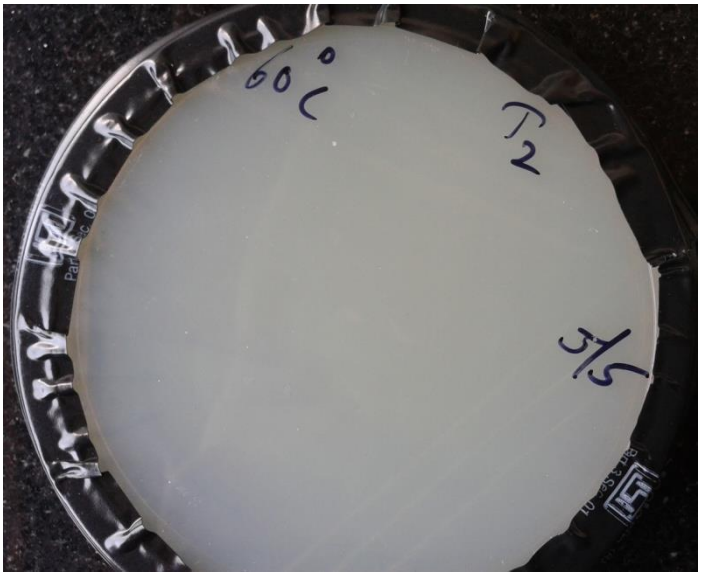


Figure 9 showing the growth of cT2 bacteria

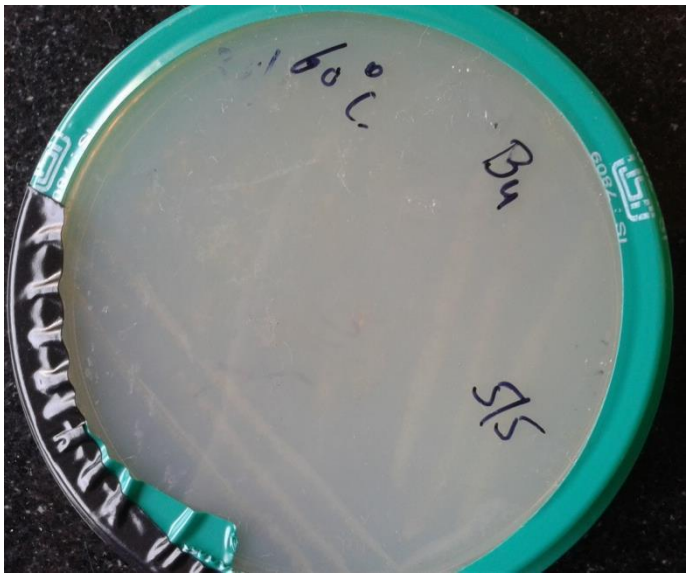


Figure 10 showing the growth of B4 bacteria

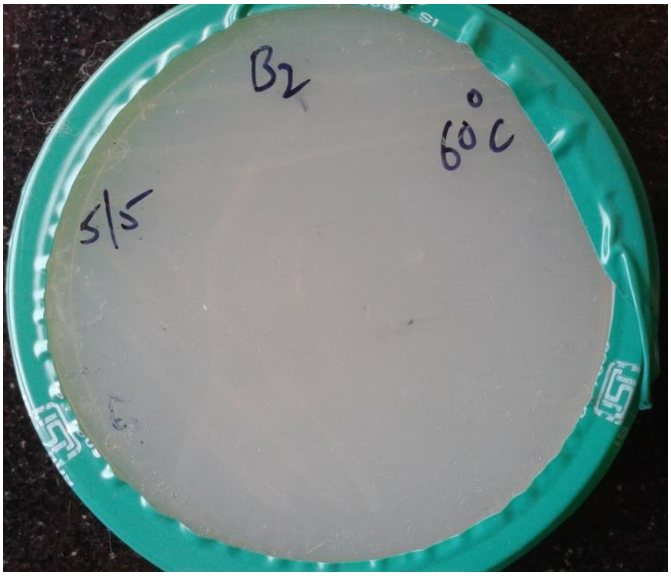


Figure 11 showing the growth of B2 bacteria

Figure 12 showing the growth of R1 bacteria

Figure 13 showing the growth of B3 bacteria

Figure 14 showing the growth of R2 bacteria

3.2.1 Morphological and Physiological Characteristics of Isolates

Morphological, cultural and biochemical characterization of pure cultures was done by applying standard techniques for bacteria. Isolated bacteria were identified by gram staining and KOH string test by using Bergey's Manual of Determinative Bacteriology

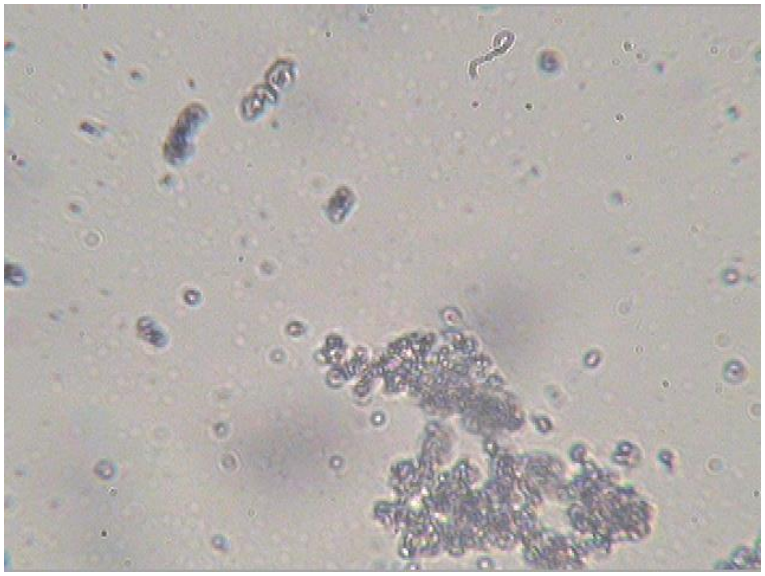


Figure 15 Showing stained B2 at 100X

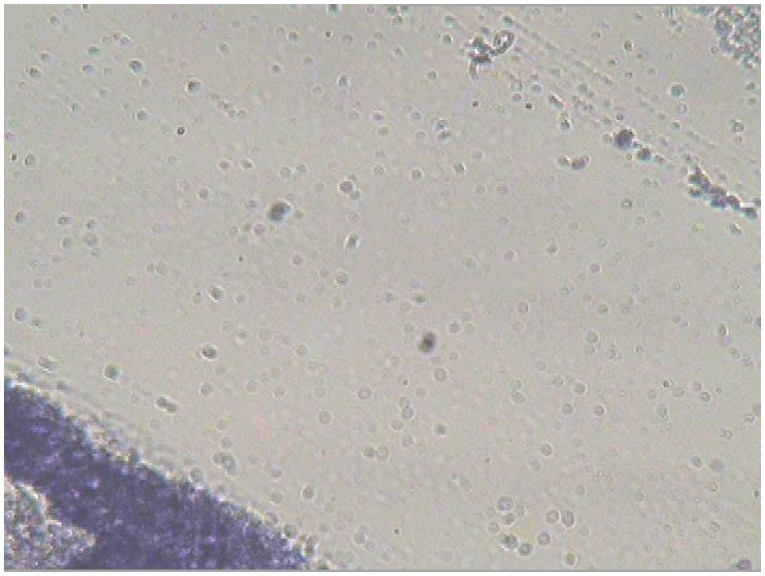


Figure 16 Showing stained B3 at 100X

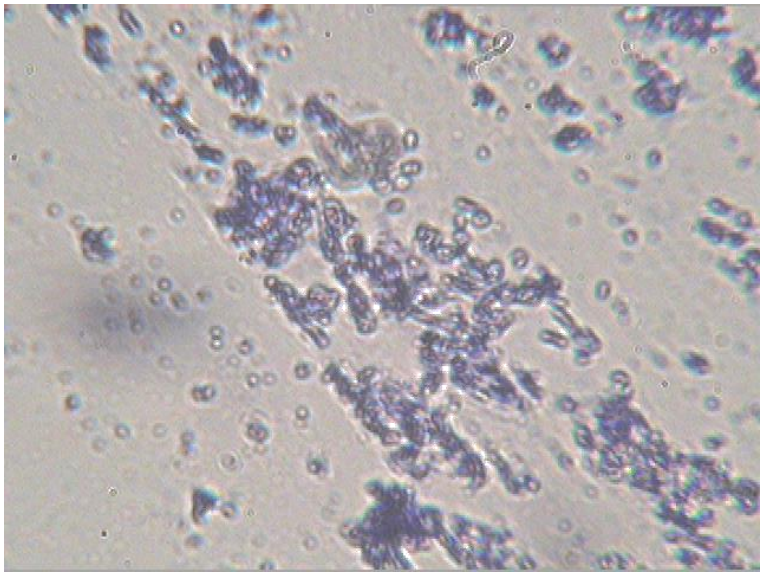


Figure 17 Showing stained B4 at 100X

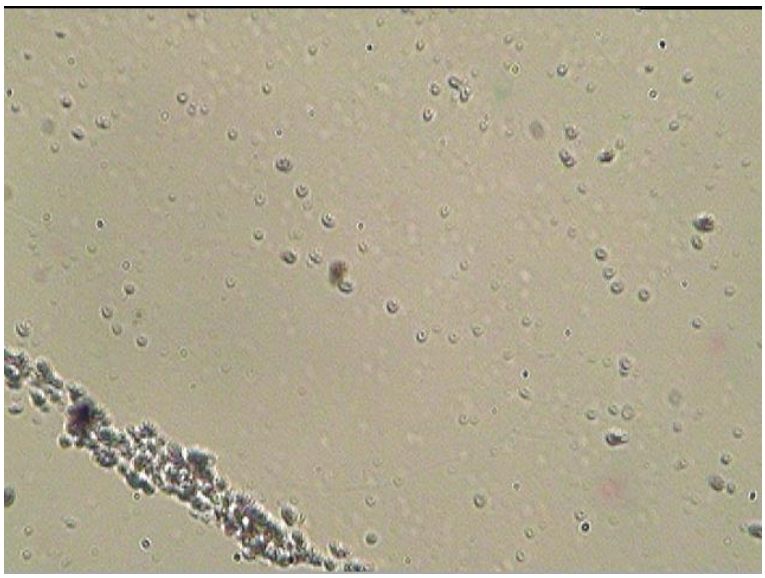


Figure 18 Showing stained R1 at 100X

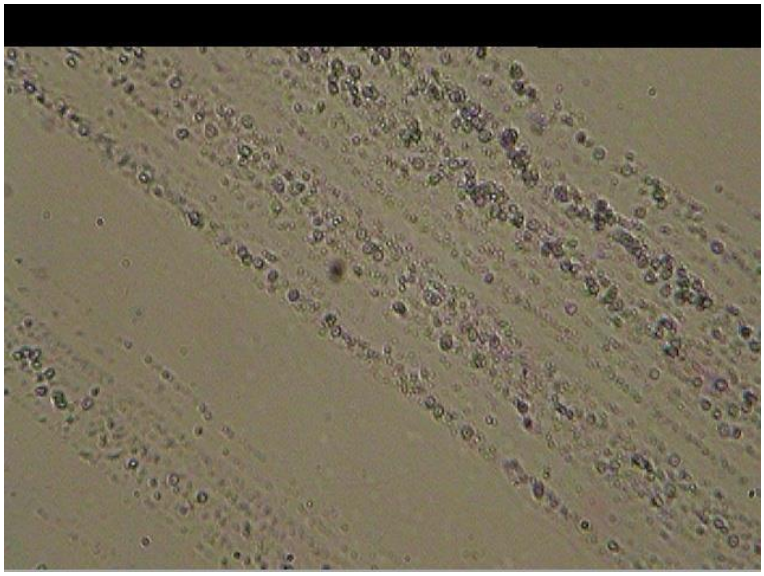


Figure 19 Showing stained R2 at 100X

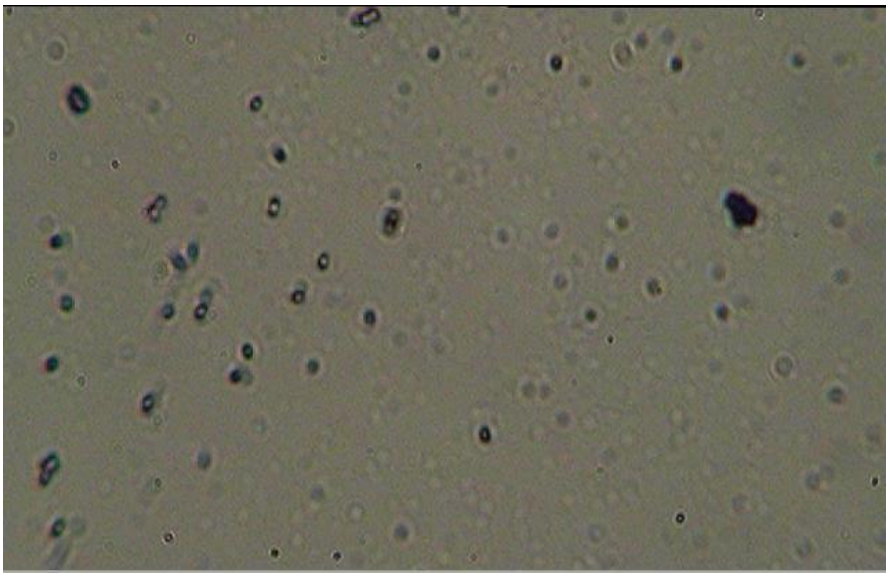


Figure 20 Showing stained cT2 at 100X

After Grams staining it was identified that all the bacteria are cocci shaped and gram positive in nature

3.3 Temperature tolerance

The isolated thermophiles are been verified for their growth at various temperature ranging from 40°C to 70°C. All the bacteria were growing at the temperature range of 50°C-60°C and for 40°C and 70°C some of the isolates are not growing

3.3.1 Growth of Bacteria at 40°C

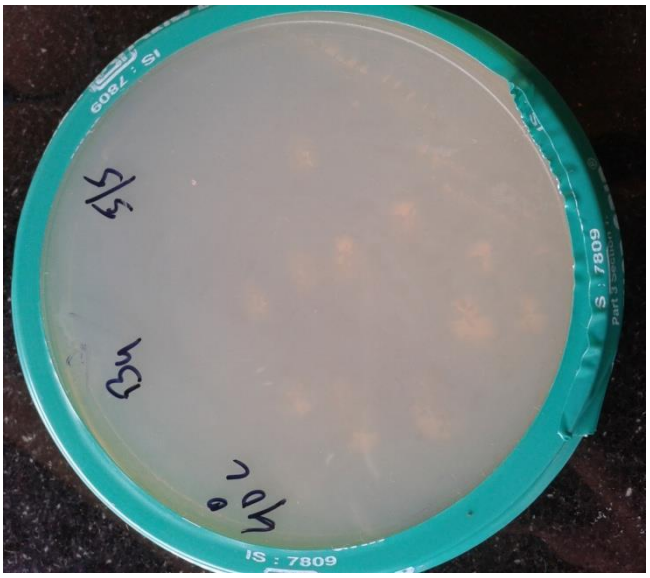


Figure 21 showing the growth of B4 bacteria at 40°C

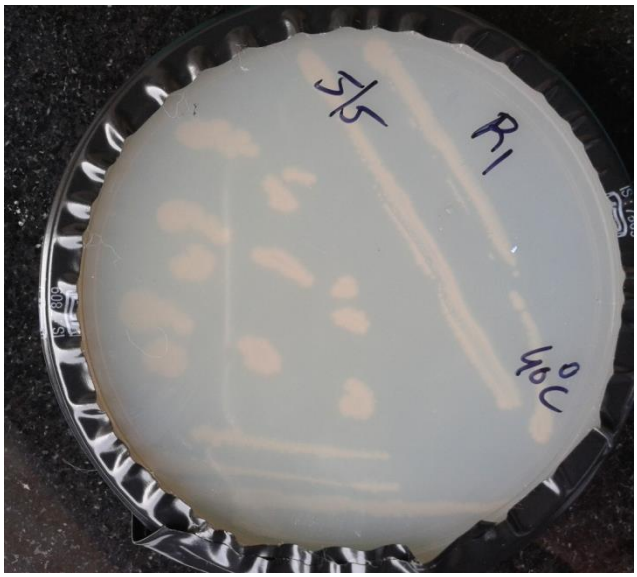


Figure 22 showing the growth of R1 bacteria at 40°C

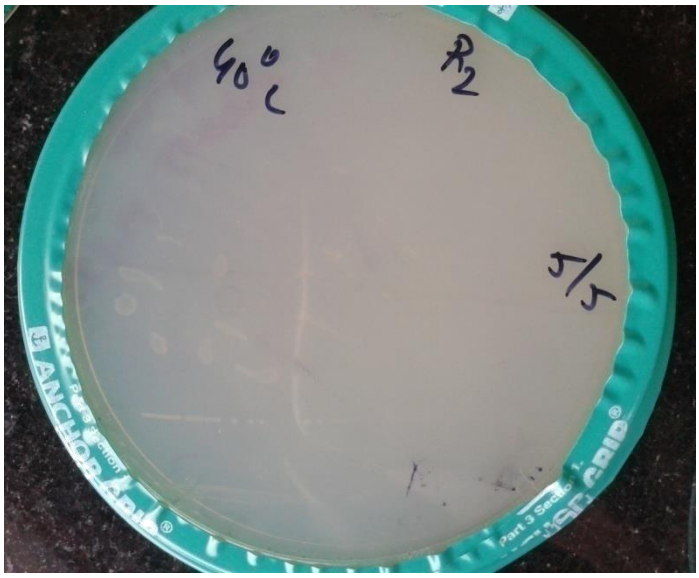


Figure 23 showing the growth of R2 bacteria at 40°C

Figure 24 showing the growth of cT2 bacteria at 40°C

Figure 25 showing the growth of B3 bacteria at 40°C

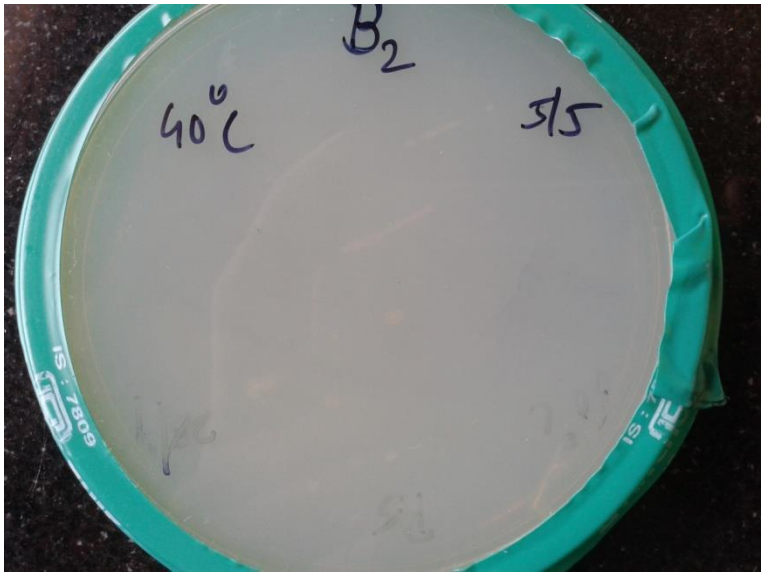


Figure 26 showing the growth of B2 bacteria at 40°C

Result Discussion: The bacteria isolated from top (outlet of water) sample (cT2) is unable to grow at this temperature, The reason cloud be as the sample location is the outlet of the water so the chances of temperature fluctuations are almost less even there is change in the surrounding climate**.**

3.3.2 Growth of Bacteria at 50°C

Figure 27 showing the growth of cT2 bacteria at 50°C

Figure 28 showing the growth of R2 bacteria at 50°C

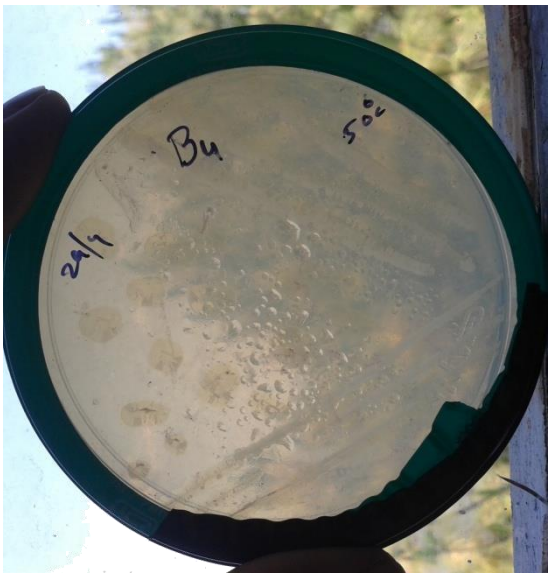


Figure 29 showing the growth of B4 bacteria at 50°C

Figure 30 showing the growth of B3 bacteria at 50°C

Figure 31 showing the growth of B2 bacteria at 50°C

Figure 32 showing the growth of R1 bacteria at 50°C

Result Discussion: All the isolated bacteria B2, B3, B4, R1, R2, cT2 are able to grow at this temperature.

3.3.3 Growth of Bacteria 70°C

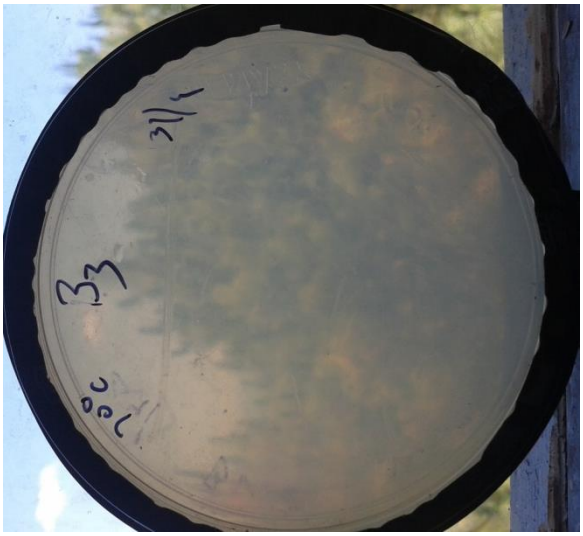


Figure 33 showing the growth of B3 bacteria at 70°C

Figure 34 showing the growth of B4 bacteria at 70°C

Figure 35 showing the growth of B2 bacteria at 70°C

Figure 36 showing the growth of cT2 bacteria at 70°C

Figure 37 showing the growth of R1 bacteria at 70°C

Figure 38 showing the growth of R2 bacteria at 70°C

Result Discussion: The bacteria isolated from Right side sample (R1, R2) are unable to grow at this temperature, the reason cloud be as the sample location if way from the point of outlet of water and that location is subjected to various fluctuations of temperature because of the change in the surrounding climate**.**

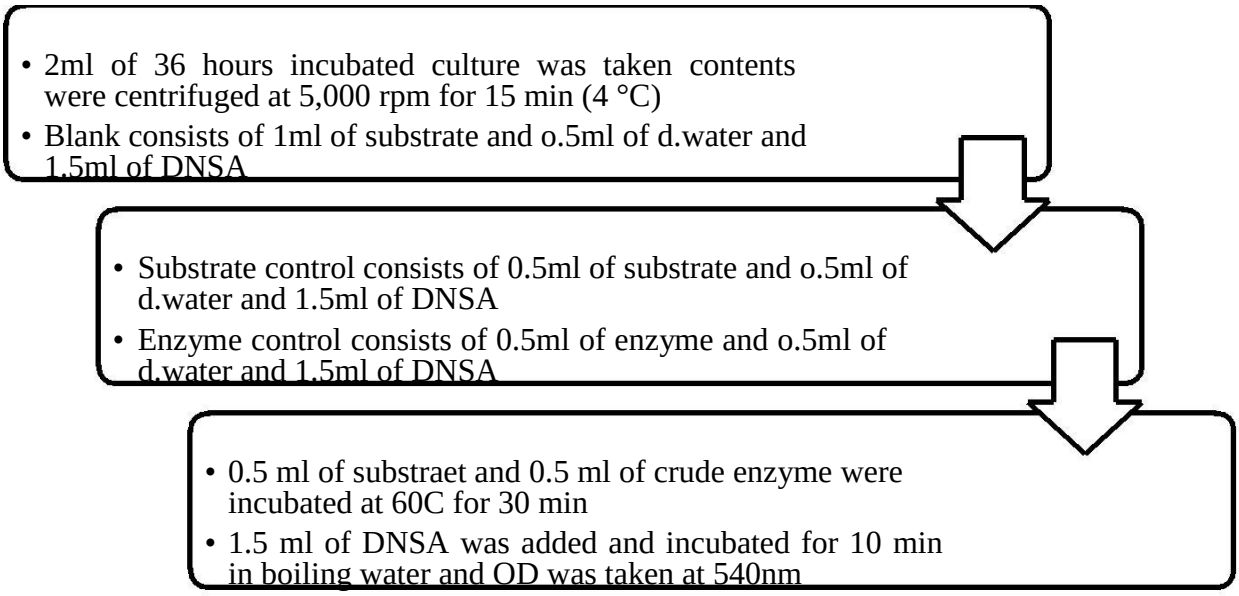
3.4 Production of extracellular cellulase

Each bacterial culture isolate was grown in 100ml of basic salt media with CMC as a carbon source. When the growth was observed in the broth, and the optical density reaches to 1.0, 3ml of inoculum was added to each 50 ml of basic salt medium broth containing 0.5% cellulose for cellulase in 250ml of Erlenmeyer flasks and the flasks were incubated for 2 days at 60±2 °C at 120rpm.

After the incubation at 60 °C for 36 hours, the culture contents were centrifuged at 5,000 rpm for 15 min (4 °C), supernatants were collected and quantitative tests were performed with the supernatants to screen out the hypercellulolytic producers among different isolates.

In order to find the activity of the cellulase produced by the bacteria 0.5% cmc which is 0.5 grams of CMC in 100 ml of distilled water was used and the protocol followed for the assay is as mentioned.

Protocol



Results of Cellulase activity

Table 6:

S.No	Name of Sample	Wavelength	Optical Density
1.	Substrate control	540 λ	0.0044
2.	Enzyme control for B2	540 λ	0.0135
3.	Enzyme control for B3	540 λ	0.0101
4.	Enzyme control for B4	540 λ	0.0184
5.	Test sample for B2	540 λ	0.0057
6.	Test sample for B3	540 λ	0.0908
7.	Test sample for B4	540 λ	0.0007

Table 7:

S.No	Name of Sample	Wavelength	Optical Density
1.	Substrate control	540 λ	0.0046
2.	Enzyme control for R1	540 λ	0.0102
3.	Enzyme control for R2	540 λ	0.0131
4.	Enzyme control for cT2	540 λ	0.0125
5.	Test sample for R1	540 λ	0.0026
6.	Test sample for R2	540 λ	0.0054
7.	Test sample for cT2	540 λ	0.0062

Discussion: The activity for the cellulase enzyme was done at 60°C as per the conditions at the sampling site. From the above results we can assume that the isolated bacteria are able to grow in the medium contain CMC as a carbon source but the cellulase produced by them doesn't have a good activity to degrade CMC.

3.5 Isolation of genomic DNA (Promega DNA Isolation Kit)

The pure culture of the isolated bacterial strains were inoculated in 50 ml of nutrient broth and grown at 60±2 ºC for 18 h. Isolation of total genomic DNA from the culture was carried out by the following standard procedure which was given below

Materials to Be Supplied by the User

1. 15ml of 18 hour incubated culture was taken into a 15ml tarson vial.
2. Centrifuged at 7000 rpm for 15 minutes to pellet the cells and the supernatant was discarded.
3. The cells were resuspended in 480µl of 50mM EDTA and 120ul of lysozymes (10mg/ml) was added to the resuspended cells
4. Incubated the sample vials at 37°C for 60 minutes. Centrifuged for 2 minutes at 13,000–16,000 × *g* and the supernatant was discarded.
5. 600µl of Nuclei Lysis Solution was added and gently pipeted until the cells get resuspended.
6. The sample vial were incubate at 80°C for 5 minutes to lyse the cells; then cool to room temperature and 3µl of RNase Solution to the cell lysate and inverted the tube slowly for 5 times to mix.
7. The sample vials incubate at 37°C for 60 minutes. After cooling the samples to room temperature 200µl of Protein Precipitation Solution was added to the RNase-treated cell lysate. Vortex vigorously at high speed for 20 seconds to mix the Protein Precipitation Solution with the cell lysate.
8. The sample vials were incubated on ice for 5 minutes and Centrifuge at 13,000–16,000 × *g* for 3 minutes.
9. The supernatant containing the DNA was transferred to a clean 1.5ml microcentrifuge tube containing 600µl of room temperature isopropanol and mixed gently by inverting the tubes until the thread-like strands of DNA form a visible mass.
10. Centrifuged at 13,000–16,000 × *g* for 2 minutes and carefully discarded the supernatant and drained the tube on clean absorbent paper.

11. 600µl of room temperature 70% ethanol was added and gently invert the tube several times to wash the DNA pellet and centrifuged at 13,000–16,000 × *g* for 2 minutes. The ethanol was aspirate carefully.
12. The tubes were drained on clean absorbent paper and allowed the pellet to air-dry for 20 minutes.
13. 100µl of DNA Rehydration Solution was added to the tube and rehydrate the DNA by incubating DNA containing vials at 65°C for 1 hour with periodical mixing tapping the tube.
14. Alternatively, rehydrate the DNA by incubating the solution overnight at room temperature or at 4°C and stored the DNA at 2°C.

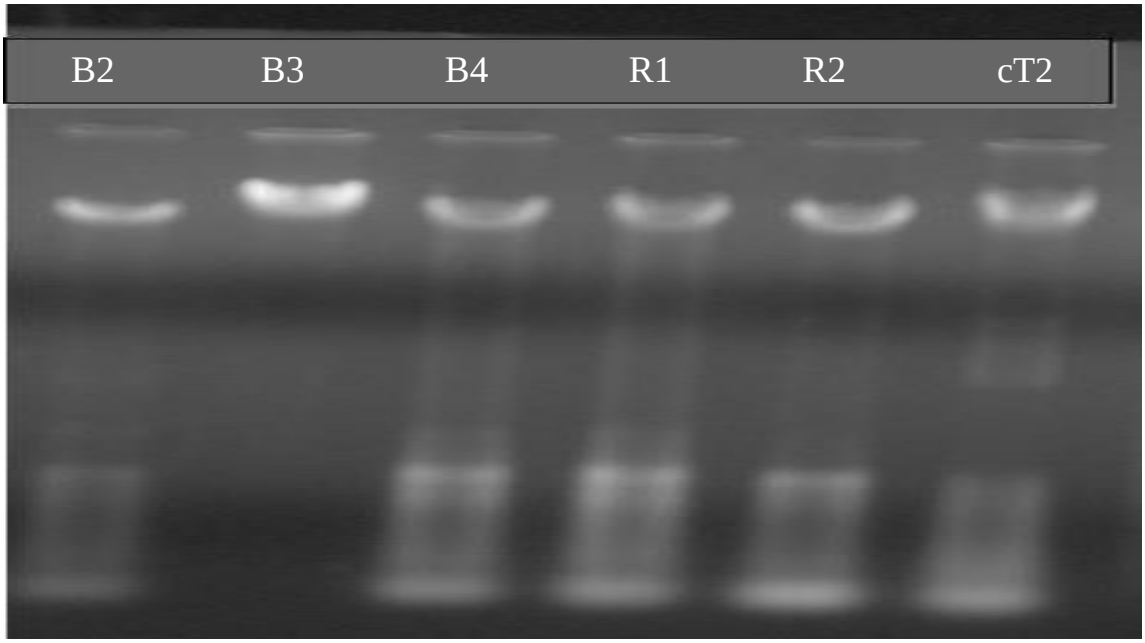


Figure 39 Showing the isolated DNA from B2, B3, B4, R1, R2, cT2

The DNA was run on 1% agarose gel with 1X TAE as buffer and 5ul of EtBr (10mg/ml) was added in gel for visualization of the DNA.

3.6 PCR amplification and sequencing

To confirm the identity of the bacterial strain PCR amplification was done, the genomic DNA was amplified the help of 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3') primers to get an amplicon size of 1500 bp.

Amplification was carried out in 25µl reaction volume consisting of

27F = 1.0 µl

1492R =1.0 µl

DNA = 1.5 ul

PCR master mix = 12.5 ul

Nuclease free water = 9ul

in a Applied bioscience thermalcycler. DNA template was amplified using PCR amplification reaction conditions specific for each primer set. The amplified product (25 µl) was size separated on 1.0% agarose gel prepared in 1X TAE buffer containing 0.5µg/ml ethidium bromide and photographed with the gel documentation system (alpha Imager 2200). A 1kb DNA ladder (NBE) was used as molecular weight size markers.

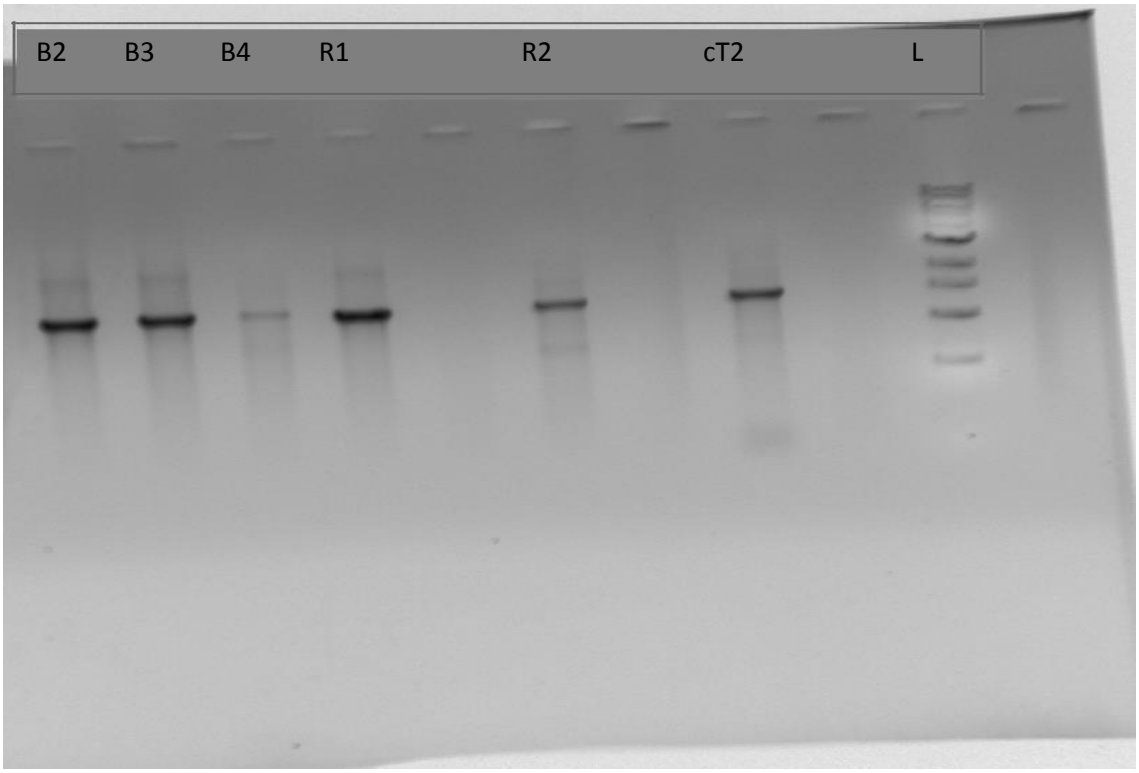


Figure 40 Showing the PCR amplification of B2, B3, B4, R1, R2, cT2

Chapter 4

CONCLUSIONS

After several years of continuous research on lignocellulosic biomass utilization, now it is considered that enzyme based technologies for biomass conversions are most efficient, cost effective and environment friendly. Good progress has been made in search of extremophiles, even their true diversity, has not yet been fully explored. Thermostable cellulases isolated from the lignocellulosic biomass degrading thermopiles have shown their potential under conditions that are appropriate for bioconversion processes which have role in industries. In summary the eight isolates were isolated from the Tattapani hot springs. No isolate was shown good activity for cellulase enzyme. On the basis of the structural identifition of bacteria we can assume that they may belong to Microccocus species For proper conformation 16S rRNA DNA amplification was done and the samples were send for sequencing.

The different isolated bacteria were able to grow at different range of temperature starting from 40°C to 70°C. The bacteria isolated from Base soil sample (B2, B3,B4) were able to grow from 40 °C to 70°C but the bacteria isolated from Right side soil sample (R1&R2) were unable to grow at the temperature of 70°C the reason cloud be as the temperature of the right side location fluctuate according to the surrounding climate so the temperature always goes less. The bacteria isolated from the top outlet was able to grow at the temperature range of 70°C-50°C and unable to grow at 40 C the reason could be that the temperature at the top is near almost constant so the bacteria were unable to grow at 40°C. A plethora of thermophilic enzymes with outstanding characteristics have not been translated into concrete applications because of the major restricting reason of the costs of the production process. Several studies have shown that the use of inexpensive media supplemented with various nitrogen sources can support microbial growth. The food wastes are so rich in nutrients such that there is a potential of producing secondary products although the end products might be compromised on the quality after using these undefined media.

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