Chapter 8
Lignocellulolytic Enzymes from Thermophiles

Vikas Sharma and D. Vasanth

Abstract Thermophilic microorganisms are considered as the important source for the production of novel enzymes for various industrial applications including degradation of lignocellulosic biomass. Bioprocessing of lignocellulosic biomass has gained significant attention for the synthesis of bio-based products by focusing on its three major components, i.e. cellulose, hemicellulose and lignin. Thermophiles (optimally grown at 60 ± 80 °C) obtained from hot springs are of great interest for providing novel thermostable enzymes that can catalyze under harsh conditions comparable to those existing in various industrial processes. Metagenomic studies helps in identifying lignocellulolytic enzymes with novel properties from the culturable and unculturable micro-organisms. In this chapter, the biotechnological significance of thermostable lignocelluloses degrading enzymes will be briefly discussed particularly cellulases, xylanases and laccases.

Keywords Extremophiles · Lignocellulosic biomass · Hotsprings Thermophiles · Cellulases · Xylanases · Laccases

8.1 Introduction

Microbial life is not limited to specific environments, some microbial communities can also withstand extreme pH, temperature, pressure and salinity conditions (Van Den Burg 2003). Such organisms are known as extremophiles. Extremophiles are further classified into different categories which include thermophiles, acidophiles, alkalophiles, psychrophiles, and barophiles (piezophiles) and others. These organisms transformed themselves to survive in immoderate places for example hot springs, sulfataric fields and deep-sea hydrothermal vents etc. Therefore, the enzymes obtained from these extremophiles can function under such conditions where mesophilic organisms cannot even survive (Demirjian et al. 2001). To date, few microbial

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communities have been explored (Van Den Burg 2003). The discovery of extremophiles and novel enzymes produced by them can contribute in the development of industrial processes (Demirjian et al. 2001). In a particular environment, nearly 10% of the organisms are cultivable, so metagenomics can play an important role in revealing these organisms which leads to the exploration of microbial diversity. Now it is possible to create gene expression libraries of microorganisms from extreme sources. The screening of these libraries with fast and precise detection technologies can discover numerous new extremozymes. Till now more than 3000 different enzymes have been explored and most of them are being used for biotechnological and industrial applications, still they are not sufficient to fulfill industry demands (Van Den Burg 2003). The main reason is that most of the enzymes are not capable to survive in extreme conditions of industrial processes. Thus, enzymes produced by the extremophiles have a great potential to be used in new bioprocessing techniques that are more specific, faster and ecofriendly.

8.2 Importance of Lignocellulosic Biomass

There is a requirement of utilizing renewable, economic and easily available biomass for the production of wide varieties of products and lignocellulose is the best suitable option existing (Turner et al. 2007). Lignocellulosic biomass contains three types of biopolymers i.e. lignin (25–30%), cellulose (35–50%) and hemicellulose (25–30%) (Wongwilaiwalin et al. 2010). Cellulose is the most opulent organic molecule present on earth and an essential component of all plant material, whereas hemicelluloses is the polysaccharides present in the plant cell wall (Turner et al. 2007). While lignin is a complex compound made up of complicated phenylpropane units that are nonlinearly and arbitrarily linked with each other. But the transformation of lignocellulosic biomass to fermentable sugars is a major task to utilize renewable resources. So many approaches including thermal, biochemical and chemical have been anticipated but none have proven to be adequate. There is a need for possibilities with new conversion technologies that are unaffected with the variation in feedstock and can face vigorous process-operating conditions (Blumer-Schuette et al. 2008) (Fig. 8.1).

8.3 Role of Thermophiles in Degradation of Lignocellulosic Biomass

In past two decades, thermophiles and thermostable enzymes have gain much importance, but the study on thermophilic mocroorganisms and their proteins started in the early 1960's by the revolutionary work of Brock and his colleagues (Turner et al. 2007). Thermostable enzymes are suitable for extreme processes, as high temperature

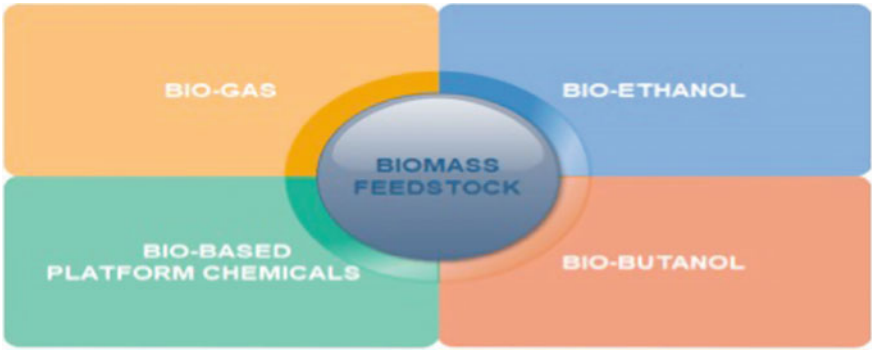

Fig. 8.1 Primary industrial biotechnology renewable product sectors supported by enzymatic release of lignocellulosic carbohydrates from biomass feedstock

often stimulates better enzyme penetration and cell-wall degradation of raw materials. Thermostable enzymes are produced by both the thermophilic and mesophilic organisms, but thermophiles are the more potential sources for such enzymes (Viikari et al. 2007). Extreme ecosystems such as hotsprings are of great interest as a source of novel extremophilic species, enzymes, metabolic functions for survival and biotechnological products (Saxena et al. 2017).

8.4 Sample Collection from Geothermal Areas

The images from Fig. 8.2a–d showcases collection of samples in the form of water, soil, rock mattings and pebbles from different sites of Tattapani hot water spring, India. These samples were placed in sterilized bottles and kept in an icebox immediately, then brought to the laboratory and stored at 4 °C in refrigerator till further processing. The temperature and pH must be measured at the time of sampling.

Thermostable enzymes obtained from thermophiles have numerous advantages over mesophiles in the degradation of lignocellulosic biomass e.g. (Viikari et al. 2007; Bhalla et al. 2013)

- the higher solvability of reactants and products, that result in higher reaction velocities thus reducing the quantity of enzyme required
- small hydrolysis period
- chances of contamination is less therefore, better productivity
- promotes restoration of evaporative compounds e.g. ethanol
- reduce the cost of power for cooling after thermal pretreatments.

The hydrolysis of lignocellulosic biomass with thermo-alkaliphilic and thermo-acidophilic enzymes could elude the neutralization phase during pretreatment (Bhalla et al. 2013). Many microbes that produced at extreme temperatures are capable of

Fig. 8.2 Sample collections from geothermal areas

utilizing a variable polysaccharides related to the transformation of lignocellulosic biomass to bioenergy (Blumer-Schuette et al. 2008). The microbial community that produces thermostable cellulases, xylanases and laccases are the most acknowledged micro-orgnisms involved in the bioprocessing of huge quantity of lignocellulosic material.

8.5 Thermostable Cellulases Obtained from Thermophilic Microbes

Cellulases (EC 3.2.1.4) are enzymes that catalyze the hydrolysis of β-1, 4 glucosyl linkages exist in the insoluble linear glucose homopolymer cellulose (Wilson 2009). Most commonly these enzymes are used in the degradation of lignocellulosic biomass and conversion of this biomass into fermentable sugar elements that can further used for the generation of other valuable products (Cerda et al. 2017). Recently, thermophilic bioprocessing techniques for bioconversion of cellulosic biomass have gained much attention, as these processes work at high tem-

perature (Rastogi et al. 2010). Considering the applications of cellulases, constant and functional thermostable cellulases would be more beneficial as compared to thermolabile enzymes in terms of time, cost reduction, and getting the appropriate product with desired yields/productivities (Franzén et al. 2017). Advancement in the proteomics, genomics, and fermentation strategies can contribute in searching more effective and unique thermostable cellulases obtained from thermophilic microorganisms of extreme environments. Most thermostable cellulases are isolated from either bacterial or fungal sources. Thermophilic bacteria are the most commonly reported source of cellulases. They have the capacity to directly ferment cellulose to ethanol and organic acids (Margaritis et al. 1986).

Thermophiles that can produce thermostable cellulase have been isolated from various hot springs around the world including Egypt, India, Thailand, Pakistan, China, Turkey and Sweden etc. These sources have harsh environmental conditions similar to those in industrial processes, so enzymes isolated from these microorganisms would be more feasible than other sources (Table 8.1).

8.6 Thermostable Xylanses Obtained from Thermophilic Microbes

Xylanases (EC 3.2.1.8) are enzymes that catalyze the hydrolysis of 1,4-β-D xylosidic linkages in xylan, therefore mainly responsible for the degradation of hemicelluloses component of the lignocellulosic biomass (Bhalla et al. 2013). Xylan requires various enzymes for its complete hydrolysis because of its complex structure, which are collectively termed as xylanases (Ellis and Magnuson 2012). Bacteria and fungi are major producers of thermostable xylanases. Thermostable xylanases produced by thermophilic bacterial strains are normally preferred for hydrolysis of lignocellulosic biomass over fungal xylanases because of their stability and better activity at elevated temperature (Viikari et al. 2007; Bhalla et al. 2015). Most of these processes require extreme conditions or extreme pre-treatment, which create a bottleneck for xylanase in industrial applications (Zhang et al. 2012). Study of extremophiles with metagenomics can further improves the understanding of xylanses to enhance its role in bioprocessing of lignocellulosic biomass (Walia et al. 2017).

Several xylanases has been produced from the thermophilic microbes isolated from geothermal areas around the world including hot springs of Argentina, China, Thailand, Japan, India, USA, Taiwan, Italy etc. Other sources of isolation of thermophilic micro-organisms are biogas reactor, local farm, mushroom compost, poultry compost, pulp samples, cow dung etc. (Table 8.1).

Table 8.1 Lignocellulolytic enzymes isolated from thermophiles

Name of the isolated organism	Extreme environment location	Temperature (°C)	MW(KDa)/pH	References
Thermostable cellulases from thermophilic microbes				
Bacillus sonorensis HSC7	Gorooh hot spring, Egypt	70	pH 4.0	Azadian et al. (2017)
Clostridium sp. DBT-IOC-C19	Puga thermal hot spring—Himalayan hot springs, India	55		Singh et al. (2017)
Bacillus subtilis J12	Hot spring soil and water sample (Thailand)	60	pH 6	Kuancha et al. (2017)
Bacillus amyloliquefaciens AK9	Hot spring of TattaPani, Pakistan	60	MW 47, pH 7	Irfan et al. (2017)
Geobacillus sp. HTA426	Hot spring of China	60	MW 40	Potprommanee et al. (2017)
Anoxybacillus kaynarcensis	Water and sludge samples from hot springs of Turkey	65		Baltaci et al. (2017)
*Bacillus licheniformis*WBS1, *Bacillus* sp. WBS3	Hot spring, India	60	pH 8 for rice and 9 for wheat	Acharya and Chaudhary (2011)
Thermoanaerobacter tengcongensis MB4	Tengcong hot springs (Yunnan, China)	75–80	MW 42.5, pH 6.0–6.5	Liang et al. (2011)
Bacillus subtilis DR	Hot spring water sample collected in YangLing, Shannxi province, China	50	MW 55, pH 6.5	Li et al. (2008)
Rhodothermus marinus	Alkaline submarine hot springs (Sweden)	95	MW 49, pH 7.0	Hreggvidsson et al. (1996)
Thermostable xylanases from thermophilic microbes				
Paenibacillus dendritiformis LT-4 and *Bacillus licheniformis* LT-5	Sediments, water, and biofilms (geothermal areas Argentina)	55	–	Cavello et al. (2017)

(continued)

Table 8.1 (continued)

Name of the isolated organism	Extreme environment location	Temperature (°C)	MW(KDa)/pH	References
Bacillus subtilis J12	Hot spring Water and soil sample from Sankamphaeng in Thailand	60	pH 5.5	Kuancha et al. (2017)
Anoxybacillus flavithermus TWXYL3 (*facultative Anaerobe*)	submerged plant material in the Mickey Hot springs area of the Alvord Basin, USA	65, retained up to 85		Ellis and Magnuson (2012)
Thermoanaerobacterium saccharolyticum NTOU1	Oceanic hydrothermal vent (Taiwan)	63	MW 50.0, pH 6.4	Hung et al. (2011)
Alicyclobacillus sp. A4	Hot spring(Yunnan Province, China)	55	MW 42.5, pH 7	Bai et al. (2010)
Acidothermus cellulolyticus 11B	Acidic hot springs in Yellowstone National Park (California)	90	MW 50, pH 6.0	Barabote et al. (2010)
Geobacillussp. MT-1	Deep-sea hydrothermal field in east Pacific (China)	70	MW 36, pH 7	Wu et al. (2006)
Bacillus thermantarcticus	Antarctic geothermal soil near the crater of Mount Melbourne (Italy)	80	MW 45, pH 5.6	Lama et al. (2004)
Bacillus sp. strain SPS-0	Hot spring in Portugal (France)	75	MW 99, pH 6	Bataillon et al. (2000)
Bacillus thermoleovorans strain K-3d	Hot spring in Kobe (Japan)	70–80	MW 40, pH 7	Sunna et al. (1997)
Bacillus flavothermus strain LB3A	Alkaline Lake Bogoria, (Kenya)	70	MW 80, pH 7	Sunna et al. (1997)
Thermotogasp. strain FjSS3-B. 1	Intertidal hot spring on savusavu beach in Fiji (New Zealand)	80	MW 31, pH 5.5	Simpson et al. (1991)

(continued)

Table 8.1 (continued)

Name of the isolated organism	Extreme environment location	Temperature (°C)	MW(KDa)/pH	References
Thermostable laccases from thermophilic microbes				
Brevibacillus sp. Z1	water and sludge samples of Diyadin Hotspring in provinces of Agri in Turkey	70	93 KDa, pH 4	Bozoglu et al. (2013)
Geobacillus thermocatenulatus MS5	Manikaran thermal hot springs, in Himachal Pradesh	55–60	pH 4.0–5.0	Verma and Shirkot (2014)
Bacillus sp. strain WT	Urmia lake, ahypersaline lake in northwest of Iran	55	pH 5	Siroosi et al. (2016)
Bacillus sp. SL-1	Aran-Bidgol Saline Lake in central region of Iran	70		Safary et al. (2016)
Anoxybacillus gonensis P39	Erzurum-Ilica Spring	60	pH 5.0	Yanmis et al. (2016)

8.7 Thermostable Laccases Obtained from Thermophilic Microbes

Laccases (E.C. 1.10.3.2; oxygen oxidoreductase) are the blue multi-copper oxidases that are responsible for catalyzing the oxidation of various phenolic and non-phenolic compounds by converting oxygen molecule to water with collateral four-electron reduction (Chauhan et al. 2017). Plants, fungi and bacteria are the major sources of this enzyme but only fungal laccases are commercially available and has been extensively studied (Muthukumarasamy and Murugan 2014). Lignin peroxidase, manganese peroxidase, and laccase are the three major enzymes associated with lignolysis (lignin component of the lignocellulosic biomass). Laccase is more readily available and easier to manipulate than both lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP). The benefit of using laccases instead of peroxidases is that laccases require O_2 rather than H_2O_2 (Sriharti et al. 2017). Laccases are considered as the lignin-modifying enzymes as they involved in the formation of lignin by promoting the oxidative coupling of monolignols, a family of naturally occurring phenols (Solomon et al. 1996). Thermophilic microbes are the promising sources of novel thermostable laccases. So far, very few thermohphilic micro-organisms have been explored for the production of lacasses. Moreover, thermostable laccase has

more resistance to alkalinity, acidity, chemical denaturants and withstand high substrate concentration without losing its catalytic efficiency (Hildén et al. 2009).

Thermostable laccase has been produced from the thermophilic microbes isolated from geothermal areas around the world including hot springs of India, China, Turkey, and Iran etc. Other sources of isolation of thermophilic micro-organisms are hypersaline lake, textile industry effluents, and rhizosphere of rice etc.

8.8 Role of Metagenomics in Mining Lignocelluloses Degrading Micorbes

Metagenomics is an approach that identifies enzymes with novel characteristics from the culturable and unculturable component of microbiomes. This methodology offers identification of enzyme at much lower price and time than conventional methods (Ausec et al. 2017; Garrido-Cardenas and Manzano-Agugliaro 2017). Metagenomics comprise a series of high-throughput DNA sequencing technologies and bioinformatics tools for the study which include sample processing, sequencing technology, assembly, binning, annotation, experimental design, statistical analysis, data storage, and data sharing (Thomas et al. 2012; Garrido-Cardenas and Manzano-Agugliaro 2017).

Metagenomics is a culture independent approach as it offers study of the genes originated from uncultured microbes encoding enzymes with remarkable biochemical and biophysical characteristics (Nimchua et al. 2012). Screening of functional activity and DNA data mining can be very beneficial for the identification of useful enzymes (Van Den Burg 2003) (Fig. 8.3).

Metagenomic study of microbial genes from hot springs in central India reveals thermophiles that degrade hydrocarbon and provided the information regarding the survival conditions required in extreme environments (Saxena et al. 2017). The first acidobacterial laccase-like multicopper oxidase studied through metagenomics expressed high salt and thermo-tolerance in an acidic bog soil metagenome. A gene that encods three-domain LMCO (LacM) was identified by using molecular screening of a small metagenomic library (13,500 clones) which shows resemblance to copper oxidases of *Candidatus Solibacter* (Acidobacteria) (Ausec et al. 2017). Metagenomics study of thermophilic cellulose-degrading microbial community reveals new thermo-stable cellulolytic genes (Xia et al. 2013). In a metagenomic study, lignocellulose-degrading microbial consortia with structural stability and aerotolerance were obtained from industrial sugarcane bagasse pile (BGC-1), fluid of cow rumen (CRC-1), and pulp mill activated sludge (ASC-1). BGC-1 isolated cellulolytic *Clostridium* and *Acetanaerobacterium* with ligninolytic *Ureibacillus* showed maximum degradation of agricultural waste and industrial pulp residues (Wongwilaiwalin et al. 2013). 2 cellulases and 12 xylanases were isolated from the microbes in the guts of wood-feeding higher termites when analyzed through metagenomics (Nimchua et al. 2012). Similarly, genes of sticky microbes on plant fiber incubated in cow rumen

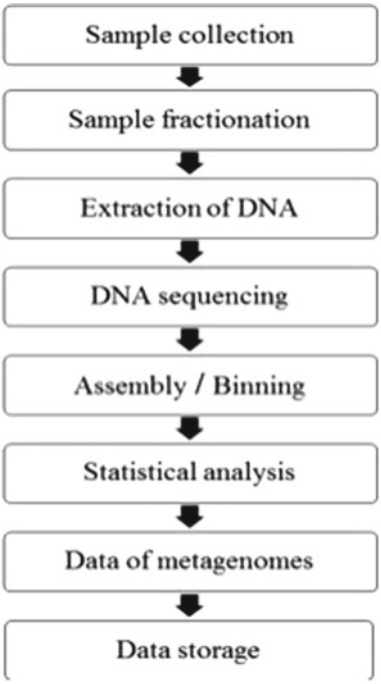

Fig. 8.3 Flow diagram of a typical metagenome study modified from Thomas et al. (2012)

were also studied through metagenomics. The study disclosed 27,755 carbohydrate-active genes out of which 57% had catalytic activity against cellulosic substrates (Hess et al. 2011). Several genes encoding cellulases, xylanases, laccases from different environments comprising termite guts, cow rumen, sugarcane bagasse pile, pulp mill activated sludge have been analyzed and identified by metagenomics studies (Hess et al. 2011; Nimchua et al. 2012; Wongwilaiwalin et al. 2013; Xia et al. 2013; Ausec et al. 2017; Saxena et al. 2017). These data sets provide information regarding genes and genomes responsible for the hydrolysis of lignocellulosic biomass. A variety of genomes from different environment have been studied but still new and suitable lignocellulose-degrading microbes are not entirely explored (Nimchua et al. 2012). So there is a need to investigate lignocellulose-degrading microbes from extreme environments through metagenomic studies that could probably provide industrial important informations applicable in bioconversion and processing.

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