Biocatalytic potential of thermophilic bacteria and actinomycetes

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Thermophilic organisms have been less explored due to the difficulties in isolation and maintenance of pure culture. Therefore, it remains to explore their diversity and biotechnological potential from majority of the thermal habitats. As a consequence of growth at high temperature and unique macromolecular properties, thermophiles can possess high metabolic rates, physically and chemically stable enzymes and lower growth but higher end product yields than similar mesophilic species. In addition, cultivation of thermophiles at high temperature is technically and economically beneficial as it reduces risk of contamination and viscosity which leads to high degree of substrate solubility.

In this chapter, we have focused on recent classification of thermophiles and various adaptive means which contribute to their survival at high temperatures. The chapter deals with different thermozymes, their unique properties, such as high operational stability, longer shelf-life and their applications in various fields under the larger umbrella of Microbial Biotechnology. Our research group is currently engaged with optimization, purification and characterization of extracellular thermostable enzymes; mainly amylases, proteases, cellulases and lipases which would have huge industrial applications, viz., starch processing, Food and Pharmaceuticals, detergent, and other related Biotechnological industries. Recent advances in the field, including cloning and overexpression of different thermozymes are also discused.

Keywords: Thermophiles, Biocatalytic Potential

Introduction

Extremophiles have provided an interesting and challenging platform for researchers since the time of their discovery. Besides growth under the extreme conditions, production of industrially valuable compounds, such as enzymes, antibiotics, hormones etc. have fascinated and focused attention in present scenario. The extremophiles majorly include; Halophiles, Thermophiles, Barophiles, Pscryophiles and Acidophiles [1, 2]. Among these, thermophilic bacteria and actinomycetes are the organisms which can grow and produce such compounds optimally high temperature [3]. Thermophiles are further subcategorized on the basis of their temperature tolerance: for instance, facultative thermophiles, can grow at temperatures between 50˚C-65˚C, but also grow also at 37˚C; obligative thermophiles have maximum growth temperatures of 65˚C-70˚C, and will not grow below 40˚C; extremely thermophiles can grow between 40˚C-70˚C with an optimal growth temperature of about 65˚C and hyperthermophiles, mainly comprising of archae, can grow over 90˚C with a range of optimal temperatures between 80˚C-115˚C. Thermophilic bacteria and actinomycetes (actinobacteria) have been less explored due to difficulties in isolation and maintenance in pure culture. The major challenge for thermophilic microorganisms is their survival and production of active and stable enzymes and other bioactive molecules at high temperatures. It largely remains to explore their diversity, molecular phylogeny, production of biotechnologically useful enzymes and other compounds. Aspects relating to specific adaptive features are also interesting to investigate. However, the trends so far have distinctly revealed that thermophilic organisms could provide answer to many basic questions relating to the stability of macromolecules, besides being a source of biotechnologically novel compounds.

How do they survive and produce enzymes at high temperature?

Microorganisms adapt to the conditions in which they have to live and survive. With reference to thermophiles, the cell membranes contain saturated fatty acids which provide a hydrophobic environment for the cell and maintain the cell rigidity at elevated temperatures [4]. Further, the hyperthermophilic archae have lipids linked with ether on the cell wall, which is responsible for heat resistance [5]. Recently, tetraether membrane lipids were reported in a thermoacidophilic euryarchaeota *Candidatus* "*Aciduliprofundum boonei*" [6]. In addition to the structural adaptations of cell wall and cell membrane, the DNA of thermophiles contains reverse DNA gyrase, which enhance the melting point by producing positive super coils in the DNA [7]. In *Sulfolobus solfataricus* a small DNA binding protein, Sso7d, not only imparts thermostability to the DNA but also promotes the annealing of complementary strands above the melting point and the ATPase-dependent rescue of the aggregated proteins [8, 9].

Thermophiles are reported to have proteins which are thermostable and resist denaturation and proteolysis [10]. In addition, thermophilic bacteria, actinomycetes and archae adapt to high temperatures by increased electrostatic, disulphide and hydrophobic interactions in their proteins [11, 12]. In addition to the above strategies, certain specialized proteins, known as 'chaperons', are produced by these organisms, which help to refold the proteins to their native form and restore their functions [13, 14, 15, 16]. Stability and folding pattern of the ferredoxin from a hyperthermophilic

archaeon *Acidianus ambivalens* has been studied using Guanidine HCl as a chemical denaturant [17]. Certain thermophilic enzymes are stabilized by certain conformational changes [18]. However, presence of certain metals [19], inorganic salts [20] and substrate molecules are also reported to impart the thermostability. Based on the thermal behavior of these enzymes, the Equilibrium model has been described to reveal the effect of temperature on enzyme activity by reversible active-inactive transition states [21].

Due to the increasing demand of highly thermostable enzymes, certain computational algorithms and bioinformatic tools are designed to predict protein rigidity and stability in order to improve the thermostability. Protein stabilization can be carried out by site-directed mutagenesis. The other powerful protein engineering method to add to the thermostability is directed evolution which involves gene shuffling through which sequence optimization leads to a combination of novel traits [22].

Why to prefer enzymes from thermophiles?

With the flourishing growth of industrialization, demand of thermostable enzymes has increased tremendously due to its high thermostability and feasibility to the processes involved [23, 24, 25, 26, 27].

One of the obvious advantages of carrying biotechnological processes at elevated temperatures is reducing the risk of contamination by common mesophiles. Besides, an operation at the high temperatures has significant influence on the bioavailability and solubility of organic compounds leading to the efficient bioremediation [28]. Reactions at higher temperatures has added benefits of decreased viscosity and hence increased diffusion coefficient of substrates leading to the favorable equilibrium displacement in endothermic reactions [29]. Such enzymes can also be used as models for understanding the basis of thermostability.

The following sections describe specific thermostable enzymes, their sources, characteristics and their respective industrial applications.

Protein degrading enzymes

Proteases, generally classified into exopeptidases (cleave off peptide bonds from the ends of the protein chain) and endopeptidases (cleave peptide bonds within the protein), are the major industrial enzymes and constitute more than 65% of the world market [30]. These enzymes are extensively used in the food, pharmaceutical, leather and textile industries [31, 32]. Among the extremophilic sources, thermostable proteases have been reported from certain haloalkaliphilic bacteria and actinomycetes [20, 33, 34]. With the increasing demand of the enzymes, there will be ever increasing need for the stable biocatalysts capable of withstanding harsh conditions of operation [23].

Starch degrading enzymes

The starch industry is one of the largest users of amylolytic enzymes comprising nearly 30% of the world's enzyme consumption [35] for the hydrolysis and modification of this useful raw material. The complete hydrolysis, however, requires a combination of enzymes, which include α-amylases, glucoamylases or β-amylases and isoamylases or pullulanases [36]. The enzymes are classified into endo and exoacting, wherein α-amylases are endoacting and hydrolyze glycosidic linkages in a random fashion leading to the formation of linear and branched oligosaccharides, while the rest are exoacting enzymes that attack the substrate from the non-reducing end, producing oligo and/or monosaccharides. The enzymatic conversion of starch includes gelatinization and saccharification and hence, it is desirable that α-amylases should be active at the high temperatures of gelatinization (100°C-110°C) and liquefaction (80°C-90°C) to economize processes. While there have been ever increasing demand for thermostable α-amylases [37], other properties of the enzymes such as catalytic efficiency, substrate range, pH profile and pH stability are also significant to enable them to function under application conditions.

In addition, one of the requirements of the starch industry is the calcium requirement of the α-amylases, which may leads to the formation of calcium oxalate, a substance likely to block process pipes and heat exchangers. Therefore, calcium independent amylases would be one of the needs to develop successful process in starch industries [23, 38, 39]. The diversity of thermophiles in high temperature environments [40] and the requirements of new and improved technological operations for enzymes with novel and fitting characteristics [41] are challenges, both for the scientific and business community.

Cellulose degrading enzymes

Cellulose, the most abundant organic source of feed, fuel and chemicals, consists of glucose units linked by β-1,4-glycosidic bonds in a linear mode. Cellulose is more resistant to digest and hydrolyze compared to starch due to the difference in the type of bond and the highly ordered crystalline form. The enzymes required for the hydrolysis of

cellulose include endoglucanases, exoglucanases and β-glucosidases [42]. In the current industrial processes, cellulolytic enzymes are employed in detergents, causing color brightening and softening, stoning of jeans, pretreatment of cellulosic biomass to improve nutritional quality of the forage and in the pretreatment of industrial wastes [43, 44, 45]. In order to attack the native crystalline cellulose, which is water insoluble and exists as fibers of densely packed structures, thermostable cellulases active at high temperature and alkaline pH would be required.

Xylan degrading enzymes

Xylan is the dominating component of hemi-celluloses and is one of the most abundant organic substances on earth and has great applications in pulp and paper industry [46]. The wood used for the production of the pulp is treated at high temperatures and basic pH, which implies that the enzymatic procedures would require xylanases exhibiting a high thermostability and activity over a broad pH range [47]. Xylanases can also be produced in solid-state fermentation, where increased concentrations of basal medium improved its production with decreased cultivation time [48]. The pulp and paper technology is one of the fastest growing industries and the use of thermostable xylanases seems attractive since they provide global environmental benefits. However, scaling up of the enzyme production from the laboratory scale to the industry scales is yet to be achieved. Further, since extreme thermophiles that are able to secrete xylanase are few; the search for suitable sources with high yield of enzyme and the desired characteristics should be one of the priorities of the research. In this context, metagenomic approaches to explore vast population of non-cultivable organisms from the natural/man made habitats would be attractive feature.

Lipid degrading enzymes

Lipases of microbial origin are the most versatile enzymes and are known to bring about a range of bioconversion reactions, which includes hydrolysis, inter-esterification, esterification, alcoholysis, acidolysis and aminolysis [49, 50, 51]. Their unique characteristics include substrate specificity, stereospecificity, regioselectivity and ability to catalyze a heterogeneous reaction at the interface of water soluble and water insoluble systems [52]. The esters produced play a significant role in the food industry as flavor and aroma constituents [53]. Whereas long chain methyl and ethyl esters of carboxylic acid moieties provide valuable oleo chemical species that may function as fuel for diesel engines, esters of long chain carboxylic acid and alcohol moieties (waxes) have applications as lubricants and additives in cosmetic formulations [54]. Other applications include the removal of the pitch from pulp produced in the paper industry, for the hydrolysis of milk fat in the dairy industry, removal of non-cellulosic impurities from raw cotton before further processing into dyed and finished products, drug formulations in the pharmaceutical industry and in the removal of subcutaneous fat in the leather industry [53, 55]. Most of the industrial processes in which lipases are employed function at temperatures exceeding 45°C. The enzymes, thus, are required to exhibit an optimum temperature of around 50°C [56]. Among the desirable characteristics that commercially important lipases should mainly exhibit are thermostability and alkalitolerance [57]. Although, few lipases are able to operate at 100°C, their half-lives are reported to be short [58]. To meet this end, there is a continuous search for sources of highly active lipolytic enzymes with specific stability to pH, temperature, ionic strength and organic solvents.

Table: Some of the industrially important enzymes with their source and unique properties

Source organism	Enzyme	Temp $_{opt}$	pH $_{opt}$	Reference
Industrially important enzymes from thermophilic bacteria and archae				
Thermophilic bacterium TSSB-6	α-amylase	80ºC (50º-100ºC)	8.0 (6.0-9.0)	[38]
B. subtilis JS-2004	α-amylase	70ºC	8.0	[59]
Thermophilic bacterium TSSB-4	Alkaline Cellulase	70ºC (50ºC-80ºC)	8.0 (6.0-9.0)	[39]
Anoxybacillus flavithermus EHP1	Cellulase	75ºC (65^0C-75ºC)	7.5 (7.0-9.0)	[60]
Thermophilic bacterium HS-08	Serine Protease	65^0C (60ºC-70ºC)	7.5 (7.0-9.0)	[61]
B. thermoleovorans CCR11	Lipase	60°C	9.0-10.0	[62]
Pyrococcus furiosus	Xylanase	100°C	6.0	[63]
Industrially important enzymes from thermophilic actinomycetes				
T. fusca NTU22	α-amylase	60°C	7.0	[64]
Streptomyces transformant T3-1	Cellulase	50°C	6.5	[65]
Thermoactinomycetes sp. HS682	Protease	70°C	11·0	[66]
Streptomyces rimosus R6-554W	Lipase	50°C	9.0-10.0 (4.0-10.0)	[67]
Thermomonospora fusca	Xylanase	60°C (60°C-80°C)	7.0 (6.0-8.0)	[68]

Thermostable DNA polymerases

The polymerase chain reaction (PCR) process has led to a huge advance in molecular biology. Developments in this process have been to a large extent facilitated by the availability of thermostable DNA polymerases, which catalyze the elongation of the primer DNA strand [69]. Taq polymerase from *Thermus aquaticus* was the first thermostable DNA polymerase characterized and commercialized in PCR [70]. Repeated exposure of the enzyme at temperatures above 96°C in PCR reactions had only limited effect on the enzyme and significant activity remained after exposure to 99°C. Although, Taq polymerase exhibits a 5'–3' exonuclease activity but the 3'–5' exonuclease activity was not detected. Thus, low base insertion fidelity of the enzyme is unable to correct erroneously incorporated nucleotides [71, 72]. It was also possible to determine the error rates of some polymerases in terms of base pairs [73]. While the PCR process has developed rapidly through the better thermal cyclers [74], lack of fidelity has remained a challenge to address. The five distinct activities in which errors can occur are the rate of phosphodiester bond formation, the binding of dNTP by the polymerase, the rate of pyrophosphate release, contamination after wrong-incorporation and the 3'–5' exonuclease proof reading capacity of the enzyme [73]. Some of the issues that have stimulated scientists, are improvements required in the fidelity of the PCR, development of strategies for more economical use of the enzymes and ensuring rapid multiplication from fewer strands. A thermostable DNA polymerase having these characteristics will indeed bring the desirable changes in PCR.

Molecular cloning of thermophilic genes into mesophilic hosts

Genetic and protein engineering are the modern techniques for the commercial production of enzymes of improved stability to high temperatures, extremes of pH, oxidizing agents and organic solvents. Use and development of molecular biology tools, permitting genetic analysis and gene transfer for recombinant production, has led to dramatically increased activities in the field of thermostable enzymes [74]. Cloning and expression of thermophilic genes into a suitable and faster growing mesophilic host have provided possibilities of producing the specific thermostable enzyme required for varied purposes [75]. Genes encoding α-amylase (*amyA*), pullulanase (*pulA*), maltodextrin phosphrylase (*agpA*) and α-glucosidase (*aglA*) were identified in the gene library of *Thermotoga maritema* [76]. The enzymes produced after expression in *E. coli* are reported to display extreme thermostability. *Pyrococus furiosus* α-amylase gene was cloned by activity screening in *E. coli* [77] and *B. subtilis* [78] was optimally active at 100°C, pH 5.5–6.0 and did not require Ca^{++} for its activity. Gene encoding α-amylase from a hyperthermophilic archaea, *Thermococcus hydrothermalis,* was cloned and expressed followed by the characterization of the recombinant enzyme [79]**.** Genes encoding pullulanases from *Pyrococus furiosus* [80] and *Ferividobacterium pennavorans* [81] are described. Simialrly, xylanases from *Thermotoga maritima* [82] and *Thermotoga neapolitana* [83] and protease from *Pyrobaculum aerophilum* and *Bacillus stearothermophilus* [84], lipases from *Bacillus thermocatenulatus* [85], esterases from *Bacillus licheniformis* [86], cellulase genes from the archaon *Pyrococus furiosus* [63], thermostable trehalose synthase (TreS) from *Meiothermus ruber* CBS-01 [87] were expressed in an *E. coli*. Earlier, cellobiose phosphorylase genes from *Cellovibrio uda* [88] and *Cellovibrio gilvus* [89] was cloned and expressed in *E. coli.* Optimization of various conditions for the over expression and production of catalytically active enzymes from extremophiles has been studied. In this context, the effect of growth temperature, induction and molecular chaperones on the solubilization of over expressed enzyme has also been analyzed [13]. Studies on the overexpression and protein folding of chimeric β-glucosidase, constructed from *Agrobacterium tumefaciens* and *Cellvibrio gilvus* was carries out and the characteristics of chimeric enzymes has been reported [90]. Similarly, genes encoding proteases were cloned from *Thermomonospora fusca* [91] and *Sterptomyces* sp. C5 [92] and expressed in *Streptomyces lividans* α-amylase gene, producing maltotriose from *Thermobifida fusca* has also been cloned and characterized [64]. These examples highlighted various examples, where genes from thermophiles were expressed in mesophilic hosts. The resultant recombinant enzymes displayed characteristics similar to those in native source.

Summary

Thermophilic bacteria and actinomycetes are the organisms which not only grow but also produce biotechnologically valuable compounds, such as thermostable enzymes at optimally high temperature through various adaptive mechanisms. Due to ever increasing industrial growth, the demand for thermostable enzymes; such as, proteases, amylases, cellulases, lipases, xylanases and DNA polymerases, has tremendously increased. Various thermostable enzymes have been described illustrating their properties, source and their respective industrial applications. The molecular cloning and overexpression of genes from theromophilic sources to produce functional enzymes into mesophilic host is the demand of the day towards fulfilling the ever increasing need for biocatalysts. This would also expand the horizon of greater applicability of bioresources, besides adding to our knowledge on adaptation and molecular stability under extreme conditions.

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