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Recent View on Heterologous Expression of Thermostable Fungal Cellulases, Focused on Expression Factory of *Pichia Pastoris*

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Abstract – Thermophilic fungal cellulases are promising enzymes play remarkable functional role in the industrial production of biofuel, detergent and textile, pulp and paper. Fungi are the most robust specie among all organisms having the ability of cellulases production, while most of them cannot tolerate to high temperature. A kind of newly discovered fungi, thermophilic fungi are the only well studied eukaryotes that can tolerate and stably cultivate at temperatures over 45°C. On behalf of these characteristics thermophilic fungi have been received mounting interest and their thermostable enzymes are much warranted. In the recent years, thermophilic cellulases have significant development in many features, such as components purification and characterization of the cellulases, perception of the degradation mode of cellulose, cloning and expression of cellulase genes, finding out the designs and shapes of cellulase components, enhancing the characteristics of cellulases through fusion and mutation processes , considering associations in the structure and function of cellulases and showing the industrial potential of cellulases. *Pichia pastoris* a methylotrophic yeast is an extensively exploited expression system for the heterologous enzymes production. With numerous beneficial characteristics such as growth to high cell density and high competence for enzymes secretion has emerged *P. pastoris* a promising expression host, besides *P. pastoris* provides a strong, methanol inducible promoter of the alcohol oxidase 1 (AOX1) gene used in the expression host to enhance the utility of expression system. This review illustrates heterologous expression of highly thermostable fungal cellulases especially focused on thermophilic endoglucanases, cellobiohydrolases and *β*-glucosidases in *P. pastoris*.

Keywords – Thermophilic fungi, thermophilic endoglucanases, thermophilic cellobiohydrolases and thermophilic *β*-glucosidases, Synergy, Thermostability. *Pichia pastoris*

1. Introduction

Cellulose is one of the main components of plant cell wall material and is the most abundant and renewable non fossil carbon source on the Earth. Degradation of cellulose to its constituent monosaccharides has attracted considerable attention for the production of food and biofuels [1]. Most plant cell walls are composed of approx. 15–40 % cellulose, 10–30 % hemicellulose and pectin, and 5–20 % lignin [2]. Recalcitrance of lignocellulosic biomass is related to its complex chemical composition (lignin, hemicelluloses and acetyl groups) and the physical features (cellulose crystallinity and degree of polymerization) of the plant cell wall [3]. The principal framework of cellulose consists of anhydroglucopyranose molecules connected by b-1,4-glycosidic linkages. The complexity of strong intra- and intermolecular hydrogen bonding found in cellulose results in the formation of microfibrillar chains which play an important role in recalcitrance of biomass and make its hydrolysis a cost intensive process [4]. Hemicellulose is a heterogeneous group of branched and linear polysaccharides, consisting mainly of D-xylose, and D-mannose, and a number of substituted sugars [5]. Both cellulose and hemicellulose can be hydrolyzed into simple sugars which may be then fermented to ethanol.

Cellulases have been commercially available for more than 30 years, and these enzymes have represented a target for both academic as well as industrial research [6, 7]. Moreover, cellulases are gaining more and more interest for agriculture, biotechnology and bioenergy uses [8], especially in the utilization of cellulosic biomass for the production of renewable liquid biofuels like ethanol, butanol or other fermentative products of sugar. With such uses, cellulases have the potential to become the largest group of industrially used enzymes worldwide [9].Cellulases are derived from certain fungi and bacteria, which are often difficult to culture on an industrial scale. Accordingly, methods to recombinantly express important cellulases and other

glycosyl hydrolase (GH) enzymes are under serious investigation. Cellulolytic enzymes are widespread in nature and are found in plants, insects, bacteria and fungi [10-12].

Fungi are important organisms for degradation of plant material in nature. It is well known that fungi produce copious amounts of cellulases [13]; cellobiohydrolases account for nearly 70 % (w/w) of secreted proteins and enzymes in cellulolytic fungi, followed by endoglucanases (~20 % w/w), while hemicellulases account for only less than <1 % of total weight of the secreted proteins [13].Thermophilic fungi are species that grow at a maximum temperature of 50°C or above, and a minimum of 20°C or above [14]. Based on their habitat, thermophilic fungi have received significant attention in recent years as a source of new thermostable enzymes for use in many biotechnological applications, including biomass degradation. Thermophilic cellulases are key enzymes for efficient biomass degradation.

Pichia pastoris (*Komagataella phaffii*) has emerged as an alternative host system for the heterologous expression of desired enzymes and proteins[15]. In contrast to other expression host, *P. pastoris* secretes endogenous cellulolytic enzymes in very low quantities[16]. A well-organized secretion tools and the relative simplicity of getting high dry cell weights >100 g/L through bioreactor fermentation make *P. pastoris* a popular alternative expression host for enzymes and proteins both at industrial and laboratory levels[17]. Despite the fact that *P. pastoris* is beneficial expression system for the heterologous expression of proteins[18] still there exist a room for betterment on transcriptional[19, 20] and posttranslational level[21, 22]. The optimization of gene and expression of enzyme can be determined on a transcriptional level through changing copy numbers of the integrated expression cassettes also by promoter selection[22, 23]. As yet highly methanol inducible promoter pAOX1 and, to a certain degree, the constitutive promoter pGAP are largely employed for enzymes and proteins heterologous expression in *P. pastoris*[18].

This review concerns basic research on highly thermostable fungal cellulases more specially discussed thermophilic endoglucanases, cellobiohydrolases and *β*-glucosidases, synergism among cellulases and their heterologouse expression in expression host *P.Pestoris*. The main thermostable cellulase producing organisms and their thermostable cellulases are also described.

2. Thermostable fungal cellulase-producing fungi

Thermophilic organisms can be classified as those organisms with an optimal growth temperature between 45 °C and 80 °C , hyperthermophiles are those with an optimum growth temperature above 80 C, and mesophiles are those that grow optimally below 45 °C [24]. Thermophily is common in bacteria and Archaea, whereas hyperthermophiles are mainly confined to the *Archaea*. Only a small fraction of the estimated 600 000 fungi [25] is considered to be thermophilic and no fungus has been described as hyperthermophilic. Most reported thermophilic fungi have been placed into the *Sordariales*, *Eurotiales*, and *Mucorales* [26]. However, [27] described the existence of two thermophilic isolates of *Basidiomycota*. Thermophilic fungi are of special interest for biomass conversion applications since they are potential sources of thermostable enzymes. The advantages of biomass conversion at high temperatures include higher reaction rates, enhanced mass transfer, lowered substrate viscosity, and reduced risk of contamination [28]. At least some thermophilic fungi possess cellulose-degrading capacities that are higher than those of mesophilic reference species [26]. The screening of thermophilic fungi and other thermophilic organisms for improved enzyme varieties may contribute to lowering the costs of enzyme preparations [29].

Cellulolytic enzymes belong to the O-glycosyl hydrolase (GH) family of the International Union of Biochemistry and Molecular Biology, which classifies enzymes based on the type of chemical reaction they catalyze. Members of the GH families hydrolyze the glycosidic bond between two or more carbohydrates, or between a carbohydrate and noncarbohydrate molecule. The enzymes are further classified into subfamilies based on their substrate specificities. Cellulases have been classified into three distinct classes, which complete the hydrolysis of cellulosic materials [30]. Endoglucanases (EGs) are endo-acting enzymes that hydrolyze the internal *β*-1,4-glycosidic bonds of amorphous cellulose chains. cellobiohydrolases (CBHs) are exoacting enzymes that cleave *β*-1,4-glycosidic bonds from the free chain ends in the crystalline regions of cellulose, producing mainly cellobiose but also cello-oligosaccharides as end products. CBHs are very important in cellulose degradation because they solubilize the insoluble crystalline regions of cellulose efficiently. The third class of enzymes is constituted by the *β*-Glucosidase (*β*-1,4-D-glucoside glucohydrolase EC 3.2.1.21) and these work in synergy with the CBHs and EGs to hydrolyze glucose oligomers and cellobiose to form monomeric glucose via breaking the *β*-glucosidic bonds between aryl or alkyl and saccharide groups. Cellobiose, however, is a strong inhibitor of both cellobiohydrolases and endocellulases, but the action of *β*-glucosidase can reduce its effect. In addition, the glucose produced also inhibits cellulolysis, albeit to a lesser extent [31-33].

Their microscopic studies clearly reveals that, 15-45 glucan chain forming a recalcitrant microfibril structure, while fusing poly microfibril forming structure of macrofibril/cellulose fibril [34]. There are some forces concerning with stability and recalcitrance of cellulose crystalline structure such as hydrogen bonding (intra-chain, inter-chain and inter-sheet) and Vander Waals forces (inter-sheet) [35]. Recalcitrance is regarded to be the chokepoint to exploiting biomass in the production of biofuels [36], which will assist to lessen our reliance on fossil energy resources. The high molecular weight and well ordered tertiary structure of cellulosic biomass created big hurdles to their solublization in water. Even though starchy energy resources need temperatures of only 60-70°C to be transformed from crystalline form into amorphous, cellulose have need of 320°C and a pressure of 25 MPa to modify from the firm crystalline

structure to an amorphous structure in water [37].

The high cost of the fungal cellulase mixtures that are commonly employed in biomass-to-biofuel conversion processes is one of the major limitations to achieving economically viable production of transportation fuel from inedible plant matter. The operating costs of cellulase treatments can be reduced by improving the thermostability of these enzyme mixtures [38].This high temperature stability is an important asset for industrial use. For example, it has been shown that a mixture of thermostable cellulases exhibits high lignocellulose degrading capacity with a temperature optimum of 65°C [38].In addition to stability, properties such as specific activity, pH dependence, product inhibition and productive versus non-productive adsorption on solid substrate surfaces all contribute to the overall performance of a cellulose mixture [39].

Fungi are important organisms for degradation of plant material in nature. They achieve this by means of secreted enzymes that are stable even under harsh environmental conditions. These same properties make the fungal enzymes suitable for industrial use. Thermophilic fungi are species that grow at a maximum temperature of 50°C or above, and a minimum of 20°C or above [14]. Thermophilic fungi are typically found in compost, wood chip piles, stored grains, animal dung and other environments that are self-heating due to degradation of plant materials [40]. Recent studies have documented the superior performance of cellulases from thermophilic fungi relative to their mesophilic counterparts in laboratory scale biomass conversion processes [38, 41], where enhanced stability leads to retention of activity over longer periods of time at both moderate and elevated temperatures. Based on their habitat, thermophilic fungi have received significant attention in recent years as a source of new thermostable enzymes for use in many biotechnological applications, including biomass degradation. Thermophilic cellulases are key enzymes for efficient biomass degradation. A numbers of thermophilic fungi have been isolated in recent years and the cellulases produced by these eukaryotic microorganisms have been purified and characterized at both structural and functional level.

Two forms of cellobiohydrolase I (CBH I, Cel7A) were purified from the culture ultrafiltrate of a mutant strain of the fungus *Chrysosporium lucknowense*, with different molecular masses 52 and 65 kDa but the same p*I* (4.5). Both enzymes displayed maximum activity at pH 5.0–5.5; they had similar specific activities against soluble substrates. However, the 65 kDa CBH I was much more efficient in hydrolysis of Avicel and cotton cellulose, and its adsorption ability on Avicel was notably higher in comparison to the 52 kDa enzyme. Both enzymes were stable at 50 °C for 24 h. At higher temperature, the 65 kDa enzyme showed better thermostability: it retained >90% of activity after 7 h at 60 °C and 50% of activity after 3 h at 65 °C[42]. Thermophilic fungus *Thermoascus aurantiacus* of order *Eurotiales* has been studied intensively. It grows readily on cellulose [43] and produces thermostable cellulases and other enzymes [44, 45]. A novel enzyme cellobiohydrolases of family 7 have been reported from the thermophilic fungi *Acremonium thermophilum*, *Thermoascus aurantiacus*, and *Chaetomium thermophilum*. lum Cel7A or the *T. reesei* Cel7A were also constructed. All these novel acidic cellobiohydrolases were more thermostable (by 4–108 °C) and more active (two- to fourfold) in hydrolysis of microcrystalline cellulose (Avicel) at 458C than *T. reesei* Cel7A. The most effective enzyme for Avicel hydrolysis at 708 °C, however, was the 2-module version of the *T. aurantiacus* Cel7A, which was also relatively weakly inhibited by cellobiose [46].Other thermophilic fungi that produce thermostable cellulases are *Talaromyces emersonii* [47], *Myceliophthora thermophila* [48], *Chaetomium thermophilum* and *Acremonium thermophilum* [46]. Busk *et al*.,2013 [49] gives a good description of fungi growing on cellulose, which is due to the production of cellulase.

3. Thermal effect on cellulases activity

Unfortunately, many naturally occurring enzymes are not suitable for industrial applications because of their poor selectivity to the substrate. For example, some family 10 xylanases display cellulase activity along with xylanase activity, and are less preferred in applications where only hemicellulase degradation is required (e.g., pulp and paper or baking industry) [50]. Textile applications such as biopolishing require endoglucanase activity. However, not all fungal endoglucanases are highly active and preferred in these applications. Endoglucanases from glycoside hydrolase families 5, 12 and 45 have found the most utility for biostoning and biopolishing applications [51-53]. Other important parameters to consider when selecting suitable enzymes are industrial process conditions, particularly pH and temperature. The range of pH and temperature conditions in various industrial enzyme application processes are shown in (Fig. 1). Fungal enzymes usually display their highest catalytic activities at slightly acidic pH and moderate temperature, while bacterial enzymes exhibit activities under a broader range of pH and temperature conditions. Although, commercial production of amylases is carried out by both bacterial and filamentous fungal cultures, bacterial α-amylases are generally preferred for starch liquefaction at high temperatures due to their high temperature stability. However, discoveries of new fungal enzymes with higher thermal stability opens new opportunities in development of novel amylases for industrial starch processing [54, 55]. Enzyme performance in industrial lignocellulosic biomass hydrolysis is also affected by temperature and pH. Many biomass pretreatment methods are based on high temperature and acidic conditions to make biomass accessible for enzyme hydrolysis. Cellulases perform well at pH 4–5, but activity decreases significantly at lower pH [56]. Thus, the pretreated biomass must be pH adjusted before enzymatic treatment. To achieve higher enzyme activities currently hydrolysis is performed at 50 °C. However, when reaching high temperatures, protein stability is affected, particularly taking in account that the lignocellulosic biomass hydrolysis process typically runs for the duration of several days. Therefore, the development of thermostable and acid tolerant enzymes for biomass degradation is also very attractive approach

for improvement of hydrolysis process and reduction of amount of enzyme required. On the other hand, using alkaline chemicals such as dilute sodium hydroxide, aqueous ammonia and lime to remove lignin prior enzymatic hydrolysis process has long been known to improve cellulose digestibility [57], thus, enzymes with improved activity and stability at higher pH are also of interest. Low enzyme stability in process streams containing variety of enzyme inhibitors, organic solvents or detergents results in slow enzymatic reaction rates. For example, in cellulosic ethanol production enzyme performance is reduced during lignocellulosic biomass hydrolysis by interaction with lignin or lignin–carbohydrate complexes [58]. Moreover, all enzymes are prone to substrate or product inhibition resulting in loss of enzymatic activity or even activation of reverse enzymatic reaction. The intermediate and end-products of lignocelluloses hydrolysis generally are not removed. This results in product inhibition of cellulolytic enzymes and significant decrease in the rate of reaction as cellulose hydrolysis proceeds [59]. For example, cellobiohydrolases and endoglucanases are inhibited by cellobiose [60], making beta-glucosidases important for avoiding product inhibition through conversion of cellobiose to glucose. On the other hand, beta-glucosidases are inhibited by their end-product, glucose [61]. Other than inhibiting beta-glucosidase reaction by occupying the active site, glucose can also take part in the reverse transglucosidase reaction using the active site capacity in non-hydrolyzing action and resulting in the production of unwanted products such as cellotriose and cellobiose [62]. Today's biotechnology and recombinant DNA technologies significantly contributed in developing of economically feasible industrial enzyme applications. In many cases, enzymes that perform under unnatural conditions, such as elevated temperatures, non-optimal pH or presence of inhibitors, were created. Several enzyme engineering approaches, such as rational design mutagenesis, gene shuffling, directed evolution and metagenome mining, have been developed over the past decades and are extensively applied in the development of novel enzymatic activities and properties. (Fig.1)

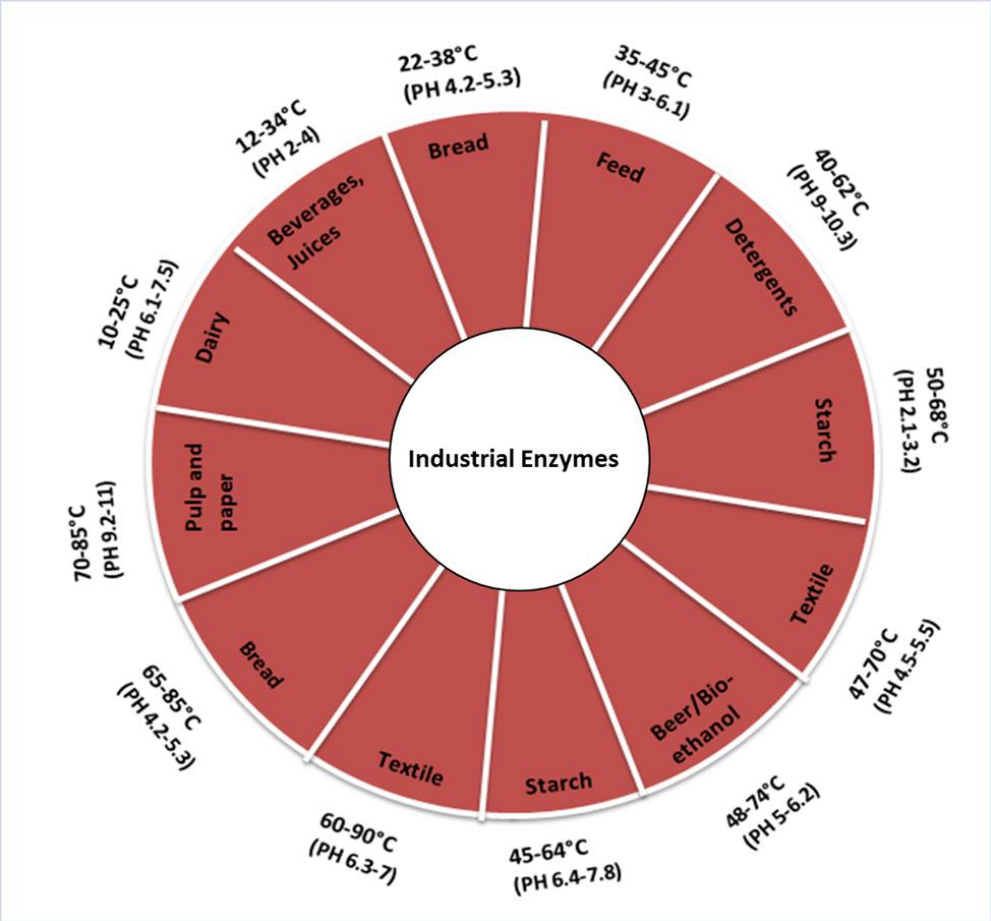

Figure 1: Approximate tendencies of PH and Temperature of various industrial enzymes

4. Effect of Cellulases Synergy on Lignocellulosic biomass degradation

Cellulose degradation is generally dependent on sets of secreted enzymes that work in concert, i.e. synergism. Most of the current commercial lignocellulolytic mixtures are based on fungal cellulases and hemicellulases [63]. An important factor for synergy is the nature of the substrate and the binding of enzymes. When more substrate is present the fixed number of enzymes is more sparsely distributed over the cellulose surface and the synergy between endo- and exo-glucanases is decreased [64]. The kinetics of synergistic degradation of a surface-like substrate, such as a cellulose microfibril, is rather different from classic kinetics since many prerequisites (e.g. soluble substrate, homogeneous solution and freely diffusing enzyme) are not fulfilled. A fractal-like kinetic model has recently been proposed as a way to analyze synergistic action [65].Important for lignocellulose degradation is also the synergy between cellulases and other enzymes. Mini-cellulosomes based on mini-scaffolding proteins and different combinations of xylanase, endoglucanases and exoglucanase were recently constructed [66]. The synergistic action of xylanases and cellulases led to increased release of both xylooligosaccharides and cellooligosaccharides, i.e. cross synergy [67].The synergistic action of a diverse set of accessory hemicellulases from different bacterial sources and core fungal cellulases resulted in high glucose (80 %) and xylose (70 %) yields with moderate enzyme loadings (~20 mg protein/g glucan) compared to commercial enzymes [68].

Recent studies on synergism stress that cellulases act mainly in a two- or one-dimensional environment and not in a three-dimensional environment since the CBM anchors the enzyme to the cellulose surface which results in a two dimensional migration with little probability for free diffusion. Furthermore, strongly processive exoglucanases are restricted to one-dimensional movement as they migrate along a cellulose chain. There are also other, more complex strategies for synergistic action, such as cellulosomes [67].Biomass degrading fungi having two different types of machineries that playing remarkable role in degradation of biomass are, direct enzymatic depolymerisation and generartion of oxidative species[69]. The architecture of fungal cellulases comprising of a catalytic domain (CD) and cellulose binding domain (CBD) linked via peptide linkers [34]. Whereas CBD remain attached with substrate letting the CD to accomplish its catalytic activity. Cellulose binding domain (CBD) is renamed as Carbohydrate binding module (CBM). CBM have many families on their similarities in amino acids sequences. 64 families of CBMs have been updated in the CAZy database [70]. CBMs comprising varying amino acids monomers from 30 up to over 200, gives single ,double or triple in one protein [71]. At least 35 different glycoside hydrolase (GH) families of fungal cellulases, including six (6) polysaccharide lyase families and three (3) carbohydrate esterase families are involved in efficient degradation of plant lignocellulosic biomass [72].CBD is not directly needed in depolymerization of cellulose but its presence indirectly increase the catalytic performance of CD [73]. *T. reesei* Cel7A carried degradation of crystalline cellulose via processivity. As the cellulobiohydrases have the ability to bind to carbohydrate chain, which decrystalize di-saccharide monomers from the end of the chain without separation [74] Cel7B endoglucase has missing four β-sheets covering their tunnel, so having open furrow type active site [74]. This enables them to bind easily to make cut internally on cellulose structure at various positions, which depends on exocellulases structural characteristics allowing them for processively binding. Cel9A structural illumination has been reported that it work as bridge between exo- and endocellulases , because it adjusted the weak binding domain of 3c CBD within the active site of catalytic domain, letting enzyme processive hydrolysis [75]. (Fig.2)

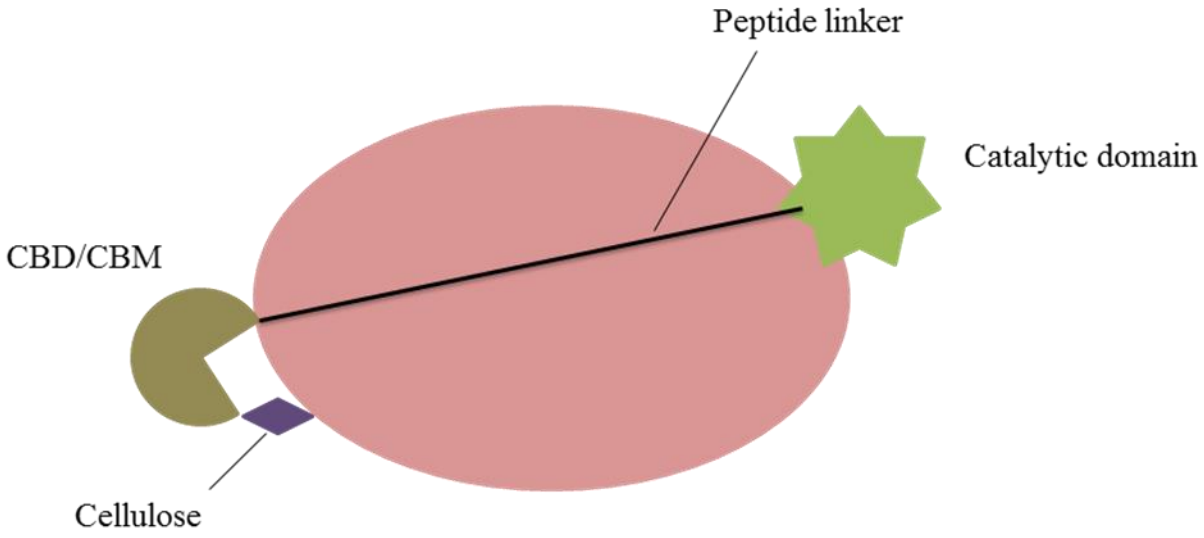

Figure 2: Fungal cellulases architecture consisting of catalytic domain and carbohydrate binding module linked through peptide linker.

5. Pichia Pastoris as an expression factory for thermostable fungal cellulases

5.1. Recombinant endoglucanases produced by P. pastoris

Endoglucanases by nature are produced by many sorts of fungal, microbal and plants species, moreover can also produce from digestive cannal of animals. Till now many thermophilic endoglucanases have been showed by *P.pastoris*. From various fungal strains the endoglucanases producing genes were collected such as fungal strain *Podospora anserine*, *T.reesei* [76], *Penicillium echinulatum* [77], *Syncephalastrum racemosum* [78], *Talaromyces emersonii* [79], *Myceliophthora thermophile* [80], *Phialophora sp. G5* [81], *Thermoascus aurantiacus* [82], *A. usamii* [83], *A. niger* [84], *Gloeophyllum trabeum* [85], *Trichoderma harzianum* [86], *A. fumigatus* [87], *Penicillium documbens* [88], *Bispora sp. MEY-1* [89], *Paecilomyces thermophile* [90], *Phaeosphaeria sp. LH21* [91]; edible straw mushroom *Volvariella volvacea* [92]; and nematode *Bursaphelenchus xylophilus [93]* (Table 1).

Better features cellulases recognition or enzymes engineering are of great importance for better result. Amongst the stated recombinant endoglucanases (r-endoglucanases) shown by *P.pastoris*, the majority thermophilic r-endoglucanase, the *T emersonii* gene product showed maximum performance at 90 °C and after 1h incubation at 70°C it retained about all the activity [94]. At 85°C the maximum performance of anther hyperthermophillic r-endoglucanase from *A.cellulolyticus* is stopped [95].At 50 °C for 12h endoglucanase obtained from *T.aurantiacus* withstand 100% of its activity [82]. At 60°C endoglucanase got from *A.fumigatus Z5* showed maximum performance and after 24h at its optimal temperature 60 °C it retained about 80% of its original activity [87].From *P.thermophile* the miximum endoglucanase activity 55,300 U/ml and uppermost reported specific activity(11,938)U/ml) against β-1,3-1,4-glucanase were achieved with recombinant barley β-glucan [90].Total extracellular protein contain about 85% of r-β-1,3-1,4-glucanase and go up to 9.1g/l protein concentration [90].

Endo-1,3-1,4-β-glucanase with *T.emersonii* CBS394.64 endoglucanase gene showed the second most high activity of 52,015U/ml [79]. At pH 6.0 neutral r-enduglucanase obtained from *phialophora sp.G5* have been showed maximum activity and 60 °C at a board pH range is quite stable, after 2h incubation maintain about 80% activity at 2.0-11.0 pH range and at 70 °C for 1h retained more than 85% of its initial activity [81].

5.2. Thermoacidophilic endoglucanases

In food, feed and biofuel industries thermoacidophilic endoglucanases have many applications. Plants biomass is mostly pre-treated at high temperature with acid before enzymatic bioconversion for biofuel production. Thermoacidophilic endoglucanase by optimizing their process conditions, temperature and pH after pre-treatment can meet all requirements for bioconversion. Thermoacidophilic endoglucanases have a wide range of industrial applications including feed, food and biofuel industries. During biofuel production, prior to enzymatic bioconversion, plant biomass is generally pre-treated with acid at high temperatures. Thermoacidophilic endoglucanases meet the requirement of bioconversion very well via optimizing their process conditions such as temperature and pH after pre-treatment.

At neutral PH of the process the thermoacidophilic endoglucanases may not work well for the efficient hydrolysis of lignocellulosic biomass if used together in the presence of neutral enzymes which might be able to cause too expensive production. The most acid loving endoglucanases amongst all fungal endoglucanases has been also expressed in *P.pastoris*, such as acidophilic endoglucanases from *P. documbens* (opt pH 3.5, Topt 60 °C) [88] and *G. trabeum* (opt pH 3.5, Topt 55 °C) [85]. Even though endo-β-1,3-1,4-glucanase from *Bispora sp. MEY-1* did not exhibited their optimal activity in the highly acidic condiotion (opt pH 5.0, Topt 60 °C), while the same endo-β-1,3-1,4-glucanase was fairly stable at acidic conditions retaining more than 85 % of its activity at pH 1.0–8.0 for 1 h [89].

5.3. Thermoalkaliphilic endoglucanases

Endoglucanases is mostly used in textile industries to produce an aged look in geans by removing dye from denims, eradicate microfibrils, reinstate bright colour, and softening of cotton fabrics [92]. Till now, acidic endoglucanases have been monetarily utilized as a part of denim industry because of their high reactivity and low costs yet they cause back recoloring and genuine harm in denim quality (Zhao et al., 2012d). Basic or neutral cellulases are highly like in material industry because of their minor or no back recoloring attributes. Additionally, thinking about the cleanser arrangements, enzymes utilized as a part of clothing applications ought to endure basic pH and furthermore high temperatures to accomplish financial esteem and reasonable application execution. As far as anyone is concerned, so far just two thermoalkaliphilic endoglucanases from *Phaeosphaeria sp. LH21*, displaying ideal movement at pH 8.0 and 60-65 °C, have been expressed by *P. pastoris* and they demonstrated in excess of 75 % of their maximal activity at pH 5.0-10.0 [91]. Additionally, they were generally steady at an expansive pH scope of 3.0-10.0 with keeping up in excess of 90 % of their unique movement for 1 h (Zhao et al., 2014).

Furthermore, some other impartial endoglucanases demonstrated resilience to alkaline environment; endoglucanase from *Phialophora sp. G5* showed in excess of 50 % of its highest activity at pH 4.0-10.0. Moreover , it was exceedingly steady at pH 2.0-11.0 for 2 h keeping up in excess of 80 % of its primary activity [81]. Another soluble base tolerant endoglucanase from *P. echinulatum* showed maximum activity at pH 5.0-9.0 [96] and endo-β-1,4-glucanase from *V. volvacea* was alkali adjusted enzymes showing 60 % of its greatest activity at pH 9.0 and stable at soluble pHs keeping up in excess of 60 % of its primary activity after 24 h at pH 4.0-11.0 [97].

5.4. Effects of codon optimization on r-endoglucanase production yield

As of late, codon optimization method has been

received much importance to enhance the productivity of recombinant proteins by *P. pastoris*. Uncommon codons have minimize mRNA firmness and interpretation rate; also, high GC substance may cause diminished translational yield or even mask existence [98]. Wild-type endoglucanase II quality (egII) of *T. reesei* was communicated by *P. pastoris* with productivity of 577.2 U/ml [52]. After improvement of the quality succession of EgII as per codon inclination of *P. pastoris*, expression yield expanded to 1783.35 U/ml [99]. The expression product of codon upgraded endoglucanase 1 (EgI) quality from *T. reesei* in *P. pastoris* was additionally enhanced to 1.24-overlap contrasted with recombinant creation level of wild-type EgI [100].

5.5. Recombinant thermophilic cellobiohydrolases produced by P. pastoris

Numerous cellobiohydrolases that had ideal action at temperatures in the range of 46 and 65 °C and optimal pH esteems inside pH 4.0-8.0 territory separated from Humicola grisea var. thermoidea [112], A. nidulans [113], T. reesei [36, 103], Phialophora sp. G5 [81], C. thermophilum [114] and Irpex lacteus MC-2 [115] were expressed by P. pastoris (Table 2). The high optimal temperature and thermostability, likewise alkaline optimal pH and expansive pH resilience, make cellobiohydrolases appropriate for modern applications, for example, in washing cleansers to remove soil stains and enhance shine and delicate quality, in textile products completing to expel microfibrils from cellulosic textures [112]. Basic and impartial cellulases are particularly critical for material industry as acidophilic catalysts lessened texture quality and tend to cause indigo back recoloring in denim (Li et al., 2011).[92].

A thermoalkaliphilic cellobiohydrolase from H. grisea var. thermoidea was efficiently expressed in P. pastoris. The recombinant cellobiohydrolase had best action at pH 8.0 and 60 °C and it was moderately balanced at 70 °C by means of keeping up 88 % of its movement after 4 h and all action was held after 6 h at pH 8.0. [112]. Cellobiohydrolase from Phialophora sp. G5 likewise showed extensive pH resilience and dependability; it Protein engineering of cellobiohydrolases In vitro coordinated development was helpful for C. thermophilum cellobiohydrolase (CBHII) to enhance chemical attributes and two mutant enzymes that contained 5 (R1S, A29T, L203Y, Q204K, and E252G) and 6 (A29T, T115I, I195V, L203Y, Q204K, and E252G) amino acids substitutions were acquired [114]. As the transformed buildups changed the isoelectric point of enzymes, this prompted best pH move; mutants principle response temperature and pH (optimal temperature 60 °C, selected pH 5.0 and pH 6.0) were prolonged contrasted with wildtype CBHII (T pick 50 °C, pick pH 4.0). Likewise transformations enhanced the thermostability of CBHII, mutants held in excess of 50 % of their action while the wild-type CBHII lost all the action at 80 °C for 1 h. [114].Number of hydrogen bonds is enhanced by mutation, and this may prompted to prolonged thermostability. As glutamine is counted as a thermolabile amino corrosive while lysine is a thermostable deposit [114],), substitution of glutamine by lysine may likewise add to the enhanced thermostability of transform enzymes. The reactivity of mutant CBHII were twice of the local CBHII; shaped hydrogen bonding may adjust the arrangement of cleavage site in the synergist space thus better interface amongst catalyst and substrate could be encouraged.

5.6. β-Glucosidases

In cellulose hydrolysis *β* -glucosidase is a key protein avoid the inhibition reaction causing through cellobiose from endoglucanase and cellobiohydrolase and at the same time also liberates the fermentable sugar [116]. *β*-glucosidases that have enhanced heat resistance, wide substrate specificity and high glucose resistance are much important as *β*-glucosidase additionally experiences input restraint by glucose.

5.7. β-Glucosidases potential application regions

β-glucosidases have numerous other applications in many industries other than utilized in biofuel industry. They change over harmful factor isoflavone glycosides to useful free isoflavone that is helpful against cancer, cardiovascular disease and osteoporosis (Yang et al., 2014). Moreover, *β*-glucosidases have been utilized to improve tea flavour, wine and fruit extract, and by transfer and condensation reaction change over glucose to functional oligosaccharides (Zhao et al., 2013). Denim bios toning, paper and detergent industries necessities are fulfill by alkaliphilic *β*-glucosidases.

5.8. Thermophilic β-glucosidases produced by P. pastoris

A large varieties of extremophilic *β*-glucosidase genes have been characterized, collected and expressed in *P.pastoris* such as from fungal specie *P. funiculosum* [117], *N. fischeri* [118], *A. niger* [116], *P. thermophile* [119], T. reesei [120], *Fomitopsis palustris* [121] and *Periconia sp* BCC2871 [122] (Table 3). At alkaline pHs higher than pH 8.0 an alkali-stable *β*-glucosidase from *Periconia sp* BCC2871 kept about all their activities for 2 h [122]. From *P. funiculosum* another glucose tolerant *β*-glucosidase was collected and up to 400 mM glucose concentration the recombinant enzyme showed tolerance [117]. A kind of fungus *F. palustris* also showed *β*-glucosidase with high thermostability of their half life of denaturation at 140.5 and 15.7 h, at 55 and 65 °C, respectively [121]. *P. thermophile* produces a kind of heat tolerant enzyme exhibited their half-life value of 1160 min at 50 °C [119].

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Table 1. Characteristics of extremophilic endoglucanases produced by *P. pastoris*

Fungal specie	Temperature opt (°C) and PH	Activity range	Stability rage	References
Thermophilic endoglucanases				
B. subtilis	65 °C, pH 6.0	>60 % at 30–70 °C	70 % EG1, 40 % EG4 after 30 min at 85 °C; pH 4.5–10.0	[101]
P. anserine	PaCel6A: 55 °C, pH 7.0	PaCel6A pH 5.0–9.0	<45 °C; PaCel6A pH <9.0	[76]
T. reesei	TrCel6A: 65 °C, pH 5.0	TrCel6A pH<6.0	TrCel6A pH < 6.0	[76]
Phialophora sp. G5	60 °C, pH 6.0	>50 % at pH 4.0–10.0	>85 % at 70 °C for 1 h; >80 % after 2 h at pH 2.0–11.0	[81]
V. volvacea	55 °C, pH 7.5		≤55 °C; pH 6.0–9.0	[92]
P. echinulatum	60 °C; pH 7.0	50–70 °C; pH 5.0–9.0	84 % after 1 h at 70 °C	[77]
P. thermophila	70 °C, pH 7.0		≤65 °C; 65 % after 30 min at 70 °C; >90 % at 50 °C for 30 min	[90]
S. racemosum	70 °C, pH 6.0; truncated: pH 5.0		>50 % after 1 h at 80 °C / 4 h at 70 °C	[78]
B. xylophilus	60 °C; Bx-ENG-1, 3: pH 5.8; Bx-ENG-2: pH 6.4	Bx-ENG-2: >70 % at pH 4.2–8.3; Bx-ENG-3: 25 % at pH 2.6		[93]
V. volvacea	55 °C, pH 7.5	>60 % at pH 9.0	>90 % at 50 °C for 1 h; >60 % at pH 4.0–11.0 for 24 h	[97]
Thermoacidophilic endoglucanases				
T. reesei	Q274V: 55 °C; others: 45 °C; pH 5.0		<20 % of EGI; >40 % of Q274V after 8 h at 65 °C	[102]
T. emersonii	70 °C, pH 4.5	>70 % at pH 4.0–5.5; >65 % at 55–75 °C	>95 % after 1 h at 65 °C; pH 2.0–12.0	[79]
T. emersonii	90 °C, pH 4.5	>70 % at 80–95 °C; >90 % at pH 4.0–5.0	almost all after 1 h at 70 °C; pH 1.0-10.0	[94]
M. thermophila	60 °C, pH 5.0	<80 °C; pH 4.0–7.0	<65 °C; t*1/2* = 9.96 h at 70°C, 6.5 h at 80 °C; pH 3.0–11.0	[80]
T. reesei	75 °C, pH 4.8	>80 % at 60–75 C; >85 % at pH 3.5–6.5	t*1/2* = 36 min at 70 °C, 2 min at 80 C	[99]
T. reesei	60 °C, pH 5.0	>60 % at pH 4.0–7.0	<60 °C; pH <6.5	[103]
Phialophora sp. G5	65 °C, pH 4.0–5.0	>50 % at pH 2.0–7.0	55 °C; >50 % at pH 2.0–9.0 for 2 h	[84]
Phialophora sp. G5	70 °C, pH 4.0–5.0	>45 % at 50–80 °C	51.6 % at 65 °C for 12 h; EgG5 and EgG5-Mut: pH 2.0–10.0; EgG5-CBM: pH 2.0–7.0	[104]
T. aurantiacus	70 °C, pH 5.0	pH 4.0–8.0	100 % at 50 °C for >12 h; >80 % at 70 °C for 2 h	[82]
A. usamii	60 °C, pH 5.0		≤55 °C; pH 3.5–7.0	[83]
T. reesei	55 °C, pH 5.0	>60 % at 40–70 °C; >50 % at pH 4.0–7.0	>90 % at 60 °C for 40 min	[52]
A. niger	70 °C, pH 4.0		>90 % at ≤60 C for 3 h; pH 3.0–10.0	[105]
G. trabeum	55 °C, pH 3.5		>80 % at 50 °C for 2 h	[85]
T. reesei	EGI: 45–55 °C; mutant: 35 °C; pH 5.0		EGI: 65 %,mutant: 57 % after 72 h at 50 °C	[106]
A. niger	55 °C, pH 5.0		<40 °C; pH 3.5–4.5	[107]
A. fumigatus	Egl2: 50 °C, pH 5.0; Egl3: 60 °C, pH 4.0		>80 % at 60 °C for 24 h;	[87]
Acidothermus cellulolyticus	80 °C, pH 5.1		pH 3.0–7.0	[95]
A. niger	70 °C, pH 5.0		>90 % at 70 °C for 30 min; at pH 3.0–9.0 for 30 min	[108]
Penicillium documbens	60 °C, pH 3.5	90 % at 70 °C		[88]

Bispora sp. MEY-1	60 °C, pH 5.0		stable at 60 °C for >1 h; >85 % at pH 1.0–8.0 for 1 h	[89]
Alicyclobacillus sp. A4	60 °C, pH 3.4		>75 % after 1 h at 75 °C and pH 3.4; pH 1.2–8.2 for 1 h	[109]
F. succinogenes	55 °C, pH 6.5	pH 5.0–8.0	>78 % after 30 min at 90 °C	[110]
Acidophilic endoglucanases				
T. harzianum	48 °C, pH 5.5		<40 °C	
A. niger	50 °C, pH 4.0	>60 % at 30–55 °C	55° C; 63. min at 60 °C; pH 2.0–7.0	[111]
T. reesei	35–55 °C, pH 5.0		96 % and 80 % at 50 °C after 48 and 72 h	[100]
Thermoalkaliphilic endoglucanases				
Phaeosphaeria sp. LH21	60–65 °C, pH 8.0	>75 % at pH 5.0–10.0	<70 °C; >90 % at pH 3.0–10.0; 50 °C for 1 h	[91]

Table 2. Characteristics of extremophilic cellobiohydrolases produced *by P. pastoris*

Fungal specie	Temperature opt (°C) and PH	Activity range	Stability rage	References
H. grisea	60 °C, pH 8.0		88 % after 4 h at 70 °C; 100 % after 6 h at pH 8.0	[112]
A. nidulans	CBHI: 52 °C, pH 4.5–6.5; 41 CBHII: 46 °C, pH 4.8; mixture: 50 °C, pH 4.9			[113]
T. reesei	55 °C; pH 5.5–6.0		<40 °C; pH 5.0–6.0	[103]
Phialophora sp. G5	65 °C, pH 7.0	>50 % at pH 4.0–10.0	>85 % at 70 °C for 1 h; >80 % after 2 h at pH 2.0–11.0	[81]
C. thermophilum	r-mutants: 60 °C, pH 5.0–6.0; r-native: 50 °C, pH 4.0		>50 % after 1 h at 80 °C	[114]
I. lacteus	50 °C, pH 5.0		80 % at 60 °C, pH 3.0–8.0 for 1 h	[115]
T. reesei	60 °C, pH 4.5–5.0			[36]

Table 3. Characteristics of extremophilic β-glucosidases produced by *P. pastoris*

Fungal specie	Temperature opt (°C) and PH	Activity range	Stability rage	References
P. funiculosum	60 °C, pH 5.0	>95 % at pH 4.0–7.0; ≤400 mM glucose	77 % after 1 h at 60 °C, pH 3.0–6.0	**[117]**
N. fischeri	80 °C, pH 5.0	>80 % at pH 4.5–5.5	70 °C; >50 % at 50–85 °C for 120 min; >80 % after 1 h at pH 3.0–9.0	[118]
A. niger	60 °C, pH 4.0	45–70 °C; 76 % at 50 mM glucose	>85 % after 30 min at 60 °C; pH 3.0–7.0	**[116]**
P. thermophila	55 °C, pH 6.0		t1/2 = 1160 min at 50 °C; >80 % at pH 5.0–11.0 for 30 min	[119]
N. takasagoensis	65 °C, pH 5.5		<60 °C; pH 2.5–8.5	**[123]**
T. reesei	70 °C, pH 5.0		>90 % after 60 min at 60 °C; pH 4.0–7.0	[120]
F. palustris	60° C, pH 5.0	>80 % at 50–70 C	t1/2 = 140.5 h at 55 °C; 15.7 h at 65 °C	[121]
Periconia sp BCC2871	70° C, pH 5.0	>50 % at 45–75 °C and pH 4.0–7.0	60 % after 1.5 h at 70 °C; 100 % after 2 h at ≥pH 8	[122]

6. Conclusions and Future Perspective

Late research has regularly showed the significance of the consistent approach for novel fungal strains which is helpful in enzymes production with special characteristics and can use for future industrial applications. For enzymes production the significance of recognizing viable expression host for recombinant cellulase articulation will be one of the most important factors. Enzyme production is a basic factor in the financial approval of this host and a critical hindrance in building up this expression host at the present level of innovation. It must be highlighted that obviously thermostable enzymes are obtained from thermophilic fungi. As the major enzymatic activity happens at 40-60 °C in most operational conditions, enzymes from hyperthermophiles are less appreciated then thermostable enzymes from thermophilic fungi. In heterologous gene expression methylotropic yeast *Pichia pastoris* has demonstrated incredible potential. Many factors are responsible for the expanding prominence of this expression host, for the molecular hereditary control of P. pastoris the straightforwardness of systems is required, high standard foreign protein and enzymes producing capacity of P. pastoris and performing eukaryotic post-translational adjustments ability.

Due to advances in biotechnological applications later on the industrial cellulose markets looking forward to expend in its volume. For the accomplishment of an extensive variety of businesses the new cellulase-based techniques will be determinant,moreover for lowering the manufacturing costs the streamlining of every specific condition will be necessary. In general for the categorization as well as designing of thermostable cellulases all the effort is engaged to increase degradation yields. To cope and overcome the hardness of the biomass, advance methodologies should be employed. Due to the recalcitrant nature of lignocellulsic biomass and employment of different physical, chemical technologies of pretreatment and biological technologies such as utilization of proteins and enzymes at low levels are much warranted and feasible to discharge high sugar and fermentative yields. The employment of mixture of various enzymes and cellulosomes designer in combination along with free enzymes has been proven optimistic to build up a novel substrate based defined enzyme cocktails. Though, nature may in any case hold the key for conveying novel enzymes.

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