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Review Article

Insights on Heterologous Expression of Fungal Cellulases in *Pichia pastoris*

Sajid Kamal[1], Shahid Ullah Khan[2], Nawshad Muhammad[3], Muhammad Shoaib[4], Mukama Omar[5, 6], Kaneza Pascal[1], Mukasekuru Marie Rose[1], Fubao Fuelbiol Sun[1, *]

[1]School of Biotechnology, Jiangnan University, Wuxi, P. R. China

[2]College of Plant Sciences and Technology, Huazhong Agricultural University, Wuhan, P. R. China

[3]Interdisciplinary Research Centre in Biomedical Materials (IRCBM) COMSATS Institute of Information Technology, Lahore, Pakistan

[4]School of Food Science and Technology, Jiangnan University, Wuxi, P. R. China

[5]Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, P. R. China

[6]Department of Biology, College of Science and Technology, University of Rwanda, Kigali, Rwanda

Email address:

fubaosun@jiangnan.edu.cn (F. F. Sun), saji_jiangnan@yahoo.com (S. Kamal)

[*]Corresponding author

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Abstract: Cellulosic biomass, the most remarkable renewable source of fuels and other compounds of potential importance, needs highly complex mixture of various enzymes for its degradation. For sustainable and economical bioprocess, the availability of these enzymes in high quantities and at a low price is much warranted. The advancements in discovery of new strains, highly valuable techniques and molecular biotechnological tools have led to *Pichia pastoris* gaining a recognized organism status both at industrial as well as laboratory level. With numerous beneficial characteristics on its credit *P. pastoris* has emerged a promising host for most of heterologous proteins production. Thus, the production of fungal cellulytic enzymes is of worth to be comprehensively illustrated. There are some activators including promoters used in the expression host to increase the utility of expression system. This review summarizes the heterologous expression of fungal cellulases (*Exo-glucanases*, *endo-glucanases* (*EGs*) and *beta-glucosidases* (*BGLs*)) and advocates the various kinds of promoters used in *P. Pastoris* for expression of these enzymes and proteins. Further, concluding remarks will provide insights revealing the scope of *P. pastoris* as a potential expression system tool in today's modern direction of research.

Keywords: *Pichia pastoris*, Heterologous Expression, Fungal Cellulases, Promoters, Synthetic Core Promoters

1. Introduction

Biomass is a principal source of food, coarse food, paper, pulp as well as delivers bioenergy ~10.2% (50.3 EJ/yr) of the total worldwide primary energy [1]. At present, the most potential and plentiful lignocellulosic biomass resulting from plant residues in the USA, South America, Asia, and Europe are corn stover, sugarcane bagasse, rice, and wheat straws, respectively [2]. From decades research is being carried out for increasing the digestibility of cellulosic biomass into various kinds of applicable products, but still no clear characteristics have been enumerated which may act as a proxy to indicate the course of successful hydrolysis of cellulosic biomass [3]. Cellulose plays the main structural function in plants and is synthesized through complex enzymatic machinery during cell wall synthesis [4]. Lignin generated by a biosynthetic process, build a protective seal around the other two constituents i.e., cellulose and hemicelluloses [5]. Cotton, chemical pulp, and flax signify new and fresh cellulosic biomass resources (up to 90%

cellulose), while wood has about 50% cellulose [6].

Novel fungal enzymes, need for high degradation of biomass, are attributed to at least 35 families of glycoside hydrolases, three of which are carbohydrate esterase and six of polysaccharide lyase [7]. The filamentous fungi are a prevalent source of fungal cellulases enzymes which are commonly exploited in the industry for biodegradation of biomass into useful products (i.e biofuels and chemical molecules) with a wide range of applications. The functional studies are progressing with such a rapidness on which genomic plus (post)-genomic data of novel fungi is being generated [8]. *Pichia pastoris* (*Komagataella phaffii*) has emerged as an alternative host system for the heterologous expression of desiredenzymes and proteins [9]. In contrast to other expression host, *P. pastoris* secretes endogenous cellulolytic enzymes in very low quantities [10].

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 [11]. Despite the fact that *P. pastoris* is beneficial expression system for the heterologous expression of proteins [12] still there exist a room for betterment on transcriptional [13] and posttranslational level [14]. 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 [15]. 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* [12]. Synthetic promoters generally render a broad range of expression levels (10−1000-fold) and finer growths [16]. The aim of designing promoters is scaling up the heterologous proteins and enzymes production. All of the recent efforts carried on to design promoters to alter the upstream regulatory sequences most commonly in *P. pastoris* and *S. cervisiae* [17].

This review gives detail elucidation on heterologous expression of fungal cellulases (*Exo-glucanases*, *endo-glucanases* (*EGs*) and *beta-glucosidases* (*BGLs*)) and various kinds of promoters commonly used in *P. Pastoris* for enzymes and proteins expression, to reveal the scope of *P. pastoris* in today's modern field of research as a good expression system tool.

2. Cellulosic Biomass

Cellulosic biomass, a complex plant derived organic compound, comprised of carbohydrates such as cellulose and hemicelluloses etc represents the most favorable choice for the production of renewable energy materials. It is a cheap, inexpensive and frequently occurring agricultural biomass, and desirable feedstock for sugar, chemicals and biofuels [18, 19]. Approximately 50-60% of celluloses, hemicelluloses biomass are accounts for a good sourceof pentose and hexose sugars using various celullases through bioprocessing [20]. Cellulose is a homogenous linear crystalline biopolymer composed of D-glucose monomers, linked covalently with (1→4) d-glycosidic bonds and also have amorphous non-crystalline regions, however the precise proportions and allocation within plant tissues and cell walls have not been entirely illuminated [21]. Cellulose has interlinked glucopyranose structure, each chain of glucan comprising over 25,000 glucose monomer [22]. 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 [22]. 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) [23]. Recalcitrance is regarded to be the chokepoint to exploiting biomass in the production of biofuels [24], 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 has need of 320°C and a pressure of 25 MPa to modify from the firm crystalline structure to an amorphous structure in water [6].

These microorganisms have been proven which containing high ability of cellulose productivity viz. *Actinomycetes*, *Butyrivibriofibrisolvens*, *Bacteroides succinogenes*, *Clostridium* species, *Aspergillus* species, *Ruminococcus albus*, *Chaetomium* species, *Myrothecium* species, *Penicillium* species, *Fusarium* species, *Methanobrevibacter ruminantium, Trichoderma* species etc [25]. The most common crystalline cellulose producing green algae include *Cladophorales (Chaetomorpha, Cladophora, Microdyction* and *Rhizoclonium*,) and some of *Siphonocladales* members, while *V. ventricosa* generated more and big microfibrils of cellulose as compared to plant microfibrils of cell wall [26].

Their modification is carried out in different ways in nature, but still limited numbers of microorganisms are involved in their degradation as compared to cellulose degradation [27]. The biomass conversion into fermentable sugars via enzymatic hydrolysis is a complex process [28]. Depolymerization of cellulose into glucose is accompanied by, both chemical and enzymatic hydrolysis. Chemical hydrolysis is carried out in harsh conditions using inorganic acids and gives toxic byproducts which need further detoxification, whereas enzymatic hydrolysis is smoothly done in mild conditions, free of byproducts [22]. That's why hydrolysis of cellulosic insoluble materials need the production of cell-associated or free extracellular cellulases [19]. In contrast, soluble substrates can be disseminated in enzymes active sites [29].

3. Major Cellulytic Fungal Species and Cellulases Productivity

The dissemination of industrial enzymes based on their

applications in food (45%), additive in surfactants (34%), textiles (11%), leather (3%), and pulp and paper industries [29]. The most commonly available commercial enzymes are derived from plants, animals, and microorganisms. The main part of industrial enzymes are approximately over 50%, however, bacteria and fungi produces also producing highly valuable enzymes, which are mainly due to simplicity of their growth, low nutritional necessities, and wide biochemical diversity. Hydrolases, such as cellulases, proteases, lipases and amylases, cover more than 75% of commercially available enzymes [30], and the demand of these enzymes are much more warranted.

Though numerous microorganisms are involved in enzymes cellulases production, but fungi are prominent microorganisms playing key role in the production of cellulases. That's why advanced studies are mainly focused on cellulase production of fungi [31-34]. The largest producer of cellulases is *Streptomyces,* some of their genera like *Micromonospora* and *Thermobifida* also used as a host for recombinant cellulases production [25, 35]. *Streptomyces thermoviolaceus* producing more thermo and PH stable recombinant cellulases, work in extreme conditions of industrial detergents and some ionic liquids [36]. *Thermomonospora fusca* generated cellulases commonly act on and degraded cotton and avicel [37]. That's why the increasing development of technology working as well discovered many organisms having the cellulose degradation machinery depolymerized cellulose into depolymerized and hydrolyzed products using in multiple applications.

In a recent study, by using six different Azurine cross-linked substrates *Penicillium chrysogenum* and *Cladosporium sphaerospermum* gives the highest production of *cellulase, arabinanase, β-glucosidase, mannase* and, *β-galactanase* activities and promote the degradation of lignocellulosic biomass. While *Stachybotrys* and *Chaetomium* showed low or had no cellulytic and xylanolytic activity by using Azurine cross-linked as a substrates [38]. *Melanoporia* sp. CCT 7736 acquired from coconut shell powder produced (7.50 IU/gds) of cellulase at optimized conditions at 24 h and pH 6.5 in a solid state fermentation, showed the same growth on both lignified and delignified substrates, avoid us from delignification [39]. *Proteobacteria* and *Firmicutes,* prevailed by the genera *Streptomyces, Arthrobacter, Bacillus, Pseudomonas, Stenotrophomonas* and *Pseudoxanthomonas* found in a chinampas saline-alkaline soils and humus content showed high cellulytic activities in a medium provided with NaCl up to 9% and at pH4.5–10.0. Furthermore, 84.8 of them degraded xylan while 71.7% degraded Avicel [40]. The most potent cellulytic fungal specie *Scytalidium thermophilum* SKESMBKU01 showed maximum cellulolytic activity of endo and exoglucanase in a media containing glucose as a carbon source complied by xylose and lactose, whilst used KNO3 as a nitrogen source for endoglucanase and urea for exoglucanase activity with the activity of (0.366 U/ml^{-1}), and exhibited high stability at 85°C and pH–8.0. Which indicates that both endo and exoglucanase from *Scytalidium thermophilum* SKESMBKU01 are highly stable at high temperature and in alkaline pH, whereas they both are also active in PH ranges of (pH 3-8) and temperature ranges up to (45–85°C) [41].

Recently about forty fungal species from Wadi El-Natrun lake areas have been studied most of them showed cellulases activity (95%) while with different degrees 15% of them exhibited xylanolytic activity and only 1% showed pectinolytic activity. Some of them produced cellulases only on the single type of media such as *Alternaria alternata* on control media*, Cladosporium cladosporioides* and *Aspergillus terreus* at PH 4 adjusted media and *Emericella nidulans*, *Cochliobolus australiensis*, *Fusarium solani*, generated cellulases on medium supplemented with 10% NaCl. Others species such as *Emericella nidulans* and *Cladosporium cladosporioides* produced cellulase on the control, 10% NaCl and the acidic media or alkaline media respectively. While some organisms like *Emericellanidulans* produced cellulase on both the control, acidic, alkaline and NaCl media. *Emericellanidulans* showed the highest xylanolytic activities. Nevertheless, only two strains belonging to *Cochliobolus australiensis* and *Fusarium solani*have not showen the capability of generating cellulase on all media used [42]. Some filamentous fungal species have been reported as *β*-glucosidase producers such as *Aspergillus, Talaromyces, Thermoascus*, *Penicillium, Trichoderma* [43, 44]. *Trichoderma reesei* is the well known organism play a pivotal role in cellulases production. Though, *T. ressei* has no ability to produce *β*-glucosidase which results in the accumulation of cellobiose and cello-oligosaccharides in media that gives catabolite repression on *endoglucanase* and cellobiohydrolase [45]. Especially, *Penicillium* sp. is widely used for *β*-glucosidase production [46, 47]. *Penicillium funiculosum* NCL1 has been accounts as an effective cellulolytic fungus and it has been also proven that up to 97% of cotton hydrolysis is accomplished by due to its high *β*-glucosidase production [48].

The production of cellulolytic enzymes and ethanol production via fermentation process needs at least 2unlike organisms conventionally, whereas recently reported single yeast isolate *Candida tropicalis* MTCC 25057 expressed xylanases and cellulases above the broad range of temperatures (32 and 42°C) through using various cellulosic substrates (carboxymethyl cellulose and wheat straw) and ferment the liberated sugars into xylitol and ethanol. It has been pointed out that the cultivation of *Candida tropicalis* at 42°C in pretreated hydrolysate having 0.5% wheat straw resulted in relative expression of cellulases (endoglucanases and exoglucanases) at concentrations of 97.8 U/gds and114.1, respectively. A high xylanase activity (689.3 U/gds) has been showen by the yeast specie in the same cultivation conditions [49]. *Trichoderma reesei* NCIM 1186 showed the maximum cellulytic activity of 30.85 IU/gds utilizing wheat bran as the substrate and coconut water as an extra dietary. The produced cellulase has been utilized for the enzymatic sacharrification of phosphoric acid pre-treated wheat straw [50]. While previous reports pertained to xylanase production showed that the strains of Penicillium sp., Aspergillus sp., etc. demonstrate

high xylanase activities ranging from 5000 to 7000 U/gds [51, 52]. *Candida tropicalis* also showed high tolerance to inhibitors for example, HMF, furfural and acetic acid, found usually in lignocellulosic hydrolysate but still producing lower ethanol and xylitol titers assigned to the accessible dietary shortage in hydrolysates. Though, in an attempt to accomplish proficient hydrolysis of pretreated cellulosic biomass, the expression and activities of the cellulolytic enzymes will encompass to be importantly meliorated by the over expression of heterologous cellulases, accessory proteins and can be improved moreover by strain adjustment to nutritionally deficient conditions and genetic engineering [49].

From few years thermophilic fungi have remarkable significant in production of thermostable cellulases and their key role in biomass degradation at high temperature [53]. The GHs families 1, 3, 5, 6, 7, 12, and 45, are frequently contributed to thermophilic fungal cellulases. Up to date, the complete repertoire of transcriptional factors involved in thermophilic fugal cellulases gene expression has not been illustrated as of *T. ressei*. They show their activities at the high temperature range in 50-80°C and in the PH range 4.0-7.0, also maintained the conspicuous thermal stability at 60°C with prolonged half-lives at 70, 80, and 90°C contrasted with other fungi [54]. Various thermophilic fungal species for example *Myceliopthora thermophila*, *Talaromycesemersonii*, *Chaetomium thermophilum*, *Melanocarpus albomyces*, *Humicola insolens*, *Humicola grisea*, and *Thermoascus aurantiacus* have been identified responsible for production of thermostable cellulases and hemicellulases [54]. Furthermore, these thermophilic fungal species have also the ability of higher production of cellobiohydrolases, endoglucansaes, β-glucosidases and xylanases [54, 55], which are the most warranted.

4. Functions of Carbohydrate Binding Modules and Cellulose Binding Domains

Mostly cellulases in bactaria are attached to their cell wall forming a structure called celllulosome [56] also known as microbial complex system, whereas fungi secrete cellulases directly into the environment, the system is called as a noncomplex or free system. The microscopic study showed that, enzymes subunits are set periodically on scaffolding proteins in cellulosome, having dockerins and cohesions [22]. Cellulosome comprises a fibillar protein scaffoldin. This protein also termed as “scaffoldins” and comprised of proteins CbpA, CipAn or Cip C [57]. Whereas dockerins are binds with enzyme subunit on one side and binds with cohesins on the other side. Theirs interactions playing a remarkable role in their structural architecture, having 6-9 different cohesions, bind to 26 various cellulosomal enzymes [22].

In fungal cellulases, the coupling module appears to perpetually have a place with the CBMI family and targets binding to cellulose surfaces [58]. The CD of exoglucanase Cel7A is made out of a β sandwich structure with a long substrate burrow lined by β-sheets, while four circles cover the passage. The CelA of *Cald. bescii* constitutes on a GH 48 and a GH 9 catalytic domain (CD), also containing type III cellulose binding modules. This carried cellulose hydrolysis not simply by surface ablation mechanism, but also carrying digging inside substrate surface, forming wide cavities therein [59]. The architecture of fungal cellulases comprising of a catalytic domain (CD) and cellulose binding domain (CBD) linked via peptide linkers [22]. Whereas CBD remains attached to 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. Until now64 families of CBMs have been updated in the CAZy database [60]. CBMs comprising varying amino acids monomers from 30 up to over 200, gives single, double or triple in one protein [61]. 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 [7]. These are either connected to the N-or C- end of the CD [62]. The nearness of CBMs is appeared to expand the binding of the substrate and focuses on the chemical towards particular substrates [61]. CBD is not directly needed in depolymerization of cellulose but its presence indirectly increase the catalytic performance of CD [63]. *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 [64]. The lower processivity of Cel6A is ascribed to a more open and shorter dynamic site burrow contrasted with Cel7A. Cel7B endoglucase has missing four β-sheets covering their tunnel, so having open furrow type active site.[64]. 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 shown that it works as a 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 [65].

The second class includes brown rot fungi (e.g *Postia placenta*) that need both CBMs and processive cellulases, in any case it is found to use crystalline cellulose as a sole carbon source, recommending the inclusion of non-enzymatic low atomic weight oxidants through the creation of receptive oxygen species (OH-, peroxide-or superoxide radicals), otherwise called Fenton response [66, 67].

5. Cellulases’ Mechanism of Action

CELs are the most important component of industrial processes having wide spread application in biotechnology, agriculture, and bio-energy uses mainly in the employment of cellulosic biomass for their corresponding products [68].

CELs are categorized into 1, 3, 5, 6, 7, 8, 9, 10, 12, 16, 44, 45, 48, 51, and 61 families. The Carbohydrate-Active Enzyme (CAZy) database is a specialized data facts system of enzymes that construct and deconstruct convoluted carbohydrates and their conjugates, comprised of 113 proteins families, have over 47% of the total glycoside hydrolases (GH) coding genes. Recently, these families are further subdivided for inconvenient grouping of proteins using their sequence similarities as specificity. Currently available subfamilies are only GH1, GH2, GH5 [60] and GH13 [69]. There are three important types of cellulases, which are used basically for the efficient degradation of cellulose [70], *Cellobiohydrolases* (CBHs), also named as exo-glucanases, attack on cellobiose producing crystalline ends of cellulose. *Endo-glucanases* (EGs) break the glycosidic bonds inside the amorphous part of the substrate. The products of cellobiohydrolases and endoglucanases are inhibitory to the activities of their enzymes, so finally, the unconfined cellobiose is split by *beta-glucosidases* (*BGLs*) into glucose monomers to facilitate organismal cellular uptake [61]. There is a high level of coordination between the enzymes, for examples exo/endo, exo/exo and endo/BGL synergy, which is required for the productive hydrolysis of cellulose crystal. (See figure 1) More recently discovered oxidative cellulases act like endoglucanases reducing long chain cellulose into short chain oligomers by free radical mechanism [22]. The enzymes *β*-glucosidases are categorized into GH1, GH3, and GH9 founded on resemblances in their amino acids sequence, moreover most of the fungal *β*-glucosidases is a part of GH1 and GH3. According to substrate specificity, *β*-glucosidases have been categorized into alkyl *β*-glucosidases that particularly hydrolyze cello-oligosaccharides (including cellobiose) and aryl *β*-glucosidases that mainly hydrolyze p-nitrophenyl-*β*-D-glucopyranoside (pNPG), cellobiase, whilst *β*-glucosidases have the capability to hydrolyze both substrates referred above [71].

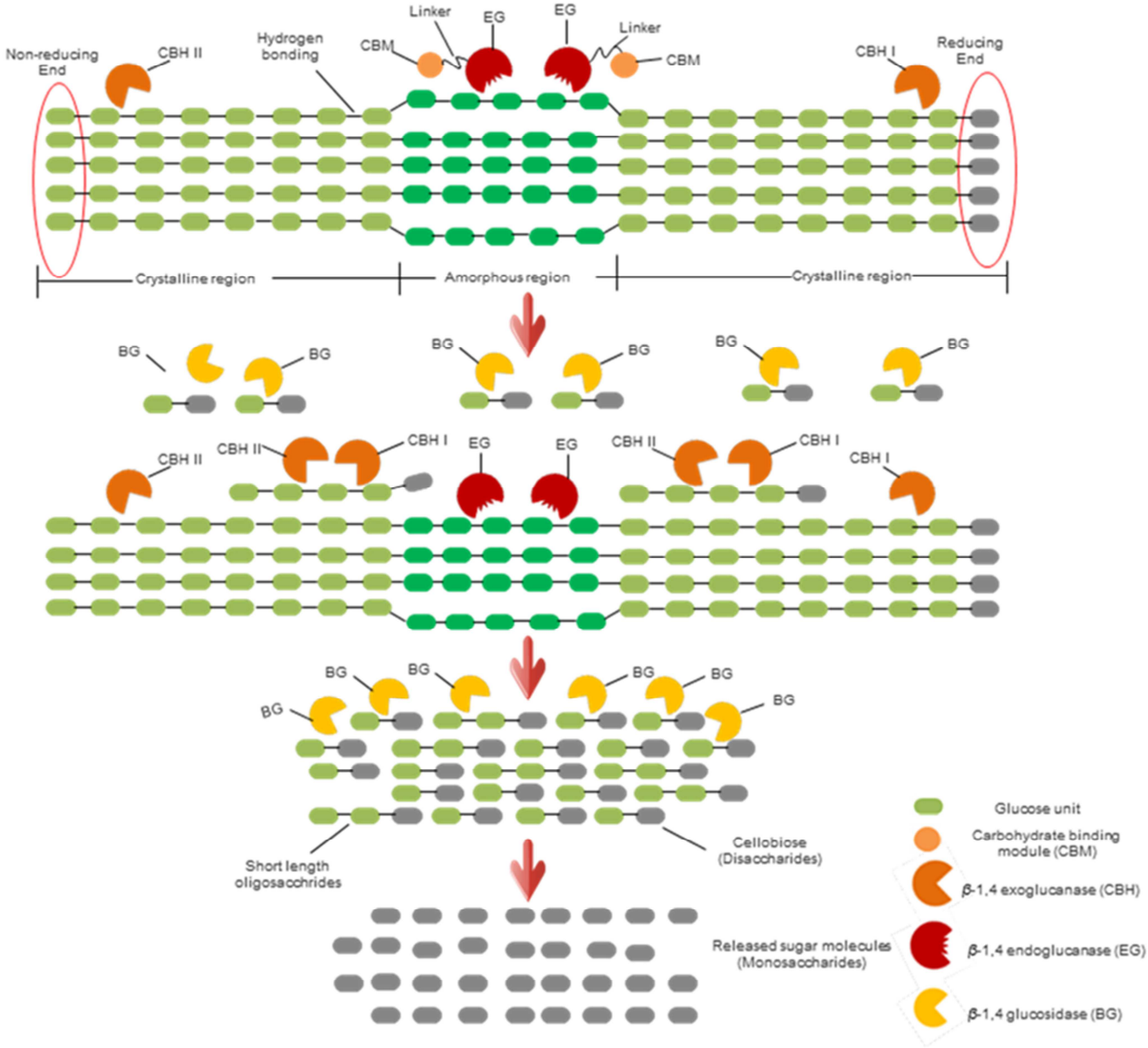

Figure 1. *Schematic Illustration of cellulose structure and cellulases' mode of action.*

CELs (*β*-1,4 glucan hydrolases) enzymes work in group act on cellulose synergistically to hydrolyze it, this extends the subject to high level specification and remarkable importance in the hydrolysis of biomass [72]. The increasing demand and high prices of CELs make constriction, resulting in wasteful production processes. These facts compelled the researchers on exploitation of low quality carbohydrates, improving strain strength through manipulating their genetic material for reduction of their high priced production [68].

Biomass degrading fungi having two different types of machineries that playing a remarkable role in the degradation of biomass are, direct enzymatic depolymerisation and generation of oxidative species [73]. The most studied fungal enzymes are saprobic ascomycetes, while enzymes in basidiomycetes are less studied but basidiomycetes showed the ability of novel and more applicable enzymes production [74]. The lack of lignin altering peroxidases enzymes of ascomycetes seldom degrade aromatic lignin chain, it merely acts on polysaccharide portion [75] but can produce laccases for lignin degradation [76]. While on the other hand, basidiomycetes are the main producer of lignin degrading enzymes, playing a key role in lignin rich biomass conversion and also have the polysaccharides degrading ability [77, 78]. Discovery of new genomic information changing this categorization of fungal species as Basidiomycota are classified as white rot and brown rot, perhaps will no longer adjust into these classes since changing the mode of action and product yield expression [79]. White rot fungi producing cellulases, also producing a suite of oxidizing enzymes of lignin deconstruction comprising on versatile peroxidase, manganese peroxidase, ligninase or laccase, or combination of these enzymes [80]. Recent insights in evolution brown rot fungi have evolved many times from, observing the current species of white rot fungi, Whereas they lost the lignocellolytic and cellulases producing characteristics [75]. *Trichoderma* genus has great importance in the high production of cellulases, especially the intension of researchers have been focused on the *Trichoderma ressei* celllulase production system for few decades [19]. Recently reported strains which are genetically engineered having the capability to generate primitive CELs (over 100 g/l) with high specificity to the elevated extent [65].

That's why the increasing development of technology working as well discovered many organisms having the cellulose degradation machinery depolymerized cellulose into depolymerized and hydrolyzed products using in multiple applications.

6. Synergy Studies Using Cellulases

Synergy among cellulases has been recognized between various cellobiohydrolases (with specifity for decreasing and non-lessening closes) amongst endo and exo-glucanase; between endo-glucanases; between cellobihydrolases, endo-glucases and *β*-glucosidases [81, 82]. The most astounding degree of synergy has been largely found on very crystalline cellulose substrates, for example, bacterial cellulose (5-10) and cotton (3.9-7.6), while more undefined cellulose by and large showed much more degrees of collaboration (0.7-1.8) [83]. Nonetheless, the examination by Andersen et al., 2008 [84] found the inverse with synergy showed by cellulases on phosphoric acid swollen cellulose (3.1), while no synergy was shown on Avicel. The degrees of synergy accordingly seem to differ contingent upon the idea of the substrate, the particular idea of the proteins and the test conditions [85]. Different audits have been distributed on the synergy between cellulases [86, 87]. While pretreatment of cellulose has been shown to enhance hydrolysis, it isn't certain whether this is because of expanded synergy or basic changes caused by the pretreatment. Pretreatment of cotton with acetone-ethanol at 1:1 ratio enhanced debasement of this cellulose with a 98.6% transformation accomplished [88]. Exceptional returns were likewise accomplished on Avicel pretreated with ionic fluids where a 100% change was accomplished inside 24 h with commercial enzyme mixtures [89]. This impact of pretreatments on the synergistic associations between cellulases has been explored by a few analysts. Samejima et al., 1998 [90] found no synergy on acid-pretreated bacterial cellulose utilizing a cellobiohydrolase and endoglucanase, while Ramirez-Ramirez et al., 2008 [91] discovered synergy between these two enzymes on acid-pretreated Avicel. Along these lines, the connection between expanded synergy and expanded hydrolysis is not clear.

The proportions of different cellulases utilized as a part of various examinations to acquire most extreme synergy showed expansive varieties. Bothwell et al., 1993 [92] utilized a proportion of *endoglucanses* to *cellobiohydrolases* of 3:1, while different investigations utilized higher proportions of *cellobiohydrolase* to *endoglucanase.* Boisset et al., 2001 [93] utilized 98.75% cellobiohydrolase to 1.25% endoglucanase; Ramirez-Ramirez et al., 2008 [91] utilized 8.1:1 cellobiohydrolase to endoglucanase; Berger et al., 2007 [94] utilized 17:1 cellobiohydrolase to endoglucanase; and Jung et al., 2008 [95] utilized a proportion of 10:1 cellobiohydrolase to endoglucanase. Hoshino et al., 1997 [96], then again, discovered ideal synergy with a mix of 1:1 cellobiohydrolase to endoglucanase. It isn't clear why such changed outcomes have been found, however, this might be because of enzyme qualities, test conditions or varieties in substrate attributes, especially coming about because of various pretreatment. These outcomes affirm that the main strategy to decide ideal synergy is through real biochemical assays with the particular enzymes and substrates.

Benoit et al., 2015 [97] evaluated the potential and approaches of plant biomass degradation of eight (8) *Aspergillus* species: *A. clavatus, A. nidulans, A. fischeri, A. flavus, A. oryzae, A. fumigatus, A. niger, and A. terreus* during cultivation using sugar beet pulp or wheat bran as a substrate. *Benoitet al*., 2015 [97] have been confirmed that the most similar fungi employ extremely varied enzymatic strategies for the similar substrates hydrolysis, although with the same

efficiency. They also presumed that in nature not all fungi degrade specifically the similar fraction of the substrate. Therefore, the classification of the enzyme sets used by different fungi can be exploited to propose proficient commercial enzyme cocktails by integrating these enzyme sets. This might be able to considerably advance the saccharification effectiveness of industrial and commercial enzyme cocktails. By integrating the whole enzymes set of two (2) fungi is probably enhance the saccharification effectiveness as compared to, by adding up specific enzymes to the cocktail produced by a single fungal species. This logic could be seen in an ecological approaching where mixed populations of fungi work together for degradation of plant cellulosic biomass. Therefore, this approach will be helpful for degradation of cellulosic biomass at industrial level [97].

7. *Pichia pastoris* as a Host Expression System

For screening and evaluation of enzyme libraries strains with desired characteristics are used, which require cloning and expressing in a suitable screening expression system. For screening of fungal enzymes, conventional yeasts such as *Kluyveromyces lactis*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and unconventional yeasts such as *Yarrowia lipolytica, P. pastoris* have been exploited equally [98, 99]. For screening of desired cellulytic enzymes *P. pastoris* has been employed as the first option for heterologous expression. Moreover, *P. pastoris* produces endogenous proteases in a very low amount by contrast to other eukaryotic expression host [10]. However, to get the highest possible product yield from *P. pastoris* might be cultured under well defined conditions, commonly manageable in continuously stirred tank fermentor. Thus the optimal exploitation of *P. pastoris* might takes more time and instrumentation resources as compared to *S. cerevisiae* expression system [100].

During the last three decades, *P. pastoris* majorly has become the best expression system for a number of heterologous proteins and enzymes because of some remarkable characteristics, these are; (1) the simplicity in genetic manipulation at molecular level and their resemblances to the most commonly used expression host *S. cerevisiae*; (2) having the ability of both intracellular or extracellular desired proteins production at maximum level; (3) A number of Post-translational modifications are carried out in it, such as di-sulfide bond formation glycosylation, and proteolytic processing; (4) the kit availability of their expression system at commercial level [12]; (5) Can also work as a useful model system tool to examine certain areas of modern cell biology including;(the implication and assemblage of peroxisomes; the selective autophagic degradation of peroxisomes; and the arrangement and function of the secretory pathway in eukaryotes) [101]. Both yeast and filamentous fungi have same phylogenetic origin, so *P. pastoris* a non-traditional yeast has the first choice for expression of various classes of active CAZymes, such as Cellulases [102, 103] GHs [104, 105], and AA9 enzymes such as lytic polysaccharide mono-oxygenases (LPMOs) [106, 107]. On behalf of much more benefits *P. pastoris* has first optionfor industrial enzymes production compared to other expression host systems [108]. Nevertheless, despite the fact that *P. pastoris* is currently well and best host for heterologous expression of various proteins and enzymes [12, 109] but still have a chance of betterment on transcriptional [13, 110] and posttranslational level [14, 111].

Recently *Scytalidium thermophilum* showed the activities of producing Oxido-reductases, namely Oxidase, Laccase, Tyrosinase and Peroxidase, and some Hydrolases like Lipase, Protease, Amylase and Cellulase. *Scytalidium thermophilum* (ST26) produced Laccase up to 1575 U/l in liquid media [112]. During study ofcellobiohydrolase I and II, endoglucanases and β-glucosidase, the highest yield production of endoglucanase from *V. volvacea* was obtained (100 mg/l) produced in *P. pastoris* [113]. The *T. ressei* produced a high yield of these proteins*TrbMan*1.142 g/l, *TrCBH2* 6.55 g/l, and *TlXynA* 1.2 g/l in fed-batch culture using *P. pastoris* as a host expression system [15]. The glucan 1,3-β-glucosidase gene A (exgA) from Aspergillus oryzae combined with α-factor of *Saccharomyces cerevisiae* was expressed in *pichia patoris* with over about one and half fold more extra-cellular enzyme activity (2 U/ml) using inducible promoter alcohol oxidase (pAOX1), while up to (1.4 U/ml) enzymatic activity was gained using GAP constitutive promoter using p-nitrophenyl-β-D-glucopyranoside as the substrate [114]. However, most fungal BGLs are set within the cells or outside the cell surface and those that are secreted are generally at very low degrees, while most of fungi express various different related BGLs [115]. While the β-glucosidase from *Aspergillus oryzae* gene GIF-10 was successfully expressed with production up to 321 mg/mL after incubation for seven days. And showed varying specificity in activity with hydrolyzing range of (1-4)-β-diglycosides and (1-4)-adiglycosides, while using Mn^{2+} as a stimulator [116]. The filamentous fungus *Penicillium funiculosum* described the maximum production ofcellulases such as carboxymethyl cellulase (13-15 U/ml), p-nitrophenyl-β-D glucopyranosidase (10-12 U/ml) and cellobiase (7.5 U/ml), showed high activity at pH (4.0) and temperature (50˚C) besides this showed high specificity in filter paper degradation. Nevertheless, it does not comprise carbohydrate binding domain and this was the first account on the expressionof cellubiohyrolase II from *Penicillium funiculosum* in *P. pastoris* [117]. The genes of the cellubiohyrolase GH6 family has been also cloned and expressed in *P. pastoris* from different fungal species such as *Irpex lacteus* MC2 [118], *Trichoderma parceramosum* [119] and *Chaetomium thermophilum* [120].

Till up to date, numerous GH3 β-glucosidases genes from fungi have been reported be expressed in *P. pastoris* [121, 122]; though, a limited accounts on heterologous expression of thermostable fungal β-glucosidase in *P. pastoris* [123, 124]. Recently discovered β-glucosidase of GH3 from *Myceliophthora thermophila* (mtbgl3b) has been expressed

successfully in *P. pastoris,* showed high stability at PH ranged over (3.0-10.0) and temperature ranges up to (60-65°C). The enzyme has proven to have maximum cellulytic activity on substrates such as pNPG, complied by p-nitrophenyl-D-cellobioside (pNPC), cellobiose, gentiobiose cellotetraose, and cellotriose. MtBgl3b has more hydrolytic ability towards the substrate cellotetaose than cellotriose and cellobiose, which showed that MtBgl3b can play a key role in cellulases cocktail, which will be work together during hydrolysis of cellulose with enzymes endoglucanase and cellobiohydrolase [125]. It has been reported that MtBgl3b has high activity at 60-65°C, alike to that of *Melanocarpus sp*. (60°C) [126] and *Penicillium purpurogenum* (65°C) [127], lower than that of *Thermoascus aurantiacus* (70°C) [128], but higher than *Fomitopsis pinicola* (50°C) [129], *Stachybotrys sp.* (50°C) [130] *β*-glucosidases activities. The isoform of *β*-glucosidase *Bgl4* of *P. funiculosum* NCL1 has been highly expressed in *P. pastoris,* with 1,354.3 U/mg of specific activity, showed maximum activity at pH 5.0 and 60°C. The isoform *Bgl4* showed possible betterment in the method of saccharification gives through the synergistic action with *T. reesei* cellulase [131]. (See table 1)

Table 1. *Charateristics of fungal cellulases produced by Pichia pastoris.*

Fungal species	Enzymes	Substrate specificity	Enzyme relative activity / Stability range	pH opt	Temp opt (°C)	References
Penicillium decumbens 114-2	*Cel45A*	konjac glucomannan, PASC, Sodium carboxymethyl cellulose (CMC-Na), barley *β*-glucan, xylan, Avicel	Ascomycota; subfamily B; 90% relative activity retained at 70°C and 30% at 80°C	5.0−3.5	60	[132]
Phanerochaete chrysosporium K-3	*Cel45A*	PASC, carboxymethyl cellulose (CMC), lichenan, barley *β*-glucan, glucomannan	-	-	-	[133]
Phialophora sp. G5	*EgG5, EgG5-Mut, EgG5-CBM, EgGH45*	Sodium carboxymethyl cellulose (CMC-Na), barley *β*-glucan, Avicel, filter paper	Activity range >45% at 50–80°C while 51.6% at 65°C for 12 h; EgG5 and EgG5-Mut: pH 2.0–10.0; EgG5-CBM: pH 2.0–7.0 showed stability	6.0−8.0	60	[134]
Syncephalastrum racemosum	*Cbh1*	Carboxymethyl cellulose (CMC), azurine cross-linked hydroxyethylcellulose	Zygomycota; retained >80% activity after 1 h at 80°C, and >50% for 4 h at 70°C	5−6	70	[135]
Penicillium decumbens	*Cel5C*	Glucomannan, tamarind seed gum, carboxymethyl cellulose (CMC), barley *β*-glucan	-	4.8	40−50	[136]
Penicillium echinulatum	*Egl1*	Carboxymethyl cellulose (CMC), filter paper, short oligosaccharides	-	5.0−9.0	60	[137]
Phialophora sp. G5	*EgG5*	Carboxymethyl cellulose (CMC), barley *β*-glucan, galactoglucomannan, filter paper, Avicel	-	4.0−5.0	70	[138]
Volvariella volvacea	*Eg1*	Carboxymethyl cellulose (CMC), PASC, filter paper	-	7.5	55	[139]
Aspergillus fumigatus	*Egl2*	Carboxymethyl cellulose (CMC), filter paper	-	5	50	[140]
Aspergillus fumigatus	*Egl3*	Carboxymethyl cellulose (CMC), filter paper, Avicel	-	4	60	[140]
Aspergillus nidulans	*EglB*	Carboxymethyl cellulose (CMC), cello-oligosaccharides	-	4	52	[141]
Aspergillus niger	*EglB*	Barley *β*-glucan, locust bean gum, cellobiose, CMC, laminarin	-	4	70	[142]
Gloeophyllum trabeum	*Cel5B*	Carboxymethyl cellulose (CMC), filter paper	-	3.5	30−70	[143]
Chaetomium thermophilum	*Cbh3*	-	*T 1/2: 45 min at 70°C*	4	60	[120]
Thermoascus aurantiacus	*Bgl1*	-	70% residual activity for 60 min at 60°C	5	70	[128]
Aspergillus nidulans FGSC A4	*Cbh1, CbhB*	Carboxymethyl cellulose (CMC), Glc3/Glc4/Glc5/Glc6	assay conditions 37°C and pH 4.5			[141]
Aspergillus nidulans FGSC A4	*EglB*	Carboxymethyl cellulose (CMC), Glc4/Glc5/Glc6, barley β-glucan, lichenan, xyloglucan	-	5.5	42	[141]
Bispora sp. MEY-1	*Bgl7A*	Barley *β*-glucan, lichenan, carboxymethyl cellulose (CMC), laminarin, xylan	stable at 60°C for at least 1 h and at pH 1.0−8.0	5	60	[30]
Chaetomium thermophilum CT2	*Cbh3*	pNPC, MCC, filter paper	-	5	60	[144]
Fusicoccum sp. BCC4124	*Cbh1*	Avicel, filter paper, 4-methylumbelliferyl-β-D-cellobioside	stable at pH 3−11 and maintains ∼50% at 70−90°C for 30 min	5	40	[35]

Fungal species	Enzymes	Substrate specificity	Enzyme relative activity / Stability range	pH opt	Temp opt (°C)	References
Myceliophthora thermophila	*Eg7A*	Barley *β*-glucan, carboxymethyl cellulose (CMC), lichenan, arabinoxylan, xylan, filter paper, hydroxyethylcellulose, Avicel, Glc3/Glc4/Glc5	stable at pH 3−11, retaining initial activity after 24 h	5	60	[35]
Trichoderma pseudokoningii	*Cel7B*	4-methylumbelliferyl-β-D-lactoside, carboxymethyl cellulose (CMC)	-		45	[145]
Chaetomium thermophilum	*Cel6A*	pNPC, BMCC, filter paper, carboxymethyl cellulose (CMC)	-	4	50	[146]
Irpex lacteus MC-2	*Cbh2 (Ex-4)*	Avicel, PASC	-	5	50	[147]
Phialophora sp. G5	*EgGH6A*	Sodium carboxymethyl cellulose (CMC-Na), barley *β*-glucan, Avicel, filter paper	retained >40% activity at pH 4.0−10	7	65	[134]
Podospora anserina S mat+	*Cel6A*	Avicel, carboxymethyl cellulose (CMC), Glc3/Glc4/Glc5/Glc6	-	7	45	[148]
Podospora anserina S mat+	*Cel6B*	Avicel, carboxymethyl cellulose (CMC), Glc3/Glc4/Glc5/Glc6	-	7	35	[148]
Podospora anserina S mat+	*Cel6C*	Avicel, carboxymethyl cellulose (CMC), Glc3/Glc4/Glc5/Glc6	-	6	25	[148]
Trichoderma reesei QM9414	*Cel6A*	Avicel, carboxymethyl cellulose (CMC), Glc3/Glc4/Glc5/Glc6, PASC	-	5	45	[148]
Penicillium funiculosum	*CMCase, pNPGase and cellobiase*	Showed more specificity in filter paper degradation	-	4	50	[117]
Myceliophthora thermophila	*β*-glucosidase (MtBgl3b)	p-nitrophenyl-*β*-D glucopyranoside (pNPG) p-nitrophenyl-D-cellobioside (pNPC), cellotriose, cellobiose, cellotetraose, and gentiobiose	-	3.0–10.0	60-65	[125]
Aspergillus oryzae	glucan 1,3-*β*-glucosidase	p-nitrophenyl-b-D-glucopyranoside	-			[114]
Aspergillus oryzae	*β*-glucosidase	hydrolyzing range of (1-4)-*β*-diglycosides and (1-4)-adiglycosides	-	6.5		[116]
Trichoderma reesei	Cbh2	Carboxymethyl cellulose (CMC)	Showed stability at 90°C	5.5	60	[149]
B. subtilis	Eg1, Eg2, Eg3, Eg4	-	Activity range >60% at 30–70°C while 70% EG1, 40% EG4 after 30 min at 85°C; pH 4.5–10.0 showed stability	6.0	65	[150]
P. anserine,	PaCel6A	-	PaCel6A pH 5.0–9.0; <45°C; PaCel6A pH<9.0	7.0	55	[137]
T. reesei	TrCel6A	-	TrCel6A pH <6.0	5.0	65	[137]
Aspergillus nidulans	Cbh1,	-	-	4.5–6.5	52	[151]
Aspergillus nidulans	Cbh2	-	-	4.8	46	[151]

8. Promoters Involved in *Pichia pastoris* Metabolic Pathway

The promoters used in fungi for heterologous expression of genes are broadly divided into constitutive and inducible promoters, though all arise from genes that are extremely expressed in the specified expression hosts. The benefit which constitutive promoters have is that, they show high expression under almost at all culture conditions, letting high flexibility in the production of the enzyme. In contrast, inducible promoters need more specific and explicit conditions for induction, but give more production of the enzyme as compared to constitutive promoters. Furthermore, inducible promoters have been exploited for expression and production of those proteins that have negative consequences on the expression host system. In such a case, the proteins producing strain can be primarily cultivated in non-inducing conditions after enough growth has taken place, the inducer can be supplemented to enhance and produce the maximal high protein in a short extent of time.

P. pastoris, the methylotrophic yeast using methanol as an inducible promoter strongly regulates the alcohol oxidase 1gene (pAOX1) has been explored and well studied in terms of regulatory sequences and factors [152]. Up to now an inducible promoter AOX1 and to a certain degree, the constitutive promoter GAP were more widely used promoters in *P. pastoris* for heterologous protein and enzymes production [12]. A large number of various proteins and enzymes have been expressed under the control of alcohol oxidase 1gene (pAOX1) regulated by methanol, with yields up to 14.8 g l^{-1} (of mouse collagen), and claims up to 20–30 g l^{-1} [153] of recombinant protein. Since *P. pastoris* prefers an aerobic rather than an anaeobic mode of culture conditions

and growth, products from an anaerobic culture such as acetic acid and ethanol do not build up fast [154], altering cultures to reach high cell densities [155]. In the pathway of methanol metabolism, the initial step is the oxidation of methanol to formaldehyde, producing hydrogen peroxide (H_2O_2) in the process, by the enzyme alcohol oxidase (AOX). During this step the toxic hydrogen peroxide is generated, to prevent their toxicity this step is carried on within peroxisome, which keeps apart hydrogen peroxide from the other components of the cell. The promoter AOX is a homo-octomer comprising on one cofactor which is non-covalently bound FAD (flavin adenine dinucleotide) to each one subunit. Alcohol oxidase has low affinity towards O_2, and methylotrophic yeasts seems to counterbalance this deficiency by synthesizing high quantities of the enzyme [156]. The yield and production quality of enzymes and proteins are exceedingly affected by culture conditions, therefore a variety of culture conditions have been developed in wild-type *P. pastoris* and glyco-engineered *P. pastoris* [157, 158]. The yield production of cellulase either at pilot scale or large industrial scale needs desirable bioprocessing, specific growth medium and optimal conditions [72]. Now days both submersed and solid state bioprocessing are very common for cellulases production [159]. The demonstration showed that at least 7 integrated expression cassettes enhance the quantity of produced enzymes in a linear manner [15]. However, to study effects of codon optimization combining with different promoters and gene copy number to supply data to make *P. pastoris* as a valuable model for codon optimization, there have been screened more than 15,000 *P. pastoris* transformants resulting by analysis of purported single copy activity landscapes. The copy number can be defined of any integrated expression cassettes using quantitative real time PCR (qRT-PCR) in *P. pastoris* [111]. There are various ways of how to realize the highest possible gene copy numbers are reviewed [160], e.g. in Gasser *et al*., 2013. Furthermore, codon optimization to alter the codon use of the desired produced gene to the desirable host codon use has been reported to show the way to get the valuable results, although there isvarious argumentation which kind of optimization is the most excellent [161]. Additionally, the folding and secretion capability has a significant effect on the yield and production of secretory enzymes [162].

A key factor in process conditions influencing the production of recombinant proteinsin *P. pastoris* is the transcriptional regulation of promoter alcohol oxidase 1 (pAOX1). The demonstration showed the regulation of AOX1 and peroxisome biogenesis based on various process conditions, whereas two types of GFP-fusion proteins, Ub-R-GFP (shortlived GFP in the cytosol) and GFP-SKL (peroxisomal targeting GFP) of AOX1 and peroxisome biogenesis respectively, were used successfully to characterized their timed (time-course). Based on various fermentation process conditions the activities of promoter alcohol oxidase 1 (pAOX1) and peroxisome biogenesis has been checked out- methanol limited condition, changed flowing of carbon sources and oxygen limited condition. The promoter alcohol oxidase 1 (pAOX1) is dynamic under limited methanol condition and peroxisome biogenesis and is also dynamic under a carbon limited fed batch culture process condition, but AOX1 promoter is less dynamic under oxygen limited condition while the growth of peroxisome biogenesis has been an account of being inhibited in a glucose containing media. The AOX1 promoter gives higher productivity of specific monoclonal antibody at 86 h of induction in the methanol limited condition than in the oxygen limited condition (0.026 vs 0.020 mg $g^{-1}h^{-1}$). Nevertheless, due to high cell fitness the oxygen limited culture condition is more suitable for longer induction process (180 h). This showed that the higher production of desired protein is influenced by the consumption rate of methanol indirectly affected by the methanol and oxygen molecules availability [163].

The AOX1 promoter of *P. pastoris* is the most prevailing and frequently applicable promoter for controlling expression and production of enzymes and proteins. However, its transcriptional regulatory mechanisms are still unclear. The Zn(II) (2) Cys (6)-type methanol-induced transcriptionfactor1 (Mit1) has been identified which regulates the expression of multi-genes required form ethanol consumption pathway, including alcohol oxidase 1 (AOX1), but did not take part in the uncontrolled growth of peroxisome and transportation of peroxisomal proteins throughout the methanol metabolism. Significantly, methanol-induced transcription factor 1 (Mit1), Mxr1, and Prm1 (also called Trm1), which positively regulated Promoter AOX1 under the control of methanol, were attached to Promoter AOX1 at various sites but they did not relate and cooperate with each other. However, these factors cooperatively activated Promoter AOX1 via cascade. Mxr1 mostly worked throughout the carbon derepression in the culture, while Mit1 and Prm1 worked in the methanol induction, with Prm1 conveying methanol signal to Mit1 by binding to the Mit1 promoter, as a result progressively expressing Mit1 and later on activating Promoter AOX1 [164].

The fungal cellulytic enzymes endoglucanase II and cellobiohydrolase II from the fungus *T. reesei* QM9414 has been expressed in *Yarrowia lipolytica* using promoters POX2 or TEF along the use of either the native or preproLip2 secretion signals. While the same enzymes have been expressed in *P. pastoris* under the methanol inducible promoter (pAOX1) and compared both *Yarrowia lipolytica* and *P. pastoris* as an expression host system, showed that *Y. lipolytica* produced endoglucanase II and cellobiohydrolase II about 15 mg/l and 50 mg/l respectively, which is higher than *P. pastoris* proteins production ability. While both systems showed the same endoglucanase II activity on account of total enzymes activity, also showed the same glycosylation, PH and temperature visibilities [99]. *P. pastoris* utilizes promoter AOX1 to assure maximum elevated levels of protein expression. Though, methanol is harmful and arises a fire hazard, consequences which befall more noteworthy at both pilot and industrial levels. It is promising to take out these risks and hazards while keep thehigh production level by switching to the constitutive promoter *(*p*GAP)*. More recently

marketed *PichiaPink*™ expression system permits and expressed two (2) endoglucanases, Af Cel12A from Aspergillus fumigatus and Ta Cel5A from Thermoascus aurantiacus, controlled by either the methanol inducible promoter *(*p*AOX1)* or the constitutive promoter (p*GAP)*. It has been demonstrated that the levels of Af Cel12A in small tubes and bioreactors achieved by using constitutive promoter might be the same or high, compared to the promoter AOX1 levels, whereas the levels of Ta Cel5A might be slightly lower. After optimization of culture conditions using a 15L bioreactor, the recombinant expression host P. pastoris employing the constitutive promoter (p*GAP*) has been produced ca. 3-5 g/l of the entire secreted protein, with enzyme Carboxymethyle cellulase activity equivalent to 1200 nkat/ml Af Cel12A and 170 nkat/ml Ta Cel5A [141].

The reported optimization study showed based on DNA level, assessing 48 various individual TrCbh2 synthetic genes, which codes the same sequence of *T. reesei* cellulase combining with various three promoters (Constitutive promoter PGAP and synthetic promoter AOX1 with random variables Pen (enhanced) and P_{DeS} (derepressed). The determined activity of secreted enzyme examines from the supernatants of 96 well plates, while their expression varied from insensible to ~300% of known gene. Eventually, based on the best optimal conditions of the gene and novel promoters, by combining them the high yielding and expression strains of *P. pastoris Cbh2* was constructed. Although, under the well controlled conditions in laboratory scale bioreactor cultivations without induction of methanol no more final titer has been produced of secreted protein than 18g/l after 60-70 h at glycerol feeding media [165]. P_{DeS} is a strong methanol inducible promoter as well active under derepressed conditions is also disclosed by protein titer finding. The highest attained titer of generated protein TrCbh2 was about over 17-20 g/l under the control of P_{DeS} in the absence of methanol [163]. This showed the latest and highest result of secreted enzyme concentration in *P. pastoris* and furthermore showed the optimization of individual parts of expression cassette and their linear effects to enhancement in protein yield production by *P. pastoris* [165]. Recent study showed that a novel strong constitutive promoter P*GCW*14 was identified, and by using green fluorescent protein (EGFP) its promoter activity was tested. P*GCW*14 were cloned upstream of the EGFP gene under different carbon sources, which showed strong promoter activity than the most common promoters P*TEF*1 and P_{GAP}. So, we can use P*GCW*14 as a strong promoter for high yield production of desired proteins and enzymes [166].

The production of desired enzymes under the control of the PADH3 has been compared with the most commonly used promoters PAOX1 and PGAP *of* P. pastoris. Regulation of methanol at production level is a fundamental parameter for sustaining the cell growth and increasing the production of recombinant desired proteins and enzymes by *P. pastoris* [167]. Instead of raising the amount of methanol to a Mut^+ strain, the mutant strains of *P. pastoris* with a slow consumption of methanol (Mut^S) have been exploited. These mutant strains utilize minute amount of methanol as well as need very low oxygen, characteristics that are beneficial in the growth of high cell density. However, it has been an account that Mut^S strains grow very slowly compared to Mut^+, while their productivity and yield is very specific [168]. An enzyme *β*-glucosidase derived from the fungus specie *Periconia* sp. BCC2871 has been revealed prospective for efficient cellulosic biomass biodegradation due to its thermal tolerance. This enzyme has been effectively expressed in *P. pastoris* KM71 in control condition of methanol with more efficiency, at the lowest incubation time of 2 h and at temperature of 70°C. The growth of *P. pastoris* KM71 at laboratory level, an extended duration of poor methanol induction can cause low down of cell concentration and restricted the productivity of this thermostable *β*-glucosidase in an expression system [122]. T. Charoenrat *et al.,* 2015 [167] has been investigated the optimization of supply rate of the primary methanol induction for high cell density fed-batch growth of fungal enzyme *β*-glucosidase in a slow methanol utilization strain (Mut^S), *P. pastoris* KM71. The primary induction at a cell concentration of 60 g/l through changing various methanol supply rates, the optimal primary methanol feeding rate has been find out up to 30.34±0.34 mg/g·h. According to the determined optimal feeding rates limitation and factors the methanol feed rate has been subjected at the elevated cell concentrations of 80 and 100 g/l for recombinant *β*-glucosidase production. The activity of recombinant *β*-glucosidase gained was of 2851.7±14.6 U/ml, which was four (4) times higher than that gained through the declared condition (40 g/l initial cell concentration). T. Charoenrat *et al.,* 2015 [167] gained the maximum activity of recombinant *β*-glucosidase in the growth medium when the maximum primary cell concentration of 100 g/l has been exploited. This approach can be implemented for the production of all kind of recombinant enzymes including endo and exoglucanases and for all other recombinant proteins production in *P. pastoris* through high cell density cultivation. (See table 2).

Table 2. *The most characterized and newly recognized promoters for recombinant fungal enzymes expression in Pichia pastoris.*

Gene Name	Gene product/function	Highest expression level	Reference
Inducible promoter			
AOX1	Alcohol oxidase 1	Strong (naturally ~5% of mRNA and ~30% of total protein)	[12]
AOX2	Alcohol oxidase 2	~5–10% of P_{AOX1}	[169]
DAS	Dihydroxyacetone synthase	Strong (similar to P_{AOX1})	[170]
FLD1	Formaldehyde dehydrogenase	Strong (similar to P_{AOX1})	[171]
PEX8	Peroxisomal matrix protein	Weak	[12]
THI11	Protein involved in synthesis of the thiamine precursor hydroxymethylpyrimidine	-	[172]

Gene Name	Gene product/function	Highest expression level	Reference
ENO1	Enolase	~20–70% of P_{GAP}	[173]
ICL1	Isocitrate lyase Repressed by glucose, induction in absence of glucose/by addition of ethanol	-	[174]
PHO89	Putative Na+/phosphate symporter Induction upon phosphate starvation	-	[175]
ADH1	Alcohol dehydrogenase Repressed on glucose and methanol, induced on glycerol and ethanol	-	[176]
GUT1	Glycerol kinase Repressed on methanol, induced on glucose, glycerol and ethanol	-	[176]
Constitutive promoters			
GAP	Glyceraldehyde 3-phosphate dehydrogenase	Strong (similar to P_{AOX1})	[177]
GPM1	Phosphoglycerate mutase	~15–40% of P_{GAP}	[173]
HSP82	Cytoplasmic chaperone (Hsp90 family)	~10–40% of P_{GAP}	[173]
ILV5	Acetohydroxy acid isomeroreductase	~15% of P_{GAP}	[178]
KAR2	ER resident chaperone (also termed Bip)	~10–70% of P_{GAP}	[173]
KEX2	Endopeptidase involved in the processing of secreted proteins	~10% of P_{GAP}	[178]
PET9	ADP/ATP carrier of the inner mitochondrial membrane	~10–1700% of P_{GAP}	[173]
PGK1	Phosphoglycerate kinase	~10% of P_{GAP}	[173, 179]
SSA4	Heat shock protein	~10–25% of P_{GAP}	[173]
TEF1	Translation elongation factor 1 alpha	Strong (similar to P_{GAP})	[152, 173]
TPI1	Triose phosphate isomerase	~10–80% of P_{GAP}	[173]
YPT1	GTPase involved in secretion	Weak	[12, 180]
GCW14	Potential glycosyl phosphatidyl inositol (GPI)-anchored protein constitutive expression on glycerol, glucose and methanol	-	[181]
G1	High affinity glucose transporter Repressed on glycerol, induced upon glucose limitation	-	[182]
G6	Putative aldehyde dehydrogenase Repressed on glycerol, induced upon glucose limitation	-	[182]
Promoters derived from literature data			
GND1	6-phosphogluconate dehydrogenase (decarboxylation)	-	[179]
MCM1	Transcription factor involved in cell-type-specific transcription and pheromone response	-	[179]
RPL1	Ribosomal protein of the large (60S) subunit	-	[179]
RAD2	Single-stranded DNA endonuclease, DNA damage repair	-	[179]
RPS2	Ribosomal protein of the small (40S) subunit	-	[179]
RPS31	Fusion protein that is cleaved to yield a ribosomal protein of the small (40S) subunit and ubiquitin	-	[179]
TKL1	Transketolase	-	[172]

9. Constructed Synthetic Core Promoters and Terminators of Pichia pastoris

Promoters covering a broad range of expression levels should be in approach to enable expression fine-tuning, ranging from tight down regulation to high over expression. To this end, either natural or synthetic promoters can be used. Synthetic promoters generally render a broad range of expression levels (10−1000-fold) and finer growths [16].

The aim of designing promoters is scaling up the heterologous proteins and enzymes production. All of the recent efforts carried on to design promoters to alter the upstream regulatory sequences most commonly in *P. pastoris* and *S. cervisiae* [17]. Cellulose is the most common inducer for production of fungal cellulases and is regulated by a repressor and inducer system [183]. In prokaryotes, where promoters are the minimal region furnishing binding sites for RNA polymerase II and some common transcriptional factors, whereas in eukaryotes promoters are more retentive and composite part of DNA [17]. The most usual promoter alcohol oxidase 1 gene (pAOX1) of *P. pastoris* regulated by methanol has been studied in terms of regulatory sequences and factors [152]. It had been also employed for variants designing of synthetic promoter rendering a wide range of expression levels and changed the regulatory profiles. These design engineering attempts have been focused on upstream regulatory sequences (URS) [110, 184] unsystematic methanol utilization agenesis of core promoter sections [185] or the 5′ untranslated region (UTR) [186]. For the first time, it has been reported to engineer a completely synthetic core promoter along with 5′ UTR for *P. pastoris* and to utilize such artificial sequences for designing of pAOX1 core promoter to acquire a sequence-diversified promoter library with diverse characteristics. Designing of URS can also obstruct the promoters regulation [110]. The way of controlled regulation stayed untouched for the fusions of synthetic core promoter/pAOX1 URS. That's why the same engineering strategy has been used for prokaryotes, where the sites of ribosome binding altered to fine-tune strong natural promoters [187], is practicable by designing eukaryotic core promoters and UTRs. As contradicted to engineering and designing strategies of the notably longer whole promoter, such short core promoters can directly contribute to a PCR primer helping library generation as well as have enough capability to acquire variable expression yields [17].

Even though, this promoter designing strategy is employed mostly in bacteria [188]. There are very few examples in which a promoter library has been designed in yeast cell [189], besides a single promoter library has been designed for fine-tuning of gene expression in *P. pastoris* [110]. Hartner., *et al* 2008 [110] designed a library comprised of 46 mutant promoters via duplication and deletion of putative transcription factor binding sites in the methanol inducible

promoter gene 1 (*pAOX1*). By induction of methanol the designed library extended from 6% to 160% of the natural promoter activity. Hartner., *et al* 2008 [110] moreover deliberate short synthetic promoters by fusing *pAOX*-derived *cis*-acting elements with a variety of basal promoters, because short promoters usually allocate to an extra efficient PCR-based production of expression cassettes or mutant libraries as compared to long promoters. However, all the designed five (5) synthetic promoters showed no more than 10% of the activity of the wild-type methanol inducible promoter (*pAOX1)*.

For expression of gene across a broad dynamic range in *P. pastoris* and to facilitate manipulation, a functional promoter library by yeast-enhanced green fluorescent protein (yEGFP) as the reporter has been created by mutagenesis of the constitutive promoter (p*GAP)*. The mutants' numbers (33) have been selected to figure the efficient promoter library which extended an activity range between 0.6% and 19.6-fold of the wild-type promoter activity by roughly linear fluorescence intensity allocation. The promoter library consent regulates the expression of gene and quantitative assessment accurately that associates gene expression level and physiologic parameters. Therefore, it is a helpful toolbox for both fundamental and practical study of *P. pastoris* [190]. These difficulties emphasized the need for engineering a promoter library derived from a constitutive promoter, which would allow regulation of steady-state gene expression and make sure transcriptional homogeneity with no inducer. The key enzyme of glycolysis Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which gives strong constitutive expression on glucose for promoter (*PGAP*) at a level similar to that seen with inducer promoter (*pAOX*) [177] and has been extensively employed for constitutive expression of heterologous proteins in *P. pastoris* [191]. A further benefit of employing constitutive promoter (*pGAP)*, is mainly for industrial fermentation, where no need of methanol for culture induction. This characteristic of *pGAP* makes simpler the strain growth with no changes of carbon source and eradicates the risks (hazards) and expenses associated with the storage and transportation of a huge methanol amount. Meanwhile, because its size (477 bp) is less than half of that of inducer promoter *(pAOX)* (960 bp), the constitutive promoter (*pGAP)* is more appropriate for the efficient production of expression cassettes or mutant libraries [190].

Recently, survey reports have been suggested on recombinant gene expression that *P. pastoris* is employed more often for single protein production than *S. cerevisiae* [11]. Lately, metabolic models have been revised on an improved annotation of the *P. pastoris* proteome [192]. However, the key knowledge of *P. pastoris* is comparatively limited as compared to *S. cerevisiae*, and have less molecular tools, such as promoters, terminators, or knockout strains, are acquirable for it [193]. In *P. pastoris* and concerned methylotrophic yeasts (for example, *Candida boidinii Hansenula polymorpha, Pichia methanolica*), methanol regulated promoters (P_{AOX1}) are typically used to drive heterologous gene expression [152, 194-197], and transcriptional regulation is the main response of these organisms during cultivation on different carbon sources [198]. As well, in the concerned methylotrophic yeasts, studies have been carried out at transcriptomics levels [199, 200]; but up to now, only few promoters have been consistently characterized (e.g., five promoters in *C. boidinii* [201]). Regardless of their similar regulation, in the recent report the promoters have been tested indicate little to no sequence similarities, perhaps because of short regulatory elements such as transcription factor binding sites may be dispersed variably over the entire promoter (as reported for P_{AOX1}, P_{DAS2}, and P_{PEX8} [152, 202, 203]). In contrast to highly resemble variants of a mutant promoter library, which are typically derived by little alterations of a single promoter, the low sequence similarity between natural methanol utilization promoters seems to be favorable for in vitro overlap-directed DNA assembly as well in vivo stability. From a fundamental research perspective, this low sequence similarity between highly co-regulated promoters is puzzling and may propose the use of methanol inducible promoters as a model system for studying transcriptional regulation. Over the last 2 decades, *P. pastoris* has become a popular expression host, surpassing *S. cerevisiae* [11] for high-level single protein production.

Regarding the latest development in genome scale metabolic models and the handiness of suitable promoters and terminators as well as the feasibility of new and easy pathway assembly methods, *P. pastoris* with its robust growth to high level cell densities as well as its low natural inclination for homologous recombination also seems to be a promising alternative to *S. cerevisiae* for metabolic engineering endeavors needing tight transcriptional regulation of large heterologous pathways, efficient cytochrome P450 (CYP) expression or other membrane protein expression, compartmentalization in peroxisomes or other organelles, and oxidative stress tolerance [178]. Realizing of these natural transcriptional regulatory systems (networks) is a useful source for metabolic engineering and synthetic biology advances that alter the design of tailor-made production strains [152].

10. Conclusion and Future Prospective

The advanced research has been mostly brought out the value of the persistent exploration for innovative fungal isolates that be able to generate enzymes and proteins with high concerning characteristics and prospect for future industrial applications. Based on the investigation analysis, a large number of various fungal isolates has been evaluated in terms of their productivity and cellulytic enzymes activities. There are many proteins that have not been successfully expressed in *Baculovirus* or *S. cerevisiae* yet, whilst nowadays they are fruitfully produced in *P. pastoris*. Which showed that *P. pastoris* is the best expression host for various kinds of proteins. Even though many more applicable key factors especially in the past three decades have been discovered for the improvement of protein production in *P. pastoris*, still the implicit fact for itshigh possible production of cellulases

remained mostly unknown. The best apprehension of secretion signals, glyco-sylation, and endogenous proteases of *P. pastoris* would be highly assisted in the development and betterment of *P. pastoris* heterologous expression system.

The advancement in molecular systems and methodologies such as rational designing, directed evolution, gene fusion, site directed mutagenesis, and DNA shuffling and new methods have to be broadening for amending the characteristics of the free fungal cellulases and all other enzymes.

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List of Abbreviations

CEL	Cellulase
CBH	Cellobiohydrolase
EG	Endoglucanases
BGLs	β-glucosidases
HC	Hemicellulase
CD	Catalytic domain
CBD	Cellulose binding domain
CBM	Carbohydrate binding module
GHs	Glycoside hydrolases
AOX1	Alcohol oxidase 1 gene
GAP	Glyceraldehyde 3-phosphate dehydrogenase
pNPG	p-nitrophenyl-β-Dglucopyranoside
UTR	5′ untranslated region
URS	Upstream regulatory sequences

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