Development of cellulolytic strain by genetic engineering approach for enhanced cellulase production

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CHAPTER 8

Development of cellulolytic strain by genetic engineering approach for enhanced cellulase production

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8.1 Introduction

The daily increase in global energy demand and the toxic effects of fossil fuel to the environment due to its nonbiodegradable property (Shaheen and Lipman, 2007) and the depletion of ozone layers (Ramakrishnan, 2015) have drawn researcher's attention to the alternative renewable sources of energy such as biofuels (Singh et al., 2017; Guerriero et al., 2018). However, there are challenges of the feedstocks, cost of enzyme, and the right technology needed for the production, which are critical factors for commercial biofuel production (Wang et al., 2012).

Moreover, the nature and cost of feedstocks have been greatly improved by making use of lignocellulosic biomass, which is cheaply available as the most abundant and ubiquitous renewable waste on Earth (Payne et al., 2015; Guerriero et al., 2016), thereby making biofuel production sustainable (Singh et al., 2017). Having solved the challenge posed by feedstock, the bottleneck is that lignocellulose (cellulose, hemicellulose, and lignin) biomass is recalcitrant to degradation (Alvarez et al., 2016). The components of biomass are deconstructed by various enzymes, namely, cellulase, β-glucosidase, hemicellulases, peroxidases, and oxidases acting synergistically to efficiently degrade lignocellulosic material (Guerriero et al., 2015; Guerriero and Siddiqui, 2017). However, the rate of degradation is still low under the conditions of catalysis; therefore, wide-ranging steps are required to improve the biomass conversion either through screening of efficient enzymes such as extremophilic (Siddiqui and Thomas, 2008; Siddiqui, 2015) and modular (Guerriero and Siddiqui, 2017) or via chemical-modification (Siddiqui et al., 1997, 2017; Rashid and Siddiqui, 1998), genetic-modification (Guerriero and Siddiqui, 2017; Siddiqui, 2015), and metabolic engineering techniques (Kubicek, 2013).

However, natural enzymes including extremophilic homologues need to be manipulated to further improve their catalytic efficiency to suit industrial applications and to reduce cost. Although numerous chemical groups other than 20 amino acids can be linked via

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chemical modification, however, the method suffers from being unpredictable (Siddiqui et al., 2017). In contrast, genetic and metabolic engineering is a key approach in enhancing the catalytic properties of cellulases, thereby reducing the cost of biofuel production. This innovation has enabled researchers to combine multiple desirable traits into a single organism (Singh et al., 2017; Singhania et al., 2010). Huge success has been achieved in this field due to rapid development in gene cloning technology through the modification of genetic makeup of organisms via in vitro processes (Pi et al., 2018). This is done simply by transferring a gene of desirable function from its host into a competent cell via a vector. The transformed cells containing the desired gene/s can be used commercially for the production of desired products (Wang and Jones, 1997; Yang et al., 2017).

Cellulase and xylanase are the two major groups of enzymes used for the conversion of lignocellulosic biomass to fermentable sugars, which can be converted into biofuels and other important value-added chemicals (Guerriero et al., 2018; Afzal et al., 2005). However, for this conversion process to efficiently takes place, lignin is a major obstacle (Singh et al., 2017; Volynets and Dahman, 2011; Saini et al., 2016), which could be partly removed by alkali pretreatment of biomass making cellulose and hemicellulose sugars accessible to enzymes. However, chemical pretreatment process is not environmental friendly. Alternately, oxidoreductases such as laccase, lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP) can be utilized for the degradation of lignin (Guerriero et al., 2016). Although LiP can cleave high-redox C—C and C—O—C bonds but cannot access bulky lignin substrate. On the other hand, MnP can only cleave low-redox bonds in lignin, but the Mn^{+3} product can penetrate deeply into the bulky lignin to bring about the degradation (Ertan et al., 2012). Hybrid VP combines the properties of both MnP and VP and therefore brings about highly efficient synergistic degradation of the substrate as compared with LiP and MnP alone (Ertan et al., 2012; Siddiqui et al., 2014). It is a known fact that cellulase alone cannot convert lignocellulosic biomass to a useful biofuel without the contribution of xylanase, which hydrolyze the hemicellulosic portion of the biomass (Guerriero et al., 2016; Hu et al., 2011).

Modular cellulases are composed of various domains such as catalytic domains (CD) with different activities (multifunctional cellulases), cellulose-binding domains (CBD), and linkers that connect CD and CBD. These multienzyme complexes are more efficient than enzymes with a single CD due to synergistic activation. In addition, binding of CBD to the cellulose brings CD in close proximity to substrate leading to increased rate (Guerriero et al., 2016; Guerriero and Siddiqui, 2017). These multifunctional enzymes are produced by both bacteria, archaea and eukaryote (Guerriero and Siddiqui, 2017). Recently, novel modular enzymes have been described in green algae. One such modular cellulase had a unique architecture with two CD (each having both endo-/exoactivities), a novel Cys-rich CBD connected via a linker (Guerriero et al., 2018). Cellulases are divided into three major components, which are endo-β-1,4-glucanase, exo-β-1,4-glucanase (or cellobiohydrolase), hybrid exo-/endohybrid cellulase, and β-glucosidase (Guerriero

et al., 2018; Biswas et al., 2014). Hemicellulose is made up of pentose monomers and is the second most abundant natural polymer on earth crust principally found in the cell wall of plants (Payne et al., 2015; Guerriero et al., 2016). Xylose is the sugar monomer of xylan, which is the major constituent of hemicellulose. Xylans are further divided into four groups based on the sugar components (Juturu and Wu, 2012); these are arabinoxylans, glucuronoxylans, glucuronoarabinoxylans, and galactoglucuronoarabinoxylans. Xylanase multienzyme system hydrolyzes these components into their corresponding monomer subunits for subsequent fermentation to produce biofuel (Burlacua et al., 2016).

Considering the various economic and desirable qualities of cellulases and xylanases in biofuel production such as stability against shear forces, resistance to end product inhibition, high thermostability, high specific activity, and catalytic efficiency against crystalline cellulose, a lot of efforts had been put by researchers over the years to develop efficient enzymes through strain improvement technology. Many of these researchers have reported the production of cellulase and xylanase from various prokaryotic and eukaryotic organisms such as aerobic and anaerobic bacteria, actinomyces, yeast, molds, algae seeds, crustaceans, and snails (Guerriero et al., 2018; Polizeli et al., 2005; Mandal, 2015; Jagadeeswaran et al., 2016; Yao et al., 2016). According to Burlacua et al. (2016) common lignocellulosic bacteria and actinomycetes producing enzymes include the following: *Bacillus pumilus*, *B. subtilis*, *B. amyloliquefaciens*, *B. cereus*, *B. circulans*, *B. megaterium*, *B. licheniformis*, *B. stearothermophilus*, *Pseudomonas* sp., *Clostridium absonum*, *Streptomyces* sp., *S. roseiscleroticus*, *S. cuspidosporus*, *S. actuosus*, and *Thermoactinomyces thalophilus*, while fungal producing ones are as follows: *Aspergillus niger*, *A. foetidus*, *A. brasiliensis*, *A. flavus*, *A. nidulans*, *A. terreus*, *Penicillium* sp., *Trichoderma reesei*, *T. longibrachiatum*, *T. harzianum*, *T. viride*, *T. atroviride*, *Fusarium oxysporum*, *Thermomyces lanuginosus*, *Alternaria* sp., *Talaromyces emersonii*, *Schizophyllum commune*, *Piromyces* sp. However, the main source for these industrially important enzymes is filamentous fungi of which *T. reesei* is the most notable (Guerriero et al., 2015; Srivastava et al., 2017).

Moreover, to make these enzymes cheaper for industrial applications, elevated expression and efficient secretion of expressed cellulases and xylanases are a necessity. These can be achieved by making use of signal sequences of DNA to obtain high level of recombinant enzyme expression for strain improvement (Juturu and Wu, 2012). Enzyme properties such as enantioselectivity, sterospecificity, substrate specificity, enzyme activity, stability, and tolerance have been reported to be amenable to genetic modification (Juturu and Wu, 2012). Strain improvement for cellulase- and xylanase-producing microbes is very important in order to achieve an industrially feasible level of production. These improvements have been carried out by researchers in various ways such as (i) classical approach using random and site-specific mutagenesis; (ii) metabolic engineering approach; (iii) genetic engineering approach; and (iv) other less popular approaches such as directed evolution, gene deletion, epigenetic, and promoter engineering.

8.2 Hydrolytic enzymes employ in the degradation of lignocellulosic biomass (cellulase and xylanase)

Lignocellulosic biomass is composed of lignin, cellulose, and hemicellulose. The percentage of these components are approximately 20%, 30%, and 50%, respectively (Fig. 8.1) (Coral et al., 2002; Walia et al., 2017). Cellulose and hemicellulose are the two important polymeric substances present in lignocellulosic biomass composed of glucose and xylose monomers, respectively, as two major simple sugars (Alvarez et al., 2016). Therefore, due to complexity of lignocellulosic biomass, multienzyme system is needed for its complete degradation. Alvarez et al. (2016) reported that in the entire processes for the bioconversion of lignocellulosic biomass to biofuel, enzyme hydrolysis step stands out as the key limiting factor.

It is noteworthy that multiple factors contribute toward successful biomass degradation. One of the key factors is biomass pretreatment step prior to enzymatic fermentation. Sometimes, chemical pretreatment may be adopted to fast-track the hydrolysis process, especially to remove the lignin, which is a complex cross-linked phenolic polymer and a major barrier to the hydrolysis of biomass. However, chemical treatment at high temperature results in the modification of the structure and (Brodeur et al., 2011) composition of lignin and hemicellulose present in the biomass. Another factor that plays a key role in the enzymatic hydrolysis of biomass is the type and source of the biomass. This could either be from softwood or hardwood trees. According to the report of Sajith et al. (2016), softwoods have very high composition of glucomannans and galactoglucomannans as a major component of hemicellulose. Therefore, mannanases, β-mannosidases, arabinofuranosidases, α-galactosidases, and acetyl xylan esterases are needed for the depolymerization of the hemicellulose to make the cellulose components accessible to the enzymes. Whereas in hardwood, the polymers are made up of xyloglucan and glucoronoxylan. As such, different sets of enzymes are needed for the degradation of the hemicellulose such as xylanase, xyloglucanase, and β-xylosidase. Table 8.1 summarizes the various classifications of

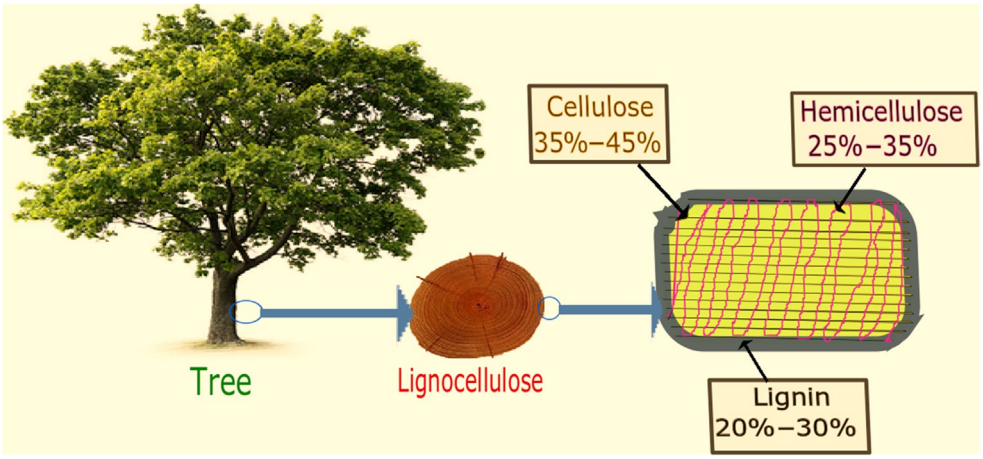

Fig. 8.1 Overview of the structure and percentage composition of lignocellulosic biomass.

Table 8.1 Overview of the classifications and functions of cellulases

Cellulases	Subdivision	Systematic names	Other common name	Location	Function
Endoglucanases (EC 3.2.1.4)	–	4-(1,3;1,4)-β-D-Glucan 4-glucanohydrolase	Cellulase; endo-β-1,4-glucanases; 1,4-β-D-glucan-4-glucanohydrolase; endo-1,4-β-D-glucanase; endo-β-1,4-D-glucan 4-glucanohydrolase β-1,4-glucanase; β-1,4-endoglucan hydrolase; cellulase A; cellulosin AP; endoglucanase D; alkali cellulase; celludextrinase; avicelase; pancellase SS	Amorphous regions of cellulose	Hydrolyzes internal β-1,4 linkages of cellulose chains and creates new reducing and nonreducing ends. It can also hydrolyze 1,4-linkages in β-D-glucans containing 1,3-linkages
Exoglucanase	Exo-1,4-β-glucosidase (EC 3.2.1.74)	4-β-D-Glucan glucohydrolase	Glucan-1,4-β-glucosidase; exocellulase; exo-β-1,4-glucosidase; exo-β-1,4-glucanase; β-1,4-β-glucanase; exo-1,4-β-glucanase; 1,4-β-D-glucan glucohydrolase	Crystalline regions of cellulose	Hydrolysis of 1,4-linkages in 1,4-β-D-glucans, to remove successive glucose units of cellobiose and other related oligosaccharides. It also has the ability to release glucose directly from cellulose
	Cellulose 1,4-β-cellobiosidase (reducing end) (EC 3.2.1.176)	4-β-D-Glucan cellobiohydrolase (reducing end)	Exo-β-1-4-cellobiosidase; CelS; CelSS; endoglucanase SS; cellulase SS; cellobiohydrolase CelS; Cel48A	Crystalline regions of cellulose	Hydrolysis of 1,4-β-D-glucosidic linkages in cellulose and similar substrates, releasing cellobiose from the reducing ends

Continued

Table 8.1 Overview of the classifications and functions of cellulases—cont'd

Cellulases	Subdivision	Systematic names	Other common name	Location	Function
	Cellulose 1,4-β-cellobiosidase (nonreducing end) (EC 3.2.1.91)	4-β-D-Glucan cellobiohydrolase (nonreducing end)	Cellobiohydrolase; cellobiosidase; exo-cellobiohydrolase; β-1,4-glucan cellobiohydrolase; β-1,4-glucan cellobiosylhydrolase; 1,4-β-glucan cellobiosidase; exoglucanase; avicelase; CBH 1; C_1 cellulase; cellobiohydrolase I; exo-β-1,4-glucan cellobiohydrolase	Crystalline regions of cellulose	of the chains using inverting reaction mechanism Hydrolysis of 1,4-β-D-glucosidic linkages in cellulose and cellotetraose, releasing cellobiose from the nonreducing ends of the chains
β-Glucosidase (EC 3.2.1.21)	–	β-D-Glucoside glucohydrolase	Gentiobiase, cellobiase, emulsin, elaterase, aryl-β-glucosidase, β-D-glucosidase, β-glucosideglucohydrolase, arbutinase, amygdalinase, *p*-nitrophenyl β-glucosidase, primeverosidase, amygdalase, linamarase, and salicilinase	Depolymerizes cellulose, cellodextrins, and cellobiose	Hydrolysis of terminal, nonreducing β-D-glucosyl residues of cellobiose units and short-chain cellodextrins into individual monomeric units of glucose

cellulases, the systematic and common names, their functions and locations in the cellulosic region of plants.

8.2.1 Hydrolytic technique in cellulases

Cellulase is an enzyme that hydrolyzes cellulose into glucose. The cellulose chain consist of 1,4-D-glucopyranose units joined by β-1,4 linkages. It is a high-molecular-weight homopolymer composed of repeating units of the disaccharide, "cellobiose" cross-linked by hydrogen bonds and van der Waals forces. These linkages form aggregation of microfibrils with a diameters of 5–15 nm (Pönni et al., 2012).

Cellulose is rarely found in pure form in nature but in association with lignin and hemicelluloses. Cellulase are made of consortium or spectrum of enzymes, which synergistically works together to ensure depolymerization of cellulose into glucose monomers (Srivastava et al., 2015). These enzymes are classified into four major groups, which are (Fig. 8.2) as follows:

(i) Endoglucanases (EG) (EC: 3.2.1.4). This is responsible for random cleavage of β-glucosidic bonds of cellodextrins (or carboxymethyl cellulose or swollen cellulose), which is the intermediate hydrolysis cellulose by converting them to cellobiose as a major product as well as glucose. EG has a preference for amorphous region of the glucan chain, thus making the reducing and nonreducing ends of the cellobiose available for further enzymatic reaction (Wang et al., 2012; Guerriero et al., 2016; Guerriero and Siddiqui, 2017).

(ii) Exoglucanase. It is grouped into two types: glucanohydrolase (GH) and cellobiohydrolase (CBH):

- **(a)** Glucanohydrolase (GH) EC 3.2.1.74: There are very few reports on the functionality of GH, but majorly, it has the ability to release glucose directly from cellulose. Some other alternative names include the following: 4-β-D-glucan glucohydrolase: exo-1,4-β-glucosidase; exo-β-1,4-glucanase; β-1,4-β-glucanase; and 1,4-β-D-glucan glucohydrolase.
- **(b)** Cellobiohydrolase (CBH): These enzymes are further grouped into two:
  - Cellobiohydrolase I (CBH 1) EC 3.2.1.76, which hydrolyzes cellobiose from the reducing ends of cellobiose.
  - Cellobiohydrolase II (CBH 2) EC 3.2.1.91, which attacks the nonreducing chain ends of cellobiose in a progressive manner splitting into glucose. However, unlike EG, it does not degrade soluble cellulose like carboxymethyl cellulose and hydroxyethyl cellulose. Also, it has partial hydrolysis for amorphous and cellodextrin. More so, these enzymes are inhibited by their own hydrolytic product of cellobiose (Wang et al., 2012).

(iii) Mixed exo-/endo- or processive cellulases: Due to different active-site architectures, many modular cellulases show characteristics of both endo- and exocellulases that produce cellobiose (C2) and C3–C4 cellooligosaccharides (Guerriero et al., 2018).

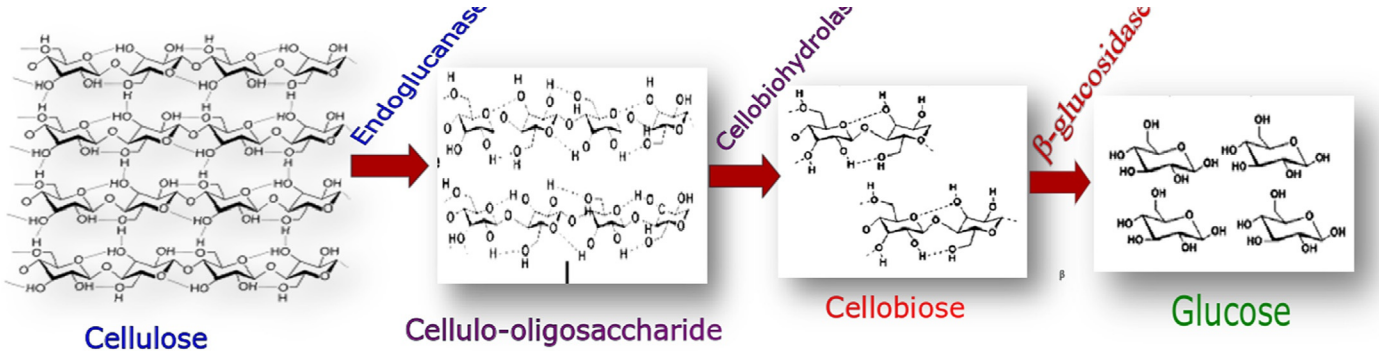

Fig. 8.2 Overview of the hydrolytic functions and stages of cellulolytic enzymes in the depolymerization of cellulose.

(iv) β-Glucosidase (BG) EC 3.2.1.21. This enzyme completes the hydrolysis of cellulose by converting cellobiose and the soluble oligosaccharide into glucose subunit. Glucosidase inhibition by its hydrolytic product has also been reported, but the effect is generally not strong as observed when D-gluconolactone was incubated with β-glucosidase (Tsai and Meyer, 2014).

8.2.2 Hydrolytic techniques in xylanases

The significant variation in composition and structure of xylan linked by β-1,4-xylopyranosyl residues from different sources is responsible for the complexity encounter during the hydrolysis as multiple enzymes are needed to bring about the process. The occurrence of these multiple enzymes has been reported in microbes (five xylanases) and plants (three xylanases). These enzymes include endoxylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), α-glucuronidase (EC 3.2.1.131), α-arabinofuranosidase (EC 3.2.1.55), and acetylxylan esterase (EC 3.1.1.6) (Fig. 8.3). Moreover, Juturu and Wu (2012) reported that endoxylanases (endo-1,4-xylanase; EC 3.2.1.8) are the most important enzymes in this group as these are responsible for the initiation and conversion of xylan to xylooligosaccharides, while β-xylosidases together with the remaining xylanases ensure the complete hydrolysis of xylooligosaccharides into their monomeric constituents. Aperl-Birkhold and Walton (1996) were of the opinion that endoxylanase and β-xylosidase are the two major enzymes responsible for the hydrolysis and are generally referred to as xylanases. Table 8.2 summarizes the various classifications of xylanases, the systematic and common names, their functions and locations in the hemicellulosic region of plants.

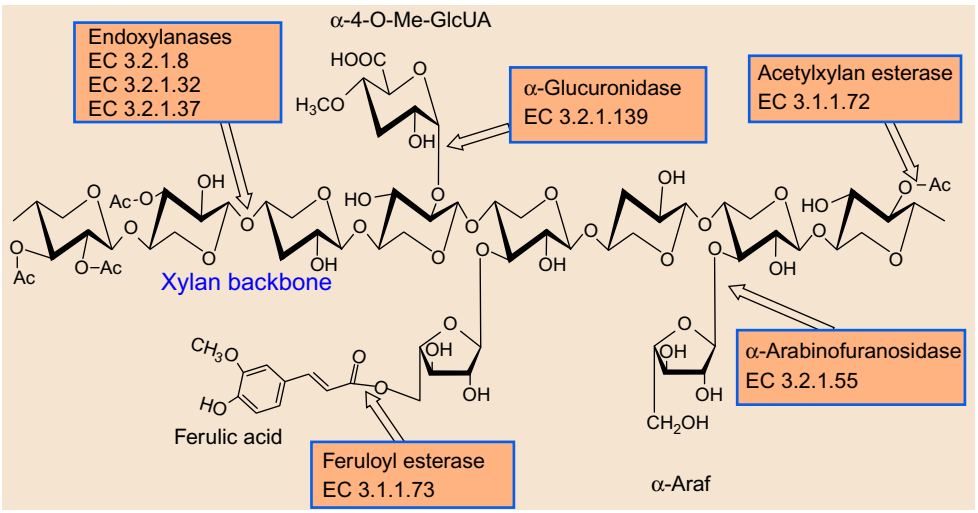

Fig. 8.3 Overview of the hydrolytic functions of xylanases in the depolymerization of xylan (*Ac*, acetyl group; *α-4-O-Me-GlcA*, α-4-*O*-methylglucuronic acid; *α-Araf*, α-arabinofuranose).

Table 8.2 Overview of the classifications and functions of xylanases

Xylanase (accepted name)	Systematic names	Other name	Location	Function
Endo-1,4-β-xylanase (EC 3.2.1.8)	4-β-D-Xylan xylanohydrolase	Endo-1,4-β-xylan 4-xylanohydrolase; endo-1,4-xylanase; xylanase; β-1,4-xylanase; endo-1,4-xylanase; endo-β-1,4-xylanase; endo-1,4-β-D-xylanase; 1,4-β-xylan xylanohydrolase; β-xylanase; β-D-xylanase	Xylan main chain of hemicellulose	It catalyzes the endohydrolysis of 1,4-β-xdylosidic linkages in xylans. It works mainly on the interior β-1,4-xylose linkages of the xylan backbone liberating short xylooligosaccharides
Endo-1,3-β-xylanase (EC 3.2.1.32)	3-β-D-Xylan xylanohydrolase	Endo-1,3-β-xylosidase; 1,3-β-xylanase; 1,3-xylanase; β-1,3-xylanase; endo-β-1,3-xylanase; 1,3-β-D-xylan xylanohydrolase; xylan endo-1,3-β-xylosidase	Xylan main chain of hemicellulose	It carries out random endohydrolysis of 1,3-β-D-glycosidic linkages in 1,3-β-D-xylans
Xylan-1,4-β-xylosidase (EC 3.2.1.37)	4-β-D-Xylan xylohydrolase	β-Xylosidase; xylobiase; exo-1,4-β-xylosidase; β-D-xylopyranosidase; β-xylosidase; exo-1,4-xylosidase; exo-1,4-β-D-xylosidase; 1,4-β-D-xylan xylohydrolase	Xylooligosaccharides of hemicellulose	It hydrolyzes 1,4-β-D-xylans by removing successive D-xylose residues from the nonreducing termini of xylooligosaccharides

α-Glucuronidase (EC 3.2.1.139)	α-D-Glucosiduronate glucuronohydrolase	α-D-Glucosiduronate	α-1,2-Linked glucuronic or 4-*O*-methyl glucuronic acid substituents attached to xylan main chain of hemicellulose	It catalyzes the hydrolysis of α-1,2-glycosidic linkages between xylose and D-glucuronic acid or its 4-*O*-methyl ether linkage
α-Arabino-furanosidase (EC 3.2.1.55)	α-L-Arabinofuranoside nonreducing end α-L-Arabinofuranosidase	Arabinosidase; α-arabinosidase; α-L-arabinosidase; α-L-arabinanase; α-arabinofuranosidase; polysaccharide α-L-arabinofuranosidase; α-L-arabinofuranoside hydrolase; L-arabinosidase (ambiguous)	α-L-Arabinofuranosyl compounds attached to the xylan main chain of hemicellulose	It hydrolyzes the terminal, nonreducing α-L-arabinofuranoside groups of α-L-arabinosides (arabinans, arabinoxylans and arabinogalactans)
Arabinan-endo-1,5-α-L-arabinanase (EC 3.2.1.99)	5-α-L-Arabinan 5-α-L-Arabinanohydrolase	Endo-1,5-α-L-arabinanase; endo-α-1,5-arabanase; endo-arabanase; 1,5-α-L-arabinanohydrolase; 1,5-α-L-arabinan	Endohydrolysis of 1,5-α-arabinofuranosidic linkages in 1,5-arabinans	It hydrolyzes the linear 1,5-α-L-arabinan. It also acts on branched arabinan, however more slowly
Acetylxylan esterase (EC 3.1.1.72)	Acetylxylan esterase	Acetylxylan esterase	*O*-acetyl groups attached to the side ends of xylan main chain of hemicellulose	It causes deacetylation of xylans and xylooligosaccharides. It also hydrolyzes the acetyl ester bonds in acetyl xylans, liberating acetic acid

Continued

Table 8.2 Overview of the classifications and functions of xylanases—cont'd

Xylanase (accepted name)	Systematic names	Other name	Location	Function
Feruloyl esterase (EC 3.1.1.73)	4-Hydroxy-3-methoxycinnamoyl-sugar hydrolase	Ferulic acid esterase; ferulic acid esterase, hydroxycinnamoyl esterase, hemicellulase accessory enzymes; FAE-III, cinnamoyl ester hydrolase, FAEA, cinnAE, FAE-I, FAE-II	Feruloyl group on the arabinofuranosyl side chain attached to the terminal nonreducing xylose of hemicellulose	It hydrolyzes the ester linkages between arabinose side chain residues and phenolic acids (ferulic acid). It is sometimes called hemicellulase accessory enzymes, since they help xylanases and pectinases to break down plant cell wall hemicellulose

Bastawde (1992) reported that accessibility of xylosidic linkages by the enzymes improved during the course of reaction as cluster of enzymes brings about hydrolysis of the substrate. This is a strategy used by some microorganisms to bring about the cleavage of xylan bonds. There have also been various reports of synergism among xylanases, whereby the enzymes complement one another for better hydrolysis of the xylosidic bonds. Wong and Maringer (1999) described the synergistic effect of xylanase A and B produced by *T. reesei* when applied for the hydrolysis of pine holocellulose. He also stated that xylanase showed more efficiency when applied in combination than singly.

8.3 Strategies used for enhancing hydrolytic enzyme production (cellulase and xylanase)

A variety of techniques have been introduced for improving the production of second-generation bioethanol with concomitant reduction in the cost of production of cellulases and xylanases. Researchers have therefore embarked on strain improvements via mutations; metabolic engineering; genetic engineering; and some less-used techniques such as directed evolution, gene deletion, epigenetic engineering, and promoter engineering (Fig. 8.4) (Adrio and Demain, 2006).

Strain improvement through mutation has been successfully used by several researchers to improve the industrial production of cellulosic and xylanase enzymes (Wong and Maringer, 1999), but the methodology is tedious and occasionally gives ambiguous results. However, bioengineering seems to be the best option for strain improvement as it is amenable to simple modification of genetic makeup of the target organism (Toyosawa et al., 2017). The availability of the whole-genome sequences of some important cellulase- and xylanase-producing strains, coupled with the detailed knowledge of parameters for modifications of genetic functions, has paved the way for huge success in industrial biocatalyst production (Mathew et al., 2008).

Currently, the research efforts are aimed at developing a consolidated bioprocessing (CBP) approach that utilizes recombinant microbes that are capable of lignocellulosic biomass degradation and sugar fermentation step for biofuel production (Mazzoli, 2012). To accomplish this, the following two strategies are employed: native and recombinant methods. Native strategy is aimed at modifying natural cellulolytic microbes by conferring high-value product properties for improved biofuel yields. For this to be achieved, several approaches have been implemented, these include (i) adaptive evolution using natural selection based on specific environmental conditions, (ii) directed evolution through the use of an error-prone PCR-based mutagenesis of cellulase genes, (iii) improving the activity of the cellulase using rational protein design, and (iv) improvement of cellulase activities using their active physiological conditions (Kricka et al., 2014). The recombinant cellulolytic strategy involves the expression of cellulase genes or conferring cellulolytic ability upon microbes having high potential

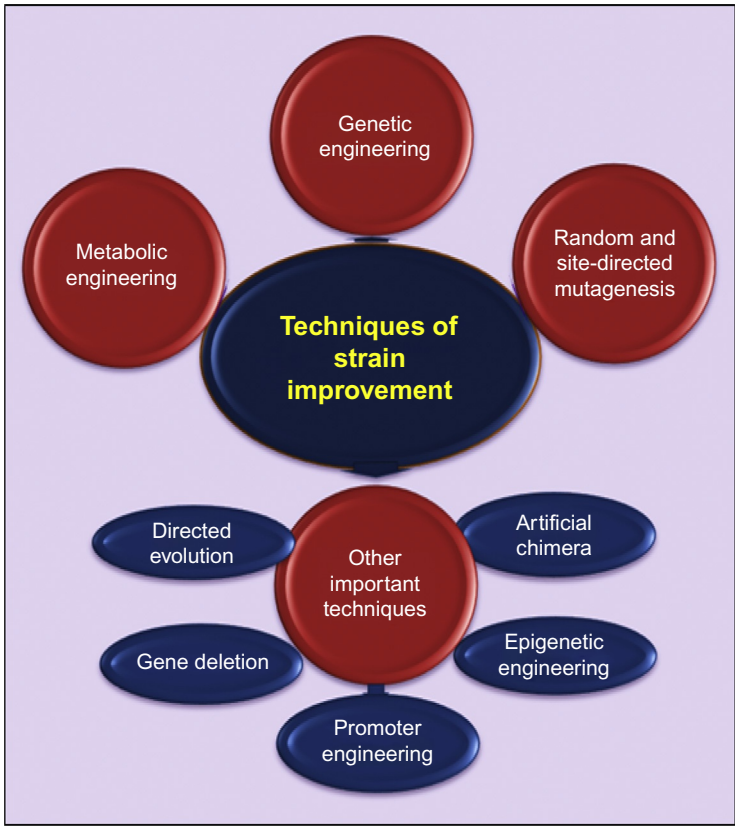

Fig. 8.4 Overview of different techniques used for improving cellulase- and xylanase-producing microbes.

for product yield. For instance, Guedon et al. (2002) were able to express heterologous genes for alcohol dehydrogenase and pyruvate decarboxylase enzymes obtained from *Zymomonas mobilis* into cellulolytic *C. cellulolyticum* with 53% increase in ethanol production.

Additionally, Singhania et al. (2010) demonstrated that microbial strain improvement for cellulase and xylanase enzyme production via mutagenesis followed by the selection of best improved strains and the application of the knowledge of recombinant DNA technology for biocatalyst production has greatly reduced the cost of production, thereby significantly benefitting the fermentation industries. Sajith et al. (2016) demonstrated the application of genetic engineering for the production of highly efficient repertoire of cellulases for the depolymerization of biomass with high saccharification efficiency using a single microbial strain. The huge success reported in the current lignocellulosic enzyme production is solely due to the ability to modify the promoter target gene sequences,

translational factors, catabolite repressor, and enzyme itself (Adsul et al., 2007). Malik (1979) reported that there is a minimum of four genes responsible for industrial microbial metabolite production with some having more than one functionalities, such as structural, regulatory, resistance, and permeability genes.

8.3.1 Genetic engineering approach

Genetic engineering is one of the most popular tools, which has enabled researchers to combine multiple desirable traits into a single organism (Siddiqui, 2015; Singhania et al., 2010). The technique can be used to engineer microbes for high metabolite production, which could vary from simple protein to highly specific therapeutic protein. However, to accomplish this, some challenges must be overcome due to the inherent complexity of the organism itself and the metabolites needed to be expressed. The most important information, which is required, is the genetic makeup of the organisms. More importantly, the detailed knowledge of whole-genome sequence and functions of the strain to be engineered makes it easier to target sites for genetic alterations (Adrio and Demain, 2006).

Yang et al. (2017) observed that the expression of recombinant enzymes in bacterial cultures such as *Escherichia coli*, lactic acid bacteria, and bacilli has been much easier compared with filamentous fungi because of adequate understanding of the genome of bacterial cells due to their simpler prokaryotic character, ability to grow better on substrates, and ability to easily adjust to cloning vectors. Terpe (2006) noted that among the various vectors used for the expression of recombinant DNA, *E. coli* happened to be the most efficient for protein expression. However, using the recombinant DNA technology for the overproduction of cellulase has been difficult to achieve due to diversity in microbial physiology (Yang et al., 2017). Furthermore, manipulating and expressing a single gene has been much easier as the cell physiology can be easily monitored during cultivation (Chou, 2007). Table 8.3 summarizes a single gene manipulation as carried out by various researchers including the target enzymes, their sources, the host organisms and the expressed genes for cellulases and xylanases improvement.

8.3.1.1 Genetic engineering approach for enhancing cellulases

Recently, much attention has been focused on finding cellulases with higher specific activity, better stability, lower susceptibility to inhibition, and improved properties using modern genetic engineering techniques (Guerriero and Siddiqui, 2017; da Silva et al., 2017; Gusakov, 2011; Bommarius et al., 2014; Trudeau et al., 2014). The ability of *T. reesei* to produce hydrolytic enzymes for the depolymerization of lignocellulosic biomass has been trialed before; however, to produce a single cellulosic strain with high saccharification efficiency and enzymatic activities for the hydrolysis of cellulose has remained a bottleneck. Knowing fully well that multiplex of enzymes is needed for

Table 8.3 Overview of the expressed cellulases and xylanases for strain improvement

Category	Target enzyme	Source	Gene	Host	Author
Cellulases	Cellobiohydrolase	*Thermoascus aurantiacus*	Cel7A	*Trichoderma reesei*	Voutilainen et al. (2008)
	Cellobiohydrolase 1	*Thermoascus aurantiacus*	CBH1	*Saccharomyces cerevisiae*	Hong et al. (2003)
	Cellobiohydrolase	*Talaromyces emersonii*	cel3A	*Trichoderma reesei*	Murray et al. (2004)
	Endoglucanase	*Humicola grisea var. thermoidea*	eg1	*Aspergillus oryzae*	Takashima et al. (1996)
	Cellobiohydrolase	*Chaetomium thermophilum*	CBH3	*Pichia pastoris*	Li et al. (2009)
	Cellobiohydrolase II	*Humicola insolens*	CBH2	*Saccharomyces cerevisiae*	Heinzelman et al. (2009)
	β-Glucanase	*Thermoascus aurantiacus*	bgl1	*Pichia pastoris*	Hong et al. (2007)
Xylanases	β-Xylanase	*Humicola grisea*	xyn2	*Trichoderma reesei*	de Faria et al. (2002)
	β-Xylanase	*Bacillus polymyxa*	xyn2	*E. coli*	Yang et al. (1988)
	β-Xylanase	*Cryptovalsa mangrovei*	BCC7197	*Pichia pastoris*	Boonyapakron et al. (2005)
	β-Xylanase	*Thermomyces lanuginosus*	Xynsig	*E. coli*	Khucharoenphaisan and Sinma (2011)
	Endoxylanase	*T. reesei*	xyn2	*Hansenula polymorpha*	Voronovsky et al. (2009)
	β-Xylosidase	*Aspergillus niger*	xlnD	*Hansenula polymorpha*	Voronovsky et al. (2009)
	β-Xylosidase	*Phanerochaete chrysosporium*	PcXylB	*Pichia pastoris*	Huy and Park (2012)
	β-Xylosidase	*Aspergillus oryzae*	XylA	*Pichia pastoris*	Kirikyali et al. (2014)
	β-Xylosidase	*Bacillus licheniformis*	xynB	*E. coli*	Aftab et al. (2017)
	β-Xylosidase gene	*Clostridium* sp.	$Bxyl_{BOH3}$	*E. coli*	Li et al. (2018)
	β-Xylosidase	*Trichoderma reesei*	Bxl1	*Saccharomyces cerevisiae*	Margolles-Clark et al. (1996)
	β-Xylosidase	*Thermoanaerobacterium* sp.	xylB	*E. coli*	Lorenz and Wiegel (1997)
	Acetyl xylan esterase 1	*Thermoanaerobacterium* sp.	axe1	*E. coli*	Lorenz and Wiegel (1997)
	α-L-Arabinofuranosidase	*Trichoderma reesei*	abf1	*Saccharomyces cerevisiae*	Margolles-Clark et al. (1996)

the degradation of cellulose, as such, multiple genetic manipulations of *T. reesei* are needed for this goal to be achieved (Druzhinina and Kubicek, 2017).

Qian et al. (2017) carried out a genetic manipulation of cellulase-producing hypercellulolytic *T. reesei* by using pyrG marker to overexpress the cellulase components for the production of highly efficient cellulase mixtures. They conducted individual overexpression and the corresponding saccharification efficiency of cellobiose hydrolase II (CBH2) and endoglucanase II (EG2). This experiment brought about an increase in activities of CBH2 and EG2 with 32- and 46-fold, respectively, with comparable protein level. Furthermore, they optimized the cellulase production of *T. reesei* by carrying out multiple genetic manipulation of β-glucosidase 1(BG1) overexpressed in EG2. They achieved this by first successfully excised the pyrG marker gene with homologous recombination as it was resistant to 5-fluoroorotic acid (5-FAO), which gave room for targeting multiple set of genes in the same strain. Later, they obtained 12-fold increase in the cellulolytic activity of BG. This double overexpression of EG2-BGL1 displayed an outstanding saccharification performance on pretreated corncob of up to 94.2% conversion after 48 h.

Harkki et al. (1991) also used the strategy of genetic engineering for the construction of a new hypercellulosic *T. reesei* strain using pAMH110 vector to express endoglucanase 1 (EG1) and cellobiohydrolase 1 (CBH1) genes. The pAMH110 containing both the promoter and the terminator sequences for CBH1 gene was used to overexpress the cDNA coding for EG1. Initially, CBH1 cDNA was incapable of coding for active enzyme but was able to inactivate other cellobiohydrolase genes especially 1,4-β-D-glucan cellobiohydrolase responsible for releasing cellobiose from nonreducing end of cellulose chains. The overall effect of CBH1 cDNA in vitro gene modification resulted in elevated production of EG1 alone.

Fujii et al. (2013) carried out a strain improvement on an efficient cellulase producer *Acremonium cellulolyticus* strain such as *T. reesei* that is at a commercial level. The creA gene with an extended homologous length similar to those found in filamentous fungi was developed for this strain. The gene was isolated using the disruption method. Moreover, to confirm the similarity of this new gene to the already sequenced cellulase producers, it was found that the amino acid sequence is similar to the wild-type creA, in which the proteins was localized in the nucleus, an indication of transcription factor of carbon catabolite. Collectively, the results indicated that the level of cellulase and xylanase production were higher as compared with the wild type.

Ellila et al. (2017) genetically engineered a *T. reesei* strain for the synthesis of cellulase in the presence of sugar repressor. This enabled the strain to use sugarcane molasses as an alternative carbon source. Additionally, to produce a strain with multienzyme functionality, Ellila et al. (2017) was able to add heterologous VTT-BR-C0020 strain obtained from *T. emersonii* expressing β-glucosidase. This greatly helped to improve the hydrolytic performance of the enzyme. Furthermore, he also ensured that the strain was able to utilize sucrose directly from sugarcane molasses by adding an invertase expressing gene

(VTT-BR-C0022) obtained from *A. niger*. The final engineered strain was able to achieve efficient hydrolytic productivity using lignocellulosic biomass with concomitant reduction in process cost.

8.3.1.2 Genetic engineering approach for enhancing xylanases

Sibbesen and Sorensen (2005) made an attempt to increase the endoxylanase of *T. reesei* using a newly discovered N-glycosylation site, which was initially created by mutating the amino acid sequence present in the wild-type coding sequence. Asn and Thr/Ser were introduced at position 131 and 133, respectively, creating Asn-Xaa-Thr/Ser complex. It was reported that Asn mutation showed 40% increase in the protein expression when compared with the wild type. Moreover, Sung and Tolan (2006) engineered endoxylanase II produced by *T. reesei* by improving its thermostability and alkaline properties. This was carried out by replacing the amino acids at a specific site. A 20°C increase in the thermostability (55–70°C) was achieved. Also, pH optimum increased from 7.5 to 9.0 pH making it more alkalophilic.

Due to the importance of xylanases in the production industries, Fang et al. (2017) examined the alternate rDNA-mediated pathway for expressing xylanase-encoding genes in *Saccharomyces cerevisiae*. He obtained xynHB gene from *Bacillus* sp., cloned into pHBM367H plasmid and expressed using *S. cerevisiae* A13. Moreover, he also used the technique of rDNA-mediated recombination to increase the number of copies of target gene. When they compared their transformants with other transformants described earlier, an increase in the number of xynHB gene was observed (13.64%) with improved degradation of xylan biomass. In addition, the transformed gene showed improved genetic and enzymatic stability.

8.3.2 Metabolic engineering approach

Metabolic engineering is another key approach that is used for the purpose of characterizing the metabolic steps involved in the signaling of the enzyme gene expression (Druzhinina and Kubicek, 2017). It is also a means of strain improvement, focusing on the modification of the biochemical reactions through the use of recombinant DNA technology for the purpose of improving the product formation (Stephanopoulos, 1999; Nielsen, 2001). Moreover, Carere et al. (2008) defined metabolic engineering as means of improving cellular function through enzymatic modulation of transport systems and other regulatory functions. In contrast to mutagenic approach, metabolic engineering facilitates the introduction of multiplex and regulatory genes, which improve or confer a special metabolic configuration to the strains (Adrio and Demain, 2006). Metabolic engineering has been used for industrial productions of numerous products, which include antibiotics, organic acids, carotenoids, propan-1,3-diol, ethanol, vitamins, and complex polyketides in bacteria and enzymes (Nakamura and Whited, 2003; Nissen et al., 2000; Pfeifer et al., 2001; Thykaer and Nielsen, 2003; Visser et al., 2003; Sybesma et al., 2004).

It is however unfortunate that despite the observed vast knowledge about the metabolic engineering processes, the production of cellulases and xylanases through

individual gene manipulation has not been adequately achieved. This is due to the fact that gene manipulations involved in central metabolism and signal transduction often result in the occurrence of pleiotropic effect, making strain improvement very difficult (Druzhinina and Kubicek, 2017). Moreover, the use of whole genome of an organism offers a better opportunity and often is the best strategy for avoiding the limiting steps posed by single gene manipulation in cellulase production.

Construction of an efficient metabolic pathway to engineer microbial enzymatic production involves two major steps (Torres and Voit, 2002): the first is metabolic system structure elucidation, whereby thorough understanding of the mechanism involves the microbial uptake of substrates, wherein its associated regulation and its metabolism should be elucidated. The second step involves the implementation of the genetic and environmental modifications of the selected organism where both the protein content and the enzymatic profile of the organism are altered. Detailed knowledge of this pathway will therefore help in the modeling for the construction of metabolically engineered strains and its enzymatic production (Gheshlaghi et al., 2009).

Several researchers have made use of heterologous proteins encoding genes to express phenotypic traits of interest. The expressed proteins according to Adrio and Demain (2014) must be associated with appropriate prosthetic groups and posttranslational modifications. Additionally, the proteins must assemble correctly by avoiding misfolding and proteolysis. Apart from these, detailed understanding of cellular functions and the various factors influencing the catabolic and anabolic flux must be taken into consideration for metabolic engineering technique to be successfully achieved. Therefore, the focal point of metabolic engineering is the metabolic flux such as pH, product inhibition, redox potential, and partial pressure, which can be analyzed using various methods as described by Eggeling et al. (1996) such as metabolite, balancing, kinetic-based models, tracer experiment, magnetic transfer method, and enzyme and genetic analysis.

Several advantages have been highlighted when a heterogeneous gene is successfully cloned and expressed: (i) the metabolic flux can be shifted for the synthesis and production of desired enzymes, (ii) the existing pathways may be extended to obtain product of interest, and (iii) the rate determining steps may be identified and accelerated for better production and modification of the various enzymatic activities responsible for the synthesis of novel products (Carere et al., 2008).

Furthermore, there have been some instances where researchers were able to use metabolic engineering technique for overproduction of cellulolytic enzymes. However, Strohl (2001) reported that overproduction of cellulase, xylanase, and other metabolite of industrial importance could be affected by factors such as addition, deletion, and modification of regulatory genes; increasing the number of copy genes responsible for encoding enzymes catalyzing difficult reactions; alteration of promoter, terminator, and regulatory sequences; increasing the number of precursor pools; and removal of competing pathways. Fig. 8.5 is a metabolic pathway used by *C. acetabulicum* for acetone, butanol, and ethanol (ABE) production starting from the decomposition of lignocellulosic biomass.

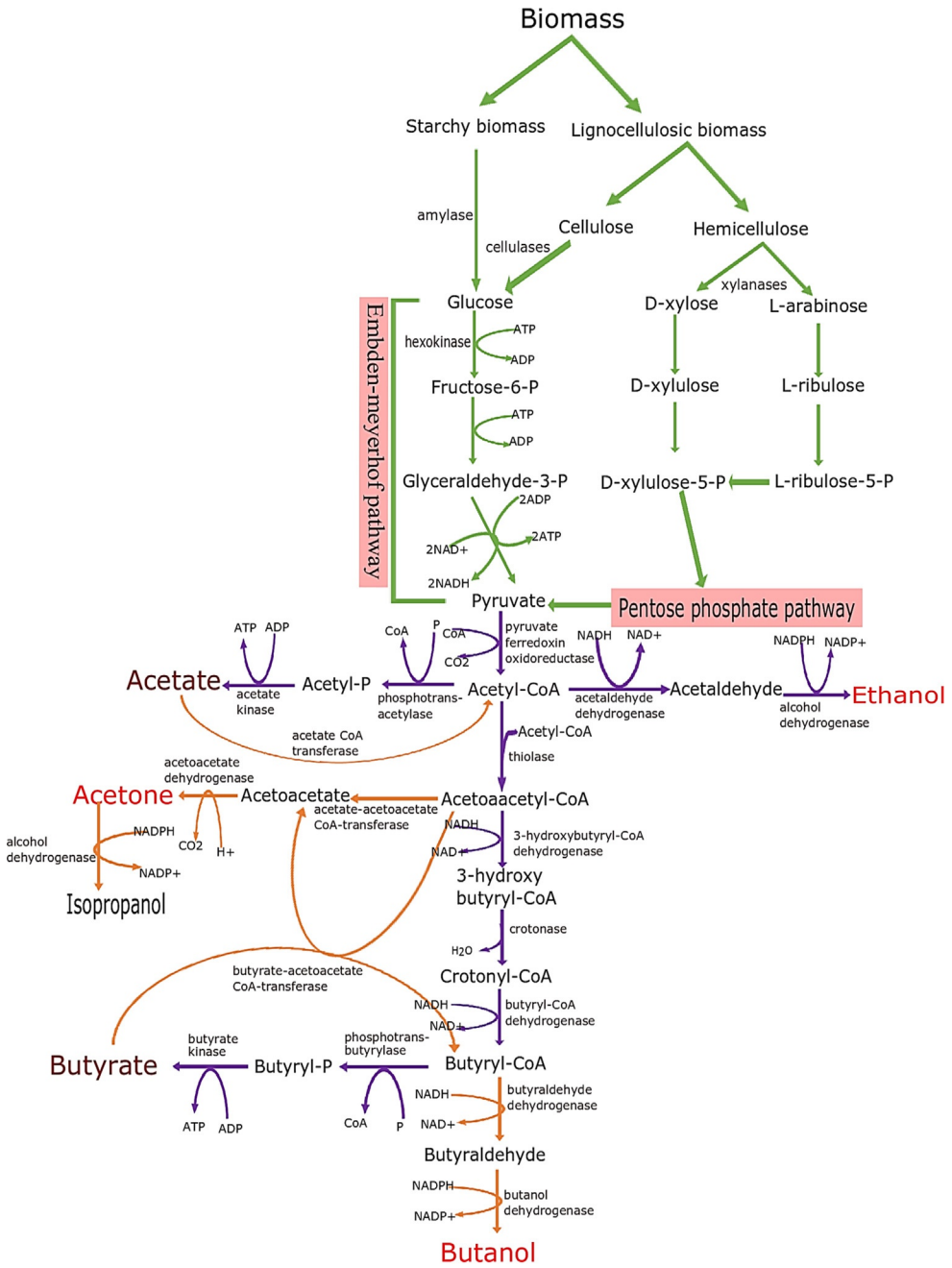

Fig. 8.5 Overview of metabolic pathway for *C. acetabutylicum* in ABE fermentation of biomass.

8.3.3 Mutagenesis approach

This approach can be regarded as the simplest method of strain improvement through which the DNA sequence of an organism is altered because the underlying mechanism or detail knowledge involved in the process is not necessary for the mutation to occur (Chand et al., 2005; Paloheimo et al., 2016). Though on many occasions, the desired expected outcome may not be achieved; in fact, it could lead to strain degeneration in the mutant, which is the accumulation of sublethal mutant in the strain lineages (Paloheimo et al., 2016). Mutagenesis approach can either be random (directed evolution) or site directed as in protein engineering (Siddiqui et al., 2017) and has been recently reviewed in case of cellulases (Guerriero et al., 2016).

8.3.3.1 Randomized mutagenesis

Various mutagens have been used to bring about the process of strain improvement for the production of desired and favorable metabolites such as ultraviolet (UV) radiation and mutagenic chemicals such as hydroxylamine, methyl methane sulfonate, 4-nitroquinoline-1-oxide, ethylmethane sulfonate, and nitrosoguanidine (Parekh et al., 2000).

Adsul et al. (2007) carried out a randomized mutagenesis on the strain of *Penicillium janthinellum* NCIM 1171 that showed the ability to produce beta-glucosidase. However, to improve production, they subjected the strain to mutagen using ethyl methyl sulfonate (EMS) for 24 h followed by UV irradiation for another 3 min. They noted that the strain showed unusual increase in cellulolytic activities when tested on a medium containing Avicel and Walseth cellulose plates even in the presence of a higher concentration of 2-deoxy-D-glucose component of the medium. Furthermore, when the mutant was transferred to fermentation broth containing 2.5% wheat bran and 1% CP-123, they observed twofold increase in the activities of both FPase and CMCase enzymes.

In another study, Kumar (2015) observed a higher yield of endoglucanase and β-glucosidase activities of the two mutant strains SGUV30 and SGUV5 of lignocellulosic degrading *S. griseoaurantiacus* when they carried out a strain improvement using UV mutagenesis. They also observed that the mutants showed higher extracellular protein secretion and stability at as high temperature as 80°C for up to 60 min. The strains were also able to utilize rice straw as a carbon source better than the wild-type strain with an increase of 57% and 12% of endoglucanase and β-glucosidase enzymes, respectively. Additionally, Druzhinina and Kubicek (2017) carried out random mutagenesis to include (a) the selection of microbial strain of interest, (b) treatment of the population with a selected mutagenic agent such as UV irradiation until a certain desired level is achieved, (c) growth of the resulting survivors on a suitable media, and (d) testing the resulting colonies for their ability to produce the desired metabolite of interest.

8.3.3.2 Site-directed mutagenesis

This is also referred to as site-specific or oligonucleotide mutagenesis used for investigating the biological structure and activities of DNA, RNA, and protein molecules purposely for protein modification. It is also defined as an in vitro method of strain improvement, whereby a specific change is made in the DNA sequence of an organism. Wang and Jones (1997) used site-directed mutagenesis to improve the carbon absorption efficiency of endoglucanase producing *Macrophomina phaseolina*. D232A mutation that lies within the substrate binding site was used to generate the mutant. The engineered mutant strain was able to utilize an oligosaccharide containing a minimum of six glycosyl units (C6) as compared with the wild type, which can only make use of C5 oligosaccharide. Additionally, the mutant did not show any loss of activity as it was able to release cellobiose (C2) from the reducing end of cellohexose and cellodextrin sugars similar to what was obtained in the wild type.

Zhang et al. (2015) also used the technique of site-directed mutagenesis to improve the activities of β-1,4-endoglucanase from hyperthermostable, *Thermotoga maritima* at high temperatures. They obtained two recombinant strains that showed 77% and 87% increase in enzyme activities. The mutants were able to retain up to 80% and 90.5% of their enzymatic activities, respectively, after incubated at temperature of 80°C for 8 h compared with wild type, which was able to retain only 45% under similar condition.

It is noteworthy to stress that the determination of biotechnological productivity of enzymes over extended duration of time at various temperatures is a more realistic way of measuring the performance compared with initial rates as the cellulase activity is continuously being affected by product inhibition, enzyme activation, and protein stability (Siddiqui et al., 2017, 2010). However, productivity measurements for enzymes in general and cellulases/xylanases in particular have seldom been reported in the literature.

8.3.4 Other approaches of strain improvement

8.3.4.1 Directed evolution

The use of directed evolution for enzymatic improvement is a technology that mimics the natural evolution process for the selection of proteins of interest (Cherry and Fidantsef, 2003; Arnold et al., 2001). The technique involves collection of related but diverse genes with naturally occurring homologues. The genes are subjected to iterative randomized mutagenesis by creating a library of variants. The screening and selection of the variant with the desirable function is followed with gene amplification. The selected enzyme genes are recombined to create "shuffling" of mutations between the selections (Kuchner and Arnold, 1997; Turner, 2009). Both in vitro-based screening method and in vivo selection technique have been used in directed evolution for improved enzyme functions and properties (Turner, 2009). The process is repeated in many rounds until the desired variant with improved properties is achieved (Voigt et al., 2000). Fig. 8.6 highlights the technique used in directed evolution for strain improvement.

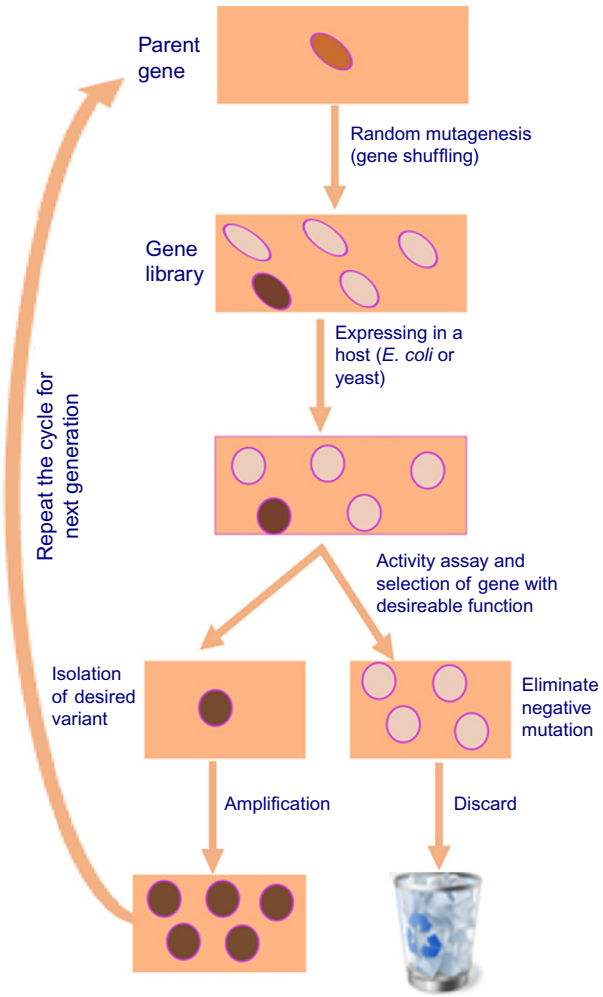

Fig. 8.6 Overview of directed evolution technique of strain improvement.

Nakazawa et al. (2009) used the technique of directed evolution to increase the specific activity of endo-β-1,4-glucanase III produced by *T. reesei* overexpressed in *E. coli* using error-prone PCR approach followed by plate assay screening. The best mutant was selected after the second round of mutagenesis producing 130-fold increase than the wild type. The mutants demonstrated broad pH activity (4.4–8.8) and increased thermostability property at 55°C for 30 min, respectively, as compared with wild type (4.4–5.2 and $T_m < 55$°C). In another development, Liu et al. (2014) used directed evolution for the 8.2-fold increase in glucose production of exoglucanase activities facilitated by coexpression of β-glucosidase. Both the exoglucanase and β-glucosidase were obtained from *Cellulomonas fimi* and *T. reesei*, respectively. The engineered exoglucanase

gene was inserted into a vector (pET30a), which was initially carrying the genes for endoglucanase and β-glucosidase. The transformant was expressed in *E. coli* and screening using insoluble cellulose (filter paper) as a test indicator. However, despite the opportunities provided by directed evolution to select the mutants with the best properties, the method has been reported to be costly and laborious (Siddiqui and Cavicchioli, 2005).

8.3.4.2 Artificial multifunctional chimeras

The use of multifunctional enzyme chimeras as means of strain improvement has been demonstrated in the deconstruction of recalcitrant plant biomass, which needs synergistic interaction of key enzymes (Guerriero and Siddiqui, 2017). The technique involves fusion of multifunctional enzyme complexes such as one or more cellulolytic and xylolytic enzymes to enhance activity, stability, and reduction in the cost of enzyme production (Elleuche, 2015). The intriguing merits of chimeras include the ability to improve efficient enzyme hydrolysis, enhance the simplicity and effectiveness of immobilized enzymes, enhance enzyme properties such as improved temperature and pH tolerance during further modification, and reduction in enzyme purification cost and provide the avenue for gene transfer to other organisms for further strain development (Siddiqui, 2015). To create an efficient chimera for biomass conversion, two important strategies are involved (Guerriero and Siddiqui, 2017). The first is intermolecular strategy, which is the use of already characterized enzyme having the ability to function in synergistic interaction. The second method is intramolecular synergy, whereby multifunctional enzymes with synergistic potential are selected from their natural diversity.

Lee et al. (2011) developed a bifunctional cellulose-degrading enzyme complex (endocellulose/β-glucosidase) with enhanced thermostability property. The thermophilic endocellulase (T_m 70.5°C) and mesophilic β-glucosidase (54.5°C) were able to generate a multifunctional thermostable enzyme complex (T_m 65.5) with twofold increase in glucose production due to hydrolysis of cellobiose by β-glucosidase before being released into the reaction system. Additionally, threefold reduction in cellobiose disaccharide was also observed. In another development, Rizk et al. (2015) generated bifunctional fusion of endoglucanase and endoxylanase with an improved deconstruction of birchwood xylan and β-glucan. However, he reported the effect of relative orientation of the enzyme complex, which could lead to decreased enzymatic activity and stability.

Moreover, artificial chimera comes with some important factors, which must be considered for the technique to be successfully achieved (Guerriero and Siddiqui, 2017) such as the following: (i) The position of the modules within the chimeras. This has a strong influence in the performance of the enzyme complex by either correcting the protein folding or interacting with other protein modules. (ii) The type of carbohydrate binding module (CBM), which plays an important role in recognizing and targeting of the specific sites of substrate-enzyme complex. (iii) The intermodular linkers, which serve as a binding force between the structured protein module in order to form appropriate

conformational protein and enhanced synergistic activities of the complex. This functionality is due to the flexibility and hydrophobicity attributes of intermodular linkers (Guerriero and Siddiqui, 2017; Hong et al., 2006; Walker et al., 2015).

8.3.4.3 Epigenetic engineering technique

This is a term used for heritable gene changes in the physiology or in the appearance of an organism without necessarily affecting their underlying DNA sequences (Waddington, 1942; Weber and Schübeler, 2007). Druzhinina and Kubicek (2017) listed the three types of epigenetic engineering that includes: (i) DNA methylation, which has yet to be identified in *T. reesei* strain. It occurs by covalent attachment of cytosine ring to 5′ end resulting in 5-methylcytosine, which causes inhibition of transcription process by extending into the major groove of DNA. According to Aghcheh and Kubicek (2015), the occurrence of DNA methylation has been cited in some fungi such as *Ascobolus* and *Neurospora* and some filamentous fungi. (ii) Chromatin remodeling using histone modification technique. This occurs when the chromatin, a complex of DNA and protein, is tightly packed within the nucleus of eukaryotic organisms and is wrapped around the histones to form nucleosome. The technique has been used by Bok and Keller (2004) to manipulate the transcription of the gene synthesis for the production of secondary metabolites by *Aspergillus nidulans*. It has also been demonstrated in *T. reesei* strain as a potential for cellulase improvement (Aghcheh and Kubicek, 2015). (iii) RNA interference occurs when the noncoding RNAs (ncRNAs) are transcribed from DNA but not translated into proteins but however regulates the expression of genes both at transcriptional and posttranscriptional levels. The use of epigenetic engineering has not been fully harnessed for the production of cellulase enzyme; nevertheless, it has a great potential for strain improvement. Wang and Chang (2011) used the technique of chemical epigenetic to manipulate the gene of *Penicillium citreonigrum* that invariably produced a profound change in the secondary metabolite profile.

8.3.4.4 Promoter engineering technique

This is another strategy used by researchers to modify the expression of cellulase genes by manipulating the binding sites for the transcriptional regulators (Druzhinina and Kubicek, 2017). Zou et al. (2012) used the principle of recombinant DNA technology by replacing the CRE1 carbon catabolite-binding site of CBH1 promoter of *T. reesei* heterologous expression with transcription of positive activator ACEII and the HAP2/3/5 complex. This invariably led to the improvement in CBH1 promoter efficiency of cellulase heterologous expression gene in *T. reesei*.

8.3.4.5 Gene deletions technique

This method has been recently used for strain improvement for the industrial production of enzymes (Paloheimo et al., 2016). This is carried out by deleting unwanted enzyme

reactions that may be detrimental or harmful to the main target enzymes of industrial importance. Occasionally, the pathways for undesirable metabolites are removed from the reaction. This deletion is usually accompanied using a selectable marker to replace the target gene with the aid of homologous recombination flanked by 5′ and 3′ genes. Paloheimo et al. (2016) used gene deletion for producing cellulase and xylanase by *T. reesei* strain in order to facilitate the monitoring of their activities (Schuster et al., 2012; Joergensen et al., 2014).

8.4 Economic outlook of hydrolytic enzymes and lignocellulose degradation

The prospect of using enzymes for the production of biofuel and other useful products has greatly increased in the 21st century as more applications are envisaged for the potential of biocatalyst in the industrial sector (Singh et al., 2016). It is estimated that over 100 billion liters of biofuel is being produced annually that account for 2.7% of total energy supply in the transportation sector (Kricka et al., 2014). Both the United States and Brazil have been very active in biofuel production accounting for 90% of total world biofuel supply in 2010, as such there is still potential for further biofuel production (Singh et al., 2016). However, operational cost of cellulase and xylanase enzymes account for more than 20% of total cost in the production of biofuel from lignocellulosic biomass (Barcelos et al., 2015). One way to overcome the cost of enzymes is by employing magnetic cellulases and xylanases that can be reused multiple times due to their magnetic susceptibility (Guerriero et al., 2015; Guerriero and Siddiqui, 2017).

Using cellulases from thermally adapted sources as starting materials for genetic manipulations can be crucial in getting efficient enzymes with high productivity as cold-adapted enzymes have high intrinsic activity but low stability compared with thermostable enzymes that have high stability but low activity around room temperature in accordance with activity-stability trade-off (Siddiqui et al., 2017; Siddiqui, 2017).

In 2016, the industrial enzymes are estimated to have value at USD 4.81 billion and are expected to increase at CAGR of 5.8%. As such, strain improvement through the use of genetic engineering is a welcomed approach to greatly reduce the cost of production. Though the financial implication of using biocatalyst in place of chemical, physical, and mechanical methods for biofuel production is cheaper when the ecological implication is considered as enzymes are far more environmentally friendly (Zhuang et al., 2007). Binod et al. (2013) reported that out of more than 3000 known enzymes, only approximately 170 are used at the commercial level of which cellulase and xylanase represent larger percentages.

We wish to propose an integrated approach for the efficient and cost-effective degradation of cellulose similar to that suggested by Siddiqui et al. (2017). This approach is based on the premise that instead of using different cellulases or organisms for improvement by employing different techniques, a single organism (such as an extremophile) is

used for maximizing the enzyme production via multiple methods. The specific cellulase/xylanase (may be modular or multifunctional) from this high-producing stain can then be used as a starting material to carry out all the subsequent genetic and chemical modifications to yield a highly efficient enzyme. This engineered cellulase mixture can then be immobilized on magnetic nanoparticles (MNP) to be reused multiple times (Alftrén and Hobley, 2014) to reduce the cost. Interestingly, more than one enzyme (such as cellulase and β-glucosidase and/or xylanase) can be immobilized on a single MNP, or a mixture of MNP with separate enzymes can be used.

The productivity of highly efficient magnetic cellulase then can be further enhanced in the presence of nonaqueous media (ionic liquids and super-critical CO_2) and/or various additives such as trimethylamine N-oxide, trehalose, betaine, amino acids, and PEG (Siddiqui et al., 2017). In this way, all methods for improvement are applied to a single-specific enzyme from a high-producing organism in a series of steps.

8.5 Conclusion and future prospects of hydrolytic enzyme production

The potential and economic value of lignocellulosic biomass as renewable feedstock for replacing a significant fraction of nonenvironmentally friendly fossil fuel consumption has been an attractive theory for many years. The progress in strain development that have been recorded so far for hydrolytic enzymes will depend on the improvement of technologies earlier mentioned and more importantly developing a mathematical method to fast-track the mechanisms for the manipulation of target genes.

Furthermore, the purpose of strain improvement using genetic engineering should be well defined as there are several reports of high-producing strains of cellulase and xylanase of industrial importance (Gupta et al., 2016). As such, if any further modification had to be done, it could either be to reduce the fermentation period for the enzymes, design an enzyme with multiplicity of actions for the hydrolysis of lignocellulose, or control the uptake of gene encoding for the cellulolytic enzymes for the degradation of the polymer into host organisms so that the modified cells will be able to secrete this multienzyme in ideal ratios (Barcelos et al., 2015; Srivastava et al., 1999). Most importantly, the aim should be to cut the cost and conveniences of enzyme production and saccharification processes.

It is quite unfortunate that most of the gene modifications that have been reported through engineering techniques have been carried out under laboratory conditions using minimal media only, followed by the determination of the relative abundance of the hydrolytic enzymes mRNA. A lot of work still needs to be done on the optimized carbon sources require for the enzymes production and saccharification of the lignocellulose at the genetic level, scaling it up for industrial use. More so, developing a bioinformatics for the molecular design, which can be used to optimize the catalytic performance of the hydrolytic enzyme, will be a good adventure to undertake (Yang et al., 2017).

Finally, improvement on already researched metabolic models will help to elucidate the physiological behavior of the strains and help in ensuring faster identification of target genes for subsequent modification (Stephanopoulos, 2007). Additionally, apart from embarking on already known techniques of genetic and metabolic engineering, application of recent advances in evolutionary engineering and synthetic biology tools such as transcriptomics, fluxomics, metabolomics, and proteomics will greatly help in elucidating the characteristics of the mutant strains as well as its optimized products (Picataggio, 2009; Dellomonaco et al., 2010). Most importantly, the urge and tenacity to make our world a better place to live through a drastically reduced greenhouse effect and various pollutants causing degradation of the ozone layer will be a driving force in ensuring the survival of an increased biofuel production.

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Further reading

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