Bioresource Technology 127 (2013) 500–507

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Review

Role and significance of beta-glucosidases in the hydrolysis of cellul[illegible] for bioethanol production

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ARTICLE INFO

Article history:
Received 27 July 2012
Received in revised form 5 September 2012
Accepted 5 September 2012
Available online 14 September 2012

Keywords:
Beta-glucosidase
Cellulase
Biomass
Trichoderma reesei
Aspergillus niger

ABSTRACT

One of the major challenges in the bioconversion of lig[illegible]llulosic biomass into liquid biofuels includes the search for a glucose tolerant beta-gl[illegible]idase. Beta-g[illegible]idase is the key enzyme component present in cellulase and completes the f[illegible]during cellulos[illegible]drolysis by converting the cellobiose to glucose. This reaction is always un[illegible]control as it gets inhibited by its product glucose. It is a major bottleneck in the efficient biomass c[illegible]ersion by c[illegible]ase. To circumvent this problem several strategies have been adopted which we have[illegible]cussed in th[illegible]rticle along with its production strategies and general properties. It plays a very signi[illegible]role in h[illegible]thanol production from biomass through enzymatic route. Hence several [illegible]endments to[illegible]he commercial preparation of cellulase for biomass hydrolysis, which co[illegible]er and improved beta-glucosidase for efficient biomass conversion. This article presents beta-g[illegible]osid[illegible]a key component for bioethanol from biomass through enzymatic route.

1. Introduction

Depletion of fossil fuel at enhanced[illegible]e and its el[illegible]n global economic and environment has ac[illegible]the researc[illegible]alternative fuels like bioethanol. Ligno[illegible]cellulos[illegible]ioethanol has been looked as a potential alternat[illegible]fuel; as the[illegible]material for it is cellulose, which is the ma[illegible]component of the[illegible]nt cells and is renewable, abundant as[illegible]ell as [illegible]quitous. Plant biomass is expected to perform the s[illegible]ro[illegible]coming years as that of oil in 20th century (Lynd et al., 2[illegible] Biomas[illegible]n be hydrolysed enzymatically to pro[illegible]cose [illegible]h c[illegible]e converted to liquid fuel; ethanol. Hyd[illegible]sis o[illegible]omass c[illegible]accomplished by fungal and bacterial b[illegible]ass hyd[illegible]yzing enzymes. There are several enzymes which are r[illegible]ed[illegible]hydrolysis of biomass such as cellulase, xylanas[illegible]inase, pectinase, etc., among which cellulase is the most importa[illegible]ne as biomass contains about 40% or above cellulose. Cellulase [illegible]multi-enzyme complex of three different enzymes; exoglucanase, endoglucanase and beta-glucosidase (BGL) which acts synergistically for complete hydrolysis of cellulose. Cellulose fibers are firstly cleaved in between by endoglucanase releasing small cellulose fragments with free reducing and non reducing ends which are attacked by exoglucanase to release small oligosaccharides, cellobiose; and is finally hydrolysed into glucose monomers by beta-glucosidase. Beta-glucosidase completes the final step of hydrolysis by converting the cellobiose (an intermediate product of cellulose hydrolysis) to glucose, hence; is the rate limiting enzyme. Most of the fungal cellulases contain these three components at different ratios. *Trichoderma reesei*, a potential cellulase producer, produces cellulase with all the components but it lacks the optimum amount of BGL required for efficient biomass hydrolysis and is further glucose sensitive too. Thus during the process, cellobiose gets accumulated due to less BGL which converts it into glucose, moreover once glucose is accumulated in the medium it also causes feedback inhibition which together exhibits inhibiting effect on the enzymatic hydrolysis of biomass. It could be desirable to construct a genetic modified strain of *T. reesei* producing optimum amount of BGL with desirable properties along with other cellulase component in it, so as to obtain cellulase with all the components in optimal amount. So BGL is the bottleneck in the overall bioethanol production technology from biomass through enzymatic route. The efficient hydrolysis of biomass necessitates the need of glucose tolerant BGL to be active at higher glucose concentration. This article presents a discussion on the significance of BGL especially on bioethanol programme.

2. Beta-glucosidases

Beta-glucosidase (beta-D-glucoside glucohydrolase, EC 3.2.1.21) is one among the earlier discovered and widely studied enzyme due to its universal distribution and well defined wide variety of substrate and simple nature of enzyme assay (Shewale, 1982).

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0960-8524/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2012.09.012

BGLs are well characterized, biologically important enzymes that catalyze the transfer of glycosyl group between oxygen nucleophiles. These transfer reaction results in the hydrolysis of beta-glucosidic linkage present between carbohydrate residues in aryl-amino-, or alkyl-beta-D-glucosides, cyanogenic-glucosides, short chain oligosaccharides and disaccharides under physiological conditions, whereas; under defined conditions, synthesis of glycosyl bond between different molecules can occur. It occurs by two modes reverse hydrolysis and transglycosylation. In reverse hydrolysis, modification of reaction conditions such as lowering of water activity (aw), trapping of product or high substrate concentration leads to a shift in the equilibrium of reaction toward synthesis. This reaction is under thermodynamic control. In transglycosylation approach, a preformed donor glycoside (e.g., a disaccharide or aryl-linked glucoside) is first hydrolyzed by the enzyme with the formation of an enzyme-glycosyl intermediate. This is then trapped by a nucleophile other than water (such as a monosaccharide, disaccharide, aryl-, amino-, or alkyl-alcohol or monoterpene alcohol) to yield a new elongated product. This reaction is under kinetic control (Bhatia et al., 2002).

Beta-glucosidases have attracted considerable attention in recent years due to their important roles in various biotechnological processes such as hydrolysis of isoflavone glucosides, the production of fuel ethanol from agricultural residues, the release of aromatic compounds from flavorless precursor, etc., BGL are widely distributed in the living world and they play pivotal roles in several biological processes. The physiological roles associated with this enzyme are diverse and depend on the location of the enzyme and the biological system in which these occur. In cellulolytic microorganisms, beta-glucosidase is involved in cellulase induction (due to its transglycosylation activities) and cellulose hydrolysis. In plants, the enzyme is involved in beta-glucan synthesis during cell wall development, pigment metabolism, fruit ripening and defense mechanisms whereas, in humans and other mammals, BGL is involved in the hydrolysis of glucosyl ceramides. Defects in BGL activity in humans are associated with Gaucher disease, a non neuropathic lysosomal storage disorder (Sidebotham, 2007). Hydrolysis of soybean isoflavone glycosides is an important application of BGL in industries. Isoflavones are known to prevent certain cancers, lowers risks of cardiovascular diseases and improve bone health but it has been revealed that the biological effects are mainly due to aglycone form of isoflavon rather than their glycosylated form because intestine absorbs aglycone forms faster (Izumi et al., 2000). Due to their wide and varied roles in nature, these versatile enzymes can be of use in several synthetic reactions as reviewed by Bhatia et al. (2002).

3. Classification of beta-glucosidases

Beta-glucosidases are a heterogeneous group of hydrolytic enzymes and have been classified according to various criteria. There is no single well-defined method for the classification of these versatile enzymes. In general, two methods for their classification appear in the literature, on the basis of (1) substrate specificity, and (2) nucleotide sequence identity (NSI) (Henrissat and Bairoch, 1996).

Based on substrate specificity, these enzymes have been classified as (1) aryl beta-glucosidases, which act on aryl-glucosides, (2) true cellobiases, which hydrolyze cellobiose to release glucose, and (3) broad substrate specificity enzymes, which act on a wide spectrum of substrates. Most of the beta-glucosidases characterized so far are placed in the last category. The most accepted method of classification is by nucleotide sequence identity scheme, proposed by Henrissat and Bairoch (1996) based on sequence and folding similarities (hydrophobic cluster analysis, HCA) of these enzymes. HCA of a variety of such enzymes suggested that the α-helices and the beta-strands were localized in similar positions in the folded conformation. Moreover, a number of highly conserved amino acids were also clustered near the active site. Such a classification is expected to reflect structural features, evolutionary relationships, and catalytic mechanism of these enzymes. Also, the identification of the nucleophile and the putative acid–base catalyst in one member of a family in effect identifies them in all members of the family. It is also expected that as the size of the family increases, the residues conserved in all members of the family usually will be important, structurally or catalytically. More sequence data and three-dimensional structure of enzymes belonging to these families are required to confirm this scheme. The sequence based classification is useful in characterizing the enzymes from the structural point of view but the substrate specificity with respect to the aglycone moiety is conserved primary, or, in some cases, the only leads isolating and characterizing unknown or structurally undefined glucosidases. The beta-glucosidases are mostly placed in either family 1 or family 3 of glycosyl hydrolases though these enzymes are also found in families 5, 9 and 30 of glycosyl hydrolases (Henrissat, 1991; Cantarel et al., 2009; Opassiri et al., 2007). Family 1 comprises nearly 62 beta-glucosidases from archaebacteria, plants, mammals, and also includes 6-phosphoglycosidases and thioglucosidases. Most family 1 enzymes, also show significant beta-galactosidase activity. The family1 beta-glucosidases are also classified as members of the 4/7 super family with common eight-fold beta/α barrel motif.

Family 3 of glycosyl hydrolases consists of nearly 44 beta-glucosidases and beta-xylosaminidases of bacterial, mold, and yeast origin. Most of the fungal beta-glucosidases studied belong to the family 3 of glycosyl hydrolases. Structural data on representatives of GH3 family is scarce, since only three of their structures are known and only one of them has been thoroughly characterized—that of a beta-D-glucan (exo1 → 3, 1 → 4) glucanase (Exo 1) from *Hordeum vulgare*, which catalyzes the hydrolysis of cell-wall polysaccharides. The enzyme consists of N-terminal (α/beta) 8 TIM barrel domain and a C-terminal domain of six stranded beta sandwich. The non-homologous region, a helix-like strand of 16 amino acid residues, connects the two domains" (Bhatia et al., 2002). The catalytic center is located in the pocket at the interface of the two domains. Asp285 in the N-terminal domain acts as a catalytic nucleophile, while Glu491 in the C-terminal domain acts as a proton donor (Varghese et al., 1999).

In general, beta-glucosidases cleave the beta-1,4-glucosidic bonds in a variety of glucosides. Two carboxylic acids are involved in catalysis at the active site. Protein engineering can be applied to increase the stability of BGL, a classic example includes the work done by Nam et al., 2008, where they have modified N and C terminal of BGL and provided an insight for increasing stability of protein.

4. BGL activities and substrate

Little is known about the interaction of beta-glucosidases with their substrates, especially with respect to the aglycone moiety, which forms the basis of tremendous diversity in terms of substrate range and is responsible for subtle differences in substrate specificity. BGL activities are measured using artificial substrates such as pNPG (para nitrophenyl beta-D-glucopyranoside) or methyl umbelliferyl beta-D-glucoside (MUG), few instances shows BGL activities based on cellobiose substrate. In most of the cases several BGL enzymes show high catalytic activity and high K_m with artificial substrate like pNPG and MUG but not with cellobiose. Kinetics of the beta-glucosidase depends on the configuration of its substrate and cellobiose requires a conformational change for

catalysis. BGL has a very rigid structure in S1 substrate binding site which will accommodate glucose of cellobiose but the second glucose of cellobiose will change the conformation using rotation of the σ-bond of the glucoside so as to fit in the substrate binding site (Nam et al., 2010). It is not needed in case of pNPG where nitrophenol follows the same binding patterns as of glucose. This is the reason behind low K_{cat}/K_m of beta-glucosidase towards cellobiose than the substrate pNPG.

Hence, it is necessary to investigate potentialities of BGL based on natural substrate as it will need to deal with cellobiose rather than pNPG or MUG in the natural process, which is the probable reason of not having significant effect on the hydrolysis even after supplementation of heterologous BGL to the cellulase employed for hydrolysis of biomass. Our understanding says that the reaction kinetics should shift drastically towards higher end on adding BGL to the cellulase of *T. reesei* which is known to be deficient in sufficient BGL; as the heterologous BGL will be sufficient enough to avoid accumulation of cellobiose. However it does not happen in the real case. There is one more limitation; that is to remove glucose, as and when it is produced, for which technologies such as membrane filters should be used to separate monomers of sugars so as to avoid feedback inhibition. Simultaneous saccharification and fermentation could also be an answer to this problem where glucose is converted to ethanol simultaneously, however there; ethanol needs to be separated as and when it is produced to have a continuous process for which again membrane separation could be a sustainable solution.

There are several reports which states that even after loading the same units of enzyme as prescribed for biomass hydrolysis and using the same conditions, same rate/extent of hydrolysis not achieved. The reason could be differences in enzyme properties, its degree of tolerance to the hydrolysis conditions and further biomass and degree of its rigidity would also affect. It is necessary to mention again that the final evaluation of BGL should be made based on assay where cellobiose has been employed as substrate rather than pNPG derivatives or MUG.

5. Microbial production of beta-glucosidase

Microbial sources have been widely exploited for beta-glucosidase production by both solid-state fermentation (SSF) and submerged fermentation. There are several reports available for beta-glucosidase productions from filamentous fungi such *Aspergillus niger* (Gunata and Vallier, 1999), *A. oryzae* (Riou et al., 1998), *Penicillium brasilianum* (Krogh et al., 2010) *P. decumbens* (Chen et al., 2010), *Phanerochaete chrysosporium* (Tsukada et al., 2006), *Paecilomyces* sp., (Yang et al., 2009) etc., though there are also various reports of beta-glucosidase production from yeasts (majority of them from *Candida* sp.) and bacteria. Microscopic fungi are the most important source of BGL. For biomass hydrolysis purpose BGL from filamentous fungal sources could be preferred as it offers an advantage of production via solid state fermentation in high titers against submerged fermentation. SSF is a cheaper technology and the difficulty in purification of metabolites produced by SSF is not a limitation for above application as the purity of enzyme is not necessary. Listed fungal strains involved in BGL production and the bioprocess for their production are enlisted in Table 1. Most of the enlisted have the potential for biomass conversion technology to generate sugar monomers from cellobiose. The world market enzymatic preparation represents a broad spectrum of cellulases, most of which are produced from *Trichoderma reesei* (*T. longibrachiatum*), however there are also preparations from *Penicillium* and *Aspergilli* (Singhania et al., 2010). There are studies based on comparison of cellulase activities but there are few reports that compare BGL actvities and properties by these fungal strains. Korotkova et al. have studied the properties of BGL produced by *Penicillium verruculosum*, *Aspergillus japonicus* and *Trichoderma reesei*, for their possible employment for biomass saccharification efficiency (Korotkova et al., 2009). They have represented specific activity of BGL from all these three fungi against different substrate at pH 5.0 and temperature 40 °C. BGL from *A. japonicus* was true cellobiase as other two exhibited exo-1,3/1,4-beta-glucosidase activity along with cellobiase activity. BGL from

Table 1
List of fungal strains producing β-glucosidases and mode of bioprocesses involved.

Microorganism	BGL characteristics/properties	Carbon source	Production process	Projected application	References
Debaryomyces pseudopolymorpha	Glucose tolerant, acidic, exocellular	Cellobiose	Shake flask fermentation	Enhancing wine aroma	Villena et al. (2006)
Trichoderma atroviride	2.5 U/g	Pretreated willow	Shake flask fermentation	Biomass hydrolysis	Kovacs et al. (2008)
Penicillium pinophillum	3 U/mg protein, V_{max} = 1120 U/mg protein, pH = 5.5, K_i = 26.6 mM, extracellular,	Cellulose	SmF, Small fermenter with working volume 200 ml	Cellobiose, cellotriose hydrolysis	Joo et al. (2010)
Penicillium decumbens	15 U/g solid, V_{max} = 85.93 U/mg, K_m = 1.2 mM, K_i = 17.9 mM with pNPG, thermoacidophillic	Rice bran	Solid-state fermentation	Cellulosic bioconversion	NG et al. (2010)
Periconia sp.	Thermotolerant, active at wide pH range	Cellulose	SmF	Biomass conversion	Harnpicharnchai et al. (2009)
Penicillium decumbens	Thrmotolerant, acidic BGL, K_m = 0.006 mM towards pNPG	Wheat bran	SmF	Biomass (corncob) conversion	Chen et al. (2010)
Penicillium echinulatum	0.85 U/mg of protein with pNPG	Microcrystalline cellulose	SmF	Biomass hydrolysis	Martins et al. (2008)
Stachybotrys sp.	K_m = 0.22 mM towards pNPG and 2.22 mM for cellobiose, active at 50 °C and pH = 5	Cellulose	Fed-batch SmF	Cellobiose hydrolysis	Amouri and Gargouri (2006)
Humicola isolens	BGL active at 50 °C and at pH 6.0, BGL activity was stimulated at 400 mM conc.	Microcrystalline cellulose	Shake flask	Cellulosic biomass hydrolysis	Souza et al. (2010)
Fomitopsis palustris	Acidic BGL active at 55 °C, K_m = 0.706 and 0.971 mM for pNPG of BGL1 and BGL2 respectively	Cellobiose	Shake flask	Cellobiose hydrolysis	Okamoto et al. (2011)
Fomitopsissp	53 U/g	Wheat bran	SSF	Rice straw and wheat straw hydrolysis	Deswal et al. (2011)
Aspergillus niger	Thermostable, acidic, glucose resistant showing 92% activity retension at 250 mM glucose	Lactose, wheat bran	SmF, shake flask	Biomass hydrolysis	Singhania et al. (2011)

A. japonicus was found superior based on its temperature stability and glucose inhibition constant. Commercial production of beta-glucosidase is often achieved by use of species of *Aspergilli*. *Aspergilli* are known to produce higher titers of the enzyme (Singhania et al., 2011). Relatively pure forms of cellulose and native as well as crude and pretreated biomass (Table 1) have been used successfully as carbon sources for production of the enzyme under both submerged (SmF) and solid state fermentations (SSF). It is also possible to obtain the desired properties in BGL or BGL with desired properties, by exploiting the conditions of fermentation as well as nutrient sources. While submerged fermentation is the most common strategy employed for commercial production of microbial enzymes due to its inherent advantages of better sterility, heat and mass transfer, easiness in process monitoring and automation etc., SSF is popular in the case of fungal fermentations for high volume low value enzymes like amylases, cellulases etc. This is because SSF has better productivity, cheaper crude substrate utilization; low capital investment, low energy requirement, lesser waste water output, higher product concentration, and lack of foam build up (Kovacs et al., 2008). SSF imitates natural habitat of these filamentous fungi and thus are better adapted and produces higher enzyme titers which can be directly employed for biomass hydrolysis (Kovacs et al., 2008; Joo et al., 2010). High water activity is projected as a probable reason for high production of metabolites in SSF by microorganisms (Joo et al., 2010). There are also reports that most of the BGL expressed by *Aspergillus* in SmF remain adhere to the cell surface (Oda et al., 2006). This fact supports SSF to be employed for BGL production. However, it is also shown by researchers that 90% of the observed activity of BGL in case of *Asperilli* in SmF was detected in supernatant (M awa et al., 2012). However, knowledge on SSF process auto is limited, and there is intense heat generation in SSF system

6. Differential expression of beta-glucosida ofor

Several filamentous fungi exhibit the prop of ferent isoforms of BGL depending on t ulture ditions or carbon sources (Singhania et al., 1). Vario oforms of endoglucanase and beta-glucosi eported to xpressed in response to carbon sources *Asper terreus* (Nazir et al., 2010). The sequential ind on of isofor as been associated with the presence of inct metabolites llas-Bôas et al., 2006). As an accepte model induction of the cellulases is mediated either by lo ole ar weight soluble oligosaccharides that are released from c ex subst s as a result of hydrolysis by constituti nes o the ducts (positional isomers) of transglyco tion ctions h ed by constituent beta-glucosidase, x ses, et adhan et a., 2007a). These metabolites enter the cell a gn e of extracellular substrates and provide the sti s for the accelerated synthesis of constituent enzymes of cellu complex. However, this process is complex in view of the fact t many fungi and bacteria are known to express functionally multiple cellulases/hemicellulases in presence of different carbon sources. This multiplicity may be the result of genetic redundancy, differential mRNA processing or post translational modification such as glycosylation, autoaggregation or/and proteolytic digestion (Collins et al., 2005). However, the regulation of expression of these multiple isoforms is still not clear which necessitates further research regarding the sequential and differential expression of the isoforms. It must be emphasized that though the regulation of cellulases is apparently mediated through induction and repression as the two major mechanisms of controlling the expression of these enzymes, the existence of highly specialized and complex nature of regulating the expression of cellulases in diverse microorganisms has also been reported (Badhan et al., 2007a,b; Sanchez-Herrera et al., 2007). There may be relationship between the metabolites present in the culture extracts and the induction of different isoforms. The understanding about regulation would be important in designing culture conditions for overproducing desired kind of isoforms or secondary metabolites. The structure and nature of carbon source can also play an important role in differential induction of the enzyme system. Culturing under submerged or solid substrate fermentation also influences the expression of distinct isoforms.

Multiplicity of cellulases and hemicellulases is well known in the case of filamentous fungi (Willick and Seligy, 1985; Nazir et al., 2010) and probably this multipli ntial, considering the vast and diverse roles these en es play fungal metabolism and survival. Beta-glucosida ultiplicity c be attributed to the presence of multiple ge es o to differe al post transcriptional modifications (C is et al., 5). Di ential expression of the various BGL teins are repo response to the carbon sources supplie the m dm or the onditions of culture (Willick and Seligy, 198 et al., 20 0) and could be a probable adaptation e fung espon the changing immediate environments s property o could be exploited for selective expres desired isofo rom a fungus by manipulating the culture conditi carbon source carefully.

7. g of beta-gluc ase genes

The beta-g sidase genes from a large number of bacterial, m yeast, pl , and animal systems have been cloned and expr in b *E. coli* and eukaryotic hosts such as *S. cerevisiae* and fi us fungi. Cloning has been performed by two methods, either by (1) formation of a genomic DNA library followed tion of the recombinant clones by screening for beta-glucosidase production, or (2) starting with a cDNA library (or a genomic library), screening of recombinant clones by specific nucleotide probes designed from *a priori* knowledge of the polypeptide sequence" (Bhatia et al., 2002). Filamentous fungi are known to be a good beta-glucosidase producer and a number of beta-glucosidase from glycosyl hydrolase family 3 have been purified and characterized from various fungi. Though fungi are known to be good producers of the enzyme, reports on cloning of BGL from fungi are relatively low. This is mostly due to the complexities associated with the presence of introns in their genes and due to complexities associated with glycosylation. Filamentous fungi are known to produce a broad range of metabolites at significant quantities. Their ability to grow on cheaper substrate have made them potential source of metabolites for industrial applications. Knowledge of fungal genetics has accumulated since years of research and industrial applications. Along with the ability to secrete proteins they can perform post transtlational modification such as glycosylation and disulphidation (Wang et al., 2005). Most of the filamentous fungi are transformed with plasmid which integrates into their genome and thereby providing stability to the fungal transformants. Thus filamentous fungi have tremendous potential to be employed as hosts for recombinant DNA. Genus *Aspergillus* and strain *Trichoderma reesei* have been used as hosts for expressing several genes of fungal as well as nonfungal origin. Few fungal beta-glucosidase of glycosyl hydrolase family 3 genes have been cloned and characterized such as from fungi like *Talaromyces emersonii* (Murray et al., 2004) *Aspergilli* (Dan et al., 2000; Kim et al., 2007) *Penicillium brasilianum* (Krogh et al., 2010) *Thermoascus aurianticus* (Hong et al., 2007). Majority of the reports also mentions the existence of multiple genes and gene products that are differentially expressed. Fungal genes have been cloned and expressed mostly in eukaryotic expression systems like *Trichoderma reesei* (Murray et al., 2004; Barnett et al., 1991), *Aspergillus* sp. (Takashima et al., 1999), *S. cerevisiae* (Dan et al., 2000), and *Pichia*

pastoris (Dan et al., 2000). *Pichia pastoris*, due to its exceptional high extracellular protein production capacity has been an ideal gene expression system for industrial enzyme production and was exploited for heterologous protein expression. Several GH 3 family BGL genes have been cloned and expressed successfully as active proteins but the amount of BGL produced always remained low (Harnpicharnchai et al., 2009; Yan et al., 2012; Xu et al., 2011). Yeasts have been also well exploited both for the source of bgl gene as well as the host organisms to express fungal beta-glucosidase. *Saccharomyces cerevisae* has been the first choice for several researchers where they have cloned genes for BGL as well as genes for other component of the cellulase to bring the simultaneous saccharification to reality (Jeon et al., 2009; Shen et al., 2008). It was realized that here too the limitation was again the expression of cellulolytic enzymes and conversion of biomass to glucose, thus the ethanol yield remained low even though having potent ethanol producers from glucose. Tri-functional mini cellulosome complex was generated consisting of *T. reesei* EG2, CBH2, and *A. aculeatus* BGL1 enzymes and expressed in *Saccharomyces cerevisae* showed 8.8-fold enhanced activity because of both enzyme–enzyme and enzyme proximity synergy (Wen et al., 2010) and was a significant study to show the consolidated bioprocessing for bioethanol production. Production of cellulosic ethanol was demonstrated by expressing heterologous endoglucanase and BGL gene from *Clostridium thermocellum* and *S. fibuligera*, respectively in *S. cerevisae* (Jeon et al., 2009). An industrial ethanol producing strain *S. cerevisae* have been constructed which could produce ethanol directly from microcrystalline cellulose by simultaneous saccharification with 88% of the theoretical yield of ethanol. Bgl 2 gene from *T. reesei* along with α-galactosidase gene was cloned into *S. cerevisae* expresses both the enzyme extracellularly (Zhang et al., 20 Thermostable native Bgl from *A. fumigatus* were cloned and e pressed in *P. pastoris* X33 stable at even 70 °C, at pH 4–7 whic proves its suitability for lignocellulose hydroly et al., 2012). Since, *T. reesei* produces the other compo in ficient quantity; it would be of high value to engine *reesei* gene of BGL having desirable properties such as t os acidic pH tolerant; which can produce ficient unt of BGL leading to better biomass hydrolysis Engineeri *reesei* in such a way that it produces the w of sachcha ng enzymes including BGL could be on of th proaches to reduce the processing cost. While c ructing *T. re* strain with high BGL expression, it is nec y to employ a d ose inducible strong promoter. The *ch* has be he widely used promoter in eukaryotic system for e sio different protein and has been extensively used for heterologous gene expression constructs in *T. reesei* (Wang and Xia, 2011). However there are reports that cbh1 and cbh2 (cel6a) promoters which are known to be very strong, were found to reduce overall cellulase activity (Rahman et al., 2009a). Use of *xyn3* promoter to drive expression of *T. reesei* BGL1 (Cel3a) was recently reported greater BGL activity than the parent strain without significant decrease in total cellulase activity (Rahman et al., 2009b). Recently, recombinant *T. reesei* was constructed with *A. aculateus* BGL1 gene that expressed under the control of *xyn1* promoter, capable of saccharification of pretreated cellulosic biomass. Recombinant strain expressed 25-fold higher BGL than that of endogenous *T. reesei* BGL ld higher than that of the parent strain, *A. aculateus* kazawa l., 2012). It could be interesting to have hyper producing e with desired properties cloned in filame tous which is eady producing other the components ellulase, ch in n will help to make the process econo Table 2 show urce and host of bgl gene and also prop s of r binant B from the recent literature have been e list accordi to authors could be a potential candidat r bio hydr s and based on our understanding a ave relev p mass hydrolysis application. Efforts a d in the dire n to obtain engineered BGL with glucose olerant perties along with the search of glucose tolerant ative BGL so In a study Liu et al. showed that 184t e of a novel B amily 1 could be responsible for its glu e tolerance which was proved based on site directed mutage s studies w e mutant strain exhibited better glucose toleranc vel from *K* .9 compared to 14.9 in the wild one (Liu et al., 2011).

Soil i ervoir of many enzymes since microbial diversity ne gram is in the range of million species. However, less than 1 icroorganisms are only culturable in laboratory conditi ns. Thus, metagenomic approach allows us to clone the microbial genome directly from the environmental samples into the culturable bacteria. Metagenomics have proven successful in exploring the diversity of indicator genes which are employed to test the hypothesis about the structural composition and structure of soil microbial flora (De Souza et al., 2008). Molecular analysis of BGL diversity and its function in soil revealed that only a narrow range of bacteria are capable to process cellobiose in soil (Canizares et al., 2011). Several beta-glucosidase have been obtained via metagenomic strategies (Bao et al., 2012). Recently, an article shows the significant role of BGL produced by *A. niger* in degrading soil organic matter and the effect of soil minerals in enzymatic activity (Lammirato et al., 2010).

Table 2
List of the so organism om where BG genes have been cloned and expressed into host organisms.

Source	organism	Properties of recombinant BGL	References
Saccharomycopsis era	*S. cerevisae*	1.02 IU/mg	Shen et al. (2008)
Talaromyces emerson	*T. reesei*	GH family 3, thermostable active at 71.5 °C, V_{max} 512 IU/mg, K_i 0.254 mM against glucose	Murray et al. (2004)
Periconia sp.	*Pitchia pasrtoris*	Thermotolerant BGL, optimal activity at 70 °C and at pH 5–7	Harnpicharnchai et al. (2009)
Penicillium decumnens	*T. reesei*	Six- to eight-fold increased BGL activity than native strain	Ma et al. (2011)
Aspergillus niger	*T. reesei*	5.3 IU/ml (106) times higher than native BGL	Wang and Xia (2011)
Caldicellulosiruptor saccharolyticus	*E. Coli*	Thermostable with 13 U/mg, having optimum activity at 70 °C and at pH 5.5	Hong et al. (2009)
Aspergillus fumigatus Z5	*Pitchia pasrtoris X33*	Active at pH 6.0 and at 60 °C with specific activity of about 100 IU/mg	Liu et al. (2012)
Neocallimastix patriciarum	*Pitchia pasrtoris*	GH family 3, 34.5 U/mg against cellobiose, optimally active at 40 °C and pH 5.0	Chen et al. (2012)
Paecilomyces thermophila	*Pitchia pasrtoris*	GH family 3, 274.4 U/ml, optimal pH 6 and temp 60 °C	Yan et al. (2012)
Periconia sp.	*T. reesei QM9414*	10.5-fold BGL activity increased from 2.2 to 23.9 IU/mg, thermotolerant and active in acidic pH	Dashtban and Qin (2012)
Fervibacterium islandicum	*E. Coli*	GH family 1, thermostable	Jabbour et al. (2012)
Chaetomium thermophilum	*Pitchia pasrtoris*	Optimally active at pH 5.0 and 60 °C	Xu et al. (2011)
Aspergillus aculateus	*T. reesei*	10 U/mg against cellobiose	Nakazawa et al. (2012)

Efforts are needed in the direction to search novel potent BGL for biomass hydrolysis and rumen of the herbivorous animals could be a good source though those will be anaerobic microorganisms which would prevent its demand in industries, but for sure this limitation could be overcome by cloning. As our interest is only the gene producing BGL and not the microorganism, the gene can be isolated, cloned and expressed. Attempts have been made to clone cellololytic enzyme producing gene from termite gut, which could also be a breakthrough. cDNA encoding novel beta-glucosidase was isolated from buffalo rumen fungus *Neocallimastix patriciarum* and was cloned and expressed in *Pitchia pasrtoris*. It performed better than Novo 188 under the living condition of the fermenting yeast such as *S. cerevisae* or *Kluveromyces marxianus* when used during simultaneous saccharification for glucose generation as well as ethanol production (Chen et al., 2012).

8. Importance of beta-glucosidase in bioethanol production

With the advent of need for alternative energy, there is resurgence in bioethanol programme through enzymatic route which was otherwise lost in oblivion and thus BGL has received immense attention of the researchers being the key enzyme for complete cellulosic hydrolysis. The cellulolytic enzyme system secreted by the filamentous fungi *Trichoderma reesei* is the one mostly used in industrial applications. The hydrolysis step converting cellulose to glucose is recognized as the major limiting operation in the development of processes for production of biofuels from lignocellulosic raw materials because of the low efficiency of cellulases and their high cost. This issue has been addressed by various ways supplementation of exogenous beta-glucosidase to the *T. reesei* cellulase to remove the cellobiose or engineering beta-glucosidase genes into the host organism so as to overproduce it, which can enable improvement in efficiency of biomass hydrolysis and cost reduction of biomass-to-ethanol conversion by reducing feedback inhibition and cellobiose mediated repression of cellulases. Enzymatic hydrolysis of cellulose is a multistep complex, the last step being a homogenous catalysis reaction involving the action of beta-glucosidase on cellobiose (Lynd et al., 2002). Cellobiose is a strong inhibitor of both cellobiohydrolases and endocellulases, and the beta-glucosidase action can reduce its effect. In addition, the produced glucose also inhibits cellulose hydrolysis and exerts feedback inhibition. Glucose at high concentration can either block the active site for the substrate or prevent the hydrolyzed substrate from leaving (Kumar et al., 2010). The amount of beta-glucosidase-1 (BGL1) generally *T. reesei* hyper-producing strains represents a very low percentage of the total secreted proteins (Lynd et al., 2002; Harpoël-Gilet et al., 2008). Though 12 BGLs have been identified in *T. reesie* genome but most are intracellular. Most of the cellulolytic filamentous fungi are characterized by low secretion of BGL (Lynd et al., 2002; Merino and Cherry, 2007) which accumulates the activity to be insufficient to convert cellobiose (an intermediate product in cellulose hydrolysis) to glucose. The less abundance of BGL even under conditions of cellulase induction and the product inhibition to which it is susceptible, limits the use of native cellulase preparations in lignocellulosic biomass hydrolysis for alcohol production. Alternative strategies such as co cultivation of fungal strains producing cellulase and BGL like *T. reesei* and *Aspergillus* strain such as *A. phoenics* or *A. niger* were done for better cellulase with increased activity of BGL (Wen et al., 2005). This limitation can be alleviated either by over expressing beta-glucosidase in *T. reesei* or by adding extra beta-glucosidase from other sources (Sukumaran et al., 2009). Supplementing the native *T. reesei* enzymatic cocktail with beta-glucosidase from other fungi is also often performed to avoid inhibition of cellobiose (Xiao et al., 2004). Enzyme cocktail prepared from cellulase producing *T. reesei* and extracellular BGL producing mutant strain of *T. atroviride* was found better than that of commercial preparation for enzymatic hydrolysis as well as for simultaneous saccharification and fermentation of pretreated spruce (Kovacs et al., 2009). There are other reports also that shows enzyme preparation from different fungal strain performs better even more than the commercial preparations such as accelerase™ 1000, and novozyme 188 and celluclast 1.5 L (Kovacs et al., 2009).

Glucose tolerant BGL can circumvent the problem of feedback inhibition, and if available in an enzyme cocktail for biomass hydrolysis can improve the efficiency of hydrolysis by shifting the equilibrium towards a higher product concentration than otherwise achievable. Few species of *Aspergillus* known to produce glucose tolerant beta-glucosidases and some of these enzymes have been cloned and characterized (Riou et al., 1998; Gunata and Vallier, 1999). It is expected that most such glucose tolerant BGLs may be present in nature ubiquitously in filamentous fungi. Isolation of such enzymes and knowledge about their properties, sequences and expression patterns can help in design of better enzyme cocktails for biomass hydrolysis as well as in targeted approaches for modifying the glucose tolerance of existing BGLs.

Commercially the enzyme suppliers Genencor and Novozymes have launched a series of cocktails of cellulolytic enzymes for biomass hydrolysis, such as Accelerase® series of enzymes (Genencor, 20[illegible] (http://www.genencor.com)) and the Cellic series of enzymes (Novozymes, 2010; (http://www.novozymes.com)). The advanced enzyme preparations from both the companies probably are from genetically modified *T. reesei* with high BGL activity. Though the detailed information on the composition of *T. reesei* cellulases is not disclosed, it is assumed to contain heterologously expressed BGL from *Aspergillus* sp. The situation with BGL secretion in *T. reesei* has been dramatically improved recently due to genetic modification. The modern generation of commercial cellulases which is meant for biomass hydrolysis, contains beta-glucosidase supplements in significant quantity; indicating the importance of this enzyme in biomass hydrolysis. Commercial enzyme giants in the market are claiming to have improved enzyme with higher titres of beta-glucosidase, efficient for hydrolysis of biomass. Still we all will agree that enzymes for biomass hydrolysis are not available in higher volumes even on excluding the matter of the prices. We can assume in lieu of the progress achieved in case of BGL research that in near future BGL will not remain a limiting factor for LC ethanol.

9. Conclusions

Beta-glucosidase is a key enzyme involved in sugar-enzyme platform for fuel ethanol production from lignocellulosic biomass. In present scenario, the commercialization of above platform is limited due to various technological challenges, which include slow enzymatic degradation rate and feed-back inhibition of the enzyme, majorly beta-glucosidase. Development of glucose-tolerant beta-glucosidase and supplementing it with cellulase from other sources, e.g. *T. reesei* could offer potential benefits in overcoming feed-back inhibition, leading to high reaction rate and also help is high solid processing. Heterologously expressed beta-glucosidase by genetically modifying host organism could also be an effective tool in developing enzyme with desired properties.

Acknowledgement

Authors (R.K.S. and A.P.) acknowledge the TIFAC, New Delhi and CSIR, New Delhi for financial support for the Centre for Biofuels at NIIST.

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