Appl Biochem Biotechnol (2010) 162:538–547
DOI 10.1007/s12010-009-8775-9

Profiling Differential Expression of Cellulases and Metabolite Footprints in *Aspergillus terreus*

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Received: 20 June 2009 / Accepted: 9 September 2009 /
Published online: 25 September 2009

Abstract This study reports differential expression of endoglucanase (EG) and β-glucosidase (βG) isoforms of *Aspergillus terreus*. Expression of multiple isoforms was observed, in presence of different carbon sources and culture conditions, by activity staining of poly acrylamide gel electrophoresis gels. Maximal expression of four EG isoforms was observed in presence of rice straw (28 U/g DW substrate) and corn cobs (1.147 U/ml) under solid substrate and shake flask culture, respectively. Furthermore, the sequential induction of EG isoforms was found to be associated with the presence of distinct metabolites (monosaccharides/oligosaccharides) i.e., xylose (X), G_1, G_3 and G_4 as well as putative positional isomers (G_1/G_2, G_2/G_3) in the culture extracts sampled at different time intervals, indicating specific role of these metabolites in the sequential expression of multiple EGs. Addition of fructose and cellobiose to corn cobs containing medium during shake flask culture resulted in up-regulation of EG activity, whereas addition of mannitol, ethanol and glycerol selectively repressed the expression of three EG isoforms (Ia, Ic and Id). The observed regulation profile of βG isoforms was distinct when compared to EG isoforms, and addition of glucose, fructose, sucrose, cellobiose, mannitol and glycerol resulted in down-regulation of one or more of the four βG isoforms.

Keywords Differential expression · Endoglucanase (EG) · β-Glucosidase (βG) · Multiple isoforms · Regulation · Metabolite footprint profile

Introduction

Cellulose is one of the major components of plant material, together with hemicelluloses and lignin. Cellulose consists of long polymers of β-1,4-linked glucose units, which in turn form higher order fibular structure. Cellulases, produced by a variety of bacteria and fungi, bring about the hydrolysis of cellulose by synergistic action of its constituent enzymes comprising (a) β-1,4-endoglucanase (1,4-β-D-glucan 4-glucanohydrolase; EC 3.2.1.4),

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which cleaves internal β-1,4-glycosidic bonds; (b) cellobiohydrolase (1,4-β-D-glucan cellobiohydrolase; EC 3.2.1.91), an exo-acting enzyme which processively releases cellobiose from reducing and non-reducing ends of cellulose; and (c) β-glucosidase (β-D-glucoside glucohydrolase; EC 3.2.1.21) that hydrolyzes cellobiose to smaller oligosaccharides and finally to monosaccharides [1, 2]. As an accepted model, the induction of the cellulases is mediated either by low molecular weight soluble oligosaccharides that are released from complex substrates as a result of hydrolysis by constitutive enzymes or by the products (positional isomers) of transglycosylation reactions mediated by constituent β-glucosidase, xylanases, etc [3, 4]. These metabolites enter the cell and signal the presence of extracellular substrates and provide the stimulus for the accelerated synthesis of constituent enzymes of cellulase complex. However, this process is complex in view of the fact that 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 [5]. However, it is still not very well understood how the expression of these multiple isoforms is regulated. The aspects regarding the sequential and differential expression of these isoforms also needs further research. It must be emphasised 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 specialised and complex nature of regulating the expression of cellulases in diverse microorganisms has also been reported [3, 6]. The present study highlights the expression of multiple cellulases in a thermotolerant fungal strain of *Aspergillus terreus* previously reported to produce endoglucanases with deinking capabilities [7] and as source of novel endoglucanase with high activity towards xyloglucan and barley β glucan from our laboratory [8]. The sequential and differential expression of isoforms of endoglucanase as well as relationship between the metabolites present in the culture extracts at different time intervals of growth and their role in inducing different isoforms of endoglucanase (EG) is being presented here. The understanding about regulation would be important in designing culture conditions for overproducing desired kind of isoforms.

Materials and Methods

Culture

A thermotolerant fungal strain isolated from composting soil of Jammu region (India) and identified as *A. terreus* (AN_1) was employed in this study [7]. The fungus was grown and maintained on yeast potato soluble starch (YpSs) of following composition (%; *w*/*v*): starch 1.5, yeast extract 0.4, KH_2PO_4 0.2, K_2HPO_4 0.23, $MgSO_4 \cdot 7H_2O$ 0.05, citric acid 0.057 and agar 2.0. The pH of medium was adjusted to 7.0. The fungus was cultured at 45 °C for 7 days and stored at 4 °C.

Induction and Repression

The induction of cellulases (endoglucanases EG and β-glucosidase) was studied in a medium containing, corn steep liquor 0.85%, $(NH_4)_2SO_4$ 1.3%, and KH_2PO_4 0.4%, along with 1% (*w*/*v*) of monosaccharides (glucose and fructose), polysaccharides (avicel, solka floc, carboxymethyl cellulose (CMC), xylan and cellobiose) and lignocelluloses (rice straw,

wheat straw, wheat bran, corn cob, paper and bagasse) as carbon source. The synergistic/negative effect of supplementing, fructose, glucose, cellobiose, sucrose and mannitol (at 1% *w*/*v*) as well as glycerol and ethanol (at 1% *v*/*v*) to the corn cob containing liquid medium was studied. Flasks were inoculated with 2 ml of spore suspension (1×10^7spores ml^{-1}) of 5-day-old culture of *A. terreus* and incubated at 45 °C/120 rpm for 120 h in an orbital shaker. The sampling was carried out at 12-h intervals up to 120 h, the contents were centrifuged (8,000×*g* for 10 min), and the supernatants were assayed for different activities.

Solid State Fermentation

For the preparation of inoculum, the culture was grown in 250 ml Erlenmeyer flasks containing 50 ml of glucose broth of the following composition (%; *w*/*v*): glucose 1.5, yeast extract 0.4, K_2HPO_4 0.2 and $MgSO_4$ 0.05. The pH of the medium was adjusted to 7.0, and flasks were incubated in an orbital shaker (120 rpm) at 45 °C for 24 h. Solid state fermentation was carried out in Erlenmeyer flasks (250 ml) that contained 5 g of different carbon source (rice straw, wheat straw, wheat bran, corn cob, paper and baggase; ground to particle size 2–7 mm) and 15 ml of basal medium of the following composition: KH_2PO_4 0.4%, corn steep liquor 0.85% and $(NH_4)_2SO_4$ 1.3% (pH 7.0). After sterilisation, the medium was inoculated with 2 ml of actively grown culture of *A. terreus* and incubated at 45 °C for 5 days. The enzyme was harvested by adding 50 ml of sodium citrate buffer (50 mM, pH 6.0) to the flasks and kept at 45 °C for 1 h under mild shaking. The resultant slurry was filtered through muslin cloth and centrifuged at 8000×*g* for 10 min. The filtrates were assayed for endoglucanase and β-glucosidase activity.

Enzymatic Assay

Endoglucanase activity was determined using 1% CMC prepared in sodium citrate buffer (50 mM, pH 6.0). The reaction mixture containing equal amounts of suitably diluted enzyme and substrate was incubated at 50 °C for 10 min. The reaction was stopped by addition of dinitrosalicylic acid followed by boiling [9]; the colour developed was read at 540 nm using Novaspec II spectrophotometer (Pharmacia). The amount of released sugars was quantified using glucose standard.

β-Glucosidase was assayed using *p*-nitro phenyl-β-D-glucopyranoside (*p*NPG) in a micro titre plate based method [10]. Appropriately diluted enzyme (25 μl) was mixed with 50 μl of sodium acetate buffer (50 mM, pH 5.0). The reaction was initiated by adding 25 μl of *p*NPG (10 mM) and incubated at 50 °C for 30 min; the reaction was terminated by adding 100 μl of NaOH–glycine buffer (0.4 M, pH 10.8), and the developed yellow colour was read at 405 nm using ELISA Reader (MULTISKAN; Lab system). One unit of β-glucosidase activity was expressed as the amount of enzyme required to release 1 μmol of *p*NP per minute under assay conditions.

Native Polyacrylamide Gel Electrophoresis and Zymogram

Crude enzyme preparations (protein 70 μg) were fractioned by native polyacrylamide gel electrophoresis (PAGE) using 10% acrylamide gel with 4% stacking gel [7]. For developing EG activity, the PAGE gel was incubated for 15 min in 0.05 M sodium acetate buffer (pH 5.0) and then was overlaid on polyacrylamide gel containing CMC (0.5%,*w*/*v*) for 2 h

at 50°C. The overlay gel was removed and stained with 0.2% Congo Red. Bands corresponding to EG appeared as clear zone against a dark background after destaining with 1 M NaCl followed by treatment with 10% (*v*/*v*) acetic acid solution. The β-glucosidase activity in gels was detected by developing zymogram against 10 mM 4-methylumbelliferyl-β-D-glucoside (Sigma) as substrate prepared in sodium citrate buffer (50 mM, pH 6.0). Upon completion of electrophoresis, the gel was immersed in substrate solution for 45 min at 50°C in the dark. The β-glucosidase bands in the gel were detected under UV light using gel documentation system (Gene Genius, Cambridge, UK).

Metabolic Profiling Using Thin Layer Chromatography

The extracts from corn cob grown shake cultures were withdrawn at intervals of 12 h. Each extract (40 ml) was lyophilised to powder (HETO, Drywinnwer-3) and dissolved in 1 ml of methanol. Hydrolyzed products in the samples were detected by thin layer chromatography (TLC; 0.25 mm layer of silica gel F-254, Merck, India) using mixture of ethyl acetate/acetic acid/water (3:2:1 *v*/*v*) as solvent system. The resolved hydrolysis products were detected by spraying the TLC plates with diphenylamine reagent followed by heating at 100°C for 10 min. Mixture of xylose (X), glucose (G_1), cellobiose (G_2), cellotriose (G_3) and cellotetraose (G_4; Sigma) was used as standard.

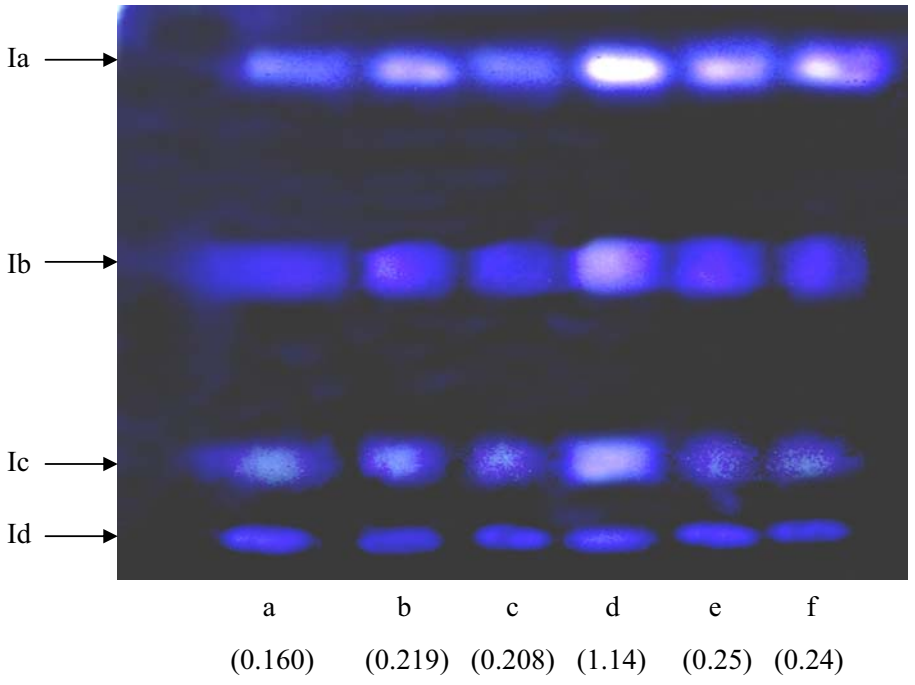

Fig. 1 Zymogram showing effect of different carbon sources on expression of multiple endoglucanases by *A. terreus* under shake flask culture. *Lanes from left to right* Rice straw (*a*), wheat bran (*b*), wheat straw (*c*), corn cob (*d*), bagasse (*e*) and paper (*f*). The values in parentheses show the endoglucanase activity (in units/ml)

Results

Expression of Multiple Endoglucanase Under Shake Flasks and Solid Substrate Culture

The results in Fig. 1 show the expression of four endoglucanase EG (Ia, Ib, Ic and Id) isoforms when *A. terreus* was grown on complex residues (rice straw, wheat straw, wheat bran, corn cob, paper and bagasse) under shake flask culture. However, corn cob led to maximal expression of EG (1.147 U/ml) as also evident from the intensity of bands. Differential expression of EG isoforms was observed when *A. terreus* was cultured on solidified medium using different carbon sources (Fig. 2.). Rice straw produced maximal level of enzyme activity (28 U/g DW substrate) and showed the presence of four EG isoforms (Ia, Ib, Ic and Id). However, corn cob and paper waste as substrate supported production of three isoforms (Ia, Ic and Id), and absence of EG Ib isoform was evident, whereas in the presence of wheat straw only two isoforms, i.e. EG (Ia and Id), were produced. In presence of wheat bran and bagasse, selective expression of sole isoform EG Ia and EG Id, respectively, was observed.

Sequential Induction of EG Isoforms

The result in Fig. 3 shows the production profile and sequential induction of EG isoforms *A. terreus* in presence of corn cob under shake flask conditions. The expression of EG Ib was initiated after 48 h of incubation which was followed by induction of isoform EG Ia observed at 60 h of incubation. At 72 h of incubation, isoform EG Ic was expressed, whereas isoform EG Id was expressed after 96 h of incubation. Maximal endoglucanase activity was observed after 120 h of incubation which coincided with the expression of all

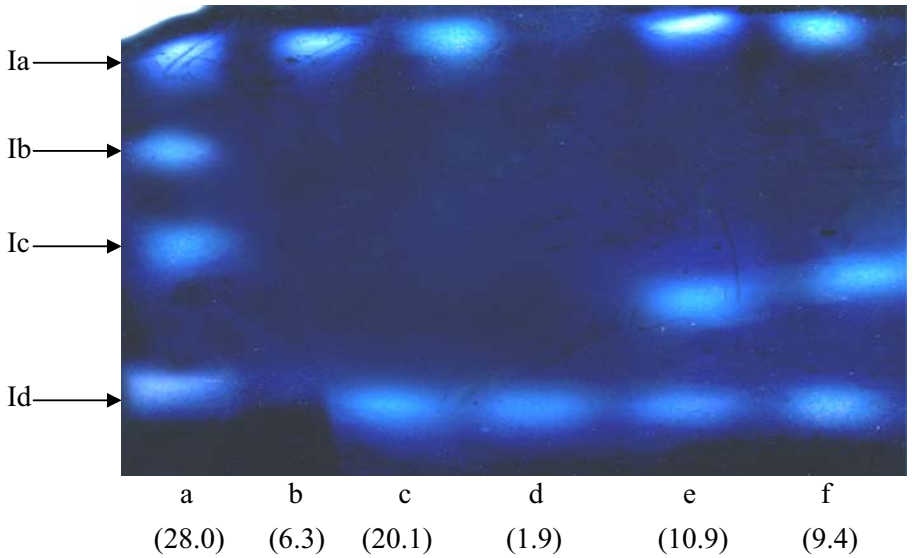

Fig. 2 Zymogram showing effect of different carbon sources on expression of multiple endoglucanase isoforms by *A. terreus* under solid state condition. *Lanes from left to right* Rice straw (*a*), wheat bran (*b*), wheat straw (*c*), bagasse (*d*), corn cob (*e*) and paper (*f*). The values in parentheses show the endoglucanase activity (in U/g DW substrate)

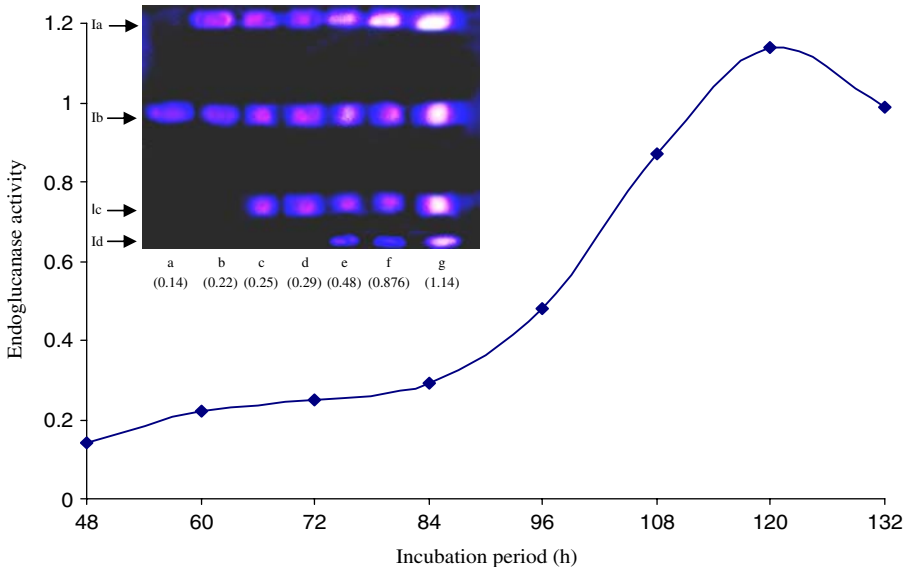

Fig. 3 Profile of endoglucanase production by *A. terreus* grown on corn cob containing medium. *Inset zymogram* shows the sequential expression of endoglucanase isoforms after 48 h (*a*), 60 h (*b*), 72 h (*c*), 84 h (*d*), 96 h (*e*), 108 h (*f*) and 120 h (*g*). The values in parentheses show the endoglucanase activity (in units/ml)

four isoforms (Fig. 3 inset). The observed sequential expression was putatively linked to the hydrolysis products/metabolite footprints (Fig. 4) present in the culture extracts. The metabolite profiling showed the presence of isomeric form of G_1 as well as G_2 at 0 h which could be the hydrolysis/degradation product formed during autoclaving of medium containing corn cob. At 48 h, the expression of EG Ib was associated to the presence of G_1, G_2/G_3, G_3 and G_4 as the putative metabolic products in the culture filtrate. At 60 h, the expression of isoform EG Ia was observed to be associated with the presence of G1, G_3 and a small unidentified molecule in the culture extracts, while the positional isomers G_2/G_3 and G_4 observed at 48 h were not detected. At 72 h, the induction of isoform EG Ic was linked to simultaneous reappearance of G_4 type positional isomer. At 96 h, when all four isoforms were expressed, the metabolic footprint showed the presence of metabolites identified as xylose (X), G_1, G_3 and G_4 as well as G_1/G_2, G_2/G_3 positional isomers in the culture filtrate.

Selective Repression of Endoglucanase and β-Glucosidase Isoforms in *A. terreus*

The addition of glucose, fructose, sucrose or cellobiose to corn cob basal medium did not cause repression of EG activity, and all four isoforms were expressed under shaking conditions. However, presence of fructose and cellobiose led to an up-regulation of EG activity (1.313 and 1.397 U/ml, respectively) when compared to growth on corn cob (1.147 U/ml) as sole carbon source, whereas addition of mannitol, ethanol and glycerol to corn cob based medium selectively repressed the expression of three EG isoforms (Ia, Ic and Id) resulting in expression of sole isoform EG Ib. The expression of isoform EG Ib is possibly constitutive (Fig. 5) and contributed nearly 50% to the observed EG activity.

The results in Fig. 6 show that corn cob supported expression of four β-glucosidases (βG Ia, Ib, Ic and Id). Addition of ethanol did not repress the expression of any of the

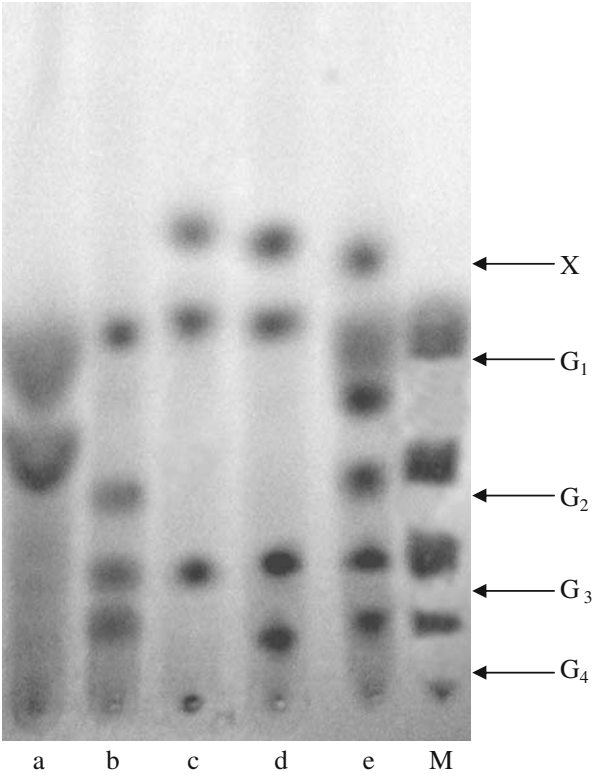

Fig. 4 TLC showing profile of the metabolites present in the culture extracts of *A. terreus* grown on corn cobs containing medium under shake flask culture. *Lanes a*, *b*, *c*, *d* and *e* denote metabolites observed in the culture extract at 0, 48, 60, 72 and 96 h, respectively. *M* denotes standards consisting of X (xylose), G_1 (glucose), G_2 (cellobiose), G_3 (cellotriose) and G_4 (cellotetraose)

isoforms, whereas addition of cellobiose and glycerol resulted in selective repression of isoform βG Id. On the other hand, glucose and fructose repressed the expression of βG Ia and Ic, whereas addition of mannitol resulted in repression of three isoforms Ib, Ic and Id, and only sole isoform βG Ia was selectively expressed.

Discussion

A. terreus, a thermotolerant fungus associated with composting material, was found to produce a battery of cellulases. The activities of cellulases in some of the strains are appreciably high when compared to others [11]. Functionally distinct endoglucanase (25 kDa) from *A. terreus* has been purified and characterised to be highly acid active [12], and another EG (78 kDa) that shows preferential activity towards xyloglucan and barley β glucan when compared to carboxymethylcellulose has also been characterised [8]. The crude cellulase preparation from *A. terreus* has been found to be efficient for biological deinking [7]. Owing to higher level of acid-active thermostable cellulase activities, it is also seen as an important source of enzymes for bioconversion of cellulosics to ethanol. The

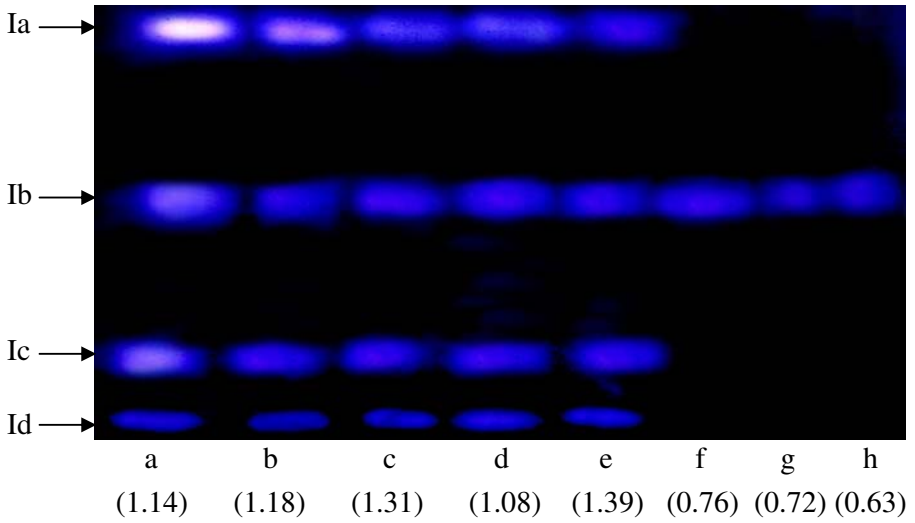

Fig. 5 Zymogram showing the effect of addition of different additives at 1% (*w*/*v*) glucose (*b*), fructose (*c*), sucrose (*d*), cellobiose (*e*), mannitol (*f*), ethanol (*g*) and glycerol (*h*) to corn cob (*a*) containing medium on the expression of endoglucanase isoforms of *A. terreus* under shake flask culture. The values in parentheses show the endoglucanase activity (in units/ml)

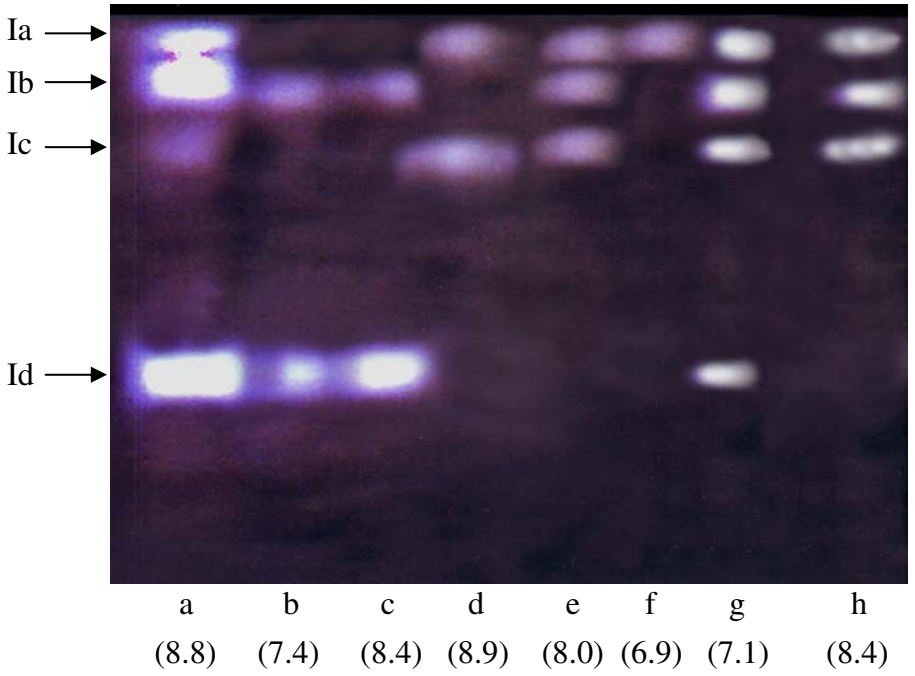

Fig. 6 Zymogram showing the expression of multiple β glucosidases in *A. terreus* grown on medium containing corn cobs (*a*) supplemented with glucose (*b*), fructose (*c*), sucrose (*d*), cellobiose (*e*), mannitol (*f*), ethanol (*g*) and glycerol (*h*) at 1% (*v*/*v*) under shake flask culture. The values in parentheses show the β-glucosidase activity (in units/ml)

activity staining of the PAGE resolved gels have shown presence of multiple endoglucanase and β glucosidase in culture filtrates of *A. terreus* [7, 13]; however, their regulation and sequential expression, which is intriguing from basic and applied research view point, has not been studied. The structure and nature of carbon source plays an important role in differential induction of the cellulase/hemicellulase system [14]. Culturing under submerged or solid substrate fermentation also influences the expression of distinct isoforms. Similar observations have been reported for multiple xylanase production in *Myceliophthora* sp. [4]. Experimental approaches based on functional aspects of proteins, i.e. developing zymograms against PAGE/sodium dodecyl sulfate-PAGE, 2D gel electrophoresis [15] and Northern blot based techniques to study the differential expression of cellulases at transcriptional level, have previously highlighted regulatory aspects in *Cellulomonas flavigena* [6], *Myceliophthora* sp. [4] and *Trichoderma reesei* [16]. In *Hypocrea jecorina*, sophorose was shown to mediate selective expression of EG and only one of the xylanases [17], whereas xylobiose led to the formation of both xylanase xyn I and II but not EG. It seems that each organism has unique machinery to regulate the expression of these cell-wall-degrading enzymes (Figs. 1, 2, 5 and 6). For example, glucose inhibits the expression of cellulase in *Aspergillus niger* which is known to dock in the D-glucose inhibition domain present at C terminal of Zn binuclear cluster domain of XYLR regulatory protein [18]. Whereas in the present study no repression in presence of glucose was observed, instead ethanol, glycerol or mannitol, which have been previously reported as neutral carbon sources [19] having no role in modulation of transcription in *T. reesei*, was found to cause repression of three out of four EGs in *A. terreus*. The regulation of β glucosidases from *A. terreus* also showed complex pattern of selective repression and de-repression in presence of distinct sugars and sugar alcohols (Fig. 6); the regulatory controls for EG and βG seems to be quite different as also observed previously [20]. The results in the present study indicated that the expression of multiple endoglucanases in *A. terreus* was sequential (Fig. 3), whereas the time course of *egl1*, *egl2* and *egl 5* mRNA accumulation studies in *T. reesei* had previously revealed the induction of all three EG transcripts to be simultaneous [16].

The sequential expression of endoglucanase was correlated to the accumulation of the distinct array of mono/oligosaccharides at different time intervals as the metabolic signatures which show possible role of distinct hydrolytic and positional isomers in the expression of these endoglucanases. There seems to be a continuous flux of these metabolites across the membrane that possibly plays an important role in the sequential expression of these EG isoforms. The dynamic role of phosphoenolpyruvate-dependent phosphotransferase system transporters that allow the flux of G_2 and G_3 across membrane in *Erwinia chrysenthemi* for the expression of cellulases has also been previously hypothesised [21]. Recent studies have shown that the metabolic footprint of extracellular metabolites accumulated in the medium [22, 23] during growth of cultures on cellulosics can be used to integrate proteome metabolome studies to the fibre degradation. However, these studies do not address to the changed profiles of the metabolites during culturing which is important to specify the role of these metabolites in differential expression.

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