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RESEARCH ARTICLE

Glycosyl Hydrolases Producing Bacterial Endophytes from Perennial Grass Species (*Neyraudia reynaudiana* L.) for biomass deconstruction

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ABSTRACT

Endophytes associated with C4 perennial grasses are a treasure trove of biocatalysts, and have emerged as a viable option for biofuel production. Studies were conducted to investigate the production of extracellular cellulases and xylanases from newly isolated *Bacillus tequilensis* BT5 and *Alcaligenes faecalis* B12 from perennial grass (*Neyraudia reynaudiana L.*). The total cellulase activity (FPase) for *B. tequilensis* BT5 and *A. faecalis* B12 were recorded as 0.61 and 0.34 FPU.ml^{-1} at 28 h and 32 h, respectively. Similarly, β-glucosidase, carboxymethyl cellulase (CMCase) and exoglucanase activities of BT5 and B12 were 1.12 and 0.94 U.ml^{-1}; 0.92 and 0.86 U.ml^{-1}; 1.98 and 1.58 U.ml^{-1}, respectively. Xylanase activities of 1.58 U.ml^{-1}and 1.22 U.ml^{-1} at 28 h and 36 h for *B. tequilensis* BT5 and *A. faecalis* B12, respectively were also recorded. Furthermore, the presence of cellulases and xylanases genes was confirmed by PCR screening using the gene-specific primers. This study revealed that the cellulases and xylanases from endophytic bacteria from perennial grasses could serve as a natural reservoir for biomass deconstruction and production of cellulose derived commodities.

Keywords: *Perennial grass, Neyraudia reynaudiana, Endophytic bacteria, Glycosyl hydrolases, Fermentation*

INTRODUCTION

The need to find sustainable alternatives to replace increasingly expensive fossil fuels has driven the interest in the production of biofuels worldwide. As a long-term solution, renewable biological resources such as plant biomass and treated municipal as well as industrial wastes are being envisaged. The use of such biomass for the production of biofuels in a biorefinery relies solely on the enzymatic hydrolysis of pretreated biomass to produce reducing sugars. The process requires a set of synergistically operating enzymes, which includes, cellobiohydrolases, endoglucanases, beta-glucosidases along with certain oxidative enzymes (Horn *et al.,* 2012). The major impediments are the cost of enzymes for enzymatic hydrolysis and the hydrolysis of pentoses from hemicelluloses (Koppram *et al.,* 2012). The potential solution is the on-site production of these enzymes in order to minimize the expenses of enzymes addition externally (Kazi *et al.,* 2010). With this perspective, many novel carbohydrate-active enzymes (CaZymes) have been explored from different niche with a wide range of attributes like temperature, pH and alkali tolerance.

The role of glycosyl hydrolases (GHs), especially cellulases and xylanases are critical in the deconstruction of lignocellulosics (Scholl *et al.*, 2015). The cellulase complex consists of endoglucanases, exoglucanases and glucosidases, which imparts functional role in biomass hydrolysis to simple sugars like glucose (Ogeda and Petri, 2010). Similarly, the enzymes of the xylanolytic complex are responsible for the conversion of the main carbohydrate xylan found in hemicellulose to xylose (Kuhad *et al.*, 1997). The cellulases and xylanases together find many biotechnological applications in the textile industry for denim fading and in pulp and paper, baking along with animal feed industries, respectively. Therefore, the use of microorganisms that produce hydrolytic enzymes such as cellulases and xylanases, using low cost abundantly available substrates, such as perennial grasses, are probable candidates for making second-generation biofuel.

The perennial grasses are considered as a rich source of lignocellulosics making it a second-generation alternative energy source. Apart from switchgrass (the energy crop), some of the traditional forage grasses like *Saccharum*

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arundinaceum, Panicum antidotale, Thysanolaena latifolia, *Erianthus* sp. and *Neyraudia reynaudiana*, can also have the potential to produce energy. These perennial grasses are abundantly available on the roadsides and forests, which can be harvested, a minimum three times a year making them suitable for the production of energy (Nair and Sekharan, 2009). Among them, the Burma reed (*Neyraudia reynaudiana*) also known as silk reed is botanically a giant C4 plant with large plumed grass, perennial in nature and native to subtropical Asia. It has a number of positive attributes such as productive long-lived perennial crop with high biomass production potential and efficient water use.

In order to break the complex macromolecules of plant biomass, hydrolytic enzyme producing endophytic bacteria, which are ubiquitous in most plant species, residing latently or actively colonizing plant tissues locally as well as systemically, are gaining importance. Such endophytes are recognized as a new source of genes, enzymes (cellulases and xylanases) and other secondary metabolites to assist their adaption and survival within higher plants (Xiong *et al.,* 2013; Castro *et al.,* 2014). It has been shown that hydrolytic enzymes produced by endophytes are involved in the initial infection process of the host (Hallmann *et al.,* 1997). However, they also produce these extracellular hydrolases in order to establish a resistant mechanism against plant invasion, thus can have a beneficial effect on host plant (Tan and Zou, 2001). *Alcaligenes faecalis* is remarkable for the degradation of hemicellulose and cellulose by 73.5 % and 67.3 %, respectively in a co-culture system (Yang *et al.,* 2011) by producing cellulases and xylanases. Earlier β-glucosidase from *A. faecalis* was purified and was found to be capable of hydrolyzing a wide variety of different chemicals. Among the endophytic microorganism, a few endophytic fungi showed lignocellulosic biomass degrading efficiency (Purahong and Hyde, 2011). However, very few reports are available on endophytic bacteria having the potential to degrade lignocellulose resources (Ma *et al.,* 2016). Recently, Xiong *et al.,* (2013) had demonstrated that an endophytic bacteria *Pantoea ananatis* Sd-1 isolated from rice seeds with strong lignocellulosic biomass degradation ability to degrade rice straw and lignin. Due to the rapid growth, environmental adaptability and biochemical versatility of bacteria (Archana and Mahadevana, 2002), developing an endophytic bacterial system may provide a variety of advantages in lignocellulosic biomass (LCB) degradation as compared to fungi due to fast growth and easy culturing techniques. Hence exploring endophytic bacteria with Glycosyl hydrolases producing ability will hold good for sustainable biorefineries. In this study, the overall goal was to decipher GHs producing endophytes from *N. reynaudiana* and assessing their efficiency for the production of glycosyl hydrolases under submerged fermentation.

MATERIAL AND METHODS

Collection of biomass sample

The above ground parts of the perennial grass *N. reynaudiana* (BPS-G109) were collected from Murlen National Park (23°37′01″N and 93°18′00″E), India using local forage chopper machine. The leaves and stems were dried separately at 55°C in a hot air oven and chopped into smaller pieces followed by grounding into smaller particles using hammer mill (Barbender Rotary Mill, Type: 880805, Germany) and sieving through 20 mesh sieve to get 0.5 to 5.00 mm size particles (Menegol *et al.,* 2014).

Isolation and screening of endophytic bacteria for glycosyl hydrolases (GHs)

Endophytic bacterial isolates were obtained in LB medium from the tissues of *N. reynaudiana* (BPS-G-109) by using the method suggested by Sturz *et al.,* (1998). The isolates emerged from the tissues were carefully selected and purified by repeated streaking and screened for cellulase and xylanase activities in BPS-CX production media (Vincent *et al.,*2016) containing xylan and carboxymethyl cellulose (CMC) as substrates.

For cellulase enzyme, cultures were initially grown in CMC induction medium containing carboxymethyl cellulose (2.0g) as cellulase inducer, peptone (1g), yeast extract (0.5g), KH_2PO_4(1g), NaCl (1g) and agar (3g) in 200mL of distilled water. The plates were incubated at 30-37°C for 24 h. The xylanase induction was also performed similarly except the inducing agent used, which was birch wood xylan used at 1% level. After 24 h of incubation, all the plates used for screening cellulase and xylanase were stained with 1% Congo red solution for 2-3 min, followed by destaining with 1M NaCl solution (Teather and Wood, 1982)

Molecular identification of GHs producing endophytic bacteria

The genomic DNA from the selected bacterial isolates was extracted using the CTAB method (Melody,1997).16S rRNA sequence as amplified using universal primers: 27 F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492 R (5'-ACGGCTACCTTGTTACGACTT-3') (Weisberg *et al.,* 1991). The 16SrRNA gene was amplified from BT5 and B12 bacterial cultures using the PCR conditions as follows: 94˚C for 5min followed by 35 cycles of 94˚C for 1min, 55˚C for 1min, 72˚C for 1min and a final extension at 72˚C for 10min. The amplified PCR products were resolved in 1% agarose gel followed by staining with ethidium bromide (10 mg. ml^{-1}) and visualized on a gel documentation system

(Bio-rad Gel DocXR+ system (Hercules, CA, USA). The amplified PCR products were visualized in 1.5% agarose gels and documented. The amplified PCR product of about 1500 bp from BT5 and B12 were purified using GeneJet PCR purification kit (Thermo Scientific, USA) and sent for sequencing to Eurofins, Bangalore, India.

GHs screening based on PCR

Two endophytic bacterial isolates, BT5 and B12 were subjected to PCR based screening for the presence of cellulase and xylanase genes using Ba_EN1F, Ba_EN1R and Ba_XlnF, Ba_XlnR primers, respectively, following the PCR conditions: 94˚C for 5min followed by 35 cycles of 94˚C for 1min, 60˚C (for cellulase)/ 50˚C for xylanase for 1min, 72˚C for 1min and final extension at 72˚C for 10min. The amplified PCR amplicons were resolved in 1% agarose gel. The primer details are as follow:

Primer name	Primer sequences	References
Ba_EN1F	5'-CCAGTAGCCAAGAATGGCCAGC-3'	Hussain *et al.,* 2011
Ba_EN1R	5'- CGAATAATCGCCGCTTTGTGC-3'	
Ba_Xln F	5'- ATGTTTAAGTTTAAAAAGAATTTC-3'	
Ba_XlnR	5'- TTACCACACTGTTACGTTAG- 3'	

GHs production under submerged fermentation (SmF)

Initial inoculum for bacterial isolates was developed using LB medium by overnight culturing until OD_{600} of 0.6 and then inoculated in a volume of 50 mL of BPS–CX production medium (containing NaCl 0.5 g, K_2HPO_4 0.5 g, peptone 1g, yeast extract 0.25 g for 100 ml at pH 7.0) supplemented with 1% CMC/xylan for cellulose/xylanase production, respectively (Vincent *et al.,* 2016). The flasks were incubated at 50°C in an orbital shaker at 110 rpm for 72 h. Enzyme activity was determined at every 24 h intervals in triplicates using analytical grade reagents.

Preparation of crude enzyme extract

After incubation, the cultures were centrifuged at 5000 rpm for 10 min, at 4°C and the cell-free culture supernatant served as the source, for determination of various enzyme activities.

Total Cellulase (FPase)

Total cellulase (FPase) activity of the crude enzyme was measured following the method suggested by Zhang *et al.,* (2009). One ml of the crude enzyme was incubated with 2 ml of 0.1 M citrate buffer (pH 4.8) containing 0.05g Whatman No.1 filter paper as a substrate. After incubation for 1 h 50 °C, the resulting reducing sugars were determined by adding 3 ml of DNS reagent as per the method of Nelson (1944). The tubes were placed in a boiling water bath for 5 min and after cooling, the absorbance of the samples was measured at 540 nm. One unit of FPase is equivalent to one micromole of glucose liberated mL^{-1} of culture filtrate min^{-1}.

Endoglucanase

Carboxymethyl cellulase (CMCase) activity was measured by the DNS method in which 1% CMC in 50 mM citrate buffer (pH 4.8) served as substrate. Different enzyme dilution series were done using citrate buffer, substrate and crude enzyme. The mixture was incubated at 50°C for 60min. The amount of reducing sugars released during hydrolysis was measured by DNS method suggested by Nelson (1944). The optical density was measured at 540 nm against glucose as standard.

Exoglucanase

Avicel, a potent substrate for exo-glucanase activity assay, was used at 1.25% in acetate buffer and the reaction performed with crude enzymes at different dilutions. The mixture was incubated at 50°C for 2 h and the end point of reaction was observed using phenol-sulfuric acid assay (Sadasivam and Manickam, 2007). The optical density was measured at 540 nm.

Xylanase

The xylanolytic activity of the cell-free culture supernatant was determined according to Satya narayana and Johri (1983), at 50°C with 1 % birch wood xylan as substrate. One unit of xylanase activity was expressed as µmole of reducing sugars (xylose equivalent) released in one min. under the assay conditions.

RESULTS AND DISCUSSION

Screening and isolation of endophytic bacteria for hydrolytic enzyme production

Six bacterial isolates were recovered from the endosphere tissue of *N.reynaudiana* (BPS-G109). All the isolates were purified by repeated streaking and screened for the production of cellulase and xylanase on CMC and Xylan (birch wood xylan) containing agar plates (Table1).

Table 1. Hydrolytic capacity of bacterial endophytes from *Neyraudia reynaudiana*

Cultures	Hydrolytic capacity	
	Cellulase	Xylanase
Bacillus tequilensis BT5	1.83	2.29
Alcaligenes faecalis B12	1.76	1.12

Among the isolates, two bacterial isolates, (BT5 and B12) were positive for both CMCase and xylanase activity, by producing clear zones on the respective medium with Congo red infusion. Based

on the hydrolytic capacity, the cellulolytic ability of BT5 and B12 were found to be 1.83 and 1.76, respectively; whereas, xylanolytic capacity was found to be 2.29 and 1.12, respectively. The isolate BT5 recorded higher cellulase and xylanase activities compared to B12.

Table 2. Quantitative assay for lignocellulosics degrading enzymes

Isolate name	Xylanase (IU.ml^{-1})	FPU ((IU.ml^{-1})	Endoglucanase ((IU.ml^{-1})	Exoglucanase ((IU.ml^{-1})	BGL* ((IU.ml^{-1})
Bacillus tequilensis BT5	7.64	0.61	0.92	1.96	1.12
Alcaligenes faecalis B12	4.02	0.34	0.86	1.24	0.94

*BGL – β- glucosidase

16s rRNA amplification showed a clear band around 1500 bp and the sequences of the two isolates were analyzed using EzTaxon database and a phylogenetic tree was constructed using MEGA 7.0. The isolate BT5 showed identity (98%) with *B. tequilensis* and the isolate B12 was found to be 98% similar to *Alcaligenes faecalis* based

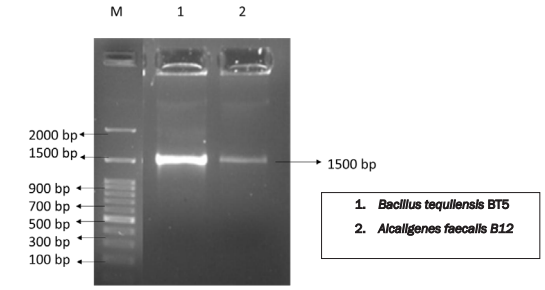

Figure. 1a

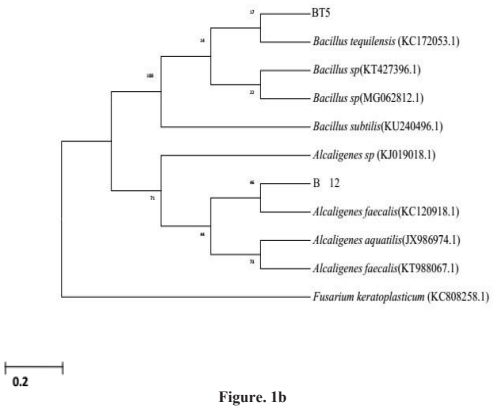

Figure. 1b

Figure 1a. PCR amplification of 16S rDNA gene showing amplification of 1500 bp. Figure 1b. Neighbourhood Joining tree constructed based on CLUSTAL W using Mega 7.0 showing homology with *Alcaligenes faecalis* for B 12 and *Bacillus tequilensis* for BT 5

on sequence homology and phylogenetic analysis (Figure1a and 1b). Several cellulose degrading bacteria like *Rhodospirillum rubrum, Cellulomonas fimi, Clostridium stercorarium, Bacillus polymyxa, Bacillus* sp. *Pyrococcus furiosus, Flavobacterium* sp., *Acidothermus cellulolyticus, Saccharophagus degradans* and *Branhamella* sp. have already been reported to exhibit GHs activity (Heather and Somerville, 2012). Similarly, there were many reports on the xylan degrading bacteria that include strains of *Aeromonas, Bacillus, Bacteroides, Cellulomonas, Microbacterium, Paenibacillus, Ruminococcus* and *Streptomyces* (Lima *et al.,* 2013).

Identification of bacterial endophytes

The promising GHs producing bacterial endophytes were identified using microscopic and molecular analyses. The isolate BT5 was Gram-positive whereas, B12 showed negative Gram reaction. Molecular identification based on partial

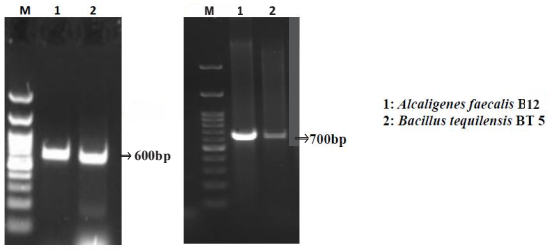

Figure 2a Figure 2b

Figure 2a. and 2b. PCR confirmation of endo glucanase and xylanase in *Alcaligenes faecalis* B12 and *Bacillus tequilensis BT5*

Cellulase and xylanase genes in bacterial endophytes

The endophytic bacterial isolates tested for glucosyl hydrolases were confirmed for the presence of cellulase (Ba_ENIF and Ba_EN1R) and xylanase genes (Ba_xlnF and Ba_xlnR), confirmed with gene-specific primers. Cellulase primer (Ba_EN1) resulted in the amplification of about 600 bp in both *B. tequilensis* BT5 and *A. faecalis* B12. Moreover, amplification of xylanase screening amplicon of about 700 bp resulted in xylanase positive thermophilic isolates (Figure 2a and 2b). This confirmed the glycosyl hydrolase activity of bacterial endophytes.

Cellulase and xylanase production under submerged fermentation (SMF)

Time course production of both cellulase and xylanase were monitored in 1% CMC medium at

600 nm at 4 h intervals, in which maximum OD_{600} (1.75) reached at 48 h. While monitoring growth, CMCase activity of cell-free culture supernatant from *B. tequilensis* BT5 showed considerable activity at 48 h (0.92 IU ml^{-1}) and the activity started to decline thereafter (Figure). The FPase activity was maintained maximum (0.614 IU ml^{-1}) at 24 h of incubation; however, on further incubation, FPase started to decline (Table 2). Interestingly, the cellulolytic bacterial strainb*B. tequilensis* BT5 showed significant β-glucosidase activity in cell-free supernatant, reaching their maximum activity (1.127 IU ml^{-1}) after 24 h of incubation and the enzyme activity started to decline on further incubation. Thus, CMCase activity was found to be an extracellular secretome and growth dependent. *Alcaligenes faecalis* B12 also recorded all the GHs activity of endoglucanase (0. 87 IU ml^{-1}), exoglucanase (1.24 IU ml^{-1}), β-glucosidase (0.942 IU ml^{-1}) and total cellulase (0.34 IU ml^{-1}) (Table 2).

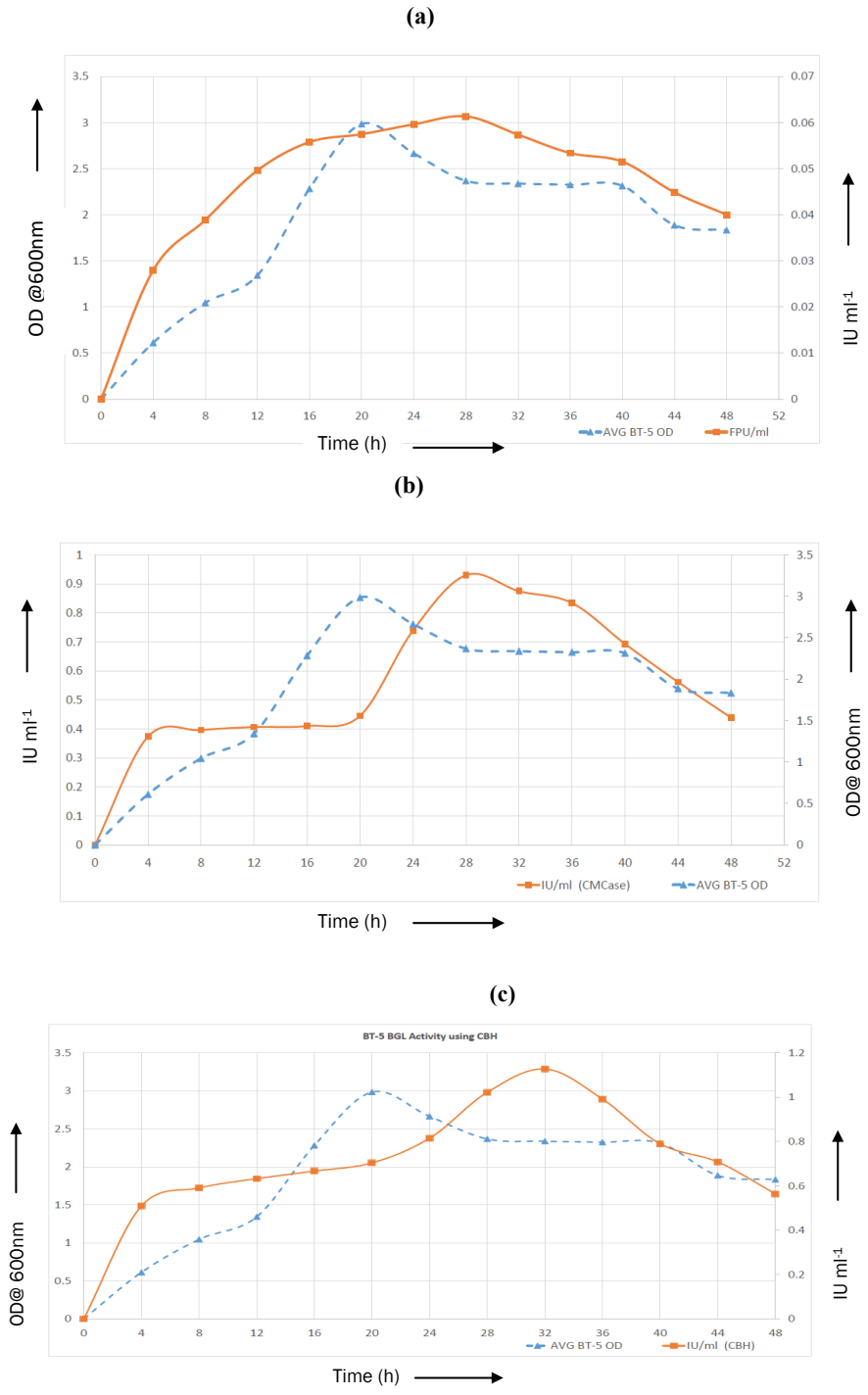

Figure 3. Time course production of cellulolytic enzymes of *Bacillus tequilensis* BT 5 showing a) endoglucanse b) exoglucanase c) betaglucosidase activities.

For xylanase, the growth of the culture in 0.5% xylan amended medium at 600nm was monitored at 4h intervals and the maximum OD_{600} of 1.75 reached at 48 h. The xylanase activity in the cell-free supernatant by the bacterial isolates showed considerable enzyme production and the strain *B. tequilensis* BT5 showed the maximum xylanase activity of 1.58 IU ml^{-1} followed by *Alcaligenes faecalis* B12 after 48 h of incubation. Extended incubation showed a decline in enzyme activity. No detectable xylanase activity was observed in the cell debris. Thus, xylanase was an extracellular enzyme and the units of xylanase activity were found to be much higher as compared to corresponding CMCase activity. Cellulase and xylanase enzyme production by several *Bacillus* species have been reported by many workers (Heck *et al.,* 2002; Sharma *et al.,* 2015).The potential of bioconversion of perennial forage grasses endemic to South Asian countries has been demonstrated widely. A few reports also indicate the application of endophytic bacteria having potential hydrolytic enzymes in bioconversion (Xiong *et al.,* 2013; Lima *et al.,* 2005; Yasinok *et al.,* 2008) of lignocellulosics. However, there is no report on the utilization of endogenous bacteria associated with perennial grasses, with the potential to valorize LCB.

CONCLUSION

Endophytic bacteria isolated from perennial grass, *Neyraudia reynaudiana* L. present an attractive potential for the exploitation of cellulases and hemicellulases due to their rapid growth rate, enzyme complexity, and extreme habitat variability. The development of rapid and reliable methods for the screening of cellulases from microorganisms within hostile environments will allow a greater number of novel bacterial cellulases to be isolated for industrial use. Improvement in the performance of cellulase can be exposed by mutagenesis and metabolic engineering techniques for the improved industrial appliances. Hence, it is concluded that endophytic microbes could harbour the great potential for various industrial processes. Till now, most of the known xylanases are obtained from fungi. Bacterial xylanases offer more competitive and milder operating conditions that can be executed as a quick substitution to fungal xylanases. An in-depth understanding of biology and biochemistry of bacterial xylanases will offer a better scope for industrial exploration to upgrade lignocellulosic biomass conversion.

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