Sugar Tech (Jan-Feb 2016) 18(1):29–38
DOI 10.1007/s12355-014-0357-7

RESEARCH ARTICLE

Xylanase Production by Isolated Fungal Strain, *Aspergillus fumigatus* RSP-8 (MTCC 12039): Impact of Agro-industrial Material as Substrate

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Received: 13 August 2014 / Accepted: 10 November 2014 / Published online: 8 March 2015

Abstract In the present investigation, the imperative role of agro-industrial biomass for improved xylanase production was evaluated using isolated fungal strain. This isolate was identified as *Aspergillus fumigatus* RSP-8 (MTCC 12039) based on morphological and 18S rRNA ribotyping and the organism was deposited in MTCC, IMTECH Chandigarh with accession number 12039. The isolated fungal strain is mesophilic in nature and produced maximum xylanase at 30 °C, at pH 7 and agitation speed of 150 rpm. Xylanase complex production titers differed with the nature and complexity of carbon source and other physiological growth parameters including aeration, growth temperature, physiological medium pH, initial inoculum levels, etc. Highest xylanase titers (73 U/mL) noticed with hemicellulose isolated from sorghum straw and least with ground nut cake as carbon source among tested agro materials such as rice bran, green gram husk, sorghum straw, groundnut cake and wheat bran. A variation of three fold enzyme titers was observed with different tested carbon sources. Supplementation of glucose as carbon source did not produce any xylanase with this fungal strain revealing the xylanase in this isolate is induced by the carbon source. Variation of hemicellulose concentration as carbon source during the fermentation altered the production xylanase titers. The study suggested that, in xylanase production by *A. fumigatus* RSP-8, one of the major limiting factors is substrate chemical complexity.

Keywords Agro material · Sorghum straw · Hemicellulose · Submerged fermentation · Xylanase enzyme

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Introduction

Commercial importance of agro industrial/photosynthetic biomass may be multiplied by the sequential conversion of the cell wall polymers to monomeric sugars initially, followed by further translation as value added compounds through fermentation process or catalyzing enzymatically (Prakasham et al. 2014). With the present technological developments, the first process is the rate limiting step due to complex and xenobiotic nature of biomass. Among different polymeric components of biomass such as cellulose (only glucose polymer), hemicellulose (unlike cellulose, this is heteropolymer with arabinose, glucose, xylose, etc. linked with alpha or beta 1–4 or 1–6 linkages making it more complex for saccharification) and lignin (aromatic alcohol or monolignol polymer), hemicellulose is a higher complex polymer compared to cellulose. Hence, the enzymatic hydrolysis of hemicellulose, the second most abundant natural polysaccharide available on earth, is one of the most important factors for economizing the biomass to biofuel program. The diversity in the chemical structure of

hemicelluloses from the cell walls of wood, cereal and other plant materials is one of the factors for existence of a large variety of xylanases with diverse catalytic activities, physicochemical properties and structures (Beg et al. 2001; Chiranjeevi et al. 2013).

Sorghum is considered as one of the important energy crops due to higher biomass productivity and carbohydrate contents over others. Sorghum straw or bagasse is an excellent source for hemicelluloses in addition to the commercial value of sucrose rich juice, which could serve as substrate for ethanol production (Yuvraj et al. 2013) and other biofuels including biohydrogen (Darmarapu et al. 2013; Prakasham et al. 2014). Xylan constitutes the major portion (70–80 %) of hemicellulose. Commercial importance of hemicellulose could be possible when it is converted to simple monomers either chemically or enzymatically or in combination of both. Xylanase play significant role in enzymatic delinking of hemicellulosic polymer to xylose monomers (Laxmi et al. 2012). Xylanases from diverse microbial sources are exploited for efficient deconstruction of xylan to xylose, xylobiose and other xylooligosaccharides (Wong et al. 1988; Laxmi et al. 2012). Xylanases also have wide application potential in production of oligosaccharides (Laxmi et al. 2008). Another approach of deconstruction of hemicellulose is application of harsh physical and chemical processes where lignocellulosic biomass results in degradation of valuable xylooligosaccharides and xylose into furfurals (Kumar and Wyman 2008; Prakasham et al. 2014) in addition to releasing toxic waste chemicals into environment, thus emphasizing the necessity to employ enzymatic treatments.

In biotechnological point of view, xylanases and xylanase producing microorganisms have potential applications in the management of waste, to degrade xylan to renewable fuels and chemicals, in addition to their use in food, agro-fiber, and paper and pulp industrial sectors (Haltrich et al. 1996; Bhatia et al. 2012; Kundu et al. 2012; Laxmi et al. 2012; Driss et al. 2013) mainly to reduce their environmental impact (Laxmi et al. 2008). In addition to xylooligosaccharides produced by the catalytic action, xylanases are of considerable importance in production of functional food additives or alternative sweeteners with beneficial properties (Laxmi et al. 2012). Since, no individual xylanase is capable of meeting all the requirements of feed and food industries, more attention has been focused on the xylanase stability under different processing conditions such as pH, temperature and inhibitory ions, in addition to their ability to hydrolyze soluble and insoluble xylan denoting requirement of research efforts for isolation of effective xylanase complex producing microbes (Harris and Ramalingam 2010; Laxmi et al. 2012; Motta et al. 2013).

Filamentous fungi are the major producers of biomass degrading enzymes such as cellulases and hemicellulases in industry. These filamentous fungi are of special interest as they secrete these enzymes in large quantities into the medium and xylanase titers are much higher than those found in yeast and bacteria (Haltrich et al. 1996). Xylanase complex differs with genetic nature of microbe either bacteria or fungi and known to be regulated by the physiological (pH, temperature, incubation time, initial inoculum size, aeration and agitation), nutritional (carbon and nitrogen sources and metal ion requirement), and genetic nature of the microbes (Kundu et al. 2012; Prakasham et al. 2005, 2006, 2007; Maheswari and Chandra 2000; Bakir et al. 2001; Rao et al. 2008). This suggests the imperative role of substrate specificity of enzyme complex which in turn plays a significant role in economics of saccharification process. Hence, in the present study xylanase complex production by fungal isolate *Aspergillus fumigatus* RSP-8 (MTCC 12039) was evaluated and reported that use of extracted hemicellulose influenced the xylanase production titers and played important role in overall process.

Materials and Methods

Isolation and Screening of Fungi for Xylanolytic Activity

The isolation of xylanolytic fungi was carried out using xylan agar medium (XA) containing Birchwood xylan (Himedia) as sole carbon source. Exotic soil samples were collected from poultry manure dump from local poultry farm near Nacharam, Hyderabad, and were used as source of fungi. One gram of soil sample was suspended in 10 mL of sterile distilled water. This suspension was serially diluted up to 10^{-4}. From this, 0.1 mL was spread on xylan agar medium plates. Bacitracin (0.1 mg/mL) was added to inhibit bacterial growth. Plates were incubated at room temperature for 7 days. Fungal colonies showing xylanolytic activity were identified by staining with 0.1 % w/v Congo red solution for 20 min followed by distaining with 1 M NaCl (Teather and WOOD 1982). Positive isolates were detected based on clear hydrolytic zones around fungal colonies.

Morphological Identification

Identification of isolated fungal strain was performed by comparing the morphological and the reproductive characteristics of the fungal strain such as color, size, texture, and reproductive structures according to Watanabe (2002).

Identification of Fungi Using 18S rRNA Gene Analysis

For 18S rRNA gene, fungal spores were cultured on potato dextrose agar medium (Himedia, India). The plates were incubated at 30 °C for 3 days. The fungal mycelium

developed on agar plates was used for isolation of DNA using Fungal DNA isolation Kit (Xcelgen).

PCR Amplification of 18S rRNA Gene

PCR amplification of 18S rRNA gene was carried out using 18S universal primer sequences; 1F (CTGGTGCCAGCAGCCGCGGCAA) and 4R (CGGAGGGCATTACAGACCTGTTAT). PCR amplification was performed in a 25 µL reaction mixture containing 1.00 µL of each forward (1F) and reverse (4R) primers (10 pmol/µL), 12.50 µL of PCR master mix (MBI Fermentas), 3.0 µL of diluted DNA (30 ng/µL) and 7.50 µL of DNase-RNase free water. The reaction conditions used for the same as follows: initial denaturation at 95 °C for 2 min, followed by 30 amplification cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and primer extension at 72 °C for 90 s; and a final extension at 72 °C for 10 min. PCR was carried out in a final reaction volume of 25 µL in 200 µL capacity thin wall PCR tube in Eppendorf Thermal Cycler. Amplified PCR product was purified using Xcelgen Gel extraction kit. The purified DNA was subjected to automated DNA sequencing on ABI 3730xl Genetic Analyzer (Applied Biosystems, USA). Sequencing was carried out using BigDye® Terminator v3.1 The reaction was carried out in a final reaction volume of 20 µL using 200 µL capacity thin wall PCR tube. After cycling, the extension products were purified and mixed well in 10 µL of Hi-Di formamide. The contents were mixed on shaker for 30 min at 300× g. Eluted PCR products were placed in as ample plate and covered with the septa. Sample plate was heated at 95 °C for 5 min, snap chilled and loaded into auto sampler of the instrument. Electrophoresis and data analysis was carried out on the ABI 3730xl Genetic Analyzer using appropriate Module, Basecaller, Dyeset/Primer and Matrix files.

Sequence Analysis

For identification of the strain, the obtained PCR amplicon of 18S rRNA gene sequence was compared to the existing sequences in the National Center for Biotechnology and Information (NCBI) GenBank library, using the alignment tool BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence data was deposited in the Gene database of DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/index-e.htmL).

Hemicellulose Quantification

One gram of selected biomass material was initially treated with 16.4 g of 72 % H_2SO_4 and incubated for 1 h at 30 °C with intermittent stirring. To this 280 mL of water was added to bring down to the acid concentration to 4 %. These reactants were subjected 121 °C for 1 h. The samples were filtered using G1 grade crucibles and filtrate pH was adjusted to pH 5 using lime. The content of hemicellulose in the biomass was calculated based on xylose, acetic acid and arabinose content present in the filtrate according NREL (Sluiter et al. 2012).

Extraction of Hemicellulose from Sorghum Biomass

Sorghum straw was procured from International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Hyderabad, India. Hemicellulose was extracted from sorghum straw by modified alkaline extraction method (Dalton et al. 2010) and was used as substrate for production of xylanase under submerged fermentation conditions.

Xylanase Production Under Submerged Fermentation Condition

Xylanase production fermentation experiments were performed in 250 mL conical flasks containing 100 mL of modified Czapek's medium (CDM) having the following composition (per liter): 1 g KH_2PO_4, 2 g $NaNO_3$, 0.5 g KCl, 0.5 g yeast extract, 0.5 g peptone, 0.5 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4$, 0.001 g $ZnSO_4$, 0.0005 g $CuSO_4$ and 1 g Hemicellulose extracted from sorghum biomass, as carbon source unless otherwise mentioned. The flasks were inoculated with 1 mL of spore suspension (1×10^7 spores/mL) and incubated at 30 °C in rotary shaker (LabTech) adjusted to 150 rpm. Production conditions are optimized by varying inoculum concentration (0.5–2.0 %); incubation temperature (26–34 °C); aeration levels (25, 50, 75, 100 and 125 mL medium in 250 mL conical flask); agitation speed (120–180 rpm); substrate concentration (0.5–3 %); initial medium pH of 5.0–9.0. The effect of different substrates (rice bran, wheat bran, green gram husk, groundnut cake) on xylanase production was studied. The cell free fermentation broth was prepared by filtering the broth with whatman 50 micron filter paper and filtrate was used as enzyme source. Results reported in this study were averages of duplicate samples.

Measurement of Xylanase Activity

Xylanase activity was determined using modified Bailey method (Bailey et al. 1992). In this, 0.1 mL of the enzyme solution was added to 1.9 mL xylan solution (1 % w/v). Xylan solution was prepared by dissolving Birchwood xylan (Himedia) in 50 mM sodium acetate buffer (pH 5.0) and incubated at 50 °C for 20 min. The reaction was terminated by adding 2 mL of DNS reagent and the contents were boiled for 5 min and volume is made up to 10 mL

with distilled water. The absorbance of final solution was read at 540 nm using UV–Visible spectrophotometer (Xplorer, XP 2001) and xylanase activity was measured in terms of International units (U) using xylose standard curve. One unit of xylanase activity was defined as 1 µmol of xylose equivalents released min^{-1} mL^{-1} of enzyme.

Optimization of Production Medium for Xylanase Production Using Plackett–Burman Design

Plackett-Burman design was employed to optimize xylanase production by varying the concentrations of medium components of modified Czapek's medium. The seven components studied were KH_2PO_4, $NaNO_3$, KCl, yeast extract, peptone, $MgSO_47H_2O$ and hemicellulose (Table 1). A total 13 experiments were carried out with 12 main batches and 1 central point's batch. Each variable was tested at two levels, high and low. High level corresponds to the increase and low level corresponds to the decrease of concentration of each medium component by 50 % of initial medium. The impact of each variable on xylanase activity was estimated based on comparison of the difference in the mean between the high level (+) and the low level (−). The central point's batch contained the unmodified medium composition as control. The resulting data was analyzed using Minitab statistical software.

Results and Discussion

Isolation and Identification of Xylanolytic Fungi

Using selective xylan agar medium several fungal colonies showing clear hydrolytic zones upon staining with 0.01 % w/v Congo red solution were selected, after 5 days of incubation. Out of 15 fungal colonies, one of the isolates with most prominent growth and hydrolytic activity was designated as RSP-8 and selected for further study. Growth and colony morphology of the fungi RSP-8 on Czapek's agar revealed white mycelial growth towards periphery with central green region bearing conidiophores (Fig. 1). Based on microscopic observation of conidia shape and ornamentation, phialide shape and branching pattern (data not shown) the fungal isolate RSP-8 was identified as *Aspergillus* species. Similar results were reported for *A. fumigatus* (Suhaib et al. 2012). On the basis of 18S rRNA gene similarity search using the tool BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the xylanolytic fungal isolate RSP-8 showed 97 % identity with *Aspergillus fumigatus* strain TMS-26. Partial sequence of 18S rRNA gene the fungal isolate *Aspergillus fumigatus* RSP-8 was submitted to the Gene database of DNA Data Bank of Japan (DDBJ) with accession number AB938216 and the organism was deposited in MTCC, IMTECH Chandigarh, with accession number MTCC 12039.

Table 1 Medium components for xylanase production and their coded values

Medium component	Concentration (g/L)	High (g/L) (+)	Low (g/L) (−)
KH_2PO_4	10	15	5
$NaNO_3$	20	30	10
KCl	5	7.5	2.5
Yeast extract	5	7.5	2.5
Peptone	5	7.5	2.5
$MgSO_47H_2O$	5	7.5	2.5
Hemi cellulose	10	15	5

Effect of Different Carbon Sources on Xylanase Production

Biotechnological potential of agro industrial waste as carbon source for production of any industrial product through fermentation is mainly associated with availability of efficient biomass deconstructing bio catalytic component (Prakasham et al. 2014). Several microbial sources are characterized in this context. However, research data revealed that substrate specific cellulose and hemicellulose deconstructing enzyme complexes and the components of the enzyme consortia differ in terms of their ratios and genetic nature of microbe employed (Laxmi et al. 2012). This is the main factor for the thriving of many novel microbial strains from various natural sources worldwide. In most of the xylanase producing microbial strains, hemicelluloses or its derivatives are frequently used to

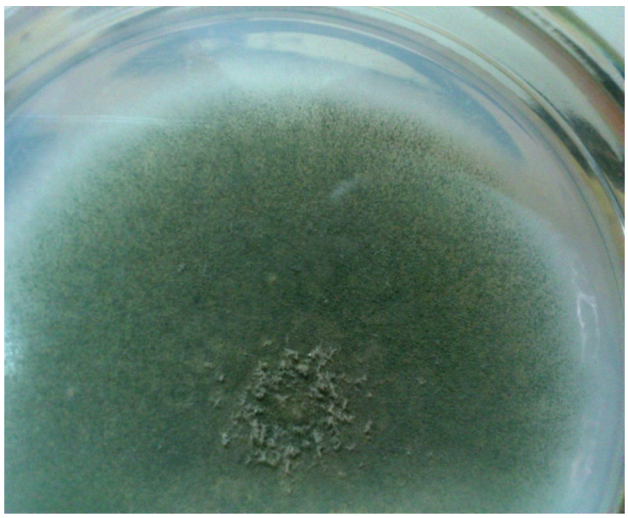

Fig. 1 Colony morphology of *Aspergillus fumigatus* RSP-8 (MTCC 12039) on czapekdox agar

induce the production of xylanase (Laxmi et al. 2008). To meet the demands of industrial application of xylanases, one needs to use cheaper substrate material as source of nutrient. With this aim, different agricultural biomass materials (rice bran, green gram husk, wheat bran, ground nut cake individually at 0.1 % w/v concentration under submerged fermentation conditions) were evaluated as substrates for xylanase production by fungal isolate *Aspergillus fumigatus* RSP-8.

Xylanase production was influenced by the type of substrate as per the data shown in (Fig. 2). Hemicellulose showed maximum influence on xylanase production yield of 59.57 U/mL, where as yield was observed to be least (8.57 U/mL) with groundnut cake as substrate. The substrate mediated variation in xylanase synthesis by this fungal strain could be interpreted in terms of catabolite repression described for other enzymes (Kermnický and Biely 1998). In addition, the xylanase enzyme production yield depends on the composition of substrate such as cellulose, and hemicellulose concentration as noticed different agro industrial wastes like palm fiber, wheat bran, and rice bran were serves as low cost substrates and sources for xylanase production (Laxmi et al. 2008; Puls and Schuseil 1993).

The observed variation in xylanase titer values in ground nut supplemented conditions may be attributed to availability of lower quantity of hemicellulose compared to other agro materials used (Fig. 2). While three fold higher titers in hemicellulose supplemented fermentation may be associated with availability of more substrate suggesting the limiting factor for production of xylanase complex by this isolate is presence of lower substrate concentration. However, absence of xylanase production in presence of glucose as carbon source (results not shown) depicts the catabolite repression refers to the repression of enzyme synthesis by glucose or other easily metabolized sugars. Similar trend is also noticed in most fungi where the addition of other carbon sources to the substrate promoted a repressive effect on xylanolytic enzyme production (Laxmi et al. 2008).

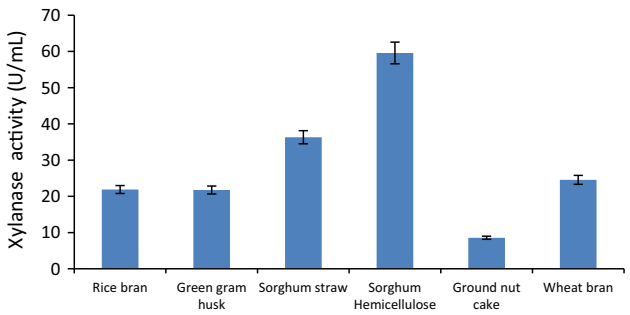

Fig. 2 Effect of different agro industrial substrates on xylanase production

Effect of incubation temperature on xylanase production

The fermentation temperature is another critical parameter to the production of xylanase and different stages of fermentation may require different temperatures to maximize the productivity of xylanase (Yuan et al. 2005). Elevation of expression levels and the efficient secretion of xylanases are crucial to ensure the viability of the process which is influenced by various fermentation factors including incubation temperature (Yuan et al. 2005). The authors reported that the fermentation time and maximum xylanase activity decreased with increasing the temperature. In steady-state condition the optimum temperature was 28 °C and 92 h were required to obtain the maximum xylanase activity. The unsteady-state operation by shifting the temperature from 33 °C for biomass growth to 27 °C for xylanase production significantly reduced production time without any adverse effect on xylanase activity. To understand the impact of incubation temperature on xylanase production by isolated fungal strain *Aspergillus fumigatus* RSP-8, the fermentation was carried out at incubation temperatures from 26 to 34 °C. Maximum production yield (74 U/mL) was observed during incubation at 30 °C as shown in (Fig. 3). This may be attributed to the mesophilic nature of the fungal strain. Similar activities were reported for xylanase production with *A. niger* with optimum incubation temperature around 30 °C (Wejse et al. 2005; Laxmi et al. 2008).

Effect of Varying Inoculum Volume on Xylanase Production

Initial biomass concentration (inoculum) play important role in over all metabolism of the microbe and subsequent production of growth or metabolism linked product. This is because higher inoculum causes imbalance in the nutrient availability hence growth and associated product production is regulated. Similarly more availability of nutrients

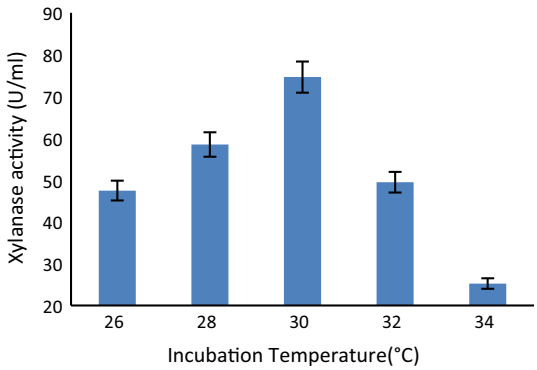

Fig. 3 Effect of incubation temperature on xylanase production

may be responsible for catabolite repressions of some of metabolic pathways leading to variation in overall production. To study the influence of inoculum volume on xylanase production, different volumes of inoculum containing 1×10^7 spores/mL is added to the production medium. Increase in inoculum size resulted in increase in xylanase production up to 1 % (v/v), where as further increase resulted in reduction of productivity up to 2 % (v/v) as shown in Fig. 4. The data is in accordance with reports on effect of spore concentration on productivity in submerged fungal cultivations, where it was observed that it must be high enough to colonize substrate particles (Sikyta 1983) but higher than optimal inoculum levels resulted in decreased xylanase productivity (Kuhad et al. 1998).

Effect of Agitation speed (rpm) and Aeration on Xylanase Production

Agitation under submerged fermentation facilitates effective distribution of spores and nutrients in the fermentation. While primary objective of aeration supply of electron acceptors is to proceed the metabolic reactions towards aerobic fermentation process and influence growth pattern of any microbial strain. Evaluation of agitation (rpm) v/s xylanase production by this isolated fungal strain indicated that xylanase production varied with rpm during fermentation. Maximum enzyme production yield (77 U/mL) was achieved with culture incubated at 150 rpm and variation of 30 rpm in either sides of this resulted in reduction of xylanase productivity (Fig. 5).

Aeration affects the growth and production yield during submerged fermentation due to the effective surface area available for diffusion of gases. In addition, it also helps in maintenance of a critical function in heat and moisture transfer between the solids and the gas phase. However, excessive aeration can produce shear stress resulting in change of the filamentous fungus morphology which is a harmful effect in fungal fermentation (Shojaosadati and Babaeipour 2002). At commercial level, balanced aeration and agitation economizes the process especially in reduction of the possibility of employing the maximum volume of bioreactors, irrespective of their design without compromise on product yield. Thus, optimization of agitation and aeration is essentially required to achieve the growth of the microorganism and the production of primary and secondary metabolites. The study in this context revealed that maximum xylanase activity was observed when 50 mL production medium was incubated in 250 mL conical flask. As per the shown data in Fig. 6, 20 % decrease in enzyme activity was observed with increase in fermentation medium from 50 to 75 mL in 250 mL flask which corresponds to the decrease is surface area. Further increase in the volume of medium resulted in slight decrease in enzyme activity from 75 to 150 mL.

Effect of Initial Medium pH on Xylanase Production

Microbial metabolism is highly sensitive to pH of medium and is regulated by variations of initial medium pH. Fermentation and metabolism mediated variations in pH, temperature, osmolarity, and electron donor availability can alter the trans-membrane pH gradient, total proton motive force and affect the internal pH of the cells

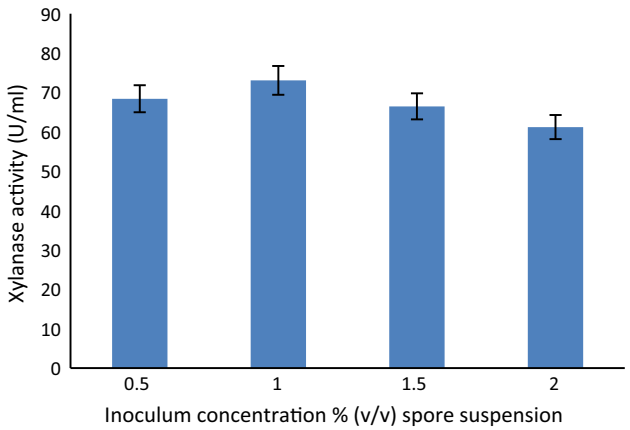

Fig. 4 Effect of Inoculum concentration on xylanase production

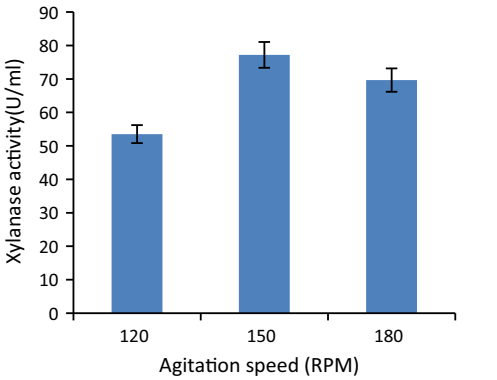

Fig. 5 Effect of agitation speed xylanase production

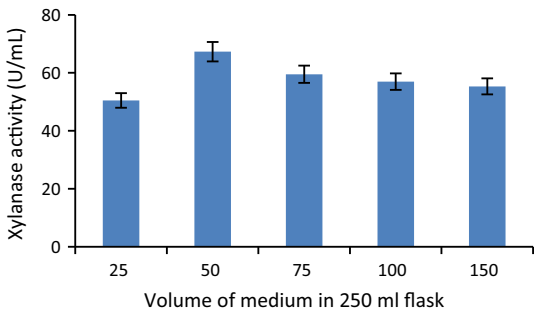

Fig. 6 Effect of aeration on xylanase production

(Repaske and Adler 1981; Srinivasan and Radhakrishnan 2010) leading to variations in growth and productivity yields. Hence, to maintain energy homeostasis, microbial cells have to actively regulate the internal pH by secreting or consuming protons. Generally, in such environment, medium can act as a source/sink for protons depending on the substrates that are present. To understand the same effect of pH on xylanase productivity, initial medium pH was adjusted to 5, 6, 7, 8 and 9 and enzyme titers are evaluated. Xylanase activity improved with increase in pH and maximum activity (~70 U/mL) was observed around pH 7 (Fig. 7). Further increase in initial medium pH up to 9 resulted in 16 % decrease in xylanase productivity. One of the factors for this reduction of xylanase complex production may be associated with pH mediated increase in hydrolysis of hemicelluloses as alkalinity is known to enhance the availability of cross-linked insoluble xylan to endo xylanases by increasing solubility of hemicellulose (Wejse et al. 2005) or pH mediated changes in microbial metabolism (Srinivasan and Radhakrishnan 2010).

Effect of Substrate Concentration (Hemicellulose %) on Xylanase Production

During microbial metabolism associated enzyme production, especially with reference to extracellular enzymes, the substrate plays a key role in improved productivity. Similarly xylanase enzyme complex production by various microbial strains is growth associated and extracellular in nature. This is mainly because xylan, a major component of hemicelluloses, and a substrate for xylanolytic enzyme complex are major nutrient source for microbial growth and metabolism. Being a high molecular mass polymer, xylan needs xylanolytic enzyme complex for its deconstruction before it is used as carbon source for microbial growth. The deconstructed low molecular mass fragments of xylan (xylose, xylobiose, xylooligosaccharides, and hetero-disaccharides of xylose and glucose and their positional isomers) liberated from xylan through the action of small amounts of constitutively produced enzymes play a key role in the regulation of xylanase biosynthesis (Laxmi et al. 2008).

Microbial production of xylanolytic enzyme complex is also regulated by xylose availability in the fermentation medium. Higher availability of xylose negatively regulates β-xylosidase activity by feedback inhibition. Xylanase, especially endo xylanase catalyzes the random hydrolysis of xylan to xylooligosaccharides, while β-xylosidase further acts on these end products of endo xylanase activity resulting in release of xylose residues from the non-reducing ends of xylooligosaccharides. However, complete degradation of xylan requires the synergistic action of other components of xylanolytic enzyme complex like acetyl xylan esterase, to remove the acetyl substituents from the β-1, 4-linked D-xylose backbone of xylan (Motta et al. 2013). Hence, it is essential to understand the requirement of substrate concentration in the fermentation medium in order to improve the xylanolytic enzyme complex productivity. In the present study, xylanase production was increased with increase in concentration of hemicellulose from 0.5 to 3 % w/v, while maximum xylanase yield was obtained with 2 % w/v of hemicellulose. Similar results were reported for xylanase production by *Streptomyces cyaneus* SN32 using corn cob as substrate (Ninawe and Kuhad 2005). The observed reduction of xylanase yields with the hemicellulose concentration beyond 2 % may be attributed to interaction of xylanase enzyme end product, xylose at the genetic repression control or this could be due to formation of a thick suspension and improper mixing of the substrates in shake flasks as suggested (Singh et al. 1991).

Analysis of xylanase titers observed in this study by fungal isolate *Aspergillus fumigatus* RSP-8 with extracted hemicellulose (Fig. 8) and other agro materials (Fig. 2) as substrate sources further suggested availability of proper substrate material is one of the major limiting factors for improved xylanase production. This could be exemplified by the observation that none of the used agro materials showed similar enzyme production titers to that of

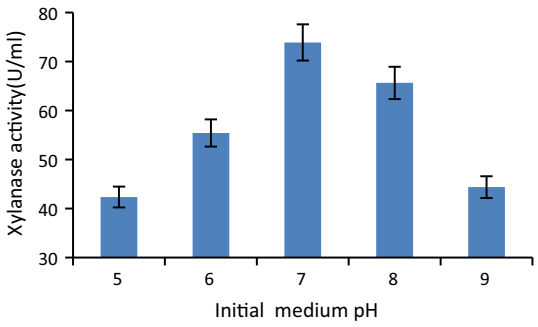

Fig. 7 Effect of initial medium pH on xylanase production

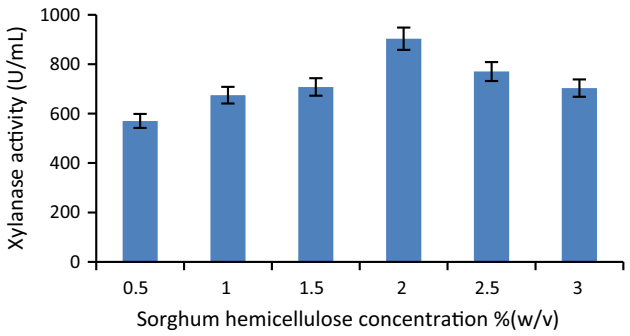

Fig. 8 Effect of varying substrate concentration on xylanase production

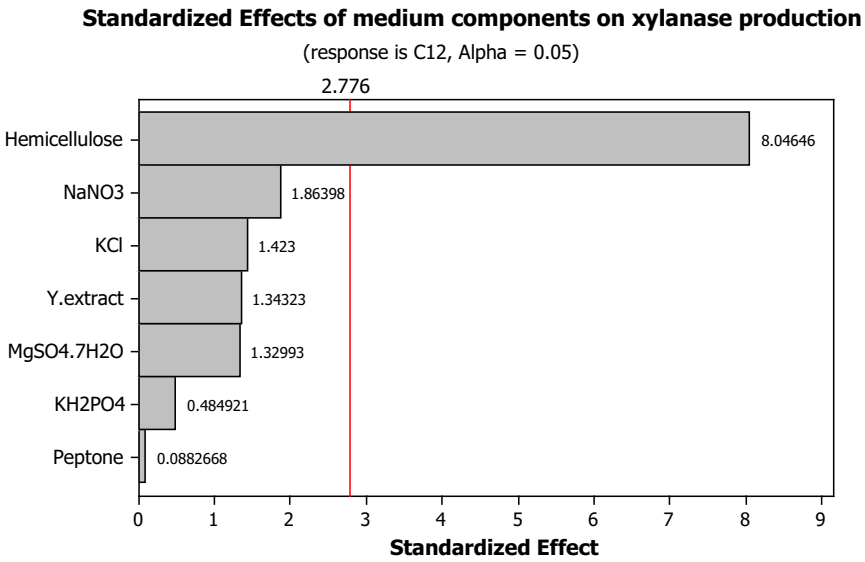

Fig. 9 Analysis of Placket–Burman design results using Minitab 16

hemicelluloses as substrate material. A variation of more than 2.5 fold was observed (Fig. 2). The above may be also evidenced by data noticed with substrate v/s xylanase enzyme production data shown in (Fig. 8) where the enzyme titer was 500 fold in 2 % hemicellulose supplemented environment compared to agro material as substrate (Fig. 8). This further confirms that the heterogeneity and complexity of xylan play significant role, as the xylosidic linkages in lignocelluloses are neither equivalent nor equally accessible. The production of an enzymatic system with specialized functions is a strategy to achieve superior xylan hydrolysis (Wong et al. 1988). In addition to heterogeneous nature of xylan, the multiplicity of xylanases in microorganisms may be caused by a redundancy in gene expression resulting in a diverse range of xylanases, which differ in their physicochemical properties, structure, and mode of action and substrate specificities as reported (Collins et al. 2005). Generally, a single xylanase gene encodes multiple xylanases, and xylanase multiplicity may arise from post translational modifications, such as differential glycosylation, proteolysis or both (Puls and Schuseil 1993) again regulated by substrate source concentration and its availability (Juturu and Wu 2012).

Analysis of PBD Results Using Minitab Statistical Software

Pareto chart of the effects in Minitab software was used to compare the relative significance of variation of medium components effecting the xylanase production. Minitab software plots the effects (xylanase production values in the present study) in decreasing order of the absolute value of the effects (medium components). The reference line (2.776) drawn on the chart with level of significance $\alpha = 0.05$, indicates which effects are significant. Analysis of Pareto chart revealed that varying hemicellulose concentration showed significant effect (8.04646) on xylanase production where as other medium components were found to be less significant (Fig. 9). Of all components tested, varying the concentration of peptone was least effective on xylanase production (0.088266).

Conclusion

Overall, large scale expression of microbial xylanases is associated with genetic nature and nutritional requirement of microbial strain employed. Our study on the isolated fungal strain, *Aspergillus fumigatus* RSP-8 (MTCC 12039) further emphasizes that the use of isolated hemicellulose (in the present study it is isolated from sorghum biomass) rather than complexes with other polymers such as cellulose and lignin is detrimental for improved productivity of xylanase complex. By understanding critical fermentation parameters such as (pH, temperature, inoculum level, type of substrate) and their concentrations would help in driving laboratory findings to applications at industrial sector. However, one needs to evaluate the process conditions at basic level with respect to high specificity, mild reaction conditions, and negligible substrate loss and side product generation during hemicellulose hydrolysis with respect to xylanase catalytic role.

Acknowledgments The authors are grateful to the Director, CSIR-IICT Hyderabad, for providing facilities to carry out this work and Council of Scientific and Industrial Research, Government of India for award of Research Fellowship to K Ravi Chandra. The authors VVN Yaswanth and B Nikhila are thankful for financial support from JCERDC project.

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