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Utilization of corncob xylan as a sole carbon source for the biosynthesis of endo-1,4-β xylanase from *Aspergillus niger* KIBGE-IB36

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Abstract

Background: Xylan is a hemicellulose polysaccharide which is composed of β-1,4-linked D-xylosyl residues. Endo-1,4-β xylanase has the ability to cleave xylan back bone chains to release xylose residues. They are produced by a number of prokaryotic and eukaryotic organisms. Among them, filamentous fungi are attracting great attention due to high secretion of xylanolytic enzymes. Endo-1,4-β xylanase has wide industrial applications such as in animal feed, bread making, food and beverages, textile, bleaching of wood pulp, and biofuel production.

Results: In this study, different *Aspergillus* species were screened for the production of endo-1,4-β xylanase, and *Aspergillus niger* KIBGE-IB36 was selected for optimum production of enzyme in submerged fermentation technique. Influence of various fermentation conditions was investigated to produce high titer of endo-1,4-β xylanase. The results indicated that *A. niger* KIBGE-IB36 showed optimum production of endo-1,4-β xylanase at 30 °C, pH 8 after 6 days of incubation. Different macro- and micronutrients were also amalgamated in the fermentation medium to increase the enzyme production. The parametric optimization of endo-1,4-β xylanase resulted in tenfold increase after hydrolysis of 20 g L^{-1} corncob xylan.

Conclusions: The use of low-cost substrate approach for high production of endo-1,4-β xylanase has been developed successfully that can be consumed in different industrial applications especially in paper and pulp industry.

Keywords: *Aspergillus* species, Corncob, Fermentation, Hemicellulose, Endo-1,4-β xylanase

Background

Substantial consideration has been given for the use of microorganisms in industrial processes particularly for the production of enzymes. Amid different microorganisms, fungi, bacteria, and actinomycetes are the abundant producers of endo-1,4-β xylanase (Lu et al. 2008). Filamentous fungi such as *Aspergillus* and *Trichoderma* species have immense significance over bacteria due to their efficient ability to degrade plant cell wall (Kaushik and Malik 2009). *Aspergillus niger* is a filamentous fungus that has been used extensively in different biotechnological applications. According to Food and Drug Administration (FDA), *A. niger* can be "generally regarded as safe" (GRAS) under good manufacturing* practices for industrial products and they can be isolated easily from soil, compost, and plant-decaying materials (Klich 2002; Schuster et al. 2002). *Aspergillus niger* has the ability to produce high yield of broad range of enzymes under both submerged and solid-state fermentation conditions, and approximately 80–90% endo-1,4-β xylanases are produced using submerged fermentation technique (Polizeli et al. 2005; Pel et al. 2007).

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Among different industrially important enzymes, xylanolytic enzymes have been used extensively in food and pharmaceutical industries. This complex enzyme includes endo-1,4-β- xylanase [EC 3.2.1.8], β-xylosidase [EC 3.2.1.37], α-arabinofuranosidase [EC 3.2.1.55], and acetyl xylan esterase [EC 3.1.1.72] (Biely 1993). Xylan is the second most abundant resource after cellulose and is the main constituent of hemicellulose which consists of long chain of 1,4-β-D-xylose monomers (Izidoro and Knob 2014). Endo-1,4-β xylanase is an enzyme which has the ability to hydrolyze β-1,4 glycosidic bonds in xylan into small series of xylooligosaccharides (Chanwicha et al. 2015). In the presence of β-xylosidase, these oligosaccharides are further hydrolyzed into xylose molecules. Alone exo-xylanase will not be able to hydrolyze the complex xylan structure. After this synergistic effect, more xylose is produced as a by-product which confirms the presence of endo-1,4-β xylanase. Being an industrially important enzyme, endo-1,4-β xylanase has several applications: in baking and in food industries, it is utilized as a taste and texture enhancer; in poultry, it is used as a food additive; and in beverages, it acts as a juice clarifying agent. Commonly, it is also used in pre-bleaching process of kraft and pulp to diminish the use of harmful chemicals (Arulanandham and Palaniswamy 2014).

To achieve entire enzymatic degradation of xylan into its monosaccharide components, a group of synergistic xylanolytic enzymes is required due to the presence of differences in xylan structure from different sources (Latif et al. 2006). Previously, corncob is reported as one of the valuable by-products of food industry which can be utilized as growth-inducing substrate for bacteria and fungi. In addition, it is also used to synthesize xylose, alcohol, xylitol, and xylooligosaccharides (Chapla et al. 2012). In this study, commercial corncob xylan was used for the synthesis of endo-1,4-β xylanase by *A. niger* KIBGE-IB36 under submerged fermentation conditions. Different physiological and chemical factors were also optimized to enhance the production of endo-1,4-β xylanase.

Results and discussion
Screening of fungal species for endo-1,4-β xylanase production

To screen the production of endo-1,4-β xylanase, four different species of *Aspergillus* were used. It was observed that *A. niger* KIBGE-IB36 is a hyper producer endo-1,4-β xylanase (837 U mg^{-1}) as compared to other species (Fig. 1a). In addition, it was also confirmed by Congo red dye method in which *A. niger* KIBGE-IB36 expressed a clear xylanolytic zone around the growing colony on medium containing corncob xylan as a sole carbon source (Fig. 1b). Further experimental studies were carried out using *A. niger* KIBGE-IB36 for the production of endo-1,4-β xylanase.
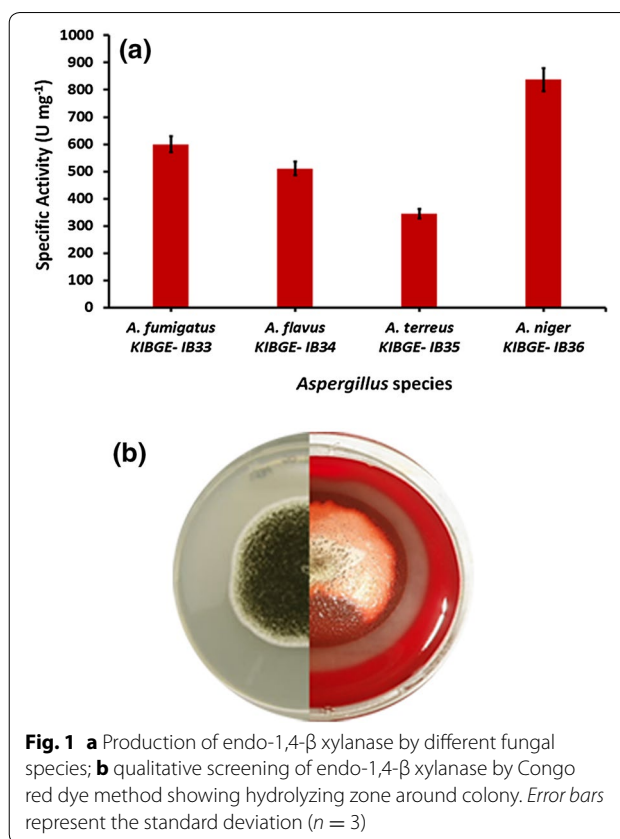

Fig. 1 **a** Production of endo-1,4-β xylanase by different fungal species; **b** qualitative screening of endo-1,4-β xylanase by Congo red dye method showing hydrolyzing zone around colony. *Error bars* represent the standard deviation ($n = 3$)

Selection of fermentation medium

To produce high titers of endo-1,4-β xylanase, five different reported media were analyzed. Among these media, maximum endo-1,4-β xylanase was synthesized in Czapek medium (1239 U mg^{-1}) as compared to other media (Fig. 2).
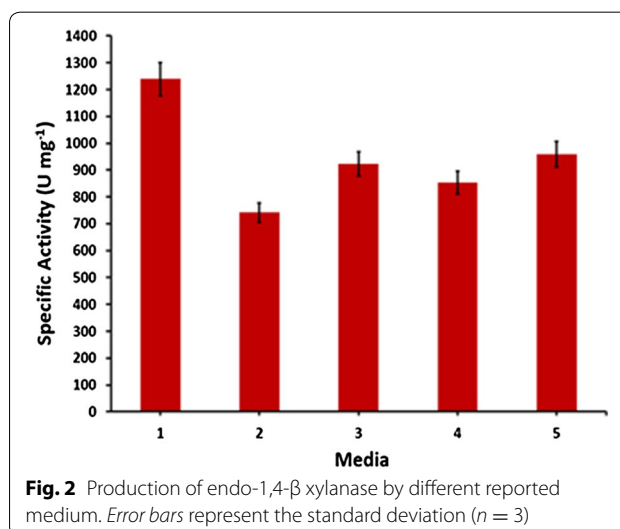

Fig. 2 Production of endo-1,4-β xylanase by different reported medium. *Error bars* represent the standard deviation ($n = 3$)

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Selection of fermentation temperature

The fermentation temperature not only influences the growth curve of an organism but it also has an impact on the production of endo-1,4-β xylanase (Senthilkumar et al. 2005). Most of the filamentous fungi grow in between 25 and 35 °C, while some thermophilic fungal species can also grow at high temperature with maximum at or above 50 °C (Suresh and Chandrasekaran 1999; Maheshwari et al. 2000). Mostly *Aspergillus niger* is reported to show growth pattern in between 25 and 30 °C (Adinarayana et al. 2003). In current study, optimum temperature for the production of enzyme was recorded at 30 °C with 1485 U mg^{-1} of endo-1,4-β xylanase. A gradual decline in enzyme titer was noted at 50 °C (50 U mg^{-1}) and this decline is due to the lower growth rate of this fungi at high temperature (Fig. 3a). The high- and low incubation temperatures cause the inhibition of fungal growth that ultimately leads to the decline in enzyme synthesis (Lenartovicz et al. 2003). This investigation synchronizes with those of the previously reported data where activity of endo-1,4-β xylanase was optimum at 30 °C (Subbulakshmi and Iyer 2014; Kanimozhi and Nagalakshmi 2014).
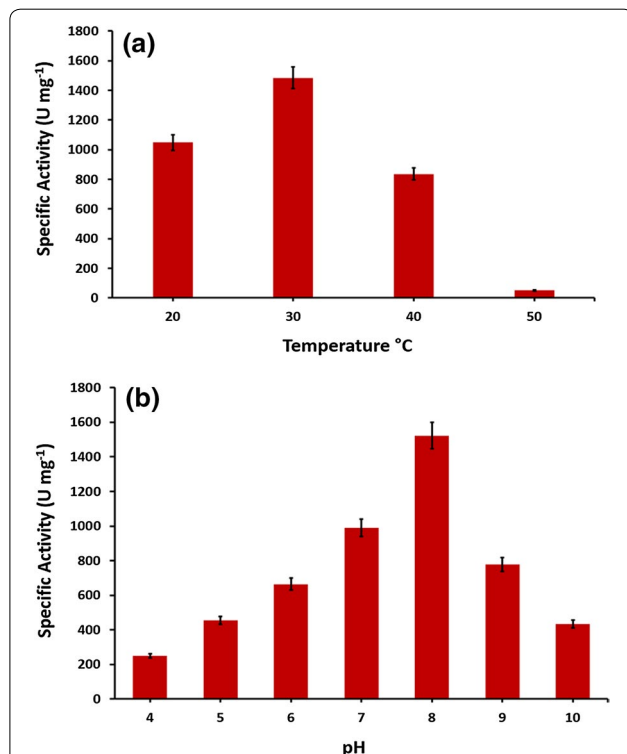

Fig. 3 a Effect of incubation temperatures on endo-1,4-β xylanase production from *Aspergillus niger* KIBGE-IB36. **b** Effect of Initial pH on endo-1,4-β xylanase production from *Aspergillus niger* KIBGE-IB36. *Error bars* represent the standard deviation ($n = 3$)

Selection of fermentation pH

The pH of the medium plays a significant role in enzyme production. It can either lower the enzyme production by effecting the growth of microorganism or by creating unsuitable toxic environment that leads to the denaturation or inactivation of enzyme produced (Bajaj and Abbass 2011). In the present study, the optimum pH for the production of endo-1,4-β xylanase was achieved at pH 8.0 with specific activity of 1523 U mg^{-1}, whereas minimum activity was observed at pH 4.0 (249 U mg^{-1}) (Fig. 3b). Most of the researchers reported maximum endo-1,4-β xylanase production by filamentous fungi in acidic pH ranging from 5.0 to 6.5 and also near pH 8.0 (Murthy and Naidu 2012; Bajaj and Abbass 2011). Some other investigators reported enzyme production at pH 9.0 and 10.0 (Kapilan and Arasaratnam 2012; Nair et al. 2008). In the present study, *A. niger* KIBGE-IB36 showed effective tolerance and potential to grow and produce endo-1,4-β xylanase at both acidic and alkaline pH values (pH 6.0–10.0).

Selection of fermentation time period

In this experiment, the production of endo-1,4-β xylanase by *A. niger* KIBGE-IB36 was determined during different intervals of time (01–10 days) along with fungal biomass estimation. The exponential phase was observed from days 01 to 06, entering the stationary phase up to day 08, and afterwards unstable decline was observed at day 10. The enzyme synthesis increased in the exponential phase (days 03 to 06) and after reaching its maxima both the enzyme activity and fungal biomass then started to decline gradually (Fig. 4a). It has been suggested that prolonged incubation period stimulates the secretion of nonspecific proteases that degrade the synthesized enzyme in the medium. Therefore, it is suggested to control the end point of fermentation period (Pal and Khanum 2010). It has been proposed previously that the optimum fermentation period relies on the nature of substrate, organism, macro- and micronutrients and many other fermentation events (Dekker 1983). This obtained result coincides with the previous studies in which maximum endo-1,4-β xylanase was achieved in 6 days of fermentation period (Sharma et al. 2015; Pal and Khanum 2010).

Effect of substrate concentration

Specific substrate plays an important role for any enzyme production. In this study, endo-1,4-β xylanase was synthesized using different concentrations (5–20 g L^{-1}) of corncob xylan which was studied in many past studies (Ahmad et al. 2012). In present study, the efficiency of corncob xylan in the maximum induction of endo-1,4-β xylanase production was established in 20 g L^{-1} of concentration, while 25g L^{-1} of corncob xylan created inhibitory effect on

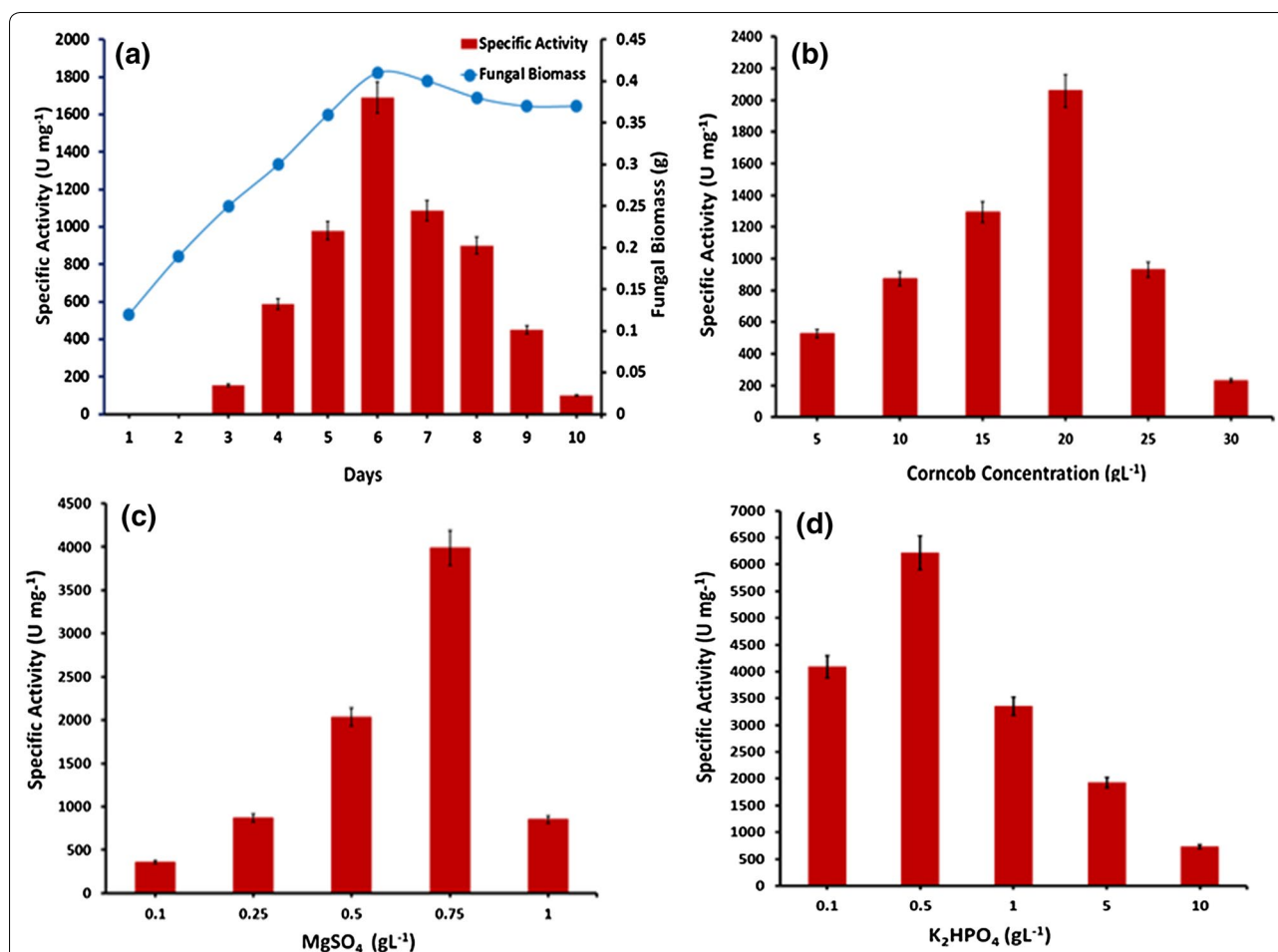Javed *et al. Bioresour. Bioprocess.* (2017) 4:19

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Fig. 4 a Optimization of fermentation period for the production of endo-1,4-β xylanase from *Aspergillus niger* KIBGE-IB36. **b** Effect of different concentrations of corncob xylan on endo-1,4-β xylanase production from *Aspergillus niger* KIBGE-IB36. Effect of different concentrations of salts on endo-1,4-β xylanase production from *Aspergillus niger* KIBGE-IB36, **c** MgSO₄, **d** K₂HPO₄. *Error bars* represent the standard deviation ($n = 3$)

the production of endo-1,4-β xylanase (Fig. 4b). It might be due to the increased viscosity of the medium that ultimately leads to feedback inhibition of enzyme (Karim et al. 2014). Our results are in line with other research in which they used 20 g L^{-1} of xylan for the induction of endo-1,4-β xylanase (Shah and Madamwar 2005).

Effect of different nitrogen sources
In the present study, different nitrogen sources (organic/inorganic) were studied (Table 1). The result showed that organic nitrogen sources have a profound effect on the production of endo-1,4-β xylanase as compared to inorganic nitrogen sources. Among different organic sources, peptone proved was the inducer for maximum endo-1,4-β xylanase production (3069 U mg^{-1}). Previously, it is also reported that endo-1,4-β xylanase yield was enhanced by the supplementation of peptone (Qinnghe et al. 2004). Other organic nitrogen sources such as tryptone, meat

extract, and yeast extract also showed endo-1,4-β xylanase production but to a lower extent as compared to peptone. When different nitrogen sources were combined, a very unique expression was observed to the production of xylanase suggesting that *A. niger* requires different types of nitrogen sources for its growth and enzyme production. Among five different combinations, meat extract and peptone produce high titers of endo-1,4-β xylanase (4219 U mg^{-1}) which was 1.37 times higher when compared with the medium incorporated solely with peptone. Various researchers have also reported the augmentation of nitrogen source into the fermentation media (Gomes et al. 1994; Bansod et al. 1993).

Effect of K₂HPO₄ and MgSO₄ concentrations
Microbial metabolism and regulation of enzyme production are reciprocal to the supplementation of salts in the medium (Maciel et al. 2008). In this study, the medium

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Table 1 Effect of different nitrogen sources on endo-1,4-β xylanase production from *Aspergillus niger* KIBGE-IB36

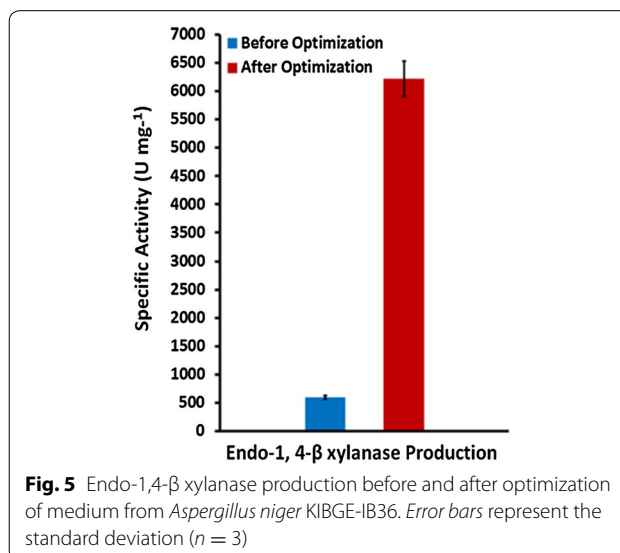| Types of nitrogen source	Nitrogen source (5 g L^{-1})	Specific activity (U mg^{-1})
Organic nitrogen sources	Yeast extract	1713 ± 9.24
	Meat extract	2166 ± 11.4
	Tryptone	2028 ± 7.75
	Peptone	3069 ± 18.49
Inorganic nitrogen sources	Urea	1090 ± 8.22
	NH$_4$Cl	642 ± 8.86
	KNO$_3$	492 ± 10.43
	NH$_4$NO$_3$	360 ± 20.85
Combination of organic nitrogen sources	Yeast extract + tryptone	3116 ± 13.54
	Yeast extract + peptone	2539 ± 18.90
	yeast extract + meat extract	1215 ± 24.37
	Meat extract + peptone	4219 ± 8.65
	Meat extract + tryptone	3056 ± 9.46

All the experiments were performed in triplicate and the results expressed are the mean values of all experimental setup

was optimized using different concentrations of K$_2$HPO$_4$ and MgSO$_4$·7H$_2$O. Magnesium ions have a significant effect on the equalization of the ribosomes and cellular membrane (Cui and Zhao 2012). In this study, with the increase of MgSO$_4$·7H$_2$O, a gradual increase was noticed in endo-1,4-β xylanase production up to 0.75 g L^{-1} (3987 U mg^{-1}), while at 1 g L^{-1} of magnesium salt the production of endo-1,4-β xylanase was declined (850 U mg^{-1}) (Fig. 4c). In contrast, Naveen and Siddalingeshwara (2015) reported 0.1 g L^{-1} of MgSO$_4$·7H$_2$O as a finest inducer of endo-1,4-β xylanase.

On the other hand, the presence of K$_2$HPO$_4$ in the growth medium also showed a positive effect on the yield of endo-1,4-β xylanase. At 0.5 g L^{-1} of K$_2$HPO$_4$, maximum endo-1,4-β xylanase production was observed (6214 U mg^{-1}) but as the concentration of salt increased, it leads towards a decline in endo-1,4-β xylanase production (729 U mg^{-1}) (Fig. 4d). According to the previous investigation, 0.3 g L^{-1} of K$_2$HPO$_4$ was found to be appropriate for the production of endo-1,4-β xylanase (Salihu et al. 2015). It is reported that K$_2$HPO$_4$ has the potential to maintain the ideal osmotic pressure for high endo-1,4-β xylanase production (Berk 2000). Hence, after optimizing all the medium formulation and physical parameters, a tenfold increase in endo-1,4-β xylanase synthesis was observed (Fig. 5).

The result obtained in the present study indicates that among different *Aspergillus* species, *A. niger* KIBGE-IB36 has more potential to saccharify corncob xylan efficiently into ample amount of endo-1,4-β xylanase through submerged fermentation. The production at low temperature (30 °C) indicates the mesophilic nature of *A. niger* KIBGE-IB36 and the production of endo-1,4-β xylanase at alkaline pH makes this enzyme a promising

Fig. 5 Endo-1,4-β xylanase production before and after optimization of medium from *Aspergillus niger* KIBGE-IB36. *Error bars* represent the standard deviation ($n = 3$)

candidate for bio-bleaching processes and other industrial applications. Hence, the current investigation provides direct comparison of enhanced production of endo-1,4-β xylanase up to tenfold after optimization of different physico-chemical parameters of fermentation medium such as pH, temperature, fermentation period, substrate concentration, suitable nitrogen source, and salt concentration.

Methods

Microorganisms

The initial screening was carried out using four strains of *Aspergillus* species namely *Aspergillus fumigatus* KIBGE-IB33 [GenBank: KF905648], *Aspergillus flavus*

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KIBGE-IB34 [GenBank: KF905649], *Aspergillus terreus* KIBGE-IB35 [GenBank: KF905649], and *A. niger* KIBGE-IB36 [GenBank: KF905650] which were isolated previously (Pervez et al. 2015).

Screening of endo-1,4-β xylanase production

All fungal isolates were grown on xylan-containing medium containing; g L^{-1}: (corncob xylan (Carbosynth, UK) 5.0; Nutrient broth 13.0; K_2HPO_4 2.5; KH_2PO_4 0.5; $CaCl_2$ 0.1; and NH_2SO_4 0.5). After incubation at 30 °C for 05 days, culture broth was centrifuged at 4000 rpm at 4 °C for 30 min and filtered through Whatman filter paper No. 1. The supernatant was used for the estimation of endo-1,4-β xylanase production. For qualitative confirmation of endo-1,4-β xylanase production, the selected strain was grown on corncob xylan agar medium for 03 days maintaining the condition same as used for the fermentation. The clear hydrolytic zone around the fungal colony was observed after flooding with Congo red (Teather and Wood 1982).

Endo-1,4-β xylanase assay

The enzyme activity of endo-1,4-β xylanase was estimated by evaluation of reducing sugars released from 10 g L^{-1} of xylan in 10 mM citrate phosphate buffer (pH 5.0) by 3,5,dinitrosalicylic acid method using xylose as a standard (Miller 1959). One unit of enzyme of enzyme activity is defined as the amount of enzyme required to release 1 μmol of xylose per minute of reaction under standard assay conditions. Specific activities were expressed as unit of enzyme per milligram of protein.

Total protein estimation

The total protein was determined in the supernatant by Lowry's method (1951) using BSA (bovine serum albumin) as a standard.

Selection of fermentation medium

Five different reported media were initially used for the production of endo-1,4-β xylanase and these reported media were designated as media 1 (Kulkarni and Gupta 2013), media 2 (Adhyaru et al. 2014), media 3 (Kocabas and Ozben 2014), media 4 (Yuan et al. 2005), and media 5 (Bibi et al. 2014).

After selection of suitable medium, fermentation conditions were optimized by varying different physicochemical parameters using *A. niger* KIBGE-IB36 for the production of maximum endo-1,4-β xylanase.

Optimization of fermentation temperature, pH, and time

For the selection of appropriate temperature for the production of endo-1,4-β xylanase, different temperatures were tested ranging from 20 to 50 °C.

In the next step, the culture was also grown in a different pH medium ranging from 4.0 to 10.0.

The time course and fungal biomass for the production of endo-1,4-β xylanase were also estimated by incubating *A. niger* KIBGE-IB36 for different time intervals ranging from 03 to 10 days.

Optimization of macro- and micronutrients

In the present study, corncob xylan was used as a substrate for endo-1,4-β xylanase production with different concentrations of xylan ranging from 5 to 30 g L^{-1}.

To determine the effect of nitrogen source, different organic and inorganic nitrogen sources were assimilated in the production medium. Furthermore, the effect of different nitrogen sources in combination was also investigated.

To analyze the influence of $MgSO_4$ and K_2HPO_4 on endo-1,4-β xylanase production, different concentrations of salts were incorporated in the production medium ranging from 0.1–1 to 0.1 to 10 g L^{-1}, respectively.

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