fermentation

MDPI

Article

Co-Cultivation of *Penicillium* sp. AKB-24 and *Aspergillus nidulans* AKB-25 as a Cost-Effective Method to Produce Cellulases for the Hydrolysis of Pearl Millet Stover

Amit Kumar *, Archana Gautam and Dharm Dutt

Department of Paper Technology, Indian Institute of Technology Roorkee, Saharanpur Campus, Saharanpur-247 001, India; archanaiitr12@gmail.com (A.G.); dharmduttiit@gmail.com (D.D.)

* Correspondence: amitkdpt@iitr.ac.in; Tel.: +91-971-999-8824

Academic Editor: Hiroshi Kitagaki

Received: 21 February 2016; Accepted: 7 June 2016; Published: 14 June 2016

Abstract: Hydrolysis of cellulose and hemicelluloses into fermentable sugars is the primary step for the production of fuels and chemicals from lignocellulosic biomass, and is often hindered by the high cost of cellulolytic and hemicellulolytic enzymes. In the present study co- and monocultures of *Penicillium* sp. AKB-24 and *Aspergillus nidulans* AKB-25 were used under a variety of fermentation conditions to optimize enzyme production. Wheat bran was found to be the optimal carbon source yielding maximum enzyme production under solid-state fermentation conditions due to its higher water retention value (175%) and minimum C/N ratio (22.7). *Penicillium* sp. AKB-24 produced maximum endoglucanase (134 IU/gds), FPase (3 FPU/gds), β-glucosidase (6 IU/gds) and xylanase (3592 IU/gds) activities when incubated for 7 days at 30 °C and pH 7 with a moisture content of 77.5%, and 1.2% yeast extract and 0.1 (*w*/*v*) sodium dodecyl sulphate supplement. Co-culturing of *Penicillium* sp. AKB-24 and *Aspergillus nidulans* AKB-25 enhanced endoglucanase, FPase, and exoglucanase activities by 34%, 18%, and 11% respectively compared to *Aspergillus nidulans* AKB-25 alone under optimum conditions. Enzymes produced by co-cultivation released equal amounts of reducing sugars at an enzyme dose of 15 FPU/g and reaction time 72 h, but the required quantity of enzyme was 14% less compared to enzyme released from *Aspergillus nidulans* AKB-25 mono-culture. In conclusion, co-cultivation of *Penicillium* sp. AKB-24 and *Aspergillus nidulans* AKB-25 to produce enzymes for the hydrolysis of pearl millet stover is more cost-effective than cultivation with *Aspergillus nidulans* AKB-25 alone.

Keywords: *Penicillium* sp.; *Aspergillus nidulans*; pearl millet stover; hydrolysis; cellulases

1. Introduction

Different species from the genus *Aspergillus* and *Penicillium* are the most commonly used for the production of pharmaceuticals, food ingredients, and enzymes [1]. In most cases, these fungal saprophytes are associated with degradation of plant polysaccharides, such as celluloses, hemicelluloses, pectins, starches and other low molecular weight carbohydrates. These enzymes are necessary to convert the natural carbon sources (mainly natural polymers) into small molecules and enable the cell to assimilate it. High cellulose conversion requires high enzyme concentrations and recycling of enzymes is difficult due to adsorption of enzymes to residual lignocelluloses [2]. The hydrolysis of plant polymers decreases rapidly with time and leads to decreased yield and long process times [3]. The surface area of cellulose is an important substrate characteristic for the determination of initial rate of hydrolysis [4].

Renewable resources are becoming increasingly important, plentifully available in nature and have a potential for bioconversion into fuels and chemicals, both natural and man-made [5], to change the world economically, socially, and environmentally [6]. Hydrolysis of the cellulosic components into fermentable sugars is the primary step for the production of fuels and chemicals from lignocellulosic biomass. The high cost of cellulolytic enzymes is the main obstruction in the cost-effective processing of lignocellulosic biomass. Considerable efforts have been aimed at mitigating cellulase production cost by improving the efficiency of known cellulase, identification of new, more active cellulase and creation of a multi-component cellulase system optimized for selected pretreated substrates [7,8]. Compared to submerged fermentation (SmF), solid-state fermentation (SSF) technology has been recognized to have lower consumption of water (concentrated enzymes) and energy and high side activities, and generally, it has been claimed that product yields are higher [9,10]. Co-culturing of fungi during SSF enhances the production of cellulolytic enzymes as they co-exited symbiotically on natural solid substrate in nature. Co-culture enables production of more efficient enzyme mixtures for industrial processes compared to monocultures [11,12]. For efficient hydrolysis of lignocellulosic biomass in to reducing sugars, enzyme preparation requires balanced activities of all the components of cellulases such as endo-β-1, 4-glucanases, exo-β-1, 4-glucanase, and β-glucosidase along with xylanases [11,13]. All these enzymes act synergistically to hydrolyze lignocellulosic biomass by the creation of new accessible sites for each other and prevention of product inhibition [14]. Products obtained after hydrolysis of lignocellulosic biomass are composed mainly of glucose, xylose, cellobiose, and oligosaccharides. Among them glucose is the most important product which can be converted into a variety of specialty chemicals such as ethanol, methane, hydrogen, organic acids, microbial polysaccharides, and single cell proteins [12,15]. Cellulolytic and hemicellulolytic enzymes have wide range of applications in pulp and paper, textile, food, brewery and wine, animal feed, detergent, and bioenergy industry [9,16,17]. The present study aimed at producing enzymes by co-culturing *Penicillium* sp. AKB-24 and *Aspergillus nidulans* AKB-25 and mono culturing *Penicillium* sp. AKB-24. Fermentation conditions were optimized in order to get higher enzyme production. Finally, the effect of enzymes produced by co-culture and monoculture techniques was investigated in terms of the hydrolysis of pearl millet stover.

2. Materials and Methods

2.1. Isolation and Identification of Fungi

The two fungal isolates AKB-24 and AKB-25 were isolated from the soil samples (rich in decaying lignocellulosic materials) and collected from district Jaipur in Rajasthan, India. Both the fungal isolates AKB-24 and AKB-25 sent to Microbial Culture Collection, National Centre for Cell Science, Pune (India) and National Fungal Culture Collection of India, Agharkar Research Institute, Pune (India) respectively for their identification. The fungal isolates were maintained over potato carrot agar (PCA) slants at 4 °C.

2.2. FE-SEM Analysis of Selected Fungal Strains

FE-SEM analysis of sexual and asexual spores of fungal strain AKB-25 was carried out for their detailed morphological study. Fungal mat with along spores was subjected to fixation using 3% glutaraldehyde (v/v) and 2% formaldehyde (4:1) for 6 h. Following the primary fixation, samples were washed with distilled water and this process was repeated three times. Samples were then treated with ethanol gradients for dehydration. After dehydration, samples were air-dried and examined under FE-SEM at suitable voltage and magnifications.

2.3. Preparation of Substrates

Various agro-residues such as sugarcane bagasse, black gram residue, corn stover, pearl millet stover, rice straw, rice husk, sugarcane tops, sun hemp residue, wheat bran, wheat straw collected from

Muzaffarnagar district in Uttar Pradesh, India. All the agro-residues washed with tap water, dried in sunlight and chopped in to 1–2 cm pieces. After chopping, all the residues dried at 70 °C overnight to remove residual moisture. Dried agricultural residues were ground in a Wiley mill in the particle size range of 250 to 1400 μm and they were used as the substrates for enzyme production. The elemental carbon and nitrogen contents in various agro-residues were estimated with organic elemental analyzer (Thermo scientific, Flash 2000, Cambridge, UK). Water retention value is the percent ratio of contained water in the sample after centrifuging at a relative centrifugal force relative to dry weight of the sample. The samples were centrifuged at relative centrifugal force of 900 G for 30 min where G is the relative centrifugal force at room temperature, which is given in multiples of earth gravity. The samples were removed onto aluminium foil, the wet weights were measured and the samples were dried at 105 ± 2 °C until they reached constant mass (dry weights). The WRV was calculated as per equation given by Cheng *et al.* [18,19].

$$\mathrm{WRV}(\%) = \frac{W_{wet} - W_{dry}}{W_{dry}} \times 100$$

where W_{wet} is the weight of wet sample after centrifugation and W_{dry} is weight of oven dried samples.

For hydrolysis studies, pearl millet stover was pretreated with 3% NaOH (as such) at 121 °C for 20 min. During pretreatment, solid to liquid ratio was maintained at 1:8. Alkali pretreated solid residue was repeatedly washed with tap water to neutral pH and finally washed with distilled water. After washing, pretreated solid residue used for enzymatic saccharification without drying.

2.4. Enzyme Production under SSF

Various agricultural residues such as sugarcane bagasse, black gram residue, corn stover, pearl millet stover, rice straw, rice husk sugarcane tops, sunhemp residue, wheat bran, and wheat straw were used as carbon sources for enzyme production during SSF. Five gram of each substrate was moistened with Mandel Weber media (g/L: 1.4 $(NH_4)_2SO_4$, 2.0 KH_2PO_4, 0.3 $CaCl_2$, 0.3 $MgSO_4 \cdot 7H_2O$, 0.1 Tween-80 and trace elements: 0.005 $FeSO_4 \cdot 7H_2O$, 0.0016 $MnSO_4 \cdot 7H_2O$, 0.0014 $ZnSO_4 \cdot 7H_2O$, 0.002 $CoCl_2 \cdot 6H_2O$). Initial moisture content and initial pH were maintained at 77.5% and 5.5% respectively. The initial pH of Mandel Weber media for enzyme production was adjusted with 1 N·HCl or 1 N NaOH before adding to the flasks. Flasks were sterilized by autoclaving at 121 °C for 30 min. Flasks were inoculated with five discs of actively growing fungi and incubated at 30 °C for 6 days [12]. The enzyme was extracted on rotator shaker at 150 rpm and 30 ± 1 °C for 60 min by adding 50 mL distilled water to the fermented medium. Following the centrifugation, different enzyme activities such as endoglucanase, filter paper activity (FPase), β-glucosidase and xylanase activities were determined in supernatant.

2.5. Optimization of Cultural Conditions

Optimization of different cultural parameters for enzyme production was performed by using one factor at a time (OFAT) approach [20]. For the selection of best carbon source, several agricultural residues were utilized for enzyme production under SSF. To optimize incubation time, harvesting of enzyme was carried out up to 10 days at an interval of 24 h. To find out the effect of temperature on enzyme production, fermentation was carried out at different temperatures (26–42 °C) at an interval of 4 °C. The effect of pH on enzyme production was assessed by adjusting initial pH of production medium varying from 3 to 10 keeping a gap of 1 with 1 N HCl or 1 N NaOH. The effect of moisture content on enzyme production was assessed by varying the initial moisture content to 55.0%, 65.0%, 70.0%, 75.0%, 77.5%, 80.0%, 82.5%, and 85%. The effect of nitrogen sources enzyme production was investigated by supplementing the medium with nitrogen sources like NH_4Cl, NH_4NO_3, $(NH_4)_2PO_4$, $(NH_4)_2SO_4$, $NaNO_3$, HN_2CONH_2 (simple) and beef extract, malt extract, peptone, soybean meal, tryptone, and yeast extract (complex) at four different concentration levels. The concentration of inorganic and organic nitrogen sources was tested at levels 0.04%, 0.08%, 0.12%, and 0.16% (as available nitrogen basis) while complex organic nitrogen sources were supplemented as 0.4%, 0.8%, 1.2%, and

1.6% (as w/v basis). In order to observe the effect of surfactant on enzyme production, various surfactants (Tween-20, Tween-40, Tween-60, Tween-80, Triton-x-100, SDS and EDTA) were added at concentrations like 0.05, 0.10, and 0.15% (w/v) to the NSS solution.

2.6. Enzyme Production with Co-cultivation of Penicillium sp. AKB-24 and Aspergillus nidulans AKB-25

All the fermentation conditions were optimized for *Penicillium* sp. AKB-24 and optimal conditions for *Aspergillus nidulans* AKB-25 were taken from PhD thesis [21] for comparing the results of mono-cultivation with mixed cultivation. The co-cultivation of *Penicillium* sp. AKB-24 and *Aspergillus nidulans* AKB-25 were carried out at optimal conditions as reported in Section 3.8. Likewise, fermentation medium was inoculated with five discs of actively growing *Penicillium* sp. AKB-24 and incubated for 3 days at 30 °C, after 3 days of incubation, spores of *A. nidulans* AKB-25 were added (1×10^5/g of substrate). The extraction of enzyme was carried out after seven days of incubation and analysed for various enzyme activities as cited above.

2.7. Saccharification Studies

The saccharification of alkali pretreated pearl millet stover (substrate) was carried out maintaining the 2% concentration. Different doses of enzymes varying from 5 to 20 FPU/g dry weight of substrate were added to the substrate making up the final reaction volume of 50 mL and maintaining the pH at pH 5.0 with citrate buffer. Further, the reaction mixture was incubated at 50 °C and 120 rpm for 96 h in an incubator shaker (New Brunswick Scientific, Innova® 43, NJ, USA). To prevent microbial contamination during saccharification process, 0.01% sodium azide was added to reaction mixture. Hydrolysate samples were withdrawn up to 84 h at regular intervals of 12 h and centrifuged to remove remaining solids. The degree of saccharification was estimated based on released reducing sugars as per method given by Miller [22].

2.8. Enzyme Assays

All the enzyme activities were assayed by incubating appropriately diluted crude enzyme with respective substrates which were dissolved in citrate buffer (50 mM, pH 5.5) at 50 °C. Endoglucanase and FPase activities were determined by the method as described by Ghose (1987). Endoglucanase activity was determined by reacting 0.5 mL appropriately diluted crude enzyme with 0.5 mL of 2% (w/v) carboxymethyl cellulose of medium viscosity (Sigma Chemical Co., St. Louis, MO, USA) for 30 min at 50 °C. FPase activity was analyzed by incubating Whatman no. 1 filter paper strip of 50 mg (approximately 1 × 6 cm) with crude enzyme totaling a reaction volume of 1.5 mL at 50 °C for 60 min [23]. Xylanase activity was measured by incubating the 500 µL appropriately diluted crude enzyme with 500 µL of 1% (w/v) beechwood xylan (Sigma Chemical Co., St. Louis, MO, USA) at 50 °C for 15 min. One unit of enzyme activity is defined as the amount of enzyme required to liberate one micromole of reducing sugars by hydrolyzing respective substrate per min per mL under reaction conditions [24]. β-glucosidase activity was determined by using 5 mM p-nitrophenyl-β-D-glucopyranoside as substrate [25]. One unit of β-glucosidase activity is defined as amount of enzyme that produces one micromole of *p*-nitro phenol per min per ml under specified assay conditions. The concentration of reducing sugars was quantified by dinitrosalicylic acid (DNS) method [22]. Enzyme activities were expressed as activity units per gram of initial dry solid substrates (IU/gds).

2.9. Statistical Analysis

All experiments were carried out in triplicate and experimental results were represented as the mean ± standard deviation of values. The significance of difference between means of each variable was analyzed with one way analysis of variance (ANOVA) at confidence limit of 95%. The means with same letters (in tables and figures) indicate that those means are not significantly different (*i.e.*, $p > 0.05$) while means with different letters show significant differences between them (*i.e.*, $p < 0.05$).

3. Results and Discussion

3.1. Identification of Microorganisms

Isolation for cellulase and xylanase producing fungi was carried out from different sites in three states, namely Uttar Pradesh, Uttarakhand and Rajasthan in India. Primary screening for cellulose-producing fungi was carried out on medium composed of various constituents expressed as g/L: 1.0 carboxymethyl cellulose (CMC), 1.0 peptone and yeast extract, 0.5 K_2HPO_4, 0.5 $MgSO_4 \cdot 7H_2O$, 0.005 $FeSO_4 \cdot 7H_2O$, 0.0016 $MnSO_4 \cdot 2H_2O$, 0.0014 $ZnSO_4.7H_2O$, 0.002 $CoCl_2 \cdot 6H_2O$, and 15 agar. The fungal isolates AKB-24 and AKB-25, originated from Rajasthan, India were selected as potential cellulase and xylanase producers [26]. ITS sequencing of fungal strains (AKB-24 and AKB-25) was carried out at Microbial Culture Collection (MCC), National Centre for Cell Science, Pune, India. The fungal isolate AKB-24 identified as *Penicillium* sp. at MCC, National Centre for Cell Science, Pune, India and deposited with accession number MCC 1031. Similar sequences obtained from GenBank and alignment of similar sequences carried out using ClustalW software. The phylogenetic tree was constructed for *Penicillium* sp. AKB-24 and closely related organisms by using neighbour joining method software MEGA 5.2 (Figure 1) [27]. Fungal strain AKB-25 was identified as *Aspergillus nidulans* based on morphological characteristics by the National Fungal Culture Collection of India, Agharkar Research Institute, Pune (India) and deposited with accession number NFCCI 2977. Scanning electron microscopy was carried out for the analysis of morphological characteristics of fungal strain *Aspergillus nidulans* AKB-25 (Figure 2). Analysis of ITS sequences of fungal strain *Aspergillus nidulans* AKB-25 also supported the results obtained in morphological identification. The ITS sequences of both the fungal strains *Penicillium* sp. AKB-24 and *Aspergillus nidulans* AKB-25 were submitted to GenBank with accession numbers KP734016 and KP734017 respectively.

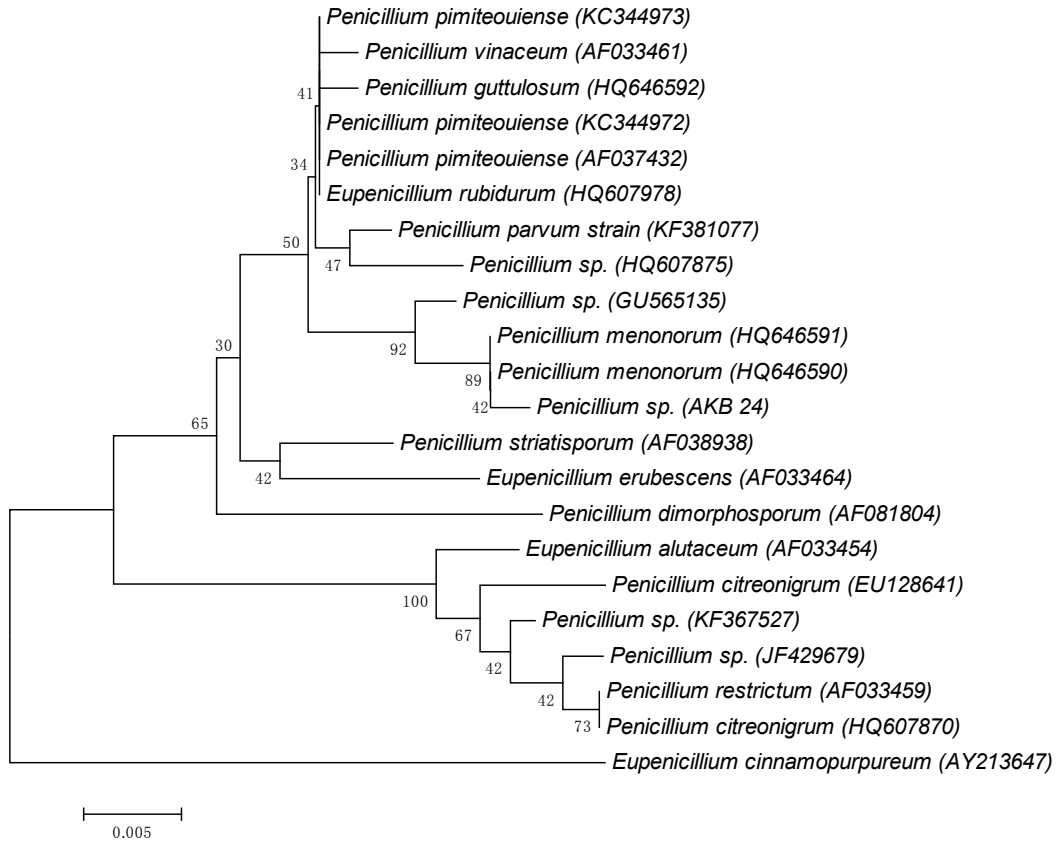

Figure 1. Phylogenetic tree for fungal strains *Penicillium* sp. AKB-24 with its closely related fungal strains based on ITS sequences.

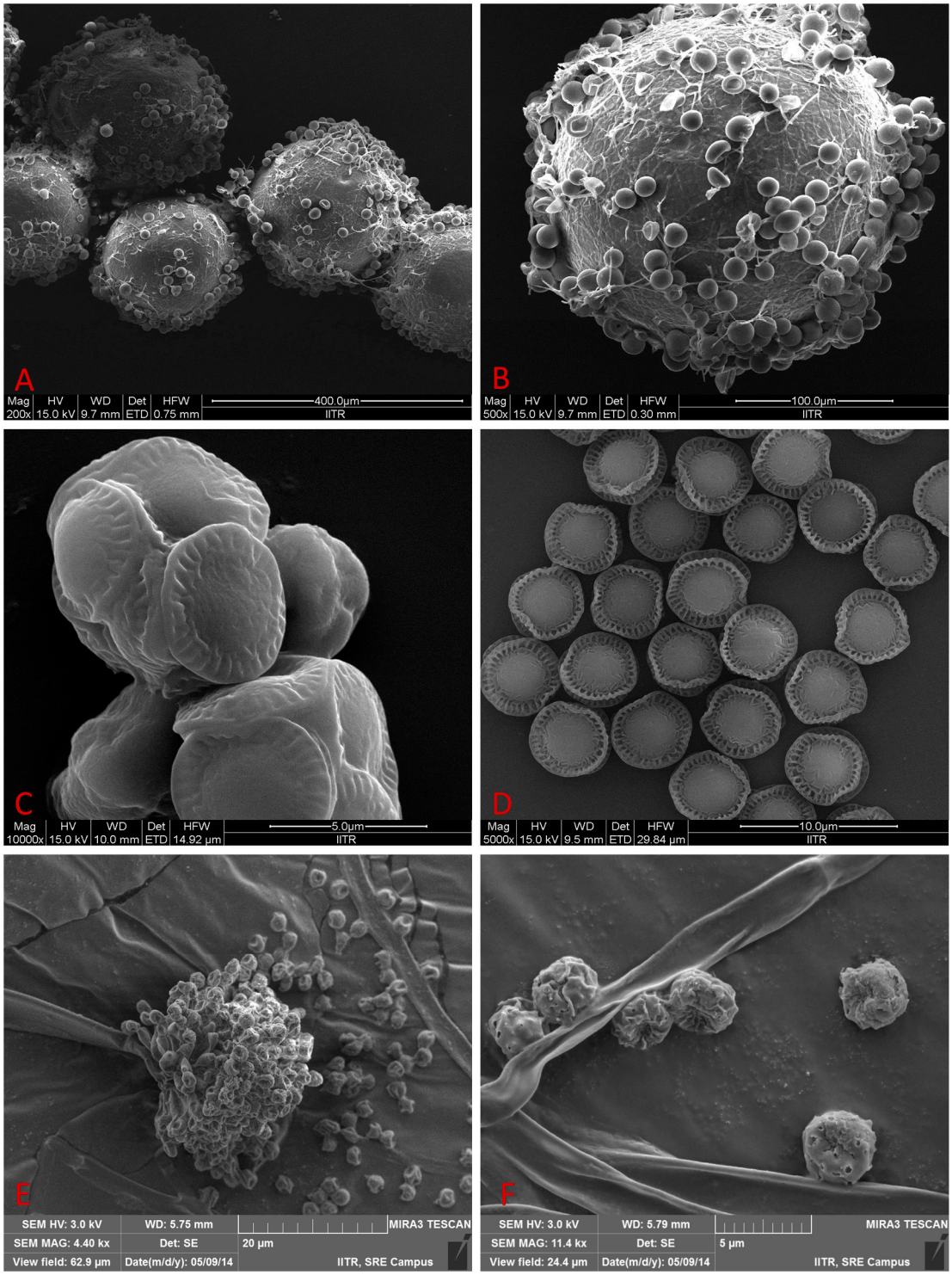

Figure 2. Sexual and asexual structures of *Aspergillus nidulanas* AKB-25: (**A**) Cleistothecia; (**B**) close-up of peridium of a cleistothecium with hulle cells; (**C**) developing ascus; (**D**) ascospores; (**E**) conidiophore; (**F**) conidia.

3.2. Agricultural Residues as the Carbon Sources for Enzyme Production

Penicillium sp. AKB-24 produced the maximal endoglucanase activity (66.96 IU/gds) with wheat bran as the carbon source and the descending order of endoglucanase production using next top two agro-residues were as follows: rice straw (58.42 IU/gds) > sugarcane bagasse (41.85 IU/gds). Likewise,

as the source of carbon, wheat bran produced the highest FPase activity (1.31 FPU/gds) and rice straw and sunhemp residue produced the second (0.87 FPU/gds) and the third (0.50 FPU/gds) highest FPase activities with fungal strain *Penicillium* sp. AKB-24.

In addition, *Penicillium* sp. AKB-24 produced the highest β-glucosidase activity using wheat bran (2.75 IU/gds) as the best carbon source and followed by rice straw (2.52 IU/gds) and sunhemp residue (1.60 IU/gds). Conversely, *Penicillium* sp. AKB-24 produced the highest xylanase activity (2150.79 IU/gds) using corn stover as the carbon source which was further followed by wheat bran (1865.15 IU/gds) and sugarcane tops (1628.95 IU/gds) (Figure 3). Based on the production of FPase, endoglucanase and β-glucosidase, wheat bran was selected as the source of carbon for further optimization. The effect of two important factors such as porosity and C/N ratio of the substrates on enzyme production under SSF conditions was investigated. Porosity of all the substrates was determined in terms of water retention value (WRV) and wheat bran and sugarcane tops showed the maximum WRV of 174.94% and 171.12% respectively. Wheat bran showed the minimal C/N ratio (22.68) compared to other substrates while sugarcane tops showed higher C/N ratio (69.49) (Table 1). The higher porosity and lower C/N ratio of wheat bran favoured enzyme production.

The chemical compositions, accessibility, physical association of components in a substrate are the most important factors for enzyme production by fungi under SSF [11,12]. Microorganisms grow on external and internal moist particles surface during SSF conditions. The space between particles is occupied by a continuous gas phase which is referred to as void volume or porosity of the substrate [28,29]. Porosity of substrate maintains the oxygen availability required for fungal growth during SSF conditions. Therefore, higher porosity of substrate improves the production of enzyme by the fungal cultures under aerobic SSF conditions [28,30].

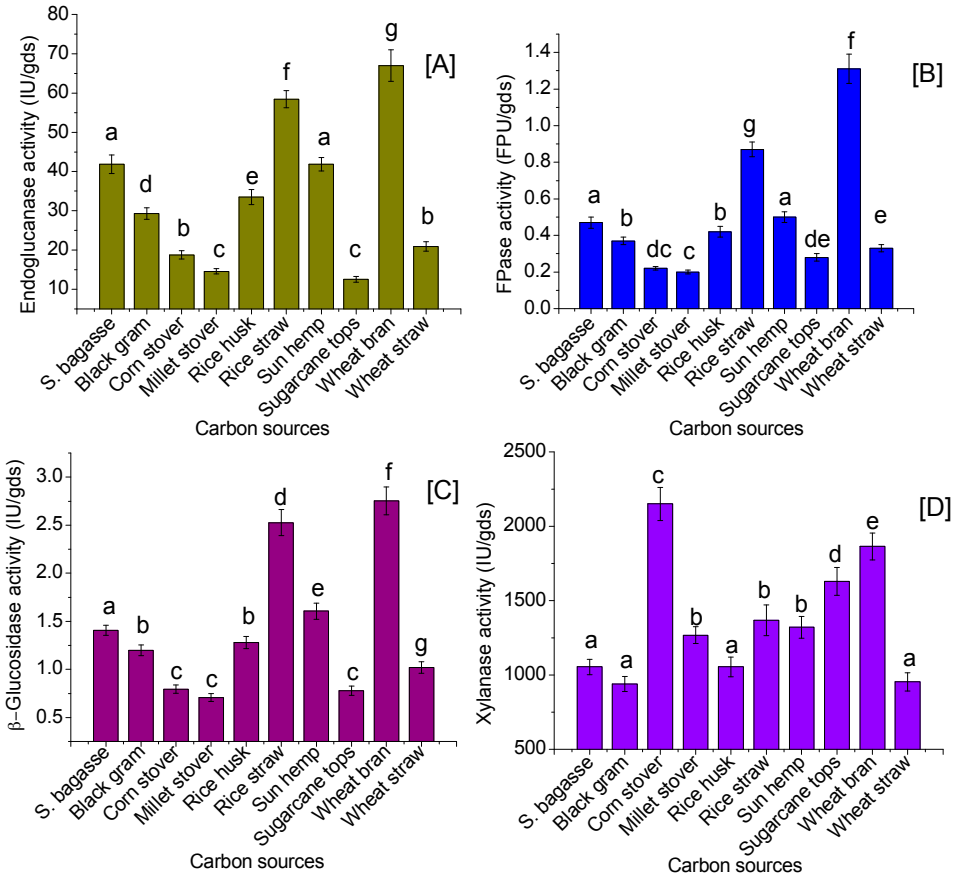

Figure 3. Screening of different carbon sources for enzyme production by *Penicillium* sp. AKB-24: (**A**) endoglucanase; (**B**) FPase; (**C**) β-glucosidase; (**D**) xylanase.

Table 1. C/N ratio and WRV of different crop residues.

S. No.	Substrates	C %	N %	C/N Ratio	WRV (%)
1	Sugarcane bagasse	43.47	0.53	82.02	126.73 ± 3.17 a
2	Black gram	42.59	0.98	43.46	145.15 ± 3.50 b
3	Corn stover	43.93	0.74	59.36	160.12 ± 2.31 d
4	Pearl millet stover	40.37	0.48	84.10	128.27 ± 3.23 a
5	Rice husk	41.32	0.63	65.59	92.05 ± 3.60 e
6	Rice straw	36.58	0.66	55.42	148.79 ± 3.61 b
7	Sunhemp	43.27	0.54	80.13	133.24 ± 3.52 a
8	Sugarcane tops	42.39	0.61	69.49	171.12 ± 4.19 c
9	Wheat bran	44.12	1.95	22.63	174.94 ± 3.42 c
10	Wheat straw	43.12	0.68	63.41	144.78 ± 3.09 b

Wheat bran is able to remain loose in moist conditions; it thereby provides a large surface area for fungal growth [16]. Wheat bran is a good source of nitrogen compared to other agro-residues tested. Several researchers have indicated the importance of C/N ratio for enzyme production [31,32]. Delabona *et al.* [31] found a C/N ratio of 16.9 for wheat bran and reported enhanced production of cellulase compared to other substrates by *Aspergillus fumigates* P40M2. Furthermore, wheat bran is a suitable carbon source for induction of enzymes in filamentous fungi under SSF conditions due to its composition [20]. Wheat bran contains starch (13.8%–24.9%), protein (13.2%–18.4%), cellulose (11.0%), hemicellulose (10.9%–26%), fats (3.5%–3.9%), phenolic acids (1.1%), and ash (3.4%–8.1%) [33,34]. Wheat bran can supply ample nutrients like proteins, hemicelluloses, iron, zinc, copper and manganese to microorganisms. In addition, hemicelluloses have proved a good inducer for the production of cellulolytic enzymes and wheat bran is a rich source of hemicelluloses [35].

In solid-state fermentation, enzyme production was carried out using lignocellulosic biomass. Cellulose and hemicelluloses are used as carbon source for the production of cellulolytic and hemicellulolytic enzymes. Cellulose and hemicelluloses are the effective inducer for cellulolytic and hemicellulolytic enzymes respectively. Cellulose and xylan are macromolecular polymers and they cannot enter through cell wall of fungal mycelia. Constitutively produced cellulases and xylanases act on these polymers to release the low molecular weight oligosaccharides, disaccharides and monosaccharides, which play essential role in the induction of cellulases and xylanases. Xylose, xylobiose, xylooligosaccharides and heterodisaccharides of xylose and glucose are released from the xylan, which regulate the biosynthesis of xylanases [36]. Several oligosaccharides and disaccharides such as cellobiose, sophorose, sorbose, δ-cellobiono-1,5-lactone, galactose, and gentiobiose induce the production of cellulases. Cellulose was also reported as an inducer for the synthesis of xylanases [37,38].

3.3. Time Course of Enzyme Production

Figure 4 shows the time course of enzyme production by *Penicillium* sp. AKB-24 using wheat bran as the substrate. Statistical analysis indicated that the increase in endoglucanase, FPase, and xylanase activities from 6th to 7th day was significant (p value < 0.05). Maximum endoglucanase (73.41 IU/gds), FPase (1.42 FPU/gds), and xylanase (1983.91 IU/gds) activities were obtained on the 7th day of incubation using wheat bran as the carbon source, and beyond that all these enzyme activities declined with time. Enzyme production in various microorganisms reached the maximum level in stationary phase and declined during the death phase. During the death phase, depletion of nutrient concentration and cellular fragmentation is very common, resulting in release of intracellular material and proteases in fermentation broth [16,39]. Sehnem *et al.* [40] reported an optimum incubation time of 7 days for cellulase production by *Penicillium echinulatum*. *Penicillium* sp. AKB-24 produced maximum β-glucosidase (4.80 IU/gds) on the 9th day of incubation and decreased thereafter. However, maximum endoglucanase, FPase and xylanase were observed on the 7th day of incubation while the level of β-glucosidase reached its maximum value on the 9th day of incubation. This may due to the existence of some soluble cellodextrins and cellobiose in the medium after exhausting of

cellulose [41]. The present results are close to the findings of Deswal *et al.* [19] who reported maximum endoglucanase and β-glucosidase production by *Fomitopsis* sp. RCK 2010 on the 11th and 15th days of incubation, respectively.

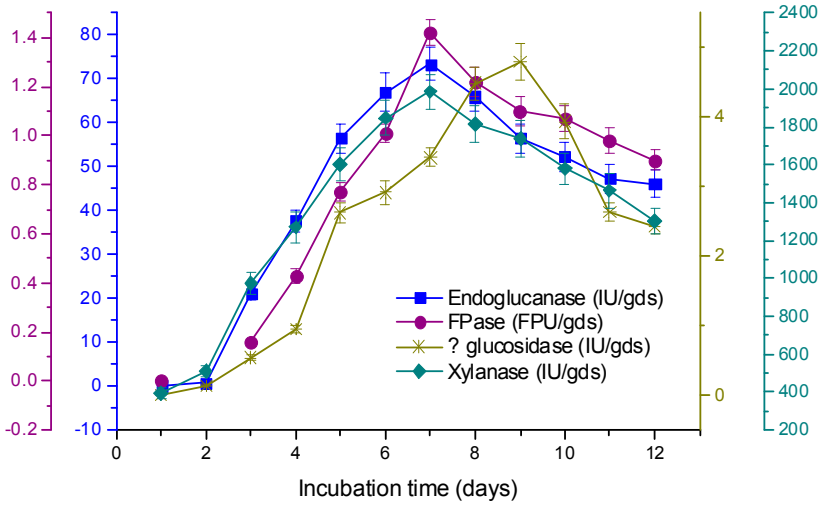

Figure 4. Effect of incubation time on enzyme production by *Penicillium* sp. AKB-24.

3.4. Effect of Temperature and Initial pH on Enzyme Production

Temperature is an important fungal-dependent parameter that affects the production of enzymes. Therefore, it is necessary to optimize temperature for particular fungi to produce maximum enzyme activity [12]. All the enzyme activities including, endoglucanase (72.85 IU/gds), FPase (1.43 FPU/gds), β-glucosidase (3.41 IU/gds), and xylanase (1963.80 IU/gds) were found to be maximized at 30 °C by *Penicillium* sp. AKB-24 (Figure 5). Increments in endoglucanase, FPase, β-glucosidase and xylanase activities from 26 to 30 were significantly different (p value < 0.05), as shown by statistical analysis. All enzyme activities declined significantly (p value < 0.05) at 34 °C. Higher temperature during enzyme production might lead to poor growth and reduction in enzyme yield due to denaturation of enzymes [12,42]. The results observed in the current study were in accordance with previous reports where a temperature of 30 °C was reported to be the optimum for cellulase, xylanase and β-glucosidase by *P. citrinum* [43] and *P. decumbens* [44].

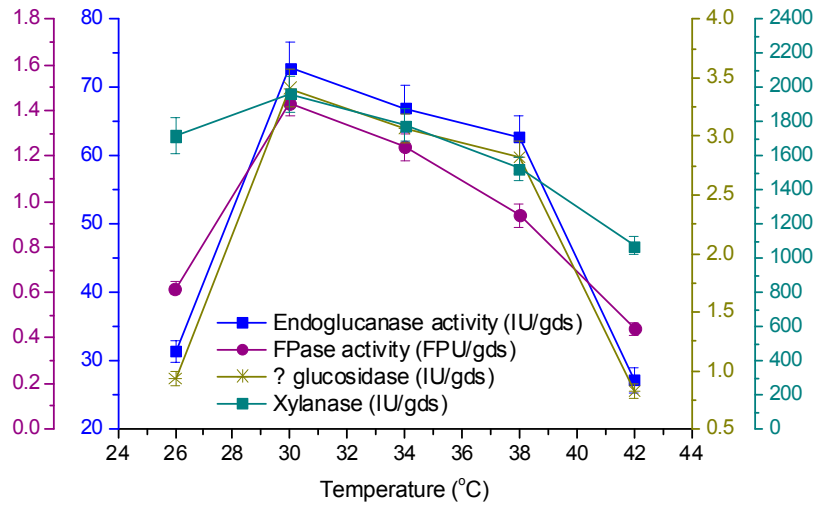

Figure 5. Effect of temperature on enzyme production by *Penicillium* sp. AKB-24.

Penicillium sp. AKB-24 showed maximum, endoglucanase (76.69 IU/gds), FPase (1.51 FPU/gds), β-glucosidase (3.75 IU/gds) and xylanase (2143.28 IU/gds) activities at an initial pH of 7.0 (Figure 6). Increasing or decreasing the initial pH from the optimum value affected the enzyme production to a lesser extent. Increases in endoglucanase, FPase, β-glucosidase and xylanase activities at pH 7.0 compared to pH 6.0 were not statistically significant (p value > 0.05). Therefore, pH adjusted in the beginning is not a controlled factor during SSF conditions. Lignocellulosic substrates have complex structures and have a buffering effect during SSF, which minimizes the variation in pH. The growth of fungi during SSF also might vary the pH due to their metabolic activities [12,45].

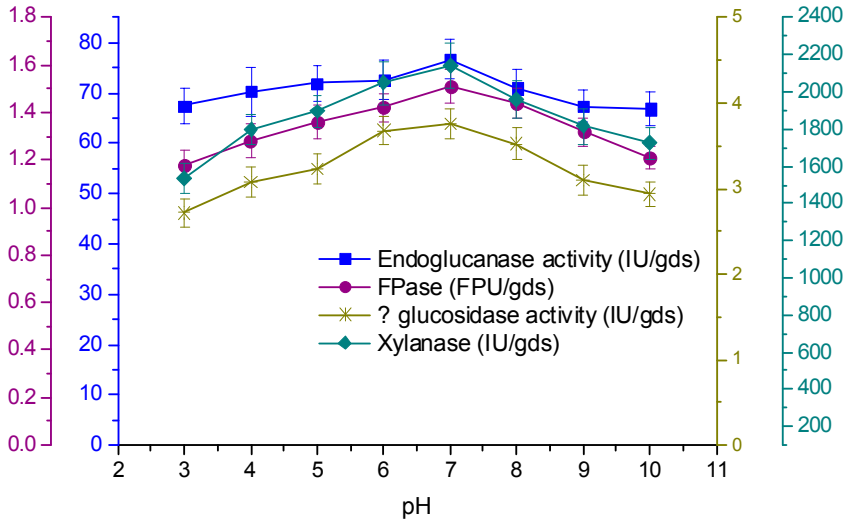

Figure 6. Effect of pH on enzyme production by *Penicillium* sp. AKB-24.

3.5. Effect of Initial Moisture Content on Enzyme Production

Influence of moisture content on enzyme production by *Penicillium* sp. AKB-24 was tested in the 55 to 85% range using wheat bran as the substrate. *Penicillium* sp. AKB-24 resulted in the maximum endoglucanase (77.60 IU/gds), FPase (1.54 FPU/gds), β-glucosidase (3.94 IU/gds) and xylanase (2214.17 IU/gds) production at 77.5% moisture content (Figure 7).

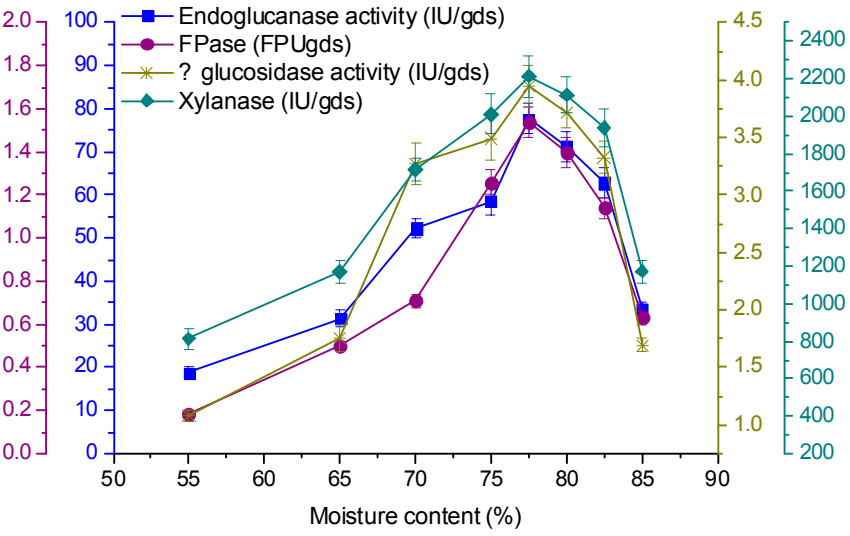

Figure 7. Effect of moisture content on enzyme production by *Penicillium* sp. AKB-24.

An increase or decrease in moisture content from the optimum value resulted in lower endoglucanase, FPase, and β-glucosidase activities (significantly different as p value < 0.05). An increase in xylanase production at 77.5% compared to 75.0% was statistically significant while a decrease to 80.0% moisture content was not significantly different. A higher than optimum moisture content led to a decrease in porosity, gummy texture, and lower oxygen transfer whereas a lower moisture content led to decreased swelling of substrate and solubility of nutrients [46]. This inhibits effective uptake of nutrients by fungi. If moisture is too high during SSF, risk of contamination by unfavorable microorganisms is greater [12]. Liu *et al.* [44] reported maximum cellulase production by *Penicillium decumbens* ML-017 at 72% moisture content using rice bran as the substrate under SSF. Ng *et al.* [43] reported maximum endoglucanase, FPase, and β-glucosidase productions by *Penicillium citrinum* YS40-5 during SSF at 90% moisture content on rice bran as the substrate. Maximum endoglucanase, FPase, β-glucosidase and xylanase production at optimum cultural conditions by *Aspergillus nidulans* AKB-25 is shown in Table 2 [21].

Table 2. Maximum enzyme production at optimum cultural conditions by *Aspergillus nidulans* AKB-25.

Variables	Endoglucanase (IU/gds)	FPase (FPU/gds)	β-Glucosidase (IU/gds)	Xylanase (IU/gds)
Incubation time, 4 days	65.79 ± 3.19	1.58 ± 0.08	23.58 ± 1.30	1449.95 ± 81.63
Temperature, 30 °C	65.79 ± 3.88	1.60 ± 0.09	23.93 ± 1.50	1472.17 ± 76.25
Initial pH, 8.0	79.00 ± 3.19	1.73 ± 0.10	27.50 ± 1.33	1832.25 ± 125.50
Moisture contents, 80.0%	104.85 ± 6.98	2.06 ± 0.13	28.94 ± 1.78	1955.24 ± 126.30

± refers standard deviation.

3.6. Effect of Nitrogen Sources

Penicillium sp. AKB-24 induced the maximum level of endoglucanase using ammonium sulphate, sodium nitrate and urea (0.08% expressed as available nitrogen) among various simple nitrogen sources (Figure 8a). Among all the nitrogen, *Penicillium* sp. AKB-24 produced maximum endoglucanase (115.05 IU/gds) with yeast extract (1.2% w/v) as the nitrogen source (Figure 8b. Among simple nitrogen sources, *Penicillium* sp. AKB-24 induced maximum yield of FPase (2.14 FPU/gds) and xylanase (2910.00 IU/gds) at urea dosing of 0.08% and 0.12% (expressed as available nitrogen) respectively (Figure 9a). *Penicillium* sp. AKB-24 was found to be more effective to induce enzyme production using complex nitrogen sources (beef extract, peptone, and yeast extract) compared to simple nitrogen sources. Among all the nitrogen sources, *Penicillium* sp. AKB-24 presented the maximum production of FPase (2.38 FPU/gds) and xylanase (2918.05 IU/gds) with yeast extract (1.2% w/v) as the complex nitrogen source (Figures 9b and 10a,b). Fungal growth and synthesis of extracellular enzymes are influenced by the availability of precursors of protein synthesis [46]. Proper combination of nitrogen sources, lignocellulosic substrate, and fungal strain is necessary for optimum production of cellulases and xylanases [12]. Maeda *et al.* [47] reported similar results with urea and yeast extract as the best nitrogen sources for cellulase production by *Penicillium funiculosum* using pretreated sugarcane bagasse as the carbon source. Adsul *et al.* [48] showed yeast extract to be the most suitable nitrogen source for cellulase production by *Penicillium janthinellum* during SSF using wheat bran and pure cellulose as the substrate.

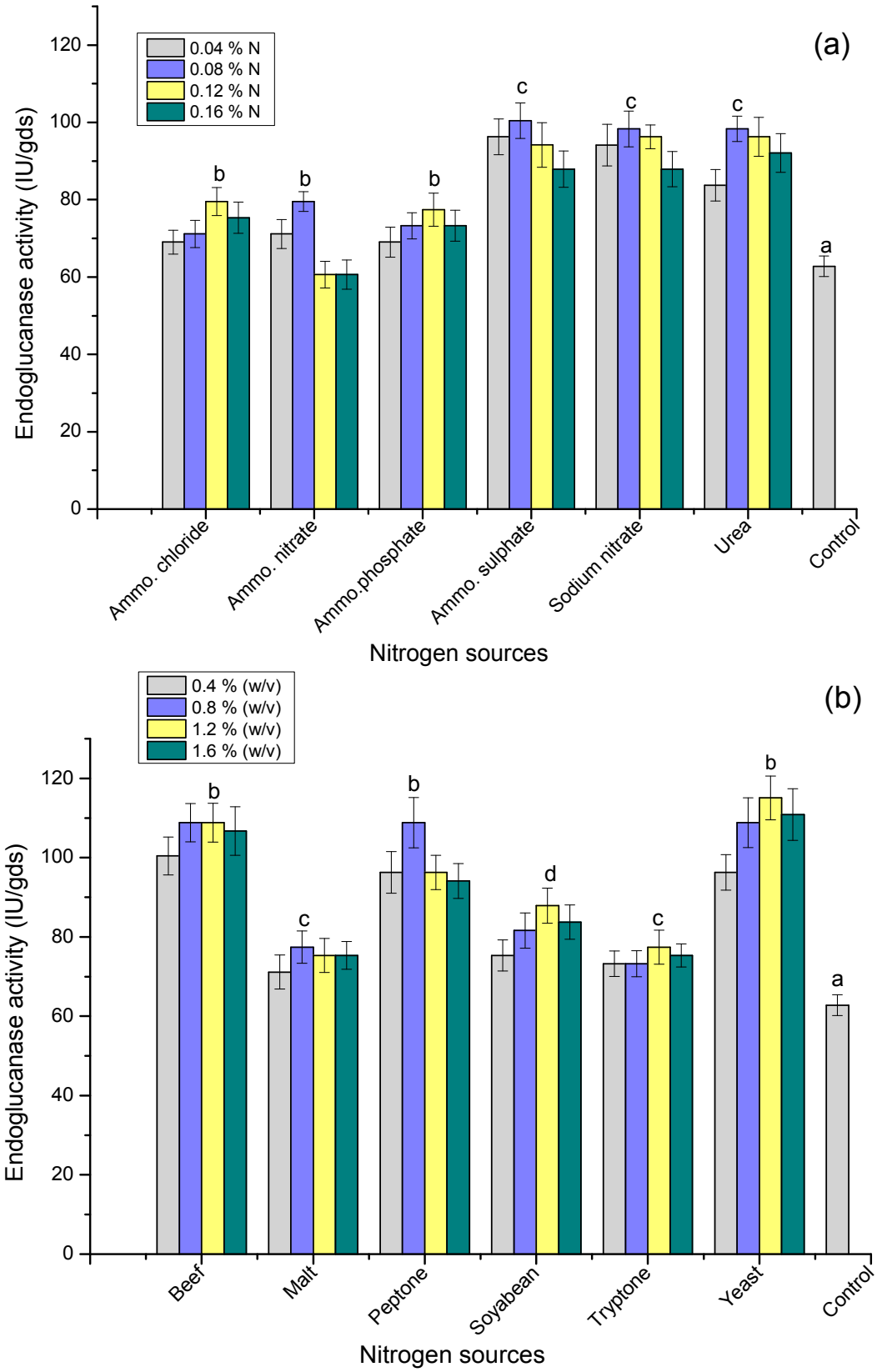

Figure 8. (**a**) Effect of simple nitrogen sources on endoglucanase production by *Penicillium* sp. AKB-24. (**b**) Effect of complex nitrogen sources on endoglucanase production by *Penicillium* sp. AKB-24.

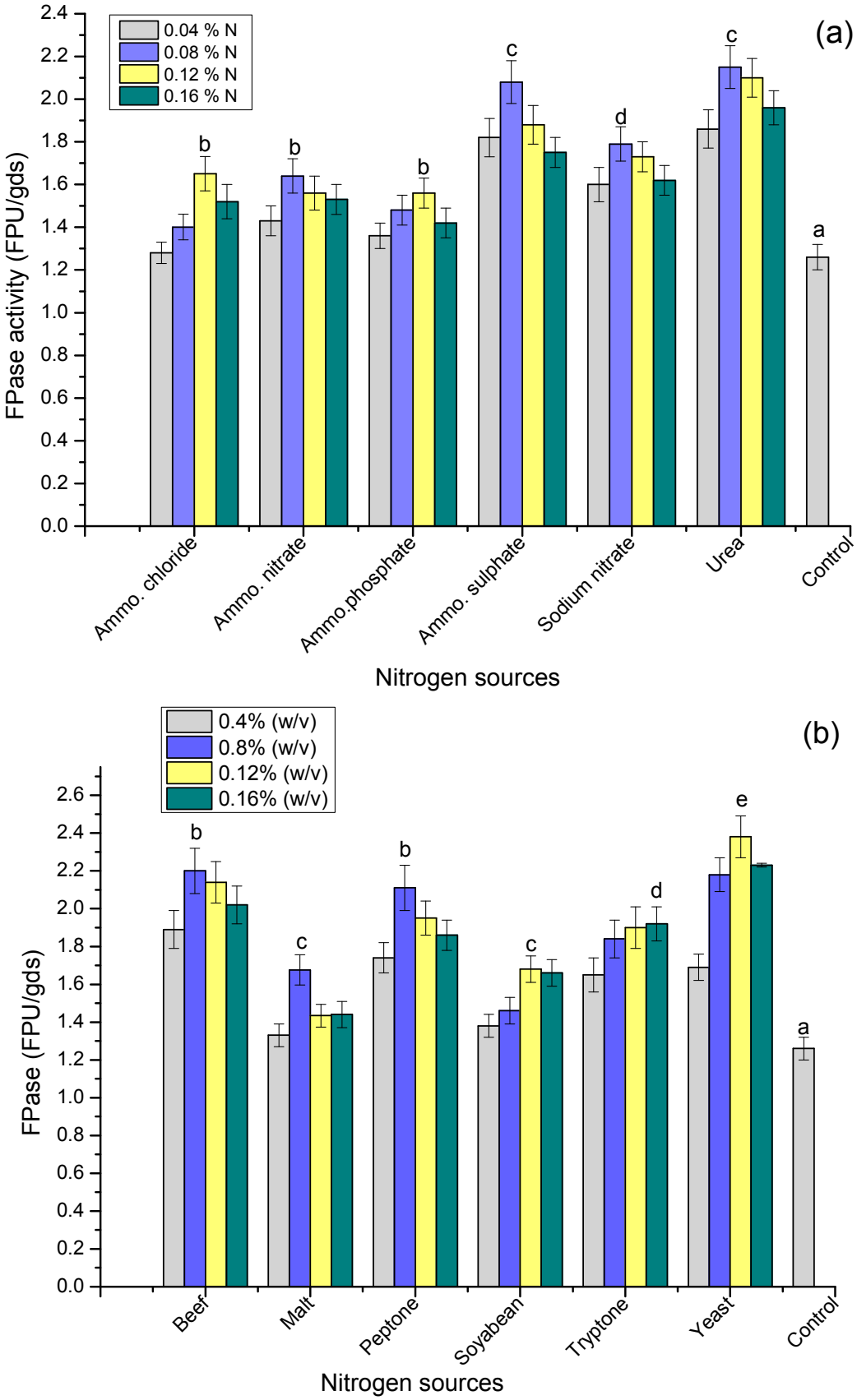

Figure 9. (**a**) Effect of simple nitrogen sources on FPase production by *Penicillium* sp. AKB-24. (**b**) Effect of complex nitrogen sources on FPase production by *Penicillium* sp. AKB-24.

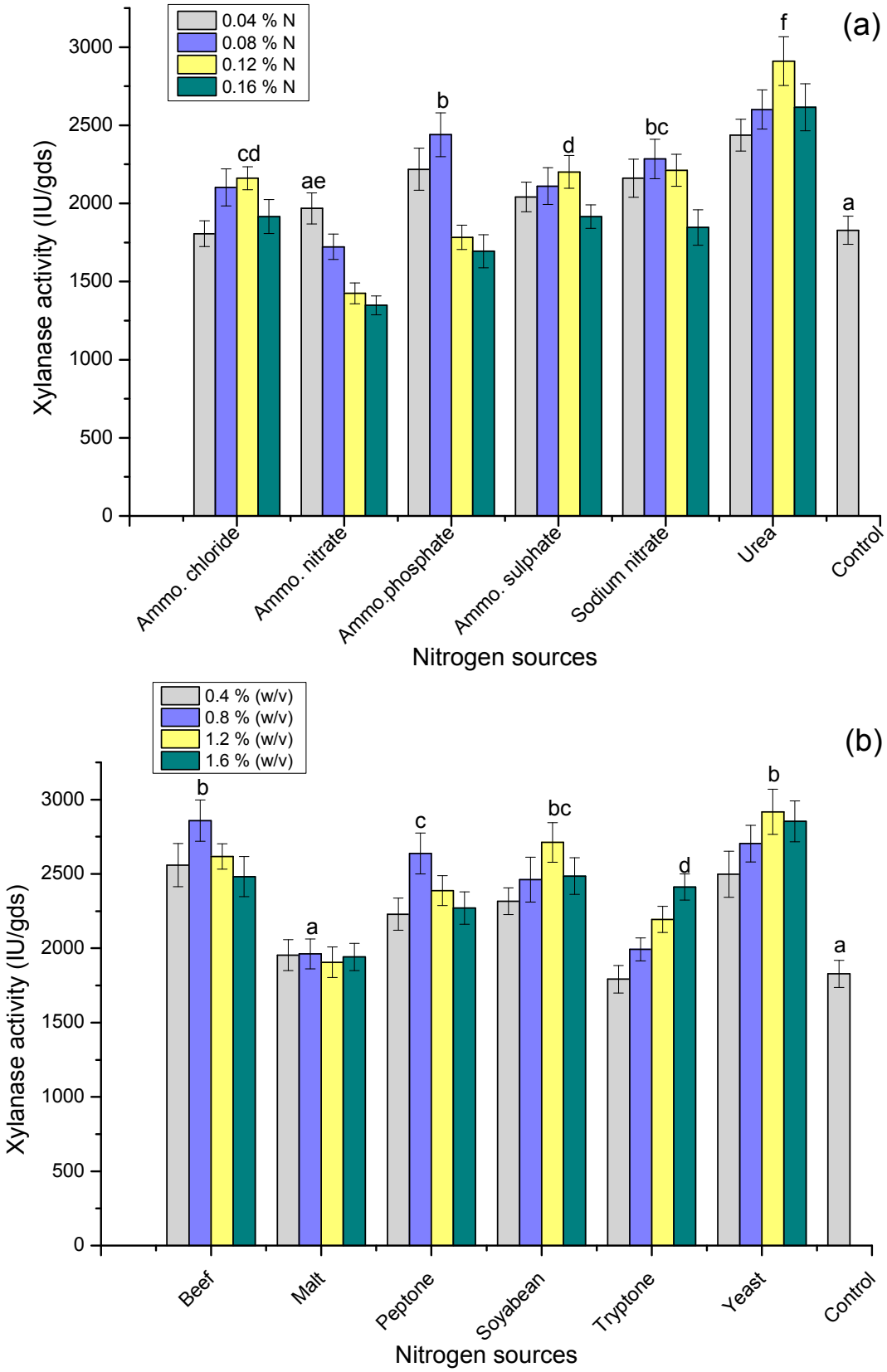

Figure 10. (**a**) Effect of simple nitrogen sources on xylanase production by *Penicillium* sp. AKB-24. (**b**) Effect of complex nitrogen sources on xylanase production by *Penicillium* sp. AKB-24.

3.7. Effect of Surfactants

Different concentrations, *i.e.*, 0.05, 0.10 and 0.20% (*w*/*v*) of surfactants namely Tween-20, Tween-40, Tween-60, Tween-80, Triton-X-100, and SDS and EDTA were added at optimum conditions to observe their effects on cellulase and xylanase production by *Penicillium* sp. AKB-24. Except EDTA, all the surfactants stimulated the production of cellulase and xylanase and among those, SDS had the maximum stimulating effect for the production of cellulase and xylanase by *Penicillium* sp. AKB-24. A SDS concentration of 0.10% (*w*/*v*) produced maximum endoglucanase (133.94 IU/gds), FPase (2.96 FPU/gds), and xylanase (3592.26 IU/gds) (Table 3). SDS at a concentration 0.10% (*w*/*v*) produced maximum β-glucosidase activity (5.88 IU/gds) by *Penicillium* sp. AKB 24 when added at optimum conditions. Surfactant facilitated the penetration of NSS into the interstices of the substrate by reducing the surface tension between NSS and substrate, wetting the substrate surface and emulsifying the extractives.

The stimulatory effect of surfactants on enzyme production may be due to an increase in the permeability of the cell membrane. Various reports show that some amount of cellulolytic and hemicellulolytic enzymes is bound to fungal hypha or found in close association with hypha. Surfactants may promote the release of cell bound enzymes [17,49]. Tween-80 and SDS have been proved effective for the production of cellulases by *Fusarium* sp. [50] and *Aspergillus* sp. SU14 [51]. Sindhu *et al.* [52] investigated the effect of surfactants on cellulase production and observed the maximum production of cellulase by *Penicillium* sp. using Tween-80 at 0.05% (*w*/*v*).

Maximum endoglucanase, FPase, exoglucanase, β-glucosidase, xylanase and amylase activities were 133.94, 2.96, 2.48, 5.88, 3592.26 and 194.90 IU/gds respectively at optimum cultural conditions by *Penicillium* sp. AKB-24 using wheat bran as substrate under SSF. Pirota *et al.* [53] reported maximum endoglucanase (113.40 IU/g), FPase (0.25 IU/g), β-glucosidase (2.00 IU/g) and xylanase (507.90 IU/g) by *Aspergillus oryzae* P27C3A using wheat bran as carbon source under SSF.

3.8. Improvement in Enzyme Production by Co-Cultivation

Co-cultivation of *Penicillium* sp. AKB-24 and *Aspergillus nidulans* AKB-25 resulted in higher production of endoglucanase, FPase, and exoglucanase compared to monocultures of *Penicillium* sp. AKB-24 and *Aspergillus nidulans* AKB-25 (Tables 4 and 5). The co-culture of fungi did not show significant improvement in xylanase activity compared to *Penicillium* sp. AKB-24. The decrease in amylase activity during co-culture (Black gram residue + wheat bran medium) may be due to lower quantities of starch fermentation medium compared to a monoculture of *Penicillium* sp. AKB-24 on wheat bran medium. The positive effect of co-culture of fungi on hydrolytic enzyme production may be due to better adaptability and substrate utilization compared to monocultures [12]. Complete hydrolysis of lignocellulosic substrate requires combined action of endoglucanase, exoglucanase and β-glucosidase. The present study reveals that β-glucosidase production was low for *Penicillium* sp. AKB-24 while *Aspergillus nidulans* AKB-25 produced higher level of β-glucosidase. The higher quantity of β-glucosidase during co-culture prevents the accumulation of cellobiose, which acts as inhibitor for endoglucanase and exoglucanase [11]. Fang *et al.* [41] reported improvement in FPase and β-glucosidase production during co-cultivation of *Trichoderma reesei* RUT-C30 and *Aspergillus niger* NL02. FPase and β-glucosidase activities were 3.30 and 1.01 IU/mL, respectively, during co-cultivation, significant higher than those of monocultures, which were 2.48 and 0.24 IU/mL of *T. reesei* RUT-C30 and 0.20 and 0.70 IU/mL of *A. niger* NL02, respectively.

Gupte and Madamwar [11] also reported improved endoglucanase, FPase, exoglucanase and β-glucosidase activity by the co-cultivation of *Aspergillus ellipticus* and *Aspergillus fumigatus* on alkali-pretreated sugarcane bagasse during SSF. Yang *et al.* [54] observed increased cellulases and ligninases activities with co-culturing of *Tricoderma reesei* RUT-C30 and *Phanerochaete chrysosporium* using pumpkin residue as the substrate.

Table 3. Effect of surfactants on enzyme production by *Penicillium* sp. AKB-24.

Surfactants	Endoglucanase (IU/gds)			FPase (FPU/gds)			Xylanase (IU/gds)		
	***Penicillium* sp. AKB-24**								
	0.05%	**0.10%**	**0.20%**	**0.05%**	**0.10%**	**0.20%**	**0.05%**	**0.10%**	**0.20%**
T-20	108.82 ± 5.69	123.47 ± 6.03 b	115.10 ± 5.47	2.47 ± 0.09	2.70 ± 0.10 b	2.24 ± 0.10	2978.88 ± 109.02	3261.69 ± 151.66 b	2855.71 ± 147.35
T-40	110.92 ± 5.71	125.57 ± 6.60 bc	115.10 ± 4.48	2.50 ± 0.11	2.64 ± 0.12 b	2.18 ± 0.12	2924.84 ± 141.26	3136.00 ± 162.13 b	2541.48 ± 133.68
T-60	108.82 ± 4.32	123.47 ± 5.96 b	113.01 ± 5.69	2.56 ± 0.11	2.79 ± 0.13 bc	2.41 ± 0.13	3002.76 ± 140.82	3262.95 ± 162.82 b	3049.27 ± 154.90
T-80	113.01 ± 5.81	129.75 ± 6.27 bc	121.38 ± 4.24	2.68 ± 0.12	2.91 ± 0.15 bc	2.78 ± 0.14	3002.76 ± 112.00	3335.85 ± 170.12 b	3081.95 ± 146.39
T-x-100	117.19 ± 5.10 b	113.01 ± 4.46	100.45 ± 4.90	2.70 ± 0.14 b	2.54 ± 0.13	2.45 ± 0.045	2435.90 ± 94.75 a	2185.77 ± 126.77	1690.55 ± 61.53
SDS	125.57 ± 5.95	133.94 ± 7.19 c	113.01 ± 3.76	2.63 ± 0.12	2.96 ± 0.14 c	2.67 ± 0.13	3094.52 ± 160.29	3592.26 ± 170.99 c	2379.34 ± 117.30
EDTA	98.36 ± 4.61 a	92.08 ± 3.48	85.80 ± 4.34	1.78 ± 0.11 d	1.61 ± 0.09	1.56 ± 0.08	2469.83 ± 130.16 a	2297.64 ± 81.79	2048.77 ± 88.71
Control		106.73 ± 5.14 a			2.08 ± 0.8 a			2510.06 ± 126.75 a	
	***Aspergillus nidulans* AKB-25**								
T-x-100		152.14 ± 8.29			3.42 ± 0.18			2441.03 ± 144.99	

± refers standard deviation.

Table 4. Co-culture of *Aspergillus nidulans* AKB-25 and *Penicillium* sp. AKB-24.

Particulars		FPase, FPU/gds	Endoglucanase, IU/gds
Carbon sources *	Wheat bran (WB)	2.93 ± 0.10	137.19 ± 5.93
	Black gram (BG)	3.48 ± 0.14	155.89 ± 5.19
	WB:BG (1:1)	3.64 ± 0.14	172.52 ± 6.80
pH **	6	3.38 ± 0.12	166.29 ± 8.13
	7	3.67 ± 0.17	174.60 ± 8.87
	8	3.83 ± 0.16	187.07 ± 9.09
Moisture content ***	77.5	3.84 ± 0.15	187.07 ± 8.19
	80.0	4.04 ± 0.19	203.70 ± 9.41

± refers to standard deviation. Fermentation conditions: * pH 7.0, moisture content 77.5%, temperature 30 °C, incubation time 7 days. ** Moisture content 77.5%, carbon sources-WB:BG (1:1), temperature 30 °C, incubation time 7 days. *** pH 8, carbon sources-WB:BG (1:1), temperature-30 °C, incubation time 7 days.

Table 5. Optimized conditions for enzyme production and comparison of production levels among monocultures and co-cultures of *A. nidulans* AKB 25 and *Penicillium* sp. AKB 24.

Particulars	*A. nidulans* AKB-25	*Penicillium* sp. AKB-24	Co-culture of *A. nidulans* AKB-25 and *Penicillium* sp. AKB-24
Optimal cultural parameters			
Carbon source	Black gram	Wheat bran	Black gram + Wheat bran
Incubation time (days)	4	7	7
Temperature (°C)	30	30	30
pH	8.0	7.0	8.0
Moisture content (%)	80.0	77.5	80.0
Nitrogen source (%)	Ammonium sulphate (0.12% N)	Yeast extract (1.2% *w*/*v*)	Ammonium sulphate (0.6% N) + Yeast extract (0.6% *w*/*v*)
Surfactant (%)	Triton-x-100 (0.05% *w*/*v*)	SDS (0.10% *w*/*v*)	Triton-x-100 (0.05% *w*/*v*)
Enzyme activities			
Endoglucanase (IU/gds)	152.14 ± 8.29	133.94 ± 7.19	203.70 ± 9.41 [(+33.88%) *, (+52.08%) **]
FPase (FPU/gds)	3.42 ± 0.18	2.96 ± 0.14	4.04 ± 0.19 [(+18.12%) *, (+36.48%) **]
Exoglucanase (IU/gds)	3.34 ± 0.14	2.48 ± 0.09	3.70 ± 0.13 [(+10.77%) *, (+49.19%) **]
β-Glucosidase (IU/gds)	44.05 ± 2.17	5.88 ± 0.26	35.28 ± 1.15 [(−19.90%) *, (+500%) **]
Xylanase (IU/gds)	2441.03 ± 144.99	3592.26 ± 170.99	3674.94 ± 183.12 [(+50.54%) *]
Amylase (IU/gds)	37.41 ± 1.95	194.90 ± 11.05	83.25 ± 4.30 [(+122.53%) *, (−57.28%) **]

± refers to standard deviation, (+/−) increase or decrease in enzyme activity in %. * Increase or decrease in enzyme activity compared to *A. nidulans* AKB-25. ** Increase or decrease in enzyme activity compared to *Penicillium* sp. AKB-24.

3.9. Saccharification by Crude Enzyme from Co-Culture

Crude enzyme produced from co-cultivation of *A. nidulans* AKB-25 and *Penicillium* sp. AKB-24 was evaluated for its ability to hydrolyze pearl millet stover at different enzyme doses varying from 5.0 to 20.0 FPU/g of substrate, hydrolysis time from 12 to 84 h for each enzyme dose and keeping other conditions constant as described in Section 2.7. Release of reducing sugars increased with increasing enzyme dose and 15 FPU/g produced maximum reducing sugars. An enzyme dose beyond 15 FPU/g did not increase reducing sugars significantly (p value > 0.05) (Table 6). Furthermore, the release of reducing sugars increased with increasing hydrolysis time and reached the maximum at a hydrolysis time of 72 h for each enzyme dose. The release of reducing sugars beyond a hydrolysistime of 72 h became insignificant (p value > 0.05) for each enzyme dose. The hydrolysis of substrate increases with increasing enzyme doses up to a certain level afterwards, and the relative number of binding sites reduces and enzyme may start competing for the same binding site leading to a reduction in overall reaction rate [55,56].

Table 6. Enzymatic hydrolysis of pearl millet stover by crude enzyme from co-cultures of *A. nidulans* AKB-25 and *Penicillium* sp. AKB-24.

Crude enzyme from co-cultures of *A. nidulans* AKB-25 and *Penicillium* sp. AKB-24				
Enzyme activity (IU/mL)	**0.42**			
Enzyme dosing (FPU/g)	**5 (11.90 mL) ***	**10 (23.80 mL) ***	**15 (35.71 mL) ***	**20 (47.61 mL) ***
Hydrolysis time (h)				
12	259.24 ± 8.92	323.55 ± 16.50	406.52 ± 18.05	420.13 ± 15.84
24	336.08 ± 14.25	396.25 ± 13.79	476.11 ± 20.57	490.55 ± 24.92
36	389.96 ± 16.11	460.37 ± 19.98	532.54 ± 17.25	547.62 ± 21.25
48	419.49 ± 16.32	514.66 ± 22.23	575.27 ± 22.72	582.85 ± 25.35
60	446.17 ± 13.30	537.16 ± 21.11	602.39 ± 29.34	606.92 ± 22.09
72	455.64 ± 16.58	550.36 ± 26.75	612.45 ± 24.17	616.75 ± 27.57
84	458.82 ± 15.31	554.15 ± 24.05	615.38 ± 28.92	618.44 ± 27.77
Crude enzyme from *A. nidulans* AKB-25				
Enzyme activity (IU/mL)	**0.36**			
Enzyme dosing (FPU/g)	**5 (13.88 mL) ***	**10 (27.77 mL) ***	**15 (41.66 mL) ***	**20 (55.55 mL) ***
Hydrolysis time (h)				
72	451.72 ± 14.44	556.14 ± 25.85	613.17 ± 20.42	610.35 ± 24.56

± refers standard deviation; * Values in bracket shows the amount of enzyme required in mL.

Crude enzyme from co-culture was found to be effective in hydrolysis of alkali-pretreated pearl millet stover due to the higher amount of β-glucosidase and xylanase along with cellulase, which plays an important role in the process of saccharification. Various researchers have indicated that supplementation of xylanase and β-glucosidase improved the cellulose digestion at reduced cellulase doses. High xylanase activity is helpful for higher conversion of cellulose and hemicelluloses into reducing sugars. Xylanase solubilises the xylan in hemicelluloses and enlarges the pore size in substrate, which assists cellulase action. Xylooligomers are the strong inhibitors of cellulases. Higher amounts of xylanase in enzyme convert the xylooligomers into monomers and neutralize the effect of inhibition by xylooligomers. The accumulation of cellobiose in the reaction mixture inhibits cellulase enzyme during hydrolysis and β-glucosidase reduces the effect of inhibition by converting cellobiose into glucose [56,57]. Chandel *et al.* [58] studied the hydrolysis of *Saccharum spontaneum* with cellulase and xylanase from *Aspergillus oryzae* MTCC1846 and observed the maximum release of reducing sugars (541.2 mg/g substrate) from alkali-pretreated substrate at an enzyme dose of 25 FPU/g.

Compared to enzymes obtained from co-cultivation of *A. nidulans* AKB-25 and *Penicillium* sp. AKB-24, *A. nidulans* AKB-25 released the same amount of reducing sugars at an enzyme dose of

15 FPU/g and reaction time 72 h. The enzyme produced by co-cultivation technique required less (14.28%) enzyme compared to the enzyme produced by *A. nidulans* AKB-25 alone in the same conditions and the same yield of reducing sugars. Therefore, enzyme production with the co-cultivation technique is cost-effective.

4. Conclusions

The maximum surface area and minimum C/N ratio made the wheat bran the best carbon source among other agro-residues for the production of cellulases. Production of endoglucanase, FPase, and exoglucanase with the co-cultivation technique was cost-effective, as enzymes produced with the co-cultivation method required less (14.28%) enzyme compared to enzymes produced with the monoculture technique (*A. nidulans* AKB-25 alone) for the hydrolysis of pearl millet stover.

Acknowledgments: The author Amit Kumar was supported by a fellowship from Jawahar Lal Nehru Memorial Fund, New Delhi (India).

Author Contributions: A.K. conceived and designed the experiments; A.K. performed the experiments; A.G. and D.D. analyzed the data; A.G. and D.D. contributed reagents/materials/analysis tools; A.K. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Saini, R.; Saini, J.K.; Adsul, M.; Patel, A.K.; Mathur, A.; Tuli, D.; Singhania, R.R. Enhanced cellulase production by *Penicillium oxalicum* for bio-ethanol application. *Bioresour. Technol.* **2015**, *188*, 240–246. [CrossRef] [PubMed]
2. Eriksson, T.; Börjesson, J.; Tjerneld, F. Mechanism of surfactant effect in enzymatic hydrolysis of lignocelluloses. *Enzyme Microb. Technol.* **2002**, *31*, 353–364. [CrossRef]
3. Gregg, D.; Saddler, J.N. Factors affecting cellulose hydrolysis and the potential of enzyme recycle to enhance the efficiency of an integrated wood to ethanol process. *Biotechnol. Bioeng.* **1996**, *51*, 375–383. [CrossRef]
4. Thompson, D.N.; Chen, H.-C. Comparison of pretreatment methods on the basis of available surface area. *Bioresour. Technol.* **1992**, *39*, 155–163. [CrossRef]
5. Sanchez, C. Lignocellulosic residues: Biodegradation and bioconversion by fungi. *Biotechnol. Adv.* **2009**, *27*, 185–194. [CrossRef] [PubMed]
6. Detroy, R.W.; Stjulian, G. Biomass conversion—Fermentation chemicals and fuels. *Crit. Rev. Microbiol.* **1983**, *10*, 203–228. [CrossRef] [PubMed]
7. Kumar, R.; Singh, S.; Singh, O.V. Bioconversion of lignocellulosic biomass: Biochemical and molecular perspectives. *J. Ind. Microbiol. Biotechnol.* **2008**, *35*, 377–391. [CrossRef] [PubMed]
8. Merino, S.T.; Cherry, J. Progress and challenges in enzyme development for biomass utilization. *Adv. Biochem. Eng. Biotechnol.* **2007**, *108*, 95–120. [PubMed]
9. Singhania, R.R.; Sukumaran, R.K.; Patel, A.K.; Larroche, C.; Pandey, A. Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. *Enzyme Microb. Technol.* **2010**, *46*, 541–549. [CrossRef]
10. Delabona, P.d.S.; Pirota, R.D.P.B.; Codima, C.A.; Tremacoldi, C.R.; Rodrigues, A.; Farinas, C.S. Using Amazon forest fungi and agricultural residues as a strategy to produce cellulolytic enzymes. *Biomass Bioenerg.* **2012**, *37*, 243–250. [CrossRef]
11. Gupte, A.; Madamwar, D. Solid-state fermentation of lignocellulosic waste for cellulase and β-glucosidase production by cocultivation of *Aspergillus ellipticus* and *Aspergillus fumigatus*. *Biotechnol. Progress* **1997**, *13*, 166–169. [CrossRef]
12. Yoon, L.W.; Ang, T.N.; Ngoh, G.C.; Chua, A.S.M. Fungal solid-state fermentation and various methods of enhancement in cellulase production. *Biomass Bioenerg.* **2014**, *67*, 319–338. [CrossRef]
13. Brijwani, K.; Oberoi, H.S.; Vadlani, P.V. Production of a cellulolytic enzyme system in mixed-culture solid-state fermentation of soybean hulls supplemented with wheat bran. *Process Biochem.* **2010**, *45*, 120–128. [CrossRef]

14. De Carvalho, M.L.A.; Carvalho, D.F.; Gomes, E.B.; Maeda, R.N.; Anna, L.M.M.S.; Castro, A.M.; Pereira, J.N. Optimisation of cellulase production by *Penicillium funiculosum* in a stirred tank bioreactor using multivariate response surface analysis. *Enzyme Res.* **2014**, *2014*, 8. [CrossRef]
15. Howard, R.L.; Abotsi, E.; Rensburg, J.V.E.L.; Howard, S. Lignocellulose biotechnology: Issues of bioconversion and enzyme production. *Afr. J. Biotechnol.* **2003**, *2*, 602–619. [CrossRef]
16. Dhillon, G.S.; Oberoi, H.S.; Kaur, S.; Bansal, S.; Brar, S.K. Value-addition of agricultural wastes for augmented cellulase and xylanase production through solid-state tray fermentation employing mixed-culture of fungi. *Ind. Crop Prod.* **2011**, *34*, 1160–1167. [CrossRef]
17. Dutt, D.; Kumar, A. Optimization of cellulase production under solid-state fermentation by *Aspergillus flavus* (AT-2) and *Aspergillus niger* (AT-3) and its impact on stickies and ink particle size of sorted office paper. *Cellul. Chem. Technol.* **2014**, *48*, 285–298.
18. Cheng, Q.; Wang, J.; McNeel, J.; Jacobson, P. Water retention value measurements of cellulosic materials using a centrifuge technique. *Bioresources* **2010**, *5*, 1945–1954.
19. Botkova, M.; Šutý, S.; Jablonský, M.; Kucerkova, L.; Vrška, M. Monitoring of kraft pulps swelling in water. *Cellul. Chem. Technol.* **2013**, *47*, 95–102.
20. Deswal, D.; Khasa, Y.P.; Kuhad, R.C. Optimization of cellulase production by a brown rot fungus *Fomitopsis* sp. RCK2010 under solid-state fermentation. *Bioresour. Technol.* **2011**, *102*, 6065–6072. [CrossRef] [PubMed]
21. Kumar, A. Production of cellulases and xylanases and their applications in industrial processes. Ph.D. Thesis, Department of Paper Technology, IIT Roorkee, Roorkee, India, 2015.
22. Miller, G.L. Use of dinitrosaiicyiic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426–428. [CrossRef]
23. Ghose, T.K. Measurement of cellulase activities. *Pure Appl. Chem.* **1987**, *59*, 257–268. [CrossRef]
24. Bailey, M.J.; Biely, P.; Poutanen, K. Interlaboratory testing of methods for assay of xylanase activity. *J. Biotechnol.* **1992**, *23*, 257–270. [CrossRef]
25. Wood, T.M.; Bhat, K.M. Methods for measuring cellulase activities. *Methods Enzymol.* **1988**, *160*, 87–112.
26. Kumar, A.; Gautam, A.; Dutt, D. Screening of fungal resources for the production of cellulases and xylanases. *Br. Biotechnol. J.* **2015**, *9*, 1–13. [CrossRef]
27. Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **2011**, *28*, 2731–2739. [CrossRef] [PubMed]
28. Brijwani, K.; Vadlani, P.V. Cellulolytic enzymes production via solid-state Fermentation: Effect of pretreatment methods on physicochemical characteristics of substrate. *Enzyme Res.* **2011**, *2011*, 10. [CrossRef] [PubMed]
29. Richard, T.L.; Veeken, A.H.M.; de Wilde, V.; Hamelers, H.V.M. Air-filled porosity and permeability relationships during solid-state fermentation. *Biotechnol. Progr.* **2004**, *20*, 1372–1381. [CrossRef] [PubMed]
30. Rahardjo, Y.S.P.; Jolink, F.; Haemers, S.; Tramper, J.; Rinzema, A. Significance of bed porosity, bran and specific surface area in solid-state cultivation of *Aspergillus oryzae*. *Biomol. Eng.* **2005**, *22*, 133–139. [CrossRef] [PubMed]
31. Delabona, P.dS.; Pirota, R.D.P.B.; Codima, C.A.; Tremacoldi, C.l.R.; Rodrigues, A.; Farinas, C.S. Effect of initial moisture content on two Amazon rainforest Aspergillus strains cultivated on agro-industrial residues: Biomass-degrading enzymes production and characterization. *Ind. Crop Prod.* **2013**, *42*, 236–242. [CrossRef]
32. Ellouz, C.S.; Belguith, H.; Hassairi, I.; M'Rad, K.; Ellouz, R. Optimization of cellulase production by *Penicillium occitanis*. *Appl. Microbiol. Biotechnol.* **1995**, *43*, 267–269. [CrossRef]
33. Apprich, S.; Tirpanalan, Ö.; Hell, J.; Reisinger, M.; Böhmdorfer, S.; Siebenhandl-Ehn, S.; Kneifel, W. Wheat bran-based biorefinery 2: Valorization of products. *LWT Food Sci. Technol.* **2014**, *56*, 222–231. [CrossRef]
34. Dornez, E.; Gebruers, K.; Wiame, S.; Delcour, J.A.; Courtin, C.M. Insight into the distribution of arabinoxylans, endoxylanases, and endoxylanase inhibitors in industrial wheat roller mill streams. *J. Agric Food Chem.* **2006**, *54*, 8521–8529. [CrossRef] [PubMed]
35. Zhang, H.; Sang, Q. Statistical optimization of cellulases production by *Penicillium chrysogenum* QML-2 under solid-state fermentation and primary application to chitosan hydrolysis. *World J. Microbiol. Biotechnol.* **2012**, *28*, 1163–1174. [CrossRef] [PubMed]
36. Kulkarni, N.; Shendye, A.; Rao, M. Molecular and biotechnological aspects of xylanases. *FEMS Microbiol. Rev.* **1999**, *23*, 411–456. [CrossRef] [PubMed]

37. Liao, H.; Li, S.; Wei, Z.; Shen, Q.; Xu, Y. Insights into high-efficiency lignocellulolytic enzyme production by *Penicillium oxalicum* GZ-2 induced by a complex substrate. *Biotechnol. Biofuels* **2014**, *7*, 162. [CrossRef] [PubMed]
38. Todero Ritter, C.E.; Camassola, M.; Zampieri, D.; Silveira, M.M.; Dillon, A.J.P. Cellulase and xylanase production by *Penicillium echinulatum* in submerged media containing cellulose amended with sorbitol. *Enzyme Res.* **2013**, *2013*, 9. [CrossRef] [PubMed]
39. Bansal, N.; Soni, R.; Janveja, C.; Soni, S.K. Production of xylanase-cellulase complex by *Bacillus subtilis* NS7 for the biodegradation of agro-waste residues. *Lignocellulose* **2012**, *1*, 196–209.
40. Sehnem, N.; de Bittencourt, L.; Camassola, M.; Dillon, A.P. Cellulase production by *Penicillium echinulatum* on lactose. *Appl. Microbiol. Biotechnol.* **2006**, *72*, 163–167. [CrossRef] [PubMed]
41. Fang, H.; Zhao, C.; Song, X.Y. Optimization of enzymatic hydrolysis of steam-exploded corn stover by two approaches: Response surface methodology or using cellulase from mixed cultures of *Trichoderma reesei* RUT-C30 and *Aspergillus niger* NL02. *Bioresour. Technol.* **2010**, *101*, 4111–4119. [CrossRef] [PubMed]
42. Sabu, A.; Sarita, S.; Pandey, A.; Bogar, B.; Szakacs, G.; Soccol, C.R. Solid-state fermentation for production of phytase by *Rhizopus oligosporus*. *Appl. Biochem. Biotechnol.* **2002**, *102–103*, 251–260. [CrossRef]
43. Ng, I.S.; Li, C.-W.; Chan, S.-P.; Chir, J.-L.; Chen, P.T.; Tong, C.-G.; Yu, S.-M.; Ho, T.-H.D. High-level production of a thermoacidophilic β-glucosidase from *Penicillium citrinum* YS40-5 by solid-state fermentation with rice bran. *Bioresour. Technol.* **2010**, *101*, 1310–1317. [CrossRef] [PubMed]
44. Liu, Y.-T.; Luo, Z.-Y.; Long, C.-N.; Wang, H.-D.; Long, M.-N.; Hu, Z. Cellulase production in a new mutant strain of *Penicillium decumbens* ML-017 by solid state-fermentation with rice bran. *New Biotechnol.* **2011**, *28*, 733–737. [CrossRef] [PubMed]
45. Mamma, D.; Kourtoglou, E.; Christakopoulos, P. Fungal multienzyme production on industrial by-products of the citrus-processing industry. *Bioresour. Technol.* **2008**, *99*, 2373–2383. [CrossRef] [PubMed]
46. Kaur, H.; Dutt, D.; Tyagi, C.H. Production of novel alkali-thermo-tolerant cellulase-poor xylanases from *Coprinopsis Cinerea* HK-1 NFCCI-2032. *Bioresources* **2011**, *6*, 1376–1391.
47. Maeda, R.; da Silva, M.; Santa Anna, L.M.M.; Pereira, N.J. Nitrogen source optimization for cellulase production by *Penicillium funiculosum*, using a sequential experimental design methodology and the desirability function. *Appl. Biochem. Biotechnol.* **2010**, *161*, 411–422. [CrossRef] [PubMed]
48. Adsul, M.G.; Terwadkar, A.P.; Varma, A.J.; Gokhale, D. Cellulases from *Penicillium janthinellum* mutants: Solid-state production and their stability in ionic liquids. *Bioresources* **2009**, *4*, 1670–1681.
49. Pardo, A. Effect of surfactants on cellulase production by *Nectria catalinensis*. *Curr. Microbiol.* **1996**, *33*, 275–278. [CrossRef] [PubMed]
50. Niranjane, A.P.; Madhou, P.; Stevenson, T.W. The effect of carbohydrate carbon sources on the production of cellulase by *Phlebia gigantea*. *Enzyme Microb. Technol.* **2007**, *40*, 1464–1468. [CrossRef]
51. Vu, V.H.; Pham, T.A.; Kim, K. Improvement of fungal cellulase production by mutation and optimization of solid state-fermentation. *Mycobiology* **2011**, *39*, 20–25. [CrossRef] [PubMed]
52. Sindhu, R.; Suprabha, N.G.; Shashidhar, S. Media engineering for the production of cellulase from *Penicillium* species (SBSS 30) under solid state-fermentation. *Biotechnol. Bioinf. Bioeng.* **2011**, *1*, 343–249.
53. Pirota, R.P.B.; Delabona, P.; Farinas, C. Simplification of the biomass to ethanol conversion process by using the whole medium of filamentous fungi cultivated under solid-state fermentation. *Bioenerg. Res.* **2014**, *7*, 744–752. [CrossRef]
54. Yang, R.; Meng, D.; Hu, X.; Ni, Y.; Li, Q. Saccharification of pumpkin residues by coculturing of *Trichoderma reesei* RUT-C30 and *Phanerochaete chrysosporium* burdsall with delayed inoculation timing. *J. Agric. Food Chem.* **2013**, *61*, 9192–9199. [CrossRef] [PubMed]
55. Banerjee, G.; Car, S.; Scott-Craig, J.S.; Borrusch, M.S.; Bongers, M.; Walton, J.D. Synthetic multi-component enzyme mixtures for deconstuction of lignocellulosic biomass. *Bioresour. Technol.* **2010**, *101*, 9097–9105. [CrossRef] [PubMed]
56. Van Dyk, J.S.; Pletschke, B.I. A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes-factors affecting enzymes, conversion and synergy. *Biotechnol. Adv.* **2012**, *30*, 1458–1480. [CrossRef] [PubMed]

57. Kumar, R.; Wyman, C.E. Effects of cellulase and xylanase enzymes on the deconstruction of solids from pretreatment of poplar by leading technologies. *Biotechnol. Progr.* **2009**, *25*, 302–314. [CrossRef] [PubMed]
58. Chandel, A.K.; Singh, O.V.; Rao, V.L.; Chandrasekhar, G.; Narasu, M.L. Bioconversion of novel substrate *Saccharum spontaneum*, a weedy material, into ethanol by *Pichia stipitis* NCIM3498. *Bioresour. Technol.* **2011**, *102*, 1709–1714. [CrossRef] [PubMed]