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Full Length Research Paper

Biohydrolysis of *Saccharum spontaneum* for cellulase production by *Aspergillus terreus*

Umbrin Ilyas[1]*, Shakil Ahmed[1], Abdual Majeed[2] and Muhammad Nadeem[3]

[1]Institue of Plant Pathology, University of the Punjab, Lahore (54000), Pakistan.
[2]Department of Botany, University of Gujrat, Gujrat (50700), Pakistan.
[3]Pakistan Council of Scientific and Industrial Research, Lahore (54000), Pakistan.

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***Saccharum spontaneum,* a wasteland weed, is utilized for cellulase production by *Aspergillus terreus* in solid state fermentation*. S. spontaneum* served as good carbon source and solid support. Various process parameters including optimal nitrogen source, initial moisture level, incubation time, initial pH, incubation temperature and inoculum size were evaluated. The maximum cellulase production was attained at 70% of initial moisture with incubation of 96 h at 30±2°C, and pH 4.5. Ammonium sulphate in concentration of 0.2% (w/w) was the most preferable nitrogen source among all tested nitrogen sources. The results indicate that *S. spontaneum* could be utilized as a substrate in solid state fermentation (SSF) for economic production of cellulase.**

Key words: Cellulase, solid state fermentation, *Saccharum spontaneum*, *Aspergillus terreus.*

INTRODUCTION

Cellulases are the third largest industrial enzyme in the world and have gained rejuvenated interest due to their applications in lignocellulose conversion. The widely accepted mechanism for biohydrolysis of cellulose involves synergistic action of three major cellulase enzymes: endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) (Thongekkaew et al., 2008). The cost of production and low yields of these enzymes are the major problems for industrial application. Lignocellulosic biomass has been regarded as a promising feedstock because of their abundance, cheapness, availability, and huge potential (Kang et al., 2004).

Considering the above, abundantly available agrowaste, *Saccharum spontaneum* was selected as a cheap substrate for cellulase production in solid state fermentation (SSF). *S. spontaneum*, one of the six species of the genus *Saccharum* (Daniels and Roach, 1987), has the widest distribution extending across three geographic zones: (i) the East Zone, which includes South Pacific islands, Philippines, Taiwan, Japan, China, Vietnam, Thailand, Malaysia, and Burma (Myanmar); (ii) the Central Zone, which includes India, Nepal, Bangladesh, Sri Lanka (Ceylon), Pakistan, Turkmenistan, Afghanistan, Iran, and Middle East; and (iii) the West Zone (African-Mediterranean), which includes Egypt, Sudan, Kenya, Uganda, Tanzania, and other countries (Panje and Babu, 1960; Daniels and Roach, 1987). This is a tall perennial grass with deep roots and rhizomes, growing up to 4 m height. It is believed to be ancestor of an important species *Saccharum officinarum* L. (cultivated sugarcane) (Sastri and Kavathekar, 1990). The presence of cellulose and hemicellulose together make the total carbohydrate content (TCC) of the substrate (67.85%), which is also the potential sugar concentration in the pretreated substrate. It can be fairly compared with the extensively explored lignocelluloses (sugarcane bagasse, 67.15%; corn stover, 58.29%; wheat straw, 54% and sorghum straw, 61%) for ethanol production (Chandel et al., 2009). Lignocellulosic biomass, such as corn stover and wheat bran, is abundant, cheap, and easily available. Various agricultural byproducts and microbial cultures have been used successfully in the SSF for cellulase production (Yang et al., 2006). SSF can be considered as a potential

*Corresponding author. E-mail: umbrin.ilyas@gmail.com . Tel: +92-344-4535023.

technology for commercial production of cellulases, taking into account its low input cost and ability to utilize naturally available sources of cellulose as substrate for enzyme production by microbial conversion. This study aimed at investigating the potential of indigenous agrowaste such as *S. spontaneum* to produce cellulase enzyme by locally isolated *Aspergillus terreus* in solid state fermentation.

MATERIALS AND METHODS

Organism

A. terreus was isolated from pulp and paper industry effluent (lignocellulolsic waste), Ferozpur Road Lahore, Pakistan, by serial dilution technique. *A. terreus* was identified by using Universal key of identification (Kenneth et al., 1965; Domsch et al., 1980; Samson et al., 2000) with the help of Fungal Culture Bank of Pakistan (FCBP) on the basis of morphological and microscopic characters. The purified culture was maintained on Potato Dextrose Agar (PDA) slants at 4°C.

Screening for cellulase activity

The purified *A. terreus* was screened for its cellulolytic potential on Carboxymethlycellulose-Agar (CMC) medium as defined by Onsori et al. (2005). A 2 µl of spore suspension (~1 × 10^6 cells/ml) of *A. terreus* was added in the small well created in the center of solidified screening plate and incubated at 28°C for 48 h. Thereafter, inoculated plates were stained with 1% Congo red for 30 min followed by destaining with 1 M NaCl solution for 15 min. A clear zone appeared in the center, indicating cellulolytic potential of *A. terreus.*

Substrate

S. spontaneum was collected from the environs of Gujrat, Pakistan. The completely mature plant, except roots, was collected, dried, chopped into small pieces and sieved well through 3 mm mesh.

Solid state fermentation

Non-optimized SSF was carried out in Erlenmeyer flasks (250 ml) with 5 g of powdered substrate. The liquid salt medium (LSM) with composition (gL^{-1}): $(NH_4)_2SO_4$, 3.5; KH_2PO_4, 3; $MgSO_4.7H_2O$, 0.05; $CaCl_2.H_2O$, 0.05 (Juhasz et al., 2005) was used. The substrate was moistened with distilled water and 5 ml of LSM to give 80% initial moisture and autoclaved at 121°C. Then, autoclaved substrate was aseptically inoculated with 1 ml of spore suspension (1×10^6 cells/ml) and incubated for 6 days at 30±2°C under static conditions.

Optimization of process parameters

The substrate was amended with different inorganic N-sources ($NaNO_3$, NH_4Cl, $(NH_4)_2SO_4$, $(NH_4)_2PO_4$ and NH_4NO_3) and organic N-sources (Malt extract, Peptone, Urea, Yeast extract and Tryptone) at concentration of 0.001 g nitrogen g^{-1} substrate (equivalent to 0.1% w/w). Varied initial moisture levels of substrate (60 to 95%) were adjusted with distilled water to trace out the optimum moisture levels. However, volumes of LSM per gram of substrate were kept constant (1 ml/g of substrate). The optimum incubation temperature for cellulolytic activity of *A. terreus* was determined by incubating inoculated substrate at different temperatures ranged from 25 to 45°C. The effect of initial pH on cellulase production was evaluated by adjusting pH of LMS up to different values (3 to 6) with 50 mM Citrate buffer prior to its sterilization. The fermentation period was limited to 192 h and the activity was determined at an interval of 24 h.

Enzyme assay

Enzyme was extracted by adding 10 ml of distilled water per gram of fermented substrate, shaken at 100 rpm for 1 h and filtered through muslin cloth. The filtrate was centrifuged at 10,000 rpm for 15 min at 4°C. The clear supernatant was used for enzyme assay. CMCase activity in extracted supernatant was determined according to Acharya et al. (2008). The amount of reducing sugar released was measured by using 3, 5-dinitrosalicyclic acid (DNSA) (Miller, 1959). One IU (International Unit) was defined as the amount of glucose (mM) released per ml of enzyme solution per minute.

Statistical analysis

The data was statistically analyzed by applying Duncan's multiple range test (Steel and Torrie, 1980) at p≤0.05 to compare means of data.

RESULTS

Biohydrolysis of *S. spontaneum* for cellulase production was carried out by *A. terreus.* The cellulolytic potential of locally isolated *A. terreus* was confirmed on screening medium as a clear halo zone appeared around the small well on the plate (Figure 1c). Among all tested nitrogen sources, $(NH_4)_2SO_4$ gave the maximum enzyme activity followed by Urea, NH_4NO_3 and Yeast extract (Figure 2). Furthermore, the concentration of ammonium sulphate was optimized and the results show that 0.2% (w/w) of $(NH_4)_2SO_4$ was optimum for the activity of *A. terreus* (Figure 3). However, the auxiliary increase in concentration of $(NH_4)_2SO_4$, up to 0.4% had no significant effect on enzyme production. The initial moisture level, a critical factor in SSF, was optimized to enhance the activity of *A. terreus*. The maximum cellulase activity (559 IU/) was attained at 70% moisture level (Figure 4). The highest cellulase activity was obtained at 96 h of incubation and decreased thereafter as shown in Figure 5. Various inoculum sizes ranging from 10 to 60% (v/w) were examined to select one at which *A. terreus* showed highest activity with the incubation of 96 h. Figure 6 shows that the highest enzyme activity was achieved at inoculums size of 20 % (v/w) of *A. terreus* while it started to decrease after increasing the inoculums size. Enzyme activity increased with increase in temperature up to 30±2°C then started to decline as shown in Figure 7. The optimum pH ranged from 3.5 to 5 (Figure 8). However, the maximum activity was attained at pH 4.5 that is, 0.667 IU/ml/min.

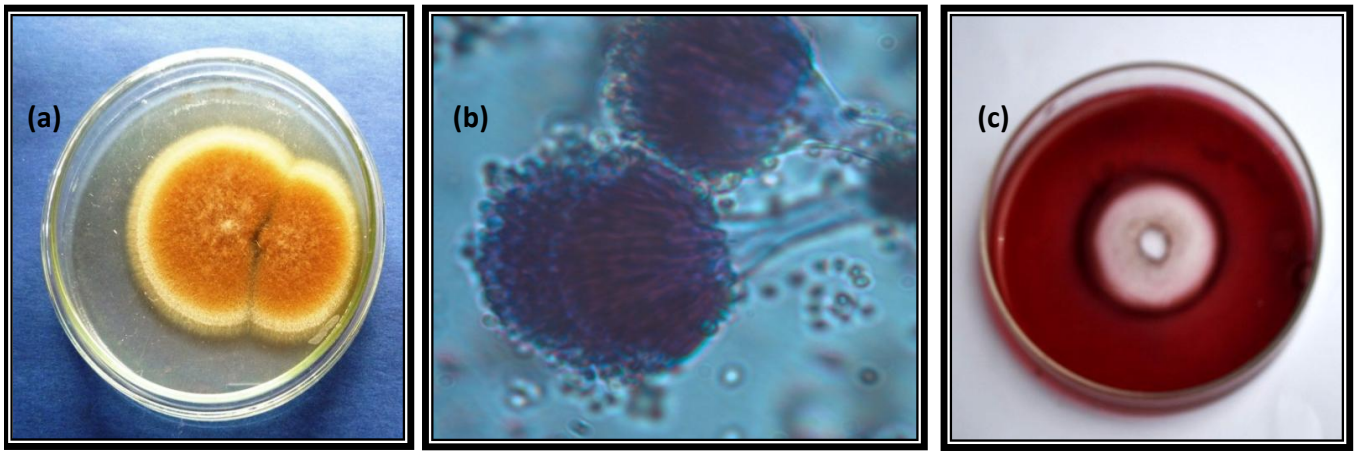

Figure 1. (a) Colony of *A. terreus* on PDA after 5 days of incubation at 28°C. (b) Microscopic view of asexual structure of *A. terreus* by Light microscope at 100X. (c) The cleared zone (indicated with arrow head) revealed cellulolytic ability of *A. terreus* on screening media.

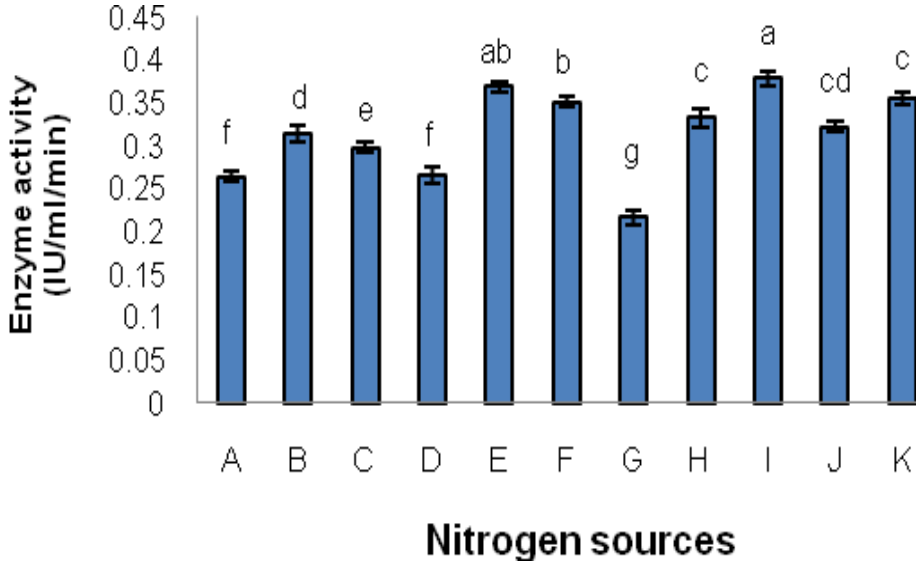

Figure 2. Effect of different nitrogen sources on cellulolytic activity of *A. terreus* in SSF on *S. spontaneum* at 30±2°C with 6 days of incubation. (A) Control; (B) Malt extract, (C) Peptone, (D) Tryptone, (E) Urea, (F) Yeast extract, (G) $NaNO_3$, (H) NH_4Cl, (I) $(NH_4)_2SO_4$, (J) $(NH_4)_2PO_4$, (K) NH_4NO_3. Verticals bars show standard error of means of three replicates. The values with different letters show the significant difference at (P≤0.05) as determined by DMRT.

DISCUSSION

The purified colony of *A. terreus* was identified on the basis of its morphological and microscopic characteristics. *A. terreus* emerged into rusty orange colonies with woolly texture (Figure 1a). Microscopic characters were examined at 100X magnification, which showed that it had hyaline hyphae with sub-spherical vesicle bearing long columnar, colorless, and biseriate conidiophores. Conidia were smooth walled and globose to sub-globose in shape (Figure 1b). Balajee (2009) while identifying the culture of *A. terreus*, observed the almost same characters as mentioned above. The indigenous agro-wastes that is, *S. spontaneum* was investigated for cellulase production by *A. terreus* in SSF. Environmental and nutritional parameters were optimized to enhance the production of cellulase by *A. terreus* on *S. spontaneum*. The nitrogen source preferred by *A. terreus* was 0.2%

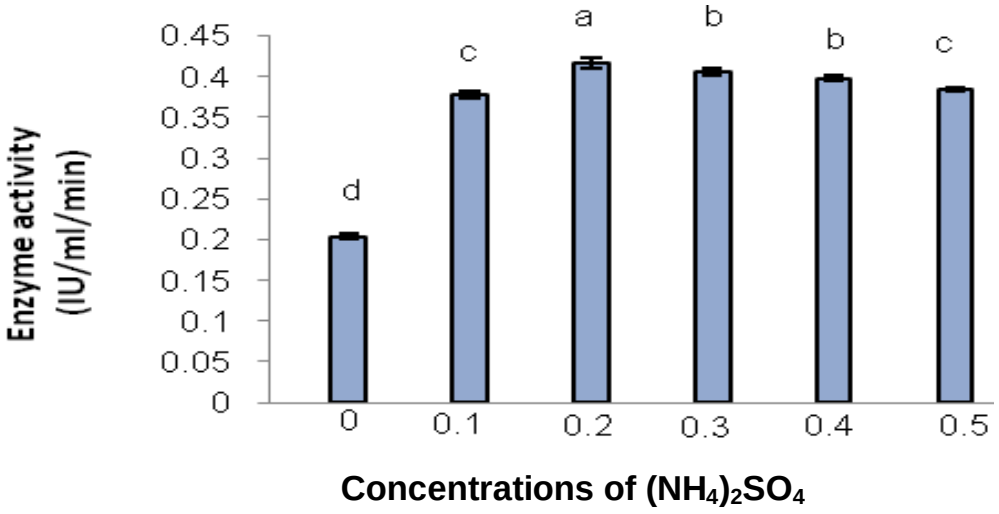

Figure 3. Effect of different concentrations of $(NH_4)_2SO_4$ on cellulolytic activity of *A. terreus* on *S. spontaneum* in SSF at 30±2°C with 6 days of incubation. Verticals bars show standard error of means of three replicates. The values with different letters show the significant difference at (P≤0.05) as determined by DMRT.

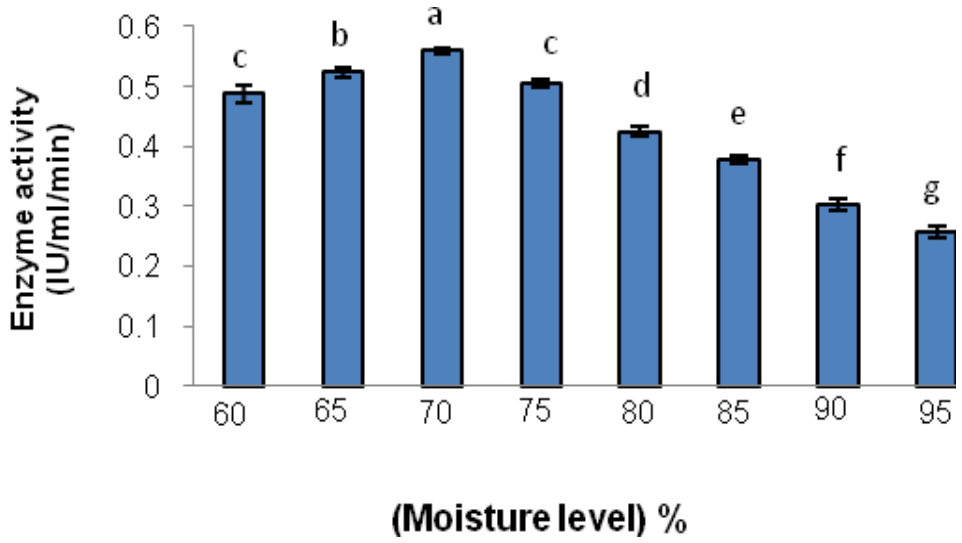

Figure 4. Effect of moisture level on cellulolytic activity of *A. terreus* on S*. spontaneum in* SSF at 30±2°C with 6 days of incubation. Verticals bars show standard error of means of three replicates. The values with different letters show the significant difference at (P≤0.05) as determined by DMRT.

ammonium sulphate and the results are similar to those reported by Vyas et al. (2005) using saw dust as the substrate; Hussain et al. (1999) reported similar results for *Arachniotus* species whereas Gao et al. (2008) reported 0.8% yeast extract for *A. terreus* on corn stover. The selection of nitrogen source greatly depends upon the nature of substrate and microorganism used to ferment it. Nitrogen is one of the major constituent of cellular proteins and stimulation of cellulose activity by ammonium salt might be due to their direct entry into protein synthesis (Mandels et al., 1975). The maximum cellulase production by *A. terreus* was achieved at 30±2°C, pH 4.5 and 70% of initial moisture with *S. spontaneum* at incubation of 96 h. Gao et al. (2008) reported the optimum cellulolytic activity of *A. terreus* on corn stover at 45°C, pH 3 and 80% moisture on the third day of inoculation. Vyas et al. (2005) optimized *A. terreus* on groundnut shells for cellulose production at 28±1°C,

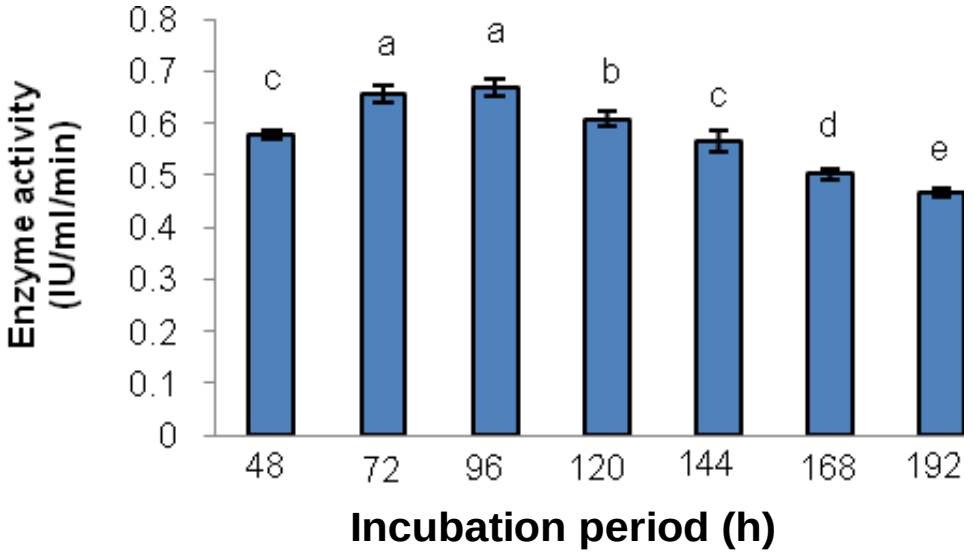

Figure 5. Effect of incubation period on cellulolytic activity of *A. terreus* on *S. spontaneum* in SSF at 30±2°C. Verticals bars show standard error of means of three replicates. The values with different letters show the significant difference at (P≤0.05) as determined by DMRT.

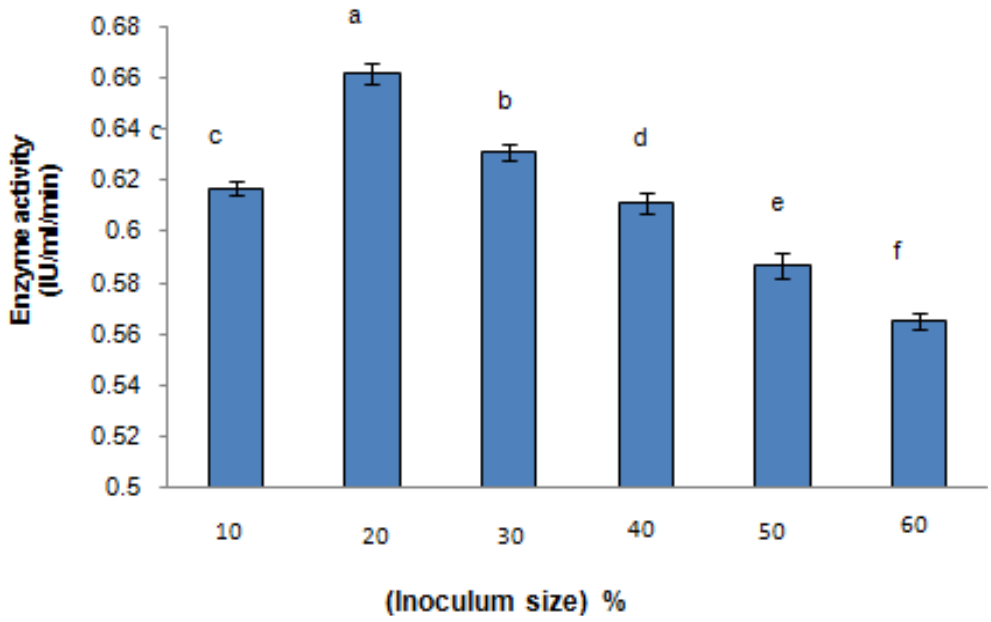

Figure 6. Effect of inoculum size on cellulolytic activity of *A. terreus* on *S. spontaneum* in SSF at 30±2°C. Verticals bars show standard error of means of three replicates. The values with different letters show the significant difference at (P≤0.05) as determined by DMRT.

pH 4 with 8 days of inocubation period. Ali et al. (1991) also reported maximum yield of cellulase from *A. terreus* at 40°C on water hyacinth after six days. The direct comparison of optimization processes would be very difficult because the afore-mentioned substrates on which SSF was carried out were different in their chemistry and nutritional values. However, the fermenting specie (*A. terreus*) was the same but was different isolates so they might have slight differences at genomic level.

Conclusion

The results in this study have shown that *S. spontaneum*

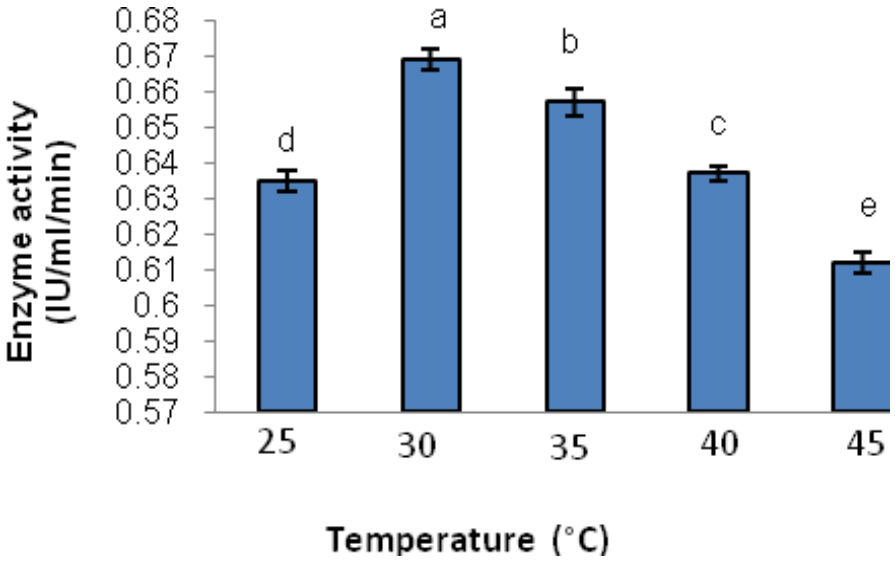

Figure 7. Effect of incubation temperature on cellulolytic activity of *A. terreus* on *S. spontaneum* in SSF with of incubation of 4 days. Verticals bars show standard error of means of three replicates. The values with different letters show the significant difference at (P≤0.05) as determined by DMRT.

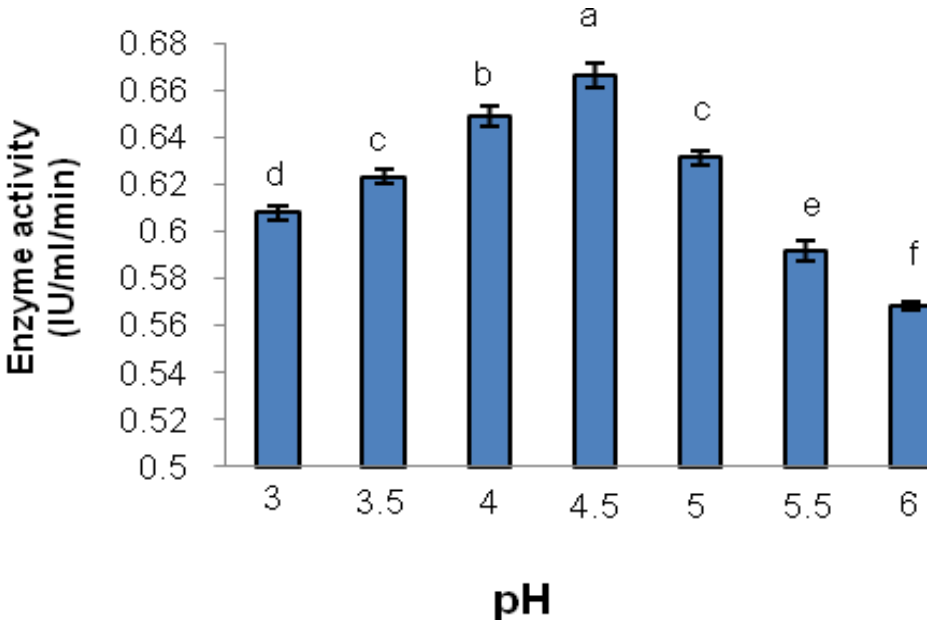

Figure 8. Effect of initial pH on cellulolytic activity of *A. terreus* on *S. spontaneum* in SSF at 30±2°C with 4 days of incubation. Verticals bars show standard error of means of three replicates. The values with different letters show the significant difference at (P≤0.05) as determined by DMRT.

can be effectively used as a substrate for cellulase production by cellulolytic *A. terreus* in SSF. Utilization of such indigenous agrowastes offers an additional reduction in the production cost of cellulases at commercial scale.

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