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Evaluation of extracellular cellulolytic potential of selected natural strains of a novel fungus *Sordaria fimicola* isolated from evolutionary canyon under submerged fermentation

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Abstract

Industrial biotechnology has a great emerging demand and sustainable expansion for mankind to utilize a variety of biodegradable material for the production of various alternative energy resources such as biogas and bioethanol. Researchers are interested in exploitation of novel fungal strains for the production of extracellular cellulase from the last few decades. This study was designed to assess the extracellular cellulase production potential of novel fungal strains of *Sordaria fimicola* first isolated from the evolution canyon, three located on South facing slope with xeric (S1, S2, S3) and other three on North facing slope with mesic (N5, N6, N7) environmental conditions. Based on initial and secondary screening two hyper producer strains from each slope were selected. The best activity for S2 was 3.125 U/ml and N6 exhibited 2.829 U/mL under optimized conditions of 14 d of incubation at 30 °C, pH 6.0, 1 mL inoculum and with 2% substrate (carboxy-methyl cellulose) concentration. Among the tested carbon and nitrogen sources, glucose proved to be best for both strains with S2 exhibiting maximum activity. Peptone and beef extract proved to be the best nitrogen sources for S2 and N6 respectively. The cellulase after characterization for temperature and pH showed slightly thermophilic nature. The cellulase was partial purification the highest cellulase activities as surviving in more xeric conditions which contribute more resistive and productive features in those microorganisms living in the harsh environment.

Introduction

Extracellular cellulases are the most important group of enzymes that hydrolyze lignocellulosic biomass to glucose subunits. Cellulases are composed of three main components: exoglucanases, endoglucanases, and β-glucosidases (Behera and Ray 2016). Hydrolytic cellulase converts lignocelluloses to sugars by the process of fermentation. Cellulose is composed of long polymers of β 1-4, linked glucose units and forms a proper crystalline structure. Cellulose, hemicellulose, and lignin are hydrolyzed by the complete action of extracellular cellulase (Fang and Xia 2015; Hansen *et al.* 2015). Enzymes isolated from microorganisms are gaining much attention due to their potential for various

industrial applications. Industrially, there are in great demand as compared to intracellular enzymes because of their cost-effectiveness as well as their high stability (Adrio and Demain 2014).
Many researchers are always interested in exploring the new potential fungal strains for the enhancement of cellulase production to complete the industrial enzyme demand in future. The enzymes isolated from the fungi are generally regarded as safe (GRAS) as compared to bacteria because they are cheap, easy to culture and more economical (Kumar 2020). The production of biofuel and bioethanol from lignocellulosic material is a potential application of cellulase which is widely used in internal combustion engines as a good substituent for gasoline (Kuhad *et al.* 2011; Gusakov and Sinitsyn 2012).
Sordaria fimicola is a well-known homothallic coprophilous fungus that regulates the nutrients in the herbivore dung. It was first isolated from the evolutionary canyon of Israel. Evolutionary canyon is considered as the model microsite to study the environmental changes and the adaptive behavior of the species (Pavlíček *et al.* 2008). A dramatic biotic contrast is displayed by the two opposite slopes which are 100 – 400 m apart from each other. African slope or South facing slope (SFS) exhibit more tropical conditions due to high solar radiation, high temperature, and more xeric conditions, more fluctuating and more heterogeneous, as they have Savannah-like biota. On the contrary, the European slope or the Nort facing slope (NFS) display a temperate environment, mesic conditions, lush green vegetation and maquis live-oak brushwood (Nevo 2006; Arif *et al.* 2017). *S. fimicola* is commonly found in animal dung and rotting vegetation, also present on wood, seed, and soil because of its slightly thermophilic nature. They are considered as the important control agents and a potential source to produce enzymes and antibiotics (Lamb *et al.* 1998).
The fermentation technology used has a significant impact on the success of cellulase production. A variety of fermentation techniques are available for cellulase production; however, the most used technologies are submerged and solid-state fermentation (Sajith *et al.* 2016). Initially enzymes were obtained from submerged fermentation due to easy handling and greater control of various physical and chemical environmental factors (Ahmed *et al.* 2017). On the contrary, SSF decreases the cost of enzyme production and improves the yield (Hareesh *et al.* 2016). But SSF is labor intensive and needs a longer lag time, large inoculum size due to which the current research was conducted on submerged fermentation (Gowthamana *et al.* 2001). The aim of this report is to study the extracellular cellulase production from *Sordaria fimicola* under SmF (submerged fermentation) by optimizing various physical and chemical parameters, characterization for pH and thermo-stability and partial purification by salting out and dialysis.

Experimental

Fungal sample collection and sub-culturing

Six different strains (S1, S2, S3, N5, N6, and N7) of *Sordaria fimicola* were collected from Fungal Culture Bank, University of the Punjab, Lahore, Pakistan. All the samples were made identified and stored at 4 °C before use. After sample collection, all the strains were sub-cultured immediately on potato dextrose agar. Fungal strains were inoculated in form of small discs in triplicates. All the cultures were incubated at 25 °C in incubator.

Screening of fungal samples for extracellular cellulase

Primary screening of cellulase was carried after the growth of all strains of *Sordaria fimicola*. The media provided for growth was prepared by adding 2 % carboxy-methyl Cellulose as a substrate with Potato Dextrose Agar and incubated with pH 5.5 at 25 °C for ten days. After ten days of incubation, all the plates were flooded with Congo red stain for 15 – 30 min. The plates were washed with distilled water and then 1M NaCl solution was added in all petri plates for 15 – 20 min. The zone of cellulase hydrolysis was clearly appeared around each colony of fungus. The zone of hydrolysis was measured in cm (Khokhar *et al*. 2012; Pavani *et al*. 2013).

Production of extracellular cellulase under submerged fermentation (SmF)

Potato Dextrose Broth was supplemented with 2 % CMC as substrate was used for cellulase production in submerged fermentation. Fungal isolates were inoculated in a 100 mL shake flask with 25 mL of Cellulase production medium (PDB and 2 % CMC). All the fungal isolates were inoculated with 1 mL of fungal spore suspension containing 10^5 spores. All the flasks were incubated in shaking incubator for 14 – 21 d in triplicates at 30 °C with the pH 5.0. The samples were withdrawn at regular intervals for determination of Enzyme Activity through Spectrophotometer at 540 nm (Sajith *et al.* 2016).

Optimization of fermentation cultural conditions and extracellular cellulase production

Different parameters were taken for the production optimization of extracellular cellulase such as temperature (20 °C, 30 °C, and 40 °C), pH (4.0, 5.0, and 6.0), incubation period (7, 14, and 21 days), inoculum concentration (spore suspension of 0.5 mL, 1 mL, 1.5 mL, 2 mL, 2.5 mL, and 3 mL), substrate concentration (0.5 %, 1 %, 2 %, 3 %, and 4 % CMC), carbon sources (glucose, maltose and lactose) and nitrogen source such as peptone, beef extract and ammonium sulphate). All the experiments were done in triplicates and values are with ± Standard deviation (Okoye *et al.* 2013; Saini *et al.* 2017).

Crude enzyme extraction

After optimization with each cultural condition, 25 mL of filtrate from all the samples was collected and centrifugation was performed at 6,000 rpm for 15 min. The supernatant was taken as crude enzyme and further used for enzyme assay. Biomass of mycelia was also calculated (Karthikeyan *et al.* 2011).

Cellulase activity by carboxy-methyl cellulase (CMCase) assay

DNS method was used for the determination of enzyme activity (Miller 1959). In this method, during the standard reaction conditions, 1 mL of crude enzyme was incubated with 1 mL of 1 % CMC as a substrate in 0.1M sodium acetate buffer for 30 min at 40 °C in water bath. The reaction was stopped by adding 3 mL DNS (dinitrosalicylic acid) reagent. Optical density was measured at 540 nm by spectrophotometer. One cellulase unit was determined as the amount of enzyme that releases 1 micromoles of glucose per minute (Martins *et al.* 2008).

Total protein estimation

Bradford Protein Assay method was used to determine the protein concentration for all samples of fungus in the crude enzyme. 1ml of crude enzyme was reacted with 2 mL of Bradford Reagent and the mixture was kept at room temperature for 45 min. Total protein of crude enzyme after optimization with each parameter was calculated at the absorbance of 595 nm (Bradford 1976).

Characterization of crude enzyme

The cellulase enzyme produced under optimized condition was characterized to check the optimum ranges of pH (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0) and temperature stability (30, 35, 40, 45, 50, 55, and 60 °C) (Hamdan and Jasim 2018).

Partial purification of crude cellulose

The crude enzyme was partially purified by salting out with ammonium sulfate and dialysis. The enzyme collected after salting out and dialysis was assessed for protein estimation and enzyme activity to check the folds of purification. Salting out was carried out by taking 20 mL of crude enzyme and brought to 80 % saturation with the solid ammonium sulfate.

The mixture was placed overnight at 4 °C in a magnetic stirrer. Centrifugation of the mixture was performed for 15 minutes at 6,000 – 8,000 rpm. The pellet formed was dissolved in 50 mM sodium acetate buffer having pH of 5.5. After salting out, the enzyme was subjected to determine the enzyme activity (Elakkiya and

Muralikrishnan 2014). The enzyme collected after ammonium sulfate precipitation was dialyzed in a dialyzing bag. 8 mL of enzyme was dialyzed against 30 Mm sodium acetate buffer with three different changes of buffer. The enzyme collected after dialysis was assessed for protein estimation and enzyme activity to check the folds of purification (Hafiz *et al*. 2011).

Statistical analysis

All the experimental work was carried out in triplicates. For statistical analyses of data Statistix program, version 8.1 was used, and data were analyzed for analysis of variance (ANOVA) using *LSD* $P \leq 0.05$. Mean values were separated ($P \leq 0.05$) and represented by different letters both in tables and figures along with ± standard error.

Results and Discussion

Primary screening of extracellular cellulase from fungal samples

The cellulolytic potential of fungal strains was observed for the primary screening of cellulases by Congo red method. A clear zone of hydrolysis was observed around each colony (Fig. 1). All the fungal strains show extracellular cellulose activity. Among S strains, S2 was observed as the hyper-producer and show the maximum zone of hydrolysis around its colony which was 4.81 cm. In the case of N strains, N6 shows the maximum zone of hydrolysis measured was 4.52 cm. It was concluded that both the strains have the potentiality for cellulase production. The results are similar with the findings of El-Nahrawy *et al*. (2017), they selected twenty isolates of *Aspergillus tubingensis*. All the isolates showed zone of hydrolysis ranges from 1.14 – 4.1 cm. The highest zone of hydrolysis was produced by F7 which was 4.1 cm.

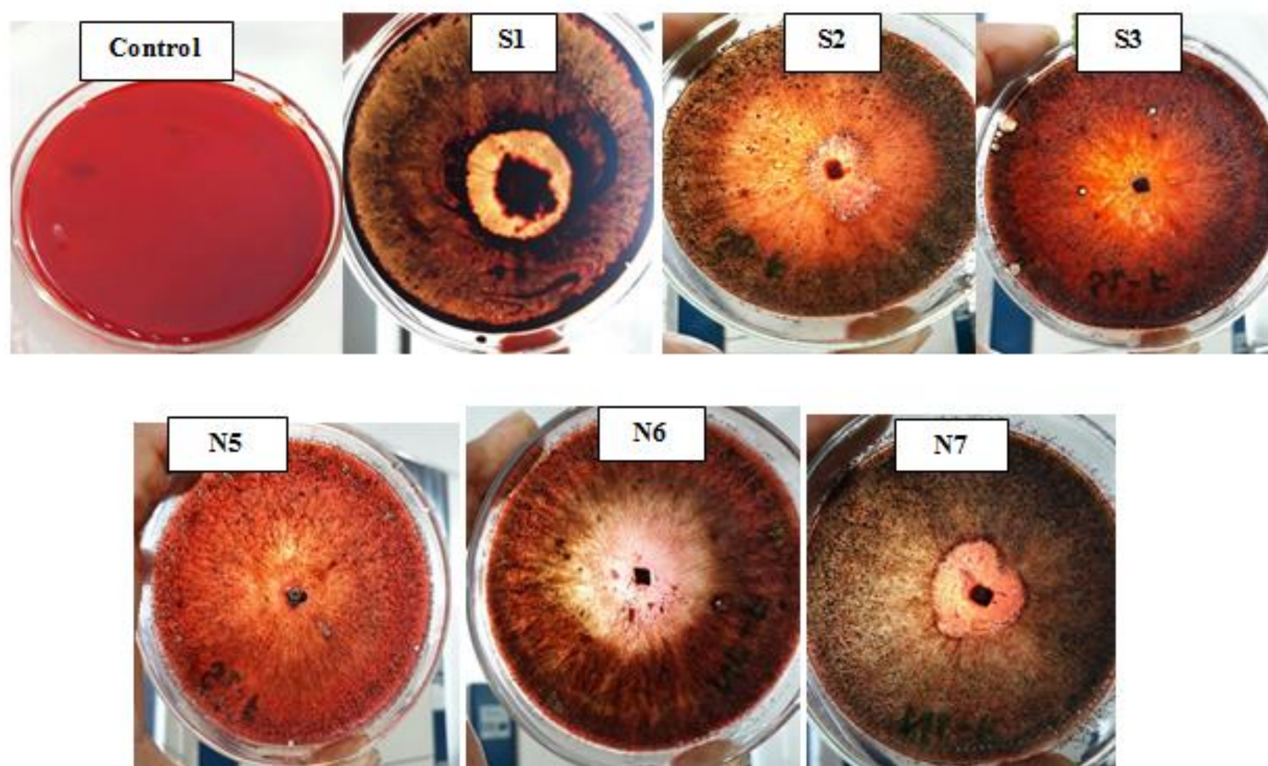

Fig. 1. Clear zone of hydrolysis (cm) shown by six different strains of *S. fimicola* in PDA containing CMC.

Secondary screening of six different fungal strains for extracellular cellulases under submerged fermentation

Submerged fermentation was chosen rather than solid state fermentation as the liquid medium is easy to handle and does not require extra labor work as in case of solid-state fermentation. Initially, all the strains were cultured in broth for fermentation. It was carried for only confirmation of results which were achieved by the primary screening of cellulase enzyme. The results showed in (Fig. 2) that among all the natural strains of *S. fimicola*, only S2 and N6 were observed as the hyper-producer celluloytic strains and the enzyme activities of S2 and N6 were 0.875 U/mL and 0.825 U/mL respectively and were selected for further investigations. Submerged fermentation is an aerobic fermentation process that provides a long culture period. During submerged fermentation adequate oxygen, cell growth and metabolism are maintained (Gomathi *et al*. 2012; Jiang *et al*. 2013).

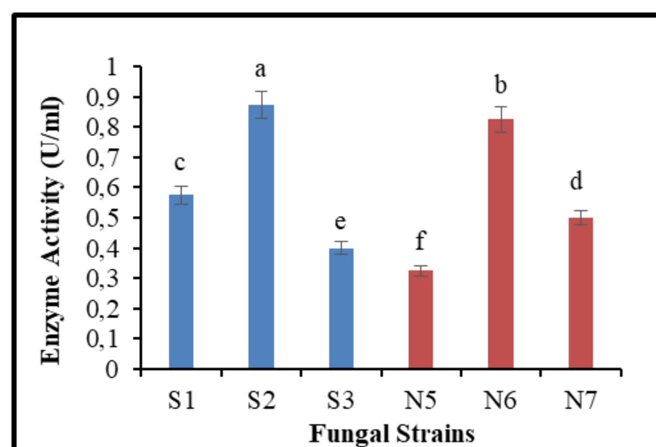

Fig. 2. Secondary screening for extracellular cellulase production from six different strains of *S. fimicola* by SmF. Means followed by different letters are significantly different according to ANOVA, LSD at $P \leq 0.05$ (Least significant difference).

Optimization of cultural conditions for cellulase production under submerged fermentation by two selected strains of S. fimicola

The enzyme production was optimized under different parameters such as pH, temperature, time of incubation, inoculum size, substrate concentration, carbon, and nitrogen sources.

Effect of different temperature on enzyme activity

Temperature is a crucial factor for the growth of microorganisms. Results showed (Fig. 3A) that the optimum temperature was 30 ºC for cellulase production in submerged fermentation for both the strains. S2 revealed maximum enzyme activity (0.95 U/mL) as compared to N6 (0.8 U/mL) because S2 survive in harsh conditions which will ultimately contribute to high yield. The results of the current study confirmed that *S. fimicola* is slightly thermophilic in nature. Findings of this study agree with Irfan *et al.* (2016) and Kumar *et al.* (2012) who reported 30 ºC optimum temperature for endoglucanase production. Higher temperature ranges from 50 to 60 ºC causes denaturation of the enzyme. The optimum temperature for cellulase production also depends on the strain variation (Murao *et al*. 1988).

Effect of different pH on enzyme activity

The effect of pH was determined for cellulase production, and the highest enzyme activity was shown by S2 (1.175 U/mL) and N6 (1.025 U/ mL) at pH 6.0 (Fig. 3B). Among S2 and N6, S2 revealed the highest enzyme production than that of N6. Similar findings were reported that initial medium pH of 6 is suitable for maximum cellulase production (Juhasz *et al* 2004; Akinola *et al* 2012). Extreme low and high pH cause instability of enzyme as they are protein which generally denatured at very extreme pH (Nidetzky *et al.* 1998).

Effect of incubation period on enzyme activity

The incubation period was determined by checking the enzyme activity from 3 to 21 d. The optimum production for both the strains showed the ideal incubation period was 14 d (Fig. 3C). S2 showed more enzyme activity (1.15 U/ mL) as compared to N6 (0.975 U/ mL) after 14 days of incubation. According to El-Hadi *at al.* (2014), the ideal incubation period for production of CMCase from *Aspergillus hortai was* 96 h under submerged fermentation. But in case of *S. fimicola,* it completes its life cycle in 10 d of incubation.

Therefore, the incubation of *S. fimicola* is different from *A. hortai* to produce extracellular cellulase enzyme.

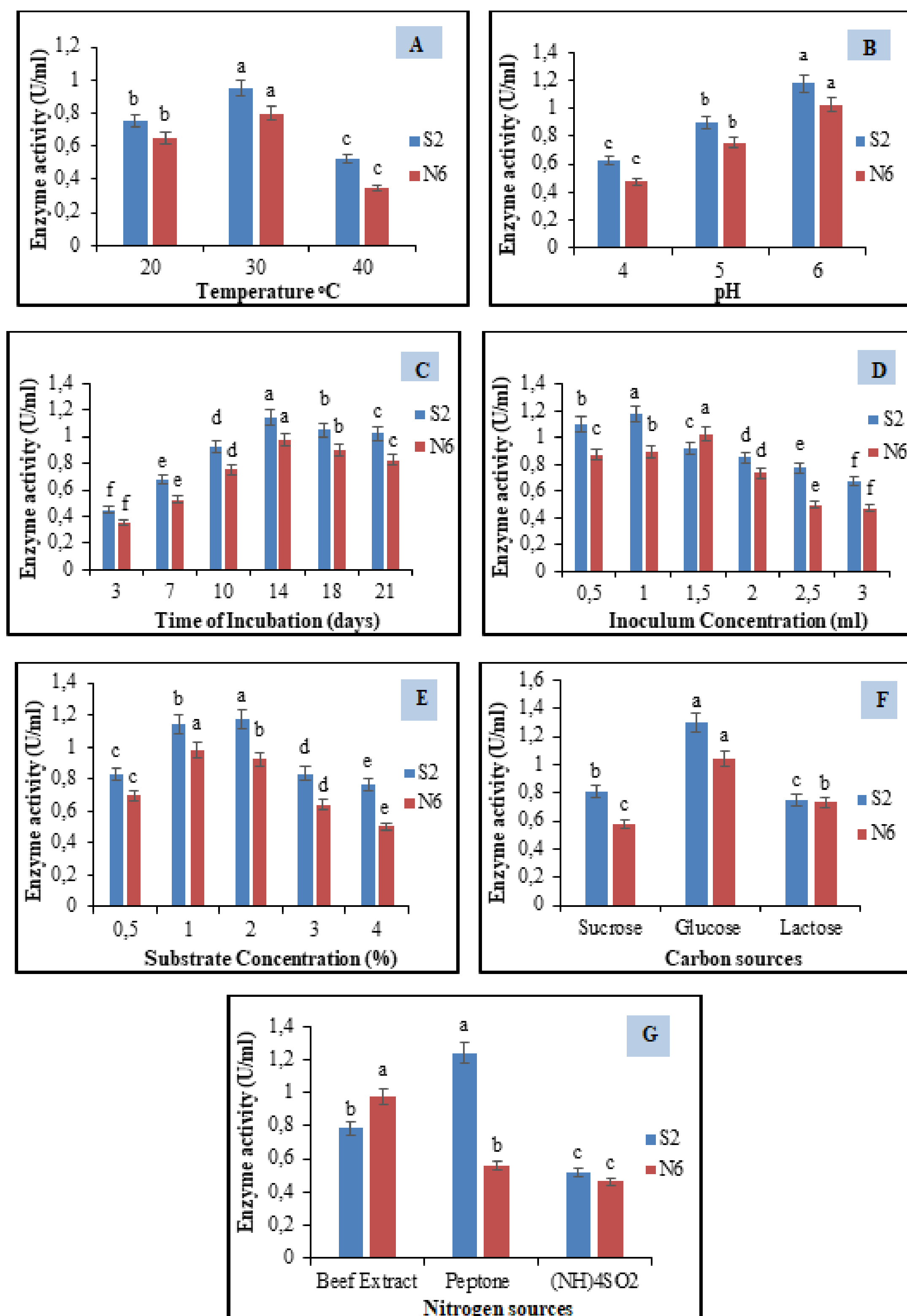

Fig. 3. Optimization of cultural conditions **(A)** Effect of different temperature, **(B)** Effect of different pH, **(C)** Effect of different Incubation period, **(D)** Effect of different Inoculum concentrations, **(E)** Effect of different substrate (CMC) concentrations **(F)** Effect of Carbon sources **(G)** Effect of Nitrogen sources. Means followed by different letters are significantly different according to ANOVA, LSD at $P \leq 0.05$ (Least significant difference).

Effect of inoculum concentration on enzyme activity

Inoculum of 1ml in both strains of fungus yields more enzyme production. S2 showed more enzyme activity as 1.175 U/mL as compared to N6 as 0.9 U/mL (Fig. 3D). Enzyme activities of both strains decrease until 3ml of inoculum due to the decrease in microbial growth. Microbial growth decreases due to competition for space and nutrient among the cells which causes nutrient depletion (Omojasola *et al.* 2008). Decreased inoculum size may not be enough for the growth initiation of the species which directly affects the enzyme production. Irfan *et al.* (2011) reported that *Aspergillus niger* showed highest CMCase production with 3 % inoculum level.

Effect of substrate concentration on enzyme activity

Cellulase activity was observed at different substrate concentrations (Fig. 3E) and it was observed that 2 % CMC act as the ideal substrate concentration for both the strains of *S. fimicola* for the highest production of extracellular cellulase. S2 showed maximum enzyme activity (1.174 U/mL) than that of N6 (0.983 U/ mL) at 2 % substrate concentration. Similar results were reported from the findings of Irfan *et al*. (2010). It reduces after a certain percentage of the substrate due to extra availability of nutrients and increased biomass and as a result, minimization in the metabolic activity. Supplementation of excessive substrate concentration causes high viscosity and decreases the probability of the substrate to bind with the active side of the cellulase enzyme (Nagah *et al*. 2016).

Effect of carbon source on enzyme activity

The effect of carbon sources was studied by adding 2 % of three different carbon sources namely, sucrose, glucose, and lactose into the culture medium. Among these the glucose proved to be the best carbon source (Fig. 3F) resulted in producing high yield for the enzyme in S2 (1.296 U/ mL) and for N6 (1.042 U/ mL). The other two carbon sources cause a reduction in enzyme activity. Similar results were obtained by the findings of Nathan *at* al. (2014) that only the glucose results in maximum cellulase yield as compared to the other carbon sources. Irfan *et al.* (2016) reported that fermentation medium supplemented with glucose results in high yielded cellulases from *Trichoderma harzianum.*

Effect of nitrogen source on enzyme activity

Effect of nitrogen source on cellulase activity revealed (Fig. 3G) that highest production of cellulase was attained by adding peptone in culture medium in case of S2 (1.241 U/mL) and beef extract in case of N6 (0.978 U/mL). Ammonium sulfate is considered as the lowest enzyme-producing nitrogen source in case of both the fungal strains. The results are in accordance with the work of Acharya *et al.* (2008) who reported that the maximum yield of cellulase is produced by adding organic solvents such as peptone and yeast extract in the medium. Prasanna *et al.* (2016) and Kathiresan and Manivannan (2010) investigated that *Penicillium sp* produced maximum cellulase enzyme when cultivated in the liquid broth containing yeast extract.

Characterization of cellulase

Crude cellulase enzyme produced under optimized conditions was also characterized for pH and temperature stability. The results revealed that it has the pH ranged from 4.5 to 5.5 (Fig. 4A) and temperature optima ranged from 40 to 50°C (Fig. 4B). Several studies have reported that endoglucanases remain active at the temperature ranges from 50 – 70 °C (Bai *et al.* 2013; Boonchuay *et al.* 2016). For example, endoglucanase from *A. niger Z10* shows thermal stability at 40 °C (Coral *et al.* 2002). Karboune *et al.* (2008) reported the optimum temperature of 65 °C for CMCase from *Penicillium funiculsoum.* The thermostability and pH stability of cellulase production also depend upon the strain variation of the microorganism. Rahnama *et al.* (2016) revealed that *Trichoderma harzinum* has maximum enzyme stability at pH of 4.5. Picart *et al.* (2007) reported the optimum pH of 4.5 for

carboxymethyl cellulase from *Penicillium* sp.

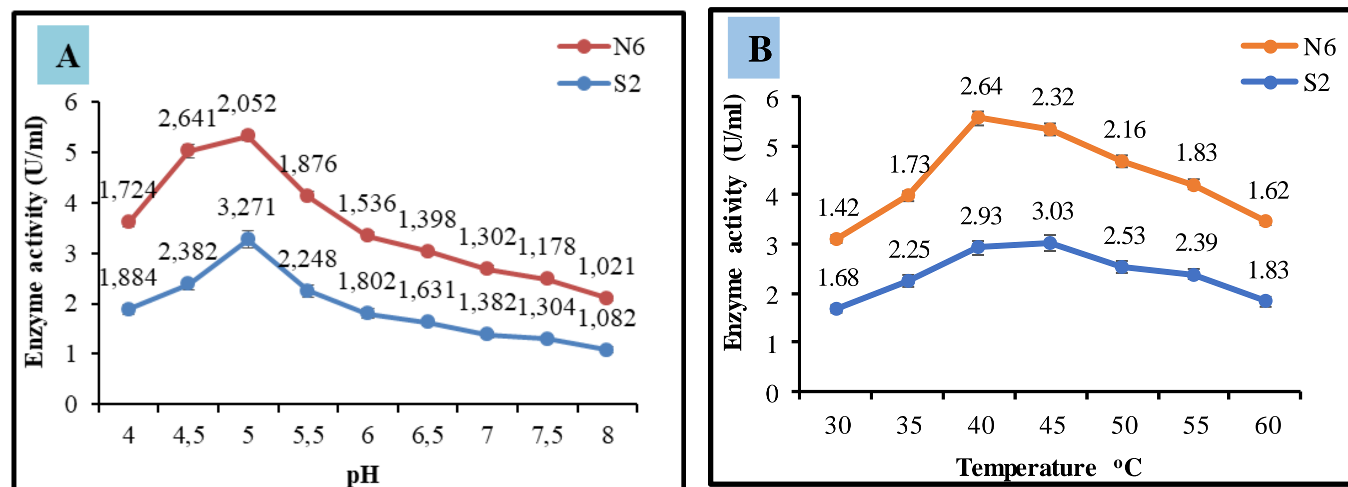

Fig. 4. Characterization of cellulose. (**A**) Enzyme stability at different pH. (**B**) Enzyme stability at different temperature. Means are significantly different according to ANOVA, LSD at $P \leq 0.05$ (Least significant difference).

Partial purification of crude cellulase

Cellulase produced under optimized cultural conditions was partially purified. From the results (Table 1), it was observed that the purification fold increases 2.7 folds in case of S2 and 1.5 folds in N6 with yield 80.2 % and 79 % respectively after dialysis. Jabbar *et al.* (2008) purified endoglucanases from *Gymnoascella citrina* by gel filtration chromatography and the result revealed that purification fold increases 27.3 folds with 25.5 % yield.

Sulyman *at al.* (2020) confirmed that the purification of cellulase from *Aspergillus niger* increases 68.1 folds when crude enzyme was treated with Sephadex G-100 gel filtration.

Table 1. Different enzyme activities and specific activities of crude enzyme, enzyme after partial purification were estimated and their folds of purification were also determined.

Purification step	Enzyme activity [U/ml]		Specific Activity [U/mg]		Yield [%]		Purification folds	
	S2	N6	S2	N6	S2	N6	S2	N6
Crude enzyme	3.03	1.88	1.07	1.16	100	100	1	1
Salting out	2.78	1.67	2.32	1.39	91	88	2.2	1.2
Dialysis	2.43	1.49	2.86	1.72	80.2	79	2.7	1.5

Conclusion

The researchers are interested in isolating novel fungal strains and exploring their potential to produce extracellular enzymes at a low cost to complete the industrial enzyme demand in future. The conducted research study demonstrates the first-time production of cellulase enzyme from *S. fimicola*. It was accomplished that S2 strain resulted in maximum cellulose production under optimized conditions by using submerged fermentation technique because it developed greater resistivity due to more harsh conditions. The biomass of both strains increased up to 14 days on incubation. After that, it starts decreasing due to nutrient depletion. The enzyme shows a slightly thermophilic and acidic nature when characterized for pH stability and thermo-stability. The partially purified enzyme shows purification folds greater than that of crude enzyme. The results showed that *S. fimicola* is a good potential fungal strain and can be exploited on an industrial scale to production second-

generation biofuel by degrading agro-industrial wastes or lignocellulosic biomass at a low cost.

Conflict of Interest

The authors declare that they have no conflict of interest.

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