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Optimization of Cellulase Production by *Chaetomium thermophilum* in Submerged Fermentation using Wheat Straw as Substrate

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The study conception and design involved the input from all authors. Conceptualization: QA. Data collection and analysis was performed by SA and FJ. First draft was written by SA. Writing, review and editing QA. The final manuscript was read and approved by all writers.

Key words
Cellulases, *Chaetomium thermophilum*, Lignocellulosic biomass, Submerged fermentation, Wheat straw

ABSTRACT

This study was carried out to produce thermally stable, economically viable and industrially important cellulase enzymes by using a thermophilic fungus i.e., *Chaetomium thermophilum*, which can efficiently produce cellulases extracellularly while growing on cellulosic substrates. Wheat straw, by product of wheat crop has been used as lignocellulosic substrate. To determine the optimum conditions for maximum production, experiment was carried out at various substrate concentrations, temperatures and pH levels. Pretreatment of wheat straw resulted in highly digestible cellulose that lead to the increased production of cellulases. Highest glucose (1.92±0.14 mg/ml), carboxymethyl cellulase (7.33±0.16 IU/ml/min) and filter paperase (10.61±0.15 IU/ml/min) activities observed in pretreated wheat straw, while untreated gave 1.89±0.19 mg/ml, 4.31±0.13 IU/ml/min and 7.88±0.18 IU/ml/min respectively. Seventy percent increase in CMC-ase and 35% increase in FP-ase activities were observed while using alkali pretreated wheat straw as compared to untreated straw. During incubation, pH 7 and 60°C temperature was found optimum for highest enzyme activities.

INTRODUCTION

Lignocellulosic biomass is the most abundant, inexpensive and renewable resource on the earth, made up of cellulose, hemicellulose, and lignin components (Liu *et al*., 2019) constantly replenished by photosynthesis. Wheat straw is byproduct of wheat farming, generated all over the world as a result of wheat growing and is a promising lignocellulosic biomass for production of renewable products. Only a small portion of wheat straw was employed as animal feed, fuel and farmyard manure, while the majority of it was thrown away or burned directly in fields, resulting in pollution (Yasin *et al*., 2010). Wheat straw contain 38- 45% cellulose, 24-40% hemicelluloses and 10-25% lignin (Sun and Tomkinson, 2005; Yasin *et al*., 2010; Trubetskaya *et al*., 2016). Cellulolytic enzymes can convert the cellulose content of lignocellulosic biomass into value-added products such fermentable sugars, bioethanol, and organic acid (Ahorsu *et al*., 2018; Jiménez-Quero *et al*., 2020). Fungi have been extensively used for the production of cellulolytic enzyme in submerged fermentation (Adsul *et al*., 2007). *Chaetomium thermophilum*, a thermophilic fungus, have high hydrolytic activity, secrete cellulase enzymes such as exoglucanases, endoglucanases and β-glucosidases while degrading lignocellulosic substrates (Payne *et al*., 2015). *C. thermophilum* has a potential to secrete higher concentration of cellulose degrading enzymes as compared to other ascomycetes (Li *et al*., 2020).

Due to lignin's cross-linking and bonding, cellulose and hemicellulose are less available to the enzymes for breakdown. Numerous pretreatment techniques have been investigated to facilitate the enzymatic hydrolysis of lignocellulosic materials (Galbe and Zaachi, 2007; Ibrahim *et al*., 2011). Alkali pretreatment involves in alteration of lignin structure, breaks the bond between lignin and partially dissolves hemicellulose, and partially decrystallizes cellulose (Badiei *et al*., 2014), resulting in increases the accessibility of cellulose and hemicellulose to enzymes.

In the present study *C. thermophilum* has been used to produce cost-effective cellulase enzymes in submerged

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fermentation using wheat straw as the lignocellulosic substrate. To find out the optimum conditions for highest enzyme activities, production of cellulases were compared at varied substrate concentrations, temperatures and pH levels.

MATERIALS AND METHODS

Fungal strain

Chaetomium thermophilum ATCC 28076 was used throughout the study, culture was revived on PDA and Vogal's media slants and plates (Rajoka *et al*., 2004).

Lignocellulosic substrate and pretreatment

Wheat straw was employed as a substrate and was gathered from the agricultural fields of University of the Punjab, Lahore. It was ground to a particle size of ~2 mm after being dried at 50°C for overnight. It was pretreated by immersing it in a solution of 1% sodium hydroxide (1:10, w/v) for 2 h at ambient temperature, autoclaved for 30 min at 121°C and 15lb/inch² (Han *et al*., 2012). After that, the substrate was filtered and rinsed with distilled water until the wash water is neutralised, then dried at 50°C and stored in polythene bag for subsequent use.

Screening

Caboxymethylcellulose-agar (CMC-Agar) medium was employed for plate screening. Conidia were softly scratched with wire loop and suspended in sterile distilled water from a one-week-old PDA slant. In the middle of the plates, a small well was made and 100 µl of conidia suspension was injected in each well. For two days, plates were incubated at 28°C. To observe cellulolytic activity, plates were stained by using 1% Congo red dye for 0.5-1 h, then destained with saline (1 M NaCl) solution for 15 min (Onsori *et al*., 2005).

Fermentation process

In their respective flasks, 2g pretreated and untreated substrates were added in 100 ml vogal's medium salt solution (Trisodium citrate, 0.25g; NH_4NO_3, 0.20g; $(NH_4)_2SO_4$, 0.40g; KH_2HPO_4, 0.50; $MgSO_4$. $7H_2O$, 0.02g; yeast extract, 0.20g and Peptone, 0.10g), respectively, having pH 5.5 and autoclaved for 30 min at 121°C and 15lb/inch² pressure. On cooling 200µl of *C. thermophilum* spore suspension was inoculated in each flask and incubated for 6 days on shaker at 40°C temperature. After every 24 h, samples were taken from each flask using a micropipette to extract 1ml from each flask under aseptic conditions. The samples were utilised to test for glucose and cellulase activities. Experiment was conducted in triplicate.

Fungal biomass

Through filtration fungal biomass was removed from the medium, which was subsequently dried at 50°C in an oven till the weight become constant and then quantified. The dry weight of the mycelial mat (mg/100 ml) was used to measure fungal growth (Narashima *et al*., 2006).

Enzyme assay

A standard curve was prepared by taking 1 ml of different concentrations of glucose (100µg, 200µg, 300µg, 400µg, 500µg, 600µg and 700µg per ml) in test tubes along with 3 mL of 3,5-dinitrosalicylic acid (DNS) and boiled for ten min in a water bath. The absorbance was measured using a spectrophotometer at 540 nm after cooling and standard curve was prepared.

To measure carboxymethyl cellulase (CMC-ase) activity, 0.5 ml of each sample and substrate was taken in test tubes. After 30 min of incubation at 50°C, the absorbance was measured at 540 nm after addition of 3ml of DNS and heated in a water bath for 10 min (Ariffin *et al*., 2006). To determine filter paper cellulase (FP-ase) activity of enzyme, filter paper strip of size 1x6cm was used as described by Vijayaraghavan and Vincent (2012). The amount of enzyme capable of releasing 1 µ mol of reducing sugars per minute is defined as one international unit (IU) of enzymatic activity (Hua *et al*., 2018). Each experiment was performed in triplicate.

To find out how varying substrate concentrations affect enzyme activity, different substrate concentrations were prepared by dissolving 0.5g, 1.0g, 1.5g, 2.0g, 2.5g, 3.0g, 3.5g, and 4.0g CMC in citrate buffer at pH 5.5, incubated with sample, respectively and enzyme activities were measured as discussed above.

The effect of pH and temperature on the activity of crude enzymes was examined. To find the optimal pH of enzyme, sample was incubated with acetate buffers pH 3-5 and sodium phosphate buffers of pH ranging from 6-9 and activity was estimated by using DNS assay. Enzyme's thermostability was determined by incubating it at temperatures ranging from 30 to 90 degrees Celsius. The ratio of residual activity to initial activity levels was used to calculate thermostability (Chen *et al*., 2018).

RESULTS AND DISCUSSION

Cellulase is one of the most commonly used industrial enzyme, that has been used in the textile, paper, pharmaceutical, brewing, food and chemical sectors (Sukumaran *et al*., 2005). To minimize the production cost of cellulases, lignocellulosic substrates were employed as they are cheaper and renewable sources than synthetic cellulose.

Table I. Activities on pretreated and untreated wheat straw.

Fermentation period (days)	Glucose activity (mg/ml)		CMC-ase activity (IU/ml/min)		FP-ase activity (IU/ml/min)	
	Pretreated	Untreated	Pretreated	Untreated	Pretreated	Untreated
1	$0.87^{d}\pm0.12$	$0.91^{c}\pm0.16$	$1.56^{e}\pm0.11$	$2.07^{e}\pm0.17$	$2.08^{e}\pm0.13$	$1.91^{e}\pm0.14$
2	$1.24^{cd}\pm0.21$	$0.95^{bc}\pm0.11$	$3.55^{c}\pm0.20$	$3.24^{c}\pm0.20$	$4.07^{c}\pm0.12$	$4.04^{de}\pm0.20$
3	$1.54^{b}\pm0.17$	$1.19^{a}\pm0.08$	$7.33^{a}\pm0.41$	$4.31^{a}\pm0.16$	$10.61^{a}\pm0.41$	$4.23^{d}\pm0.17$
4	$1.93^{a}\pm0.16$	$1.15^{a}\pm0.17$	$5.15^{b}\pm0.43$	$3.61^{b}\pm0.25$	$9.78^{b}\pm0.20$	$7.88^{a}\pm0.29$
5	$1.39^{c}\pm0.15$	$1.10^{b}\pm0.16$	$2.81^{d}\pm0.16$	$2.55^{d}\pm0.12$	$4.11^{c}\pm0.33$	$7.23^{b}\pm0.20$
6	$0.31^{e}\pm0.02$	$0.60^{d}\pm0.12$	$1.80^{e}\pm0.10$	$1.04^{f}\pm0.02$	$3.02^{d}\pm0.18$	$6.64^{c}\pm0.16$
$LSD_{0.05}$	0.16	0.17	0.34	0.21	0.32	0.25
CV(%)	1.18	1.70	0.89	0.74	0.55	0.46

CMCase, carboxymethyl cellulase; FPase, filter paperase..

In the present study pretreated and untreated wheat straw was used as substrate in submerged fermentation by using *C. thermophilum* to evaluate CMC-ase and FP-ase activities (Table I). Activities were measured daily for six consecutive days. Maximum cellulase activity 4.31 IU/ml/min was observed at 3rd day whereas high FP-ase activity 7.88 IU/ml/min at 4th day in untreated wheat straw while in case of pretreated wheat straw highest CMC-ase activity was 7.33 IU/ml/min and 10.61 IU/ml/min FP-ase activity respectively on 3rd day of fermentation. There was a 70% increase in CMC-ase activity and a 35% rise in FP-ase activity while using alkali pretreated wheat straw as compared to untreated straw. Steffien *et al*. (2014), observed five h earlier and better enzymatic hydrolysis in pretreated substrate as compared to non-treated wheat straw. Han *et al*. (2012), found that enzymatic hydrolysis of wheat straw was strongly impacted by the substrate grinding and sodium hydroxide (NaOH) pretreatment, leading to an increase in cellulose content of 45% and a decrease in hemicellulose and lignin content of 44% and 42%, respectively. They observed a 52% increase in carboxymethyl cellulase activity and a 74% increase in filter paper activity when contrasting the treated substrate to the untreated one.

Highest mycelial weight 559 g/100ml was noted at pH 6.84 (Table II) on the 3rd day in pretreated wheat straw after that decline in the weight was noted. Muthuvelayudham and Viruthagiri (2006) reported the same findings and hypothesised that that this drop in mycelial weight may be due to the reason that substrate levels fall below the concentration required to support cell maintenance, resulting in cell death and lysis. Alkali pre-treated substrates showed better results as compared to untreated wheat straw.

To investigate the effect of temperature on enzyme activity, samples were incubated with substrate at temperatures ranging from 30°C-90°C. Forty three percent increase in CMC-ase activity (10.53 IU/ml/min) and 15% increase in FP-ase activity (12.1 IU/ml/min) was observed at 60°C (Fig. 1A). These findings are in line with Li *et al*. (2003) who reported that optimum temperature for production of endocellulases by using *C. thermophilum* was 60°C. Kellner *et al*. (2016), determined 50°C as optimum temperature for growth of *C. thermophilum* whereas Jiang *et al*. (2020) reported maximum cellulase activity at 65°C by using *C. thermophilum*.

Table II. Changes in pH and mycelial weight during fermentation period.

Fermentation period (days)	pH		Dry mycelial wt. (g/100ml)	
	Pretreated	Untreated	Pretreated	Untreated
1	$5.16^{c}\pm0.16$	$5.2^{d}\pm0.17$	$290^{c}\pm2.62$	$93.8^{d}\pm2.86$
2	$5.85^{b}\pm0.12$	$5.62^{c}\pm0.20$	$332^{c}\pm6.68$	$165^{c}\pm4.18$
3	$6.84^{a}\pm0.17$	$5.93^{b}\pm0.12$	$559^{a}\pm3.32$	$231^{a}\pm8.16$
4	$5.71^{b}\pm0.24$	$6.38^{a}\pm0.36$	$340^{b}\pm7.58$	$199^{b}\pm6.68$
5	$4.92^{cd}\pm0.28$	$5.17^{d}\pm0.24$	$224^{d}\pm4.11$	$31.7^{e}\pm2.44$
6	$4.52^{d}\pm0.29$	$4.61^{e}\pm0.20$	$71^{e}\pm6.2$	$19^{f}\pm2.0$
$LSD_{0.05}$	0.27	0.29	6.75	6.18
CV(%)	0.48	0.51	0.21	0.49

Figure 1B illustrate the effect of different pH on enzyme activities. Maximum CMC-ase and FP-ase activities were observed at pH 7. Further increase in pH resulted in decrease in the enzyme activities. This may be due to the denaturation of enzymes. Kapoor *et al*. (2010) found that *C. thermophilum* produce cellulases best at pH 6.5. Coral *et al*. (2002) observed that *Aspergillus niger* (Z10, wild type strain) showed maximum CMCase activity

at pH 4.5 and pH 7.5 among the tested pH range 4.0 to 9.0.

Dose response of cellulose supplementation within a range of 0.5-4 g on cellulase production of *C. thermophilum* was examined (Fig. 1C). Increase in CMCase activity observed by increasing concentration of substrate upto 3g in case of pretreated straw whereas maximum CMCase activity observed at 3.5 g substrate in case of untreated straw.

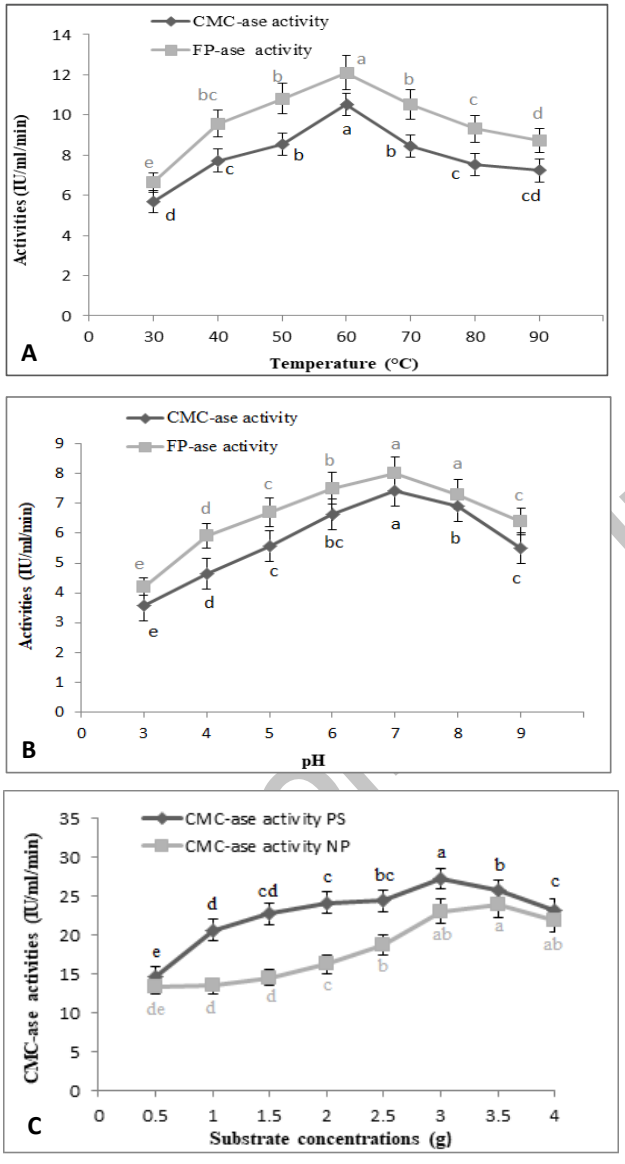

Fig. 1. Effect of temperature (A), pH (B), and substrate concentration (C) on CMC-ase activity.

The result clearly showed that the supplementation of cellulose of 3-3.5g was optimum for cellulase production. In contrast, Narasimha *et al.* (2006) found maximum cellulase activity at seventh day in medium supplemented with 1% synthetic cellulose while studying the nutrient effect on production of cellulolytic enzymes by *Aspergillus niger*. Decrease in enzymatic activity beyond the maximal substrate concentration may be blame to certain inhibitors. According to Hsieh *et al.* (2015), cellulases are known to be inhibited by the buildup of glucose, cellobiose and other monomers, the byproducts of hydrolysis. Further scientific investigations are needed to understand these aspects during fermentation.

CONCLUSION

This study provides a sustainable solution for producing cost effective cellulases in submerged fermentation by using wheat straw and thermophilic fungus *C. thermophilum*. It has been found that *C. thermophilum* can efficiently degrade wheat straw to produce cellulases. A pH of 7 and a temperature of about 60°C were determined to be the best conditions for maximum activity, while a substrate concentration of 3% was shown to be best for maximum enzyme activity. Alkali pretreatment of substrate yield better outcomes as compared to without pretreatment. This new understanding of cellulose degradation could lead to the more effective exploration of biomass resources in thermophilic conditions.

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Statement of conflict of interest

The authors have declared no conflict of interest to declare.

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