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Cotton Saccharifying Activity of Cellulases by *Trichoderma harzianum* UM-11 in Shake Flask

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Abstract: The present study deals with the optimization of cultural conditions for Cotton Saccharifying Activity (CSA) of cellulases by *Trichoderma harzianum* UM-11 using shake flask technique in 250 mL Erlenmeyer flasks. Among the different fermentation media tested, Carboxymethyl Cellulose (CMC) with Mineral Salts Solution (MSS) gave the best CSA. Cotton saccharification was optimal when 25 mL of the medium was incubated at 28°C for 96 h. The maximum CSA (0.528 U/mL/h) was found at initial pH 6.0. The level of inoculum was optimized at 5.0×10^7 conidia/25 mL medium.

Key words: Cellulases, CSA, *Trichoderma harzianum*

INTRODUCTION

Saccharification of the cellulosic materials can be achieved chemically, enzymatically, or by the combination of both. Enzymatic saccharification is more economical and favourable[1,2]. Microbial cellulases are multienzyme complexes composed of several acidic proteins with significant carbohydrate content[3]. Cellulases consist of three major components: endo-β-glucanase (EC 3.2.1.4), exo-β-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). These enzymes act together synergistically to convert crystalline cellulose to oligosaccharides and glucose. Endo-β–glucanase (CMCase) causes random scission of cellulose chains yielding glucose and cello-oligosaccharides. Exo-β-glucanase (avicelase) attacks on the non-reducing end of cellulase yielding cellobiose. β-glucosidase (cellobiase) hydrolyses aryl- and alkyl-glycosides as well as cellobiose and cellodextrin[4-6].

Cultural conditions and their optimization such as carbon sources, cultivation time, temperature, pH and inoculum size are essential as these affect the microbial growth and subsequent product formation[7]. Different carbon sources such as wheat bran, wheat straw, rice husk, newspaper, paper waste and bagasse have been studied for the induction and biosynthesis of cellulases. However, crystalline cellulose is a superior carbon source for induction of cellulases in fungi than amorphous or impure forms[8]. Cotton Saccharifying Activity (CSA) determines the synergistic effect of all the major components of microbial cellulases for the hydrolysis of cellulose. The present study is concerned with the determination and optimization of cultural conditions for cotton saccharifying activity of cellulases produced by *Trichoderma harzianum* UM-11 using shake flask technique.

MATERIALS AND METHODS

Organism and culture maintenance: The strain of *Trichoderma harzianum* UM-11 was obtained from stock culture of Biotechnology Research Centre, Department of Botany, G.C. University, Lahore and culture was maintained on sterilized potato dextrose agar medium, pH 5.6 (E-Merck, Germany).

Inoculum preparation: Conidial suspension was prepared from a 3-5 day old slant culture by adding 10 mL of 0.005% (w/v) sterile dioctyl ester of sodium sulpho succinic acid (Monoxal O.T.). The number of conidia per milliliter of inoculum was determined with a Haemocytometer (Neubauer Precicdor HBG, Germany).

Fermentation media: Mineral Salt Solution (MSS) and Eggins and Pugh (E and P) medium were varied with reference to their different carbon sources, %, w/v (Table 1).

MSS: $(NH_4)_2SO_4$ 0.14, KH_2PO_4 0.20, urea 0.03, $MgSO_4.7H_20$ 0.03, $ZnSO_4.7H_2O$ 0.00014, $FeSO_4.7H_2O$ 0.0005, $MnSO_4$ 0.00016, $CoCl_2$ 0.0002, $CaCl_2$0.0002, polypeptone 0.10, tween-80 2.0 mL, pH 6.0[9].

E and P: $KH_2PO_4$0.10, $(NH_4)_2SO_4$, 0.05, KCl 0.05, $MgSO_4$ 0.02, L-asparginine 0.05, yeast extract 0.05, pH 5.0[10].

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Fermentation technique: Twenty-five milliliter of the fermentation medium was transferred to individual 250 mL cotton plugged conical flask. The flasks were autoclaved at 15.0 lbs/in^2 pressure (121°C) for 15 min. After cooling at room temperature, the flasks were inoculated with 1.0 mL of the conidial suspension (5.0×10^7 conidia/mL) and incubated at 28°C in a rotary shaking incubator (200 rpm) for 96 h. The fermented broth was then centrifuged at 10,000 rpm for 10 min and the supernatant was used for further analysis.

CSA: It was determined after Takao *et al.*[11]. To a mixture of 1.0 mL of diluted enzyme solution and 1.0 mL of 0.1 M acetate buffer (pH 4.5), 50 mg of absorbent cotton was added and incubated at 50°C for 24 h. The released reducing sugar was estimated by dinitrosalicylic acid (DNS) method[12]. A double beam UV/VIS-scanning spectrophotometer (Model: Cecil-CE 7200-series, Aquarius, UK) was used for measuring the % transmittance.

One unit of activity is defined as the amount of enzyme required to liberate one μmol of reducing sugars per hour.

Statistical analysis: Treatment effects were compared after Snedecor and Cochran[13]. Significance difference among the replicates has been presented, as Duncan's Multiple Range in the form of probability (<p>) value.

RESULTS AND DISCUSSION

Cellulases are inducible enzymes and their induction and activity depends on the nature of substrate[14]. In the present study, eighteen different culture media were investigated (Table 1). Among them, *Trichoderma harz*ianum UM-11 gave the best CSA (0.418 U/mL/h) when CMC with MSS was used as a basal carbon source. CMC is a better inducer of cellulases[8] and MSS contained a balance supply of essential macro- and micronutrients for the growth of organism, optimal cellulases production and saccharifying activity. Various concentrations of CMC (0.25-4.0 %, w/v) were also tested (Table 2). The maximum saccharification (0.427 U/mL/h) was attained at CMC concentration 1.75 %, when added in the fermentation medium as a basal substrate. Further increase in CMC concentration resulted in the decreased CSA. It might be because increase in the level of substrate declines the agitation rate and oxygen supply due to thickness of fermentation medium which is very essential for the optimal growth of organism[15].

The rate of saccharification of cellulases produced by *Trichoderma harzianum* UM-11 was carried out from 8-152 h. The maximum CSA was achieved after an incubation period of 96 h (Fig. 1). It might be due to that

Table 1: Production of enzyme cellulases by *Trichoderma harzianum* UM-11 on different media

Different media	Cotton saccharifying activity (U/mL/h)
BMC+E and P	0.2267±0.0058a-c
BMC+MSS	0.25±0.01a-c
Cellulose powder+E and P	0.133±0.0057bc
Cellulose powder+MSS	0.1580±0.0035bc
Wheat bran+E and P	0.283±0.0153a-c
Wheat bran+MSS	0.32±0.0105ab
Wheat Straw+E and P	0.222±0.0190a-c
Wheat Straw+MSS	0.224±00.0089a-c
Rice husk+E and P	0.119±0.0153bc
Rice husk+MSS	0.127±0.0176bc
CMC+E and P	0.272±0.0176a-c
CMC+MSS	0.4180±0.496a
Newspaper +E and P	0.104±0.0148bc
Newspaper+MSS	0.285±0.00503a-c
Cotton wool +E and P	0.1397±0.182bc
Cotton wool+MSS	0.0617±0.0126c
Bagasse+ E and P	0.08±0.01bc
Bagasse+MSS	0.064±0.0461bc
LSD	0.2095

Each value is an average of three replicates±denotes standard deviation among replicates. Numbers followed by different letters differ significantly at p = 0.05. Incubation time 72 h, Temperature 30±1°C

Table 2: Effect of different concentration of carboxymethyl cellulose (CMC) on the biosynthesis of cellulases by *Trichoderma harzianum* UM-11

CMC (%)	Cotton saccharifying activity (U/mL/h)
0.25	0.004±0.00045g
0.5	0.023±0.00528f
0.75	0.098±0.13300d
0.1	0.271±0.01153c
1.25	0.336±0.00551b
1.5	0.420±0.02000a
1.75	0.427±0.01528a
2	0.423±0.00577a
2.5	0.270±0.01002c
3	0.209±0.01060d
3.5	0.049±0.00503e
4	0.0235±0.0013f
LSD	0.0164

Each value is an average of three replicates±denotes standard deviation among replicates. Numbers followed by different letters differ significantly at p = 0.05. Incubation time 72 h, Temperature 30±1°C

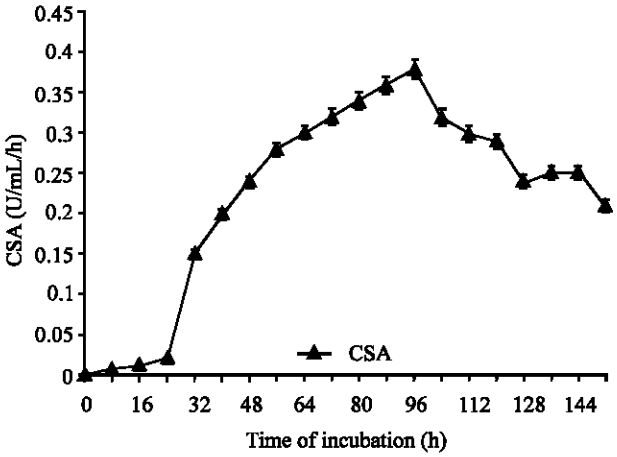

Fig. 1: Rate of enzyme cellulases synthesis by *Trichoderma harzianum* UM-11

Note: Y error bars indicate the standard error of means among the three parallel replicates

Table 3: Effect of different incubation temperatures on the production of cellulases by *Trichoderma harzianum* UM-11

Temperature (°C)	Cotton saccharifying activity (U/mL/h)
20	0.120±0.0100f
22.5	0.250±0.0100d
25	0.370±0.0100c
28	0.417±0.0208a
30	0.390±0.0100d
32.5	0.367±0.0058c
35	0.230±0.0100e
37.5	0.097±0.0058g
40	0.009±0.0006h
LSD	0.0179

Each value is an average of three replicates±denotes standard deviation among replicates. Numbers followed by different letters differ significantly at p = 0.05. Incubation time 96 h

Table 4: Effect of different initial pH on the production of cellulases by *Trichoderma harzianum* UM-11

Initial pH	Cotton saccharifying activity (U/mL/h)
3	0.004±0.00045g
3.5	0.023±0.00528f
4	0.098±0.13300d
4.5	0.271±0.01153c
5	0.336±0.00551b
5.5	0.420±0.02000a
6	0.427±0.01528a
6.5	0.423±0.00577a
7	0.270±0.01002c
7.5	0.209±0.01060d
8	0.049±0.00503e
8.5	0.0235±0.0013f
LSD	0.0164

Each value is an average of three replicates±denotes standard deviation among replicates. Numbers followed by different letters differ significantly at p = 0.05. Incubation time 96 h, Incubation temperature 28±1°C

Table 5: Effect of inoculum size of *Trichoderma harzianum* UM-11 on the production of cellulases

No. of conidia	Cotton saccharifying activity (U/mL/h)
$2.5x10^7$	0.436±0.0153bc
$5.0x10^7$	0.528±0.0416a
$7.5x10^7$	0.493±0.02a
$1.0x10^8$	0.486±0.015ab
$1.25x10^8$	0.42±0.005c
LSD	0.0516

Each value is an average of three replicates±denotes standard deviation among replicates. Numbers followed by different letters differ significantly at p = 0.05. Incubation time 96 h, Incubation temperature 28±1°C

organism entered in the stationary phase of growth producing maximum cellulases and hence maximum CSA[16]. Further increase in the incubation period reduced CSA. It may be due the age of fungi, nutrients depletion and other inhibitory by-products accumulation in medium. Incubation temperature of the fermentation medium is a critical factor has insightful influence on metabolic activities of microorganisms. The effect of different incubation temperature (20-40°C) on the CSA was also investigated (Table 3). The optimal saccharification (CSA 0.417 U/mL/h) was found to be at 28°C. Any change in this temperature decreased CSA. A higher temperature modifies the membrane composition[17], stimulates protein catabolism and thus causes cell death[18]. Initial pH of fermentation medium has a direct effect on the uptake of mineral nutrients present in the medium and mould metabolism consequently, CSA of cellulases produced by *Trichoderma harzianum* UM-11 was studied by varying the initial pH from 3.0-8.5 (Table 4). The optimal CSA (0.427 U/mL/h) was observed at pH 6.0. Any change in pH resulted in decreased CSA. It might be due to the fact that organism requires vaguely acidic pH for growth and cellulases biosynthesis[19].

Inoculum size certainly has an effect on the rate of production[20]. In present study, effect of different sizes of conidial inoculum ($2.5x10^7$ to $1.25x10^7$ conidia) was also explored (Table 5). CSA found to be optimal (0.528 U/mL/h) when flasks were inoculated with $5.0x10^7$ conidia per 25mL medium. Variations in inoculum size from this optimal range resulted in decreased CSA. It might be due to as at low inoculum size, cells were not in adequate quantity for substrate consumption and enzyme production within optimal incubation period. Moreover, at high inoculum level it could be because tremendous growth of organism led to anaerobic condition and nutritional imbalance in fermentation medium and subsequently less CSA[9].

CONCLUSION

From the present study, it may concluded that *Trichoderma harzianum* strain UM-11 has better CSA in the culture medium containing 1.5 % (w/v) CMC with MSS using shake flask technique. The maximum CSA was found when 250 mL Erlenmeyer flask containing 25 mL of fermentation medium having slightly acidic initial pH (6.0), was inoculated with $5.0x10^7$ conidia for an incubation period of 96 h at 28°C. CSA can be further enhanced by the mutagenic treatment of fungus (UV, MNNG and EMS) and optimizing the cultural conditions of selected mutant.

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