INT. J. BIOL. BIOTECH., 11 (4): 537-545, 2014.

OPTIMIZATION OF GROWTH CONDITIONS FOR ENHANCED CELLULASE ENZYME ACTIVITY FROM WILD AND IMPROVED MUTANTS OF *TRICHODERMA VIRIDE* PERS.

Shazia Shafique* and Sobiya Shafique

Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan.
*Corresponding author: shazia.iags@pu.edu.pk

ABSTRACT

The production of cellulase enzyme using an economical medium has been a significant achievement in the field of industrial biotechnology. Cellulase enzyme activity of *Trichoderma viride* FCBP–142 and its mutant derivatives, Tv-UV-5.6 and Tv-Ch-4.3 was evaluated by growing them on different substrates at different incubation temperatures, initial pH levels, incubation periods and nitrogen sources. Optimization growth assays illustrated 2% wheat straw, 4.0 pH, 72 hours of incubation period and ammonium sulfate as nitrogen supplement for the best enzymatic activity by all the test strains. The suitable temperature for the best mycelial growth and enzyme activity was 30 °C for each of *T. viride* FCBP–142 and Tv-UV-5.6, and 32.5 °C for Tv-Ch-4.3. Mass production of selected test strains indicated the aptness of wheat straw for rapid fungal proliferation and viability under optimized conditions. Stability of mutants concerning the best cellulase activity potential, evaluated up to 10 generations, revealed that Tv-UV-5.6 and Tv-Ch-4.3 were highly stable for enzymatic activity under pre-optimized conditions.

Key Words: *Trichoderma viride* FCBP–142, optimization, mass production, cellulase enzyme activity

INTRODUCTION

Pakistan being an agricultural country has wide diversity of rich lignocellulosic agricultural by–products that are low cost and easily available. These lignocellulosics by-products can be exploited as substrate for mass production of potential enzyme producers to enhance enzyme production (Solomon *et al*., 1999; Ojumu *et al*., 2003; Yang *et al*., 2006; Membrillo *et al*., 2008). But, at the same time cultural conditions and their optimization such as carbon and nitrogen sources, their concentrations, cultivation time, temperature and pH are the critical parameters that greatly affect microbial growth and subsequent enzyme secretion or product formation (Dahot and Noomrio, 1996; Senthilkumar *et al*., 2005). Thus an ideal substrate to improve fungal enzyme production should combine a biomass increasing property with enzyme synthesis induction as the cellulase secretion in fungi is directly proportional to the mycelial growth (Bhat and Maheshwari, 1987).

The intention of this contemporary study was to stabilize the conditions for mass production of inoculum on economically feasible substrate to attain large amount of active enzyme from fungi.

MATERIALS AND METHODS

Optimization: The agricultural wastes, wheat straw var. Maxi Pak, rice straw of IRRI Pak–6 were obtained from Government Agriculture Farm, Shiekhupura, sugar cane waste was obtained from Juice corner of Campus area of University of the Punjab, Lahore while wood waste of *Delbergia sissoo* was taken from Campus area of University of the Punjab, Lahore. All the substrates were dried and chopped into small pieces of 30–50 mm. Two g of each substrate was taken into a 250 mL Erlenmeyer flask in 100 mL distilled water and pH was adjusted to 4.0. The contents of the flasks were mixed thoroughly and autoclaved (121 °C and 15 lb $inch^{-2}$ for 15 min). After cooling, each medium was inoculated with adjusted spore inoculum (5 x 10^5 conidia mL^{-1}). The flasks were incubated on orbital shaker incubator at 100 rpm and 30 ± 2 °C for 96 hours and were assayed after every 12 hours interval for their maximum activities according to Shafique *et al*. (2007). Different parameters like the best substrate concentration, temperature, initial pH, incubation period and nitrogen source were optimized on the best substrate for active fungal strains.

Mass Production: In order to use mutant strains of fungi on large scale for enzyme production, the mutant strains were mass produced. The wheat straw was drenched in water for about 12 to 14 hours, for softening of substrate. Afterwards, 70% moisture content was maintained by using following formula:

$$\text{Moisture (\%)} = \frac{\text{Difference in wt. of substrate before and after soaking (g)}}{\text{Initial wt. of substrate(g)}} \text{ x } 100$$

Then 200 g of substrate was filled in plastic bags measuring 20x30 cm, tied with nylon rope or rubber bands and was sterilized at 121 °C and 15 lb inch^{-2} for 15 min. After 3–4 hours cooling each bag was inoculated with 0.5 g of ammonium sulfate (optimized nitrogen source) and two discs of 5 mm diameter from the freshly grown cultures of native, UV and chemical mutant derivatives. The experiment was run in triplicate. The inoculated bags were incubated in growth room at 30 ± 2 °C for 15 days until the mycelium has fully penetrated the bottom of the substrate and mycelium ramified the whole substrate.

Evaluation of Sporulation of Mass Cultivated Strains: After 15 days, bags were opened and 2 g of material was suspended in 20 mL of distilled sterilized water. The concoction was shaken and allowed to settle for 15 min in order to facilitate the loosening of conidia. The resulting suspension was filtered through muslin cloth to remove large mycelial masses and the remnants of substrates. The concentration of the conidia was measured using haemacytometer and expressed as number of conidia per 10 µL of suspension (Siddiqui, 2004).

Viability of Mass Produced Inoculum: To check the viability of the conidia, mass produced on selected suitable substrate (wheat straw + ammonium sulfate), trails were carried out with *T. viride* FCBP–142 and its mutants Tv-UV-5.6 and Tv-Ch-4.3 under preset conditions. The conidial suspension @ 5 x 10^5 conidia mL^{-1} was prepared. The flasks were inoculated with 1 mL of the adjusted inoculum and their enzyme activity was scrutinized after 72 hours of inoculation using the same assay procedure of Shafique *et al*. (2007).

RESULTS

Effect of Carbon Source: Data regarding the influence of variable carbon sources including wheat straw, rice straw, sugar cane waste and wood waste on enzymatic activity of the selected mutant strains of *Trichoderma viride* FCBP–142 is presented in Fig. 1. Evaluation of the data suggested that the candidate microorganisms exhibited maximal cellulase activity (53.33, 80.34 and 112 Units mL^{-1} for *T. viride* FCBP–142, Tv-UV-5.6 and Tv-Ch-4.3, respectively) on wheat straw followed by rice straw (47, 76.39 and 104.13 Units mL^{-1} for *T. viride* FCBP–142, Tv-UV-5.6 and Tv-Ch-4.3, respectively) and differ significantly ($P \leq 0.05$) than other substrates. Wood waste was found to be comparatively less effective substrate. It only supported 27.15, 33.49 and 56.97 Units mL^{-1} of enzyme activity by *T. viride* FCBP–142, Tv-UV-5.6 and Tv-Ch-4.3, respectively. Consequently, wheat straw was deemed to be the most suitable fermentation substrate for the biosynthesis of cellulase enzyme by wild as well as its mutant strains thus was selected for further studies.

Effect of Substrate Concentration: As maximum activity of enzyme was recorded on wheat straw by *T. viride* FCBP–142, Tv-UV-5.6 and Tv-Ch-4.3, so the applied dilutions (0.5–4.0%) of wheat straw were evaluated for further improvement in activity of cellulase enzyme and the results are presented in Fig. 2. The response of the two mutants differed significantly ($P \leq 0.05$) from one another as well as from wild strain with each employed concentration. Among these tested concentrations of wheat straw, enzyme activity was significantly ($P \leq 0.05$) high at 2% concentration by all the test strains. However, further increase in concentration of wheat straw resulted in a gradual reduction in enzyme formation.

Effect of pH: Impact of preliminary pH of the medium in the range of 3.5–9.0 using citrate and tris buffers on the cellulase enzyme activity was studied for wild strain of *T. viride* FCBP–142 and its mutant derivatives Tv-UV-5.6 and Tv-Ch-4.3. The optimal pH recorded for enzyme activity was pH 4.0 at which maximum enzyme activities of 58.83, 88.84 and 122.68 Units mL^{-1} were attained by *T. viride* FCBP–142, Tv-UV-5.6 and Tv-Ch-4.3, respectively (Fig. 3). Beyond that, a continuous impede in enzyme activity was observed. These studies signify that all these are acidophilic microorganisms and produce extra cellular cellulase enzyme at acidic pH more efficiently.

Effect of Incubation Temperature: To evaluate the effect of different incubation temperatures for maximum cellulase enzyme activity, wild and improved strains of *T. viride* FCBP–142 were developed at diverse temperatures in the range of 20–40 °C with an augmentation of 2.5 °C. The results pertaining to enzyme activity with respect to change in temperature and their statistical analysis are presented in Fig. 4. The results revealed that maximum value

for cellulase enzyme activity of 58.83 and 87.5 Units mL^{-1} was achieved at 30 °C for *T. viride* FCBP–142 and Tv-UV-5.6 strain, respectively while Tv-Ch-4.3 mutant displayed the maximum enzyme activity of 120.35 Units mL^{-1} at 32.5 °C. The temperature regimes above and below this level showed lesser enzyme titer.

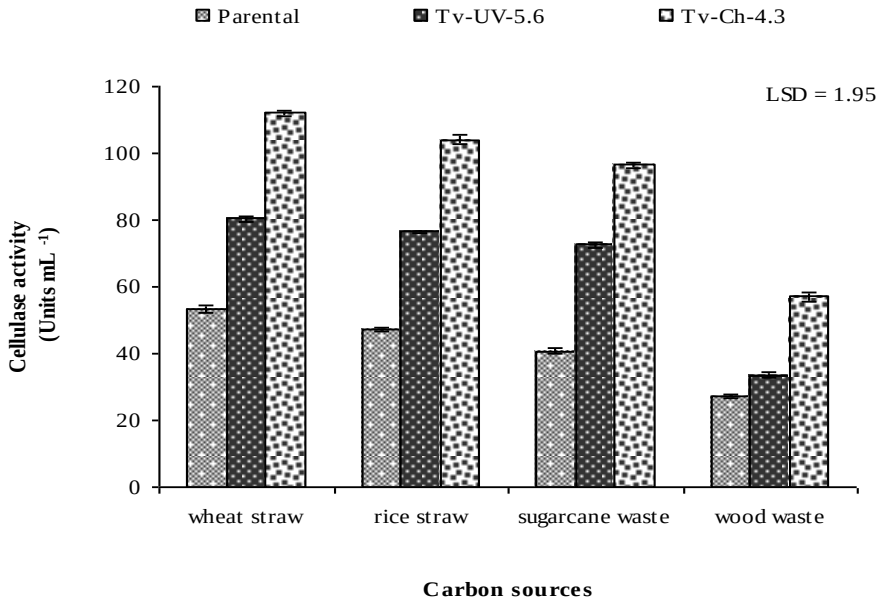

Fig. 1. Effects of carbon sources on cellulase enzyme activity by *T. viride* FCBP–142 and mutants Tv-UV-5.6 and Tv-Ch-4.3.

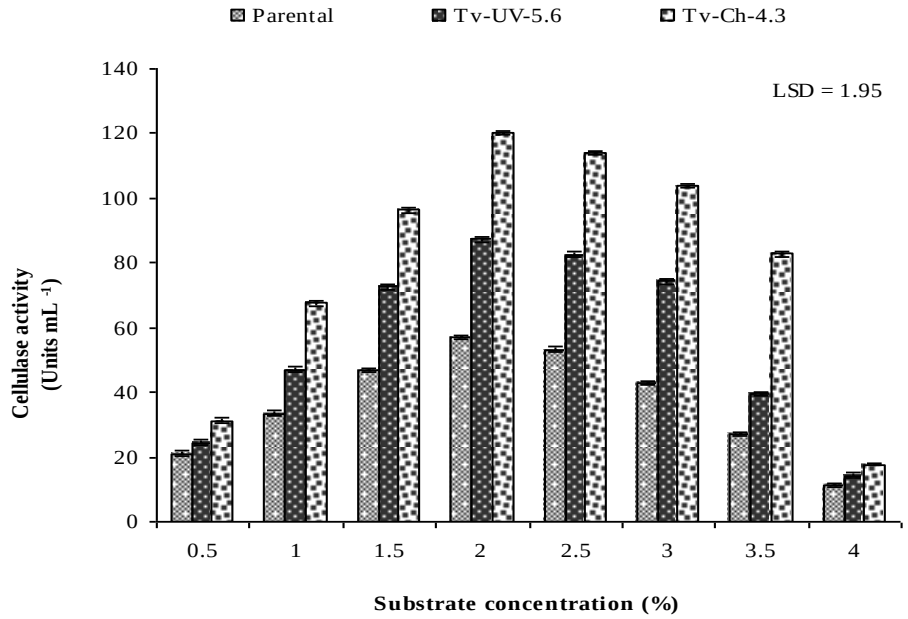

Fig. 2. Effects of different concentrations of substrate on cellulase enzyme activity by *T. viride* FCBP–142 and mutants Tv-UV-5.6 and Tv-Ch-4.3.

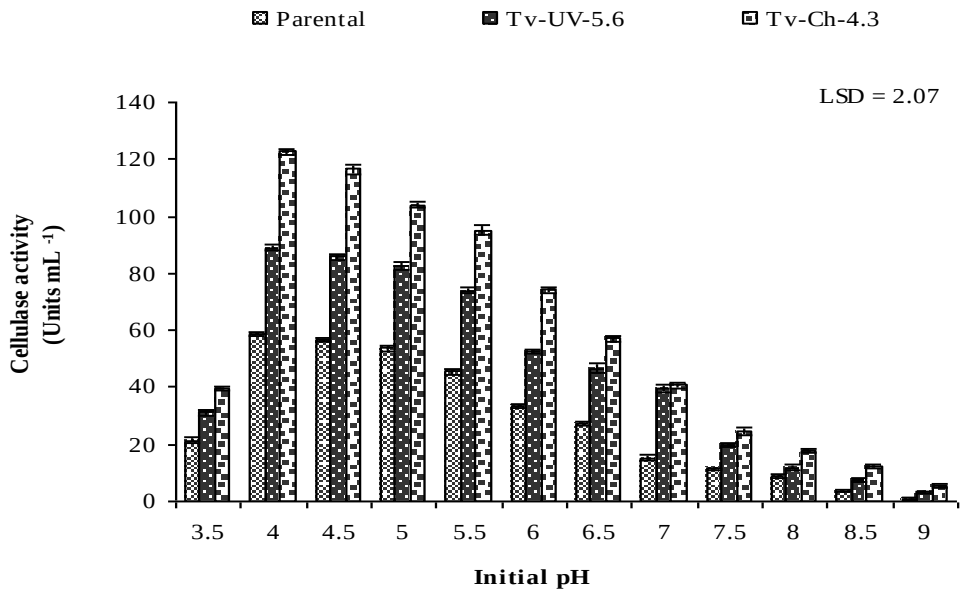

Fig. 3. Effects of initial pH of basal medium on cellulase enzyme activity by *T. viride* FCBP–142 and mutants Tv-UV-5.6 and Tv-Ch-4.3.

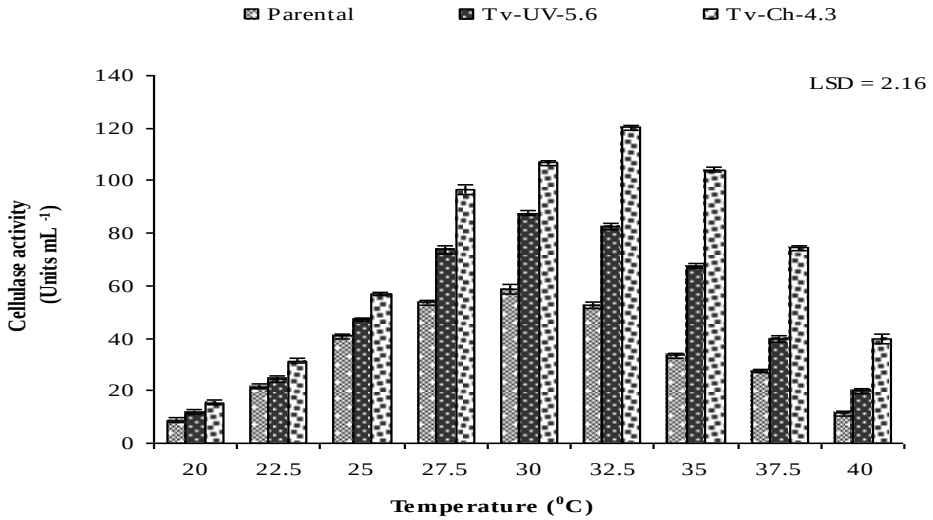

Fig. 4. Effects of incubation temperature on cellulase enzyme activity by *T. viride* FCBP–142 and mutants Tv-UV-5.6 and Tv-Ch-4.3.

Effect of Incubation Period: Changes in the activity of typical cellulase enzymes by *T. viride* FCBP–142, Tv-UV-5.6 and Tv-Ch-4.3, over a range of different time periods up to 96 hours, are plotted in Fig. 5. Data shows that enzyme activities of fungal isolates varied considerably at different incubation periods with LSD value of 1.81. Maximum enzyme activity was attained after 72 hours of incubation. A decrease in enzyme production was observed beyond that period. Similar trend was exhibited by Tv-UV-5.6 and Tv-Ch-4.3 in response to change in time course. The maximum enzyme activities were 53.48, 93.8 and 128 Units mL^{-1} for parental, UV and chemical mutant strains, respectively.

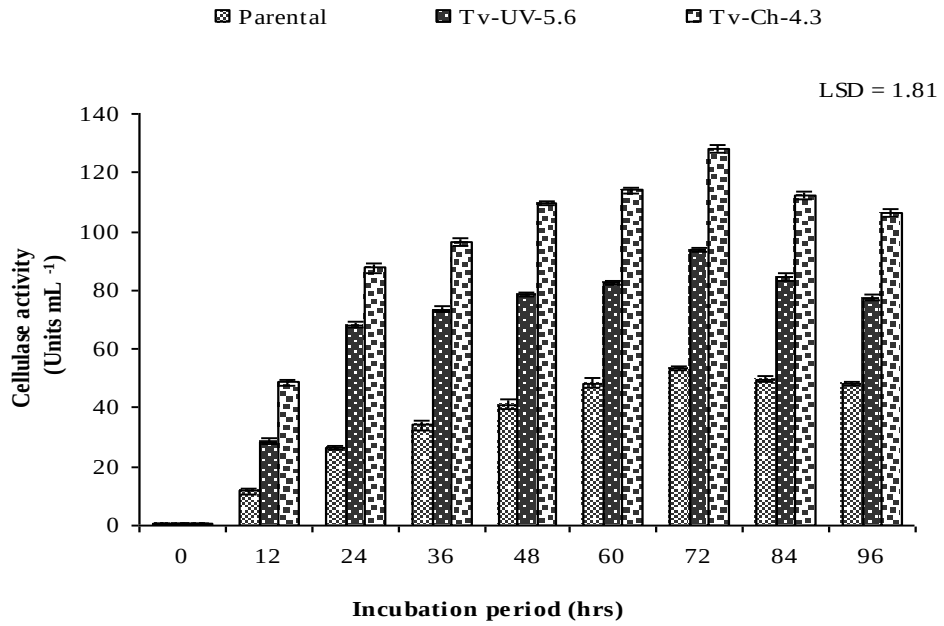

Fig. 5. Effects of incubation period on cellulase enzyme activity by *T. viride* FCBP–142 and mutants Tv-UV-5.6 and Tv-Ch-4.3.

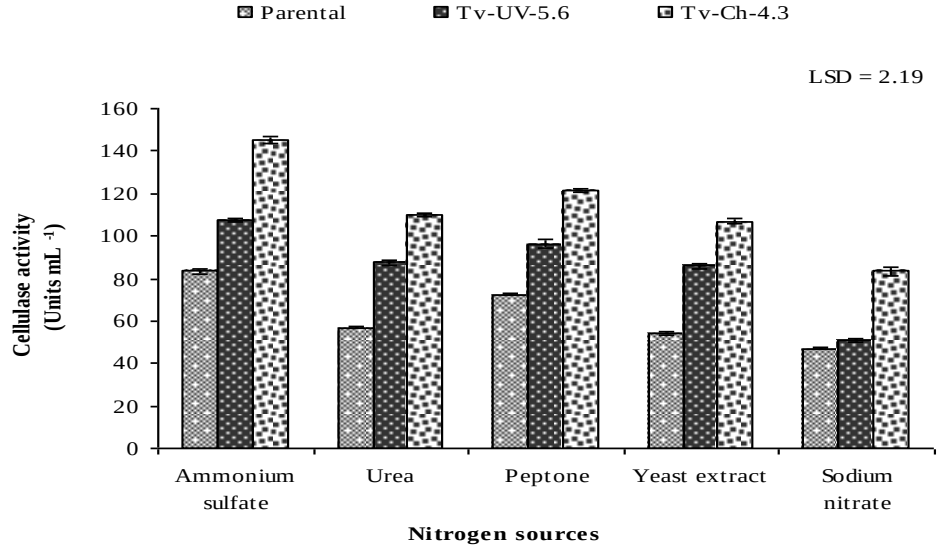

Fig. 6. Effects of different nitrogen sources on cellulase enzyme activity by *T. viride* FCBP–142 and mutants Tv-UV-5.6 and Tv-Ch-4.3.

Effect of Nitrogen Source: Growth and enzyme production of any organism are greatly influenced by the nutrients available in growth medium. The results of cellulase activity using ammonium sulfate, urea, peptone, yeast extract and sodium nitrate as nitrogen supplements in wheat straw are presented in Fig. 6. Both organic and inorganic nitrogen sources were found effective for the production of cellulase enzyme. Ammonium sulfate and peptone were found to be the promising nitrogen sources for the enzyme activity. Nevertheless, maximum activity of 83.33, 107.22 and 145.1 Units mL^{-1} of cellulase enzyme by *T. viride* FCBP–142, Tv-UV-5.6 and Tv-Ch-4.3, respectively was achieved when 2% wheat straw was supplemented with ammonium sulfate (0.5%). Yeast extract and urea also proved good sources of nitrogen for better enzyme activity of *T. viride* FCBP–142, Tv-UV-5.6 and Tv-Ch-4.3. Sodium nitrate was considered to be the least effective as it resulted in 47, 50.86 and 83.39 Units mL^{-1} of enzyme titer for *T. viride* FCBP–142, Tv-UV-5.6 and Tv-Ch-4.3, respectively.

Stability of Mutant Derivatives: Stability of mutant derivatives of *T. viride* FCBP–142 i.e., Tv-UV-5.6 and Tv-Ch-4.3 was evaluated up to 10 generations after every two months to check their ability for synthesis of hyper active enzyme under previously adjusted assay conditions. The results revealed that these improved isolates exhibited same yield with insignificant difference up to the last test generation (Fig. 7).

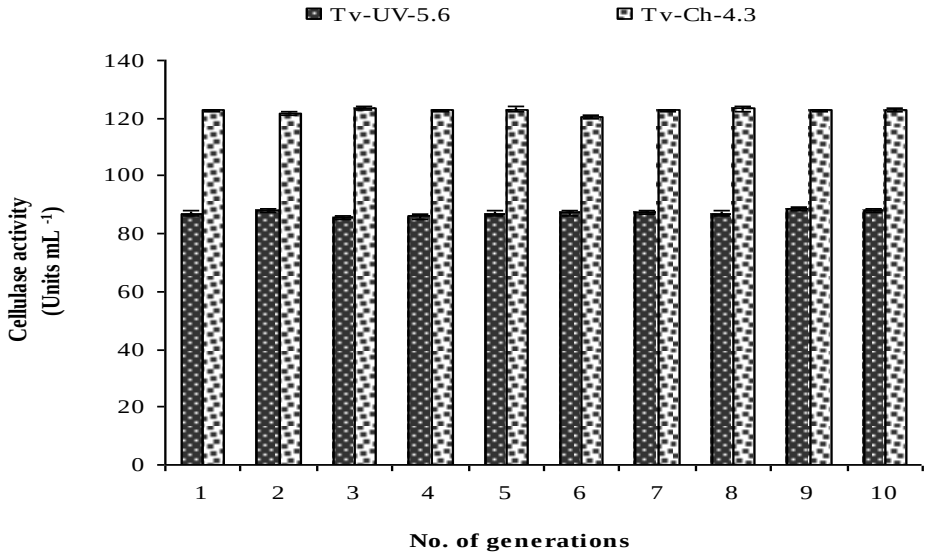

Fig. 7. Stability of Tv-UV-5.6 and Tv-Ch-4.3 mutant derivatives of *T. viride* FCBP–142 for cellulase activity.

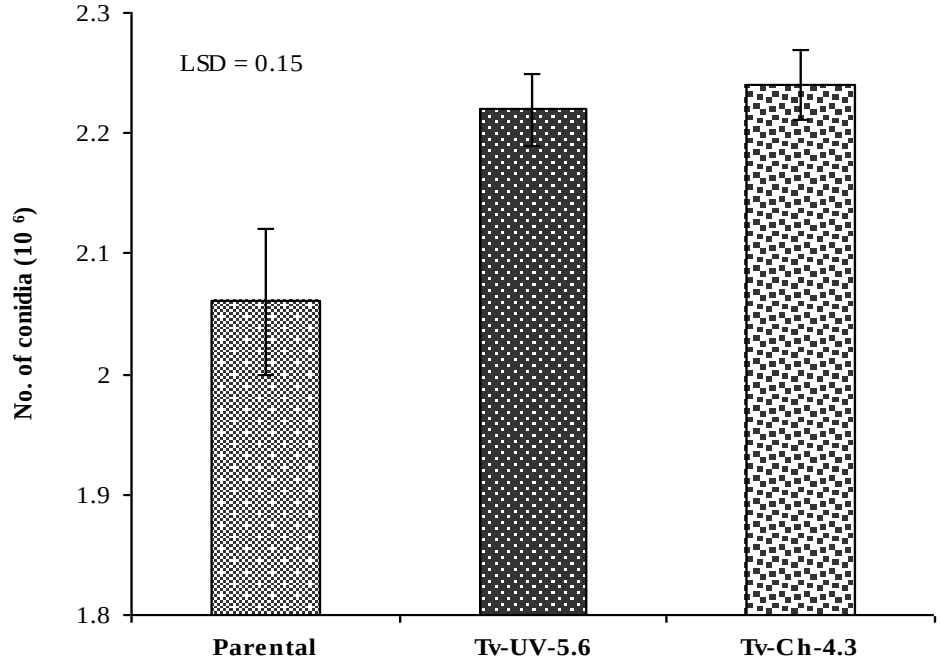

Fig. 8. Mass production of *T. viride* FCBP–142, Tv-UV-5.6 and Tv-Ch-4.3 mutant derivatives.

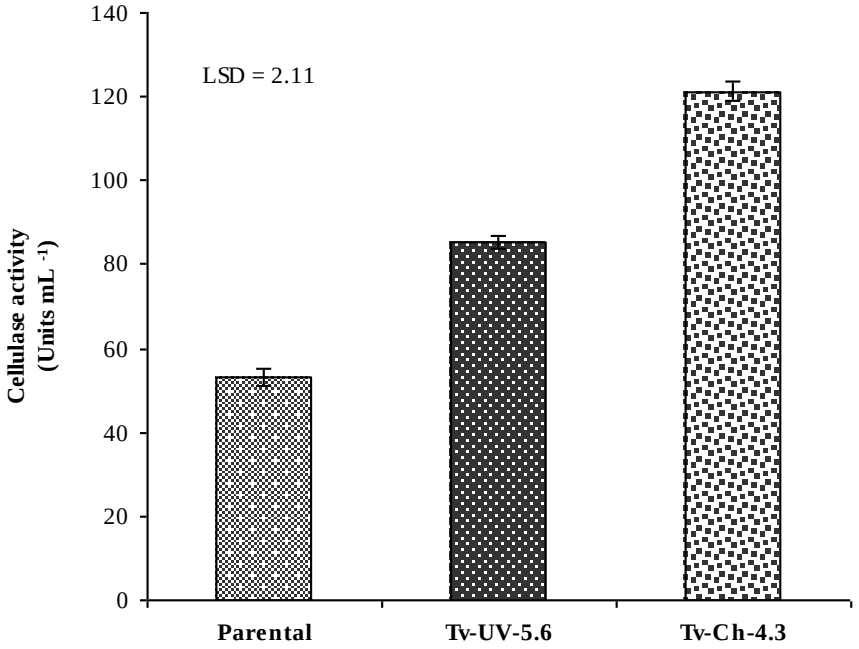

Fig. 9. Viability of *T. viride* FCBP–142, Tv-UV-5.6 and Tv-Ch-4.3 mutant derivatives.

Pilot Scale Mass Production of Mutants Under Optimized Conditions: The assessments to categorize the most suitable substrate for mass production of *T. viride* FCBP–142 and its mutant derivatives Tv-UV-5.6 and Tv-Ch-4.3 demonstrated that among all the substrates wheat straw, with ammonium sulfate as nitrogen supplement at 70% moisture content and 30±2 °C temperature, was the best carbon source for rapid mycelial colonization, maximum conidial production and the highest enzyme activity of these test strains. It was biologically the most active source and propped up healthy growth of the fungus which was supported in terms of conidial productivity. The mutant derivatives Tv-UV-5.6 and Tv-Ch-4.3 produced conidia almost at the same rate i.e., 2.22 x 10^6 and 2.24 x 10^6 conidia 10 μL^{-1} on wheat straw. However, among these, Tv-Ch-4.3 yielded slightly higher number of conidia. By contrast, relatively low mycelial colonization and conidial production was attained by native strain *T. viride* FCBP–142 on the same substrate i.e., up to 2.06 x 10^6 conidia 10 μL^{-1} (Fig. 8). Thus, wheat straw was considered as an active biological source for the maximal and conidial productivity of the fungal strains to be used as inoculum for enzyme production.

Viability of Mass Produced Inoculum: The viability tests of the conidia of *T. viride* FCBP–142, Tv-UV-5.6 and Tv-Ch-4.3, mass produced on selected suitable substrate (wheat straw + ammonium sulfate), for cellulolytic enzyme production (quantitatively) revealed that the efficacy of the inocula was not affected during mass culturing. All the strains exhibited almost similar activity of cellulase enzyme i.e., 53, 85.33 and 121.33 Units mL^{-1} by *T. viride* FCBP–142, Tv-UV-5.6 and Tv-Ch-4.3 respectively, under similar conditions as described earlier (Fig. 9).

DISCUSSION

Stepwise trials were conducted in the present study to select an inexpensive and easily available substrate together with the optimization of culture conditions to reduce the cost of enzyme preparation. Agricultural wastes and by–products are not only cheap and easily available but also supply sufficient nutrients to grow microbial cultures (Thiry and Cingolani, 2002; Narasimha *et al*., 2006; Bhatti *et al*., 2007; Latifian *et al*., 2007). Therefore, these economically feasible substrates were employed for the optimization of growth conditions of mutant strains of fungi for large scale enzyme production.

Cellulase enzyme activity data of native test fungal species *T. viride* FCBP–142 and its mutants, Tv-UV-5.6 and Tv-Ch-4.3, testified that cellulolytic activity of the fungal strains was significantly dependent on carbon source and nitrogen supplement in the medium. The maximum activity of enzyme was observed when 2% wheat straw was

used as fermentation medium, further increase in amount of wheat straw led to declined enzyme activity. Apparently the extra accumulation of basal substances in fermentation medium with resultant richness caused decline in anxiety and poor mixing of air which was crucial for organism's growth as well as enzyme production as has earlier been suggested by Haq *et al.* (1998; 2002). In a similar kind of study, role of carbon and nitrogen sources on cellulose enzyme synthesis by *A. niger* was investigated by Hanif *et al.* (2004). They reported wheat bran and cellulose as the most operational promoters of β–cellobiohydrolase and filter peperase (FPase) activities, respectively, followed by rice bran. These findings are supported by more recent reports on the effect of nitrogen source on production of cellulase enzyme where results were found to vary on the basis of fungi as well as nutrient sources tested (Kachlishvili *et al*., 2006; Membrillo *et al*., 2008).

Presently, optimization assays further revealed that all the test strains exhibited maximum enzyme activity at pH 4.0 after 72 hours of incubation period with ammonium sulfate as the best nitrogen supplement. The temperature of 30 °C was found effective for *T. viride* FCBP–142 and Tv-UV-5.6 whereas Tv-Ch-4.3 exhibited the best mycelial growth and enzyme production at 32.5 °C. However, the effect or range of these parameters was found to vary with the species/strains involved. Shi and Cui (2001) in similar studies have optimized fermentation conditions for the mutant strain of *Trichoderma koningii* (Oud) T–199 for β–glucanase production. The mutant obtained by exposing wild strain to ultraviolet light and N-methyl-N-nitro-N-nitrosoguanidine, produced about 8 times more β–glucanase at pH 5.0 and 60 °C than its parent strain. Barley β–glucan was reported as the most promising substrate for growth and enzyme production.

A variety of substrates have been employed by several workers for the better production of inoculum (Buswell *et al*., 1995; Solomon *et al*., 1996; Stepanova *et al*., 2003). The results of present findings indicate that for selected test strains wheat straw was the most suitable for rapid fungal proliferation and viability under optimized conditions. Thus both wild and mutant strains expressed great potential to utilize cellulosic substrate for mass production. In earlier findings it has been attributed to bioconversion of indigestible cellulose, hemicelluloses and lignin in wheat, rice straw and cotton waste into digestible material (Kirk, 1983; Ali, 1986; Margaritis and Merchant, 1986; Puniya and Singh, 1995). Differential response of fungal enzyme activities could be due to difference in their nutritional requirement and better response on wheat straw could be due to large surface area and appropriate moisture content contributing in extensive vegetative and reproductive growth.

The stability of mutants regarding cellulase production potential was investigated up to 10 generations. Tv-UV-5.6 and Tv-Ch-4.3 revealed enormous stability for production of enzyme under pre-optimized conditions. These results are consistent with previous report of Mohsin (2006) who also tested the mutant stability with respect to enzyme secretion and discerned markedly stable mutants under defined conditions.

REFERENCES

Ali, A. (1986). *Crop residues and their treatment for livestock. Presented in seminar on: application of new technology in livestock nutrition organized by livestock and Dairy Development*. Deptt. Govt. of the Punjab. Pakistan, 1 - 3.

Bhat, M.K. and R. Maheshwari (1987). *Sporotrichum thermophile*: Growth, cellulose degradation and cellulase activity. *Appl. Env. Microbiol*., 53: 2175 - 2182.

Bhatti, H.N., M.H. Rashid, R. Nawaz, M. Asgher, R. Perveen and A. Jabbar (2007). Optimization of Media for Enhanced Glucoamylase Production in Solid-State Fermentation by *Fusarium solani. Food. Technol. Biotechnol*., 45 (1): 51 56.

Buswell, J.A., Y.J. Cai and S.T. Chang (1995). Fungal and substrate associated factors affecting the ability of individual mushroom species to utilize different lignocellulosic growth substrates, In: Mushroom Biology and Mushroom Products (eds. S.T. Chang, J.A. Buswell, S.W. Chiu). Pp 141.

Dahot, M.U. and M.H. Noomrio (1996). Microbial production of cellulases by *Aspergillus fumigatus* using wheat straw as a carbon source. *J. Isl. Acad. Sci*., 9 (4): 1 - 4.

Hanif, A., A. Yasmeen and M.I. Rajoka (2004). Induction, Production, repression and derepression of exoglucanase synthesis in *Aspergillus niger*. Biores. Technol., 94: 311 -319.

Haq, I., H. Ashraf, R. Zahara and M.A. Qadeer (1998). Biosynthesis of α-amylase by *Bacillus subtilis* GCB-12 using agricultural by-products as substrates. *Biologia*, 44 (1 & 2): 154 - 163.

Haq, I., S. Rani, A. Hamad and M.A. Qadeer (2002). Biosynthesis of α-amylase by chemically treated mutant of *Bacillus subtilis. J. Biol. Sci*., 2 (2): 73 -75.

Kachlishvili, E., M.J. Penninckx, N. Tsiklauri and V. Elisashvili (2006). Effect of nitrogen source on lignocellulolytic enzyme production by white-rot basidiomycetes under solid-state cultivation. *Wor. J. Microbiol. Biotechnol*., 22: 391 -397.

Kirk, T.K. (1983). Degradation and conversion of lignicellulosics, In: *The filamentous fungi* (T.E. Smith, D.R. Berry, B. Rristiansen, eds.). Fungal Technology, London. 4: 266 - 295.

Latifian, M., Z. Hamidi-Esfahani and M. Barzegar (2007). Evaluation of culture conditions for cellulase production by two *Trichoderma reesei* mutants under solid-state fermentation conditions. *Biores. Technol*., 98: 3634 - 3637.

Margaritis, A. and R.F. Merchant (1986). Optimization of fermentation conditions for thermostable cellulase production by *Thielavia terrestris. J. Ind. Microbiol*., 1: 149 - 156.

Membrillo, I., C. Sánchez, M. Meneses, E. Favela and O. Loera (2008). Effect of substrate particle size and additional nitrogen source on production of lignocellulolytic enzymes by *Pleurotus ostreatus* strains. *Biores. Technol*., 99 (16): 7842 - 7847.

Mohsin, M.J. (2006). *Improvement of a thermophilic fungal strain for cellulase production by chemical and UV mutagenesis*. Ph.D. thesis, Dept. of Botany, GC University, Lahore.

Narasimha, G., A. Sridevi, V. Buddolla, C.M. Subhosh and R.B. Rajasekhar (2006). Nutrient effects on production of cellulolytic enzymes by *Aspergillus niger*. *Afr. J. Biotechnol*., 5 (5): 472 -476.

Ojumu, T.V., B.O. Solomon, E. Betiku, S.K. Kayokun and B. Amigun (2003). Cellulase production by *Aspergillus flavus* Linn. isolate NSPR 101 fermented in sawdust, bagasse and corncob. *Afr. J. Biotechnol*., 2 (6): 150 - 152.

Puniya, A.K. and K. Singh (1995). Biochemical changes during the solid substrate fermentation of wheat straw. *Ind. J. Microbiol*., 35: 211 - 215.

Senthilkumar, S.R., B. Ashokkumar, K.C. Raj and P. Gunasekaran (2005). Optimization of medium composition for alkali-stable xylanase production by *Aspergillus fischeri* Fxn 1 in solid-state fermentation using central composite rotary design. *Biores. Technol*., 96: 1380 - 1386.

Shafique, S., R. Bajwa and S. Shafique (2007). Cellulase production potential of selected strains of *Aspergilli*. *Pak. J. Phytopathol*., 19 (2): 219 -.

Shi, J. and F. Cui (2001). Selection of β-glucanase producing *Trichoderma koningii* T-199 and its fermentation conditions. *Wei. Sheng. Wu. Xue. Bao*., 41 (6): 750 - 752.

Siddiqui, I. (2004). *Fungal pathogens as biological control agents of weeds of wheat*, Ph.D. thesis, Dept. of Botany, University of the Punjab, Lahore.

Solomon, B.O., B. Amigun, E. Betiku, T.V. Ojumu and S.K. Layokun (1999). Optimization of cellulase production by *Aspergillus flavus* Linn. Isolate NSP 101 grown on bagasse. *J. Niger. Soc. Chem. Eng*., 16: 61 - 68.

Stepanova, E.V., G.V. Koroleva, U.P. Gaurilova, E.O. Landesman, A. Makower and D.B. Papkousky (2003). Comparative stability assessment of Laccase from Basidiomycetes, *Coriolus hirsutus* and *C. zonatus. App. Biochem. Microbiol*., 39: 482 - 487.

Thiry, M. and D. Cingolani (2002). Optimization of scale up fermentation processes. *Trends. Biotechnol*., 20 (3): 103 - 105.

Yang, S.Q., Q.J. Yan, Z.Q. Jiang, L.T. Li, H.M. Tian and Y.Z. Wang (2006). High-level of xylanase production by the thermophilic *Paecilomyces themophila* J18 on wheat straw in solid-state fermentation. *Biores. Technol*., 97: 1794 - 1800.

(*Accepted for publication September 2014*)