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FUSANT *Trichoderma HF9* WITH ENHANCED EXTRACELLULAR CHITINASE AND PROTEIN CONTENT

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Strain improvement was carried out to obtain higher chitinase and protein by inter-specific protoplast fusion between *Trichoderma harzianum* and *Trichoderma viride*. Fusant HF9 and parental strains of *Trichoderma* were compared for chitinase and protein production. 1% of glucose, sucrose and fungal cell wall (*Rhizoctonia solani*), were used as carbon source for cultivation of *Trichoderma* and fungal cell wall was the best to induce chitinase and protein. Usage of 0.5% colloidal chitin for the fungal growth under aerated conditions at pH 6.5 and 28°C led to higher chitinase and protein production. In these conditions fusant *Trichoderma* HF9 in comparison with parent strains had 3-, 2.5- and 1.5-fold increase of total chitinase, specific chitinase and protein, respectively. SDS-PAGE analysis revealed that it had 9 major protein bands with up-regulation compared to parent strains. Amino acid analysis showed that protein of culture filtrate of *T. harzianum, T. viride* and fusant *Trichoderma* HF9 had 8, 6 and 10 amino acids, respectively. The results obtained suggested that fusant HF9 could be an integration of *T. harzianum* and *T. viride* through protoplast fusion.

Chitin, an insoluble linear β-1,4-linked polymer of N-acetyl glucosamine (GlcNAc), is one of the most abundant polysaccharides found in nature and the most important constituent of insects, exoskeletons, shells of crustaceans and fungal cell walls. Chito-oligomers produced by enzymatic hydrolysis of chitin and chitosan have been of interest in the past few decades due to their broad range of medical, agricultural and industrial applications [1]. Chitinases have received increasing attention from the last decade due to the potential action in biological control program. This can be achieved by either using the chitinase producing microorganism or indirectly, using purified protein or chitinase encoding genes [2]. In addition, chitinase producing organisms along with β-glucanases or cellulases are effective biocontrol agents against phytopathogenic and soil borne fungi [3]. Chitinases from bacteria and fungi are extremely important for maintaining a balance between the large amount of carbon and nitrogen trapped in the biomass as insoluble chitin in nature [4] and also they are needed for fungi to disrupt existing cell walls when normal cells divide [5].

Recent research has been focused on chitinases owing to its multifarious usage in different fields, where they degrade chitin into its monomer of GlcNAc, used for preparation of protoplasts from fungi and are indirectly involved through protoplast fusion for improvement and development of new strains which are economically viable [6, 7]. Furthermore, chitinases and chitooligomers produced by enzymatic hydrolysis of chitin can also be used as chitohexaose and chitoheptaose for antitumor activity in human health [8], to hydrolyze chitinous material in industries, in single cell protein production for yeast *Pichia kudriavzevii* [9], in decomposing chitinous wastes from shellfish [10] and in genomic and genetic studies like chitinase gene transformation to plants [11].

Chitinase could also be involved in the penetration of a host by mycoparasites or entomopathogenic fungi. However, extracellular enzymes are important for both pathogenic and saprophytic fungi to utilize organic and inorganic materials to overcome resistance in the environment. *Trichoderma* is one of the potential species that excretes hydrolytic enzymes such as chitinases, β-1,3-glucanases and cellulases into the medium when supplemented with chitin, laminarin and cellulose, respectively [12]. This filamentous fungus has long been recognized as a source of industrial enzymes and has the ability to produce several enzymes in culture.

Strain improvements are generally concentrated to obtain higher yields of specific product of interest. Increased antibiotic production in *Penicillium* sp. [13], enhanced cellulase production than the parental strain of *Trichoderma* sp. [14], interspecific hybrids with high cellulolytic activity [15], increased citric acid [16] and antagonistic potential with antibiotic production [17], following protoplast fusion were reported. Protoplast fusant of *Aspergillus carbonarius* and *Aspergillus niger* was characterized by higher production of pectinase with increased growth rates and efficient substrate colonization [18]. The aim of study was to use parent strains of *T. harzianum* and *T. viride* and their fusant strain HF9 for maximum chitinase and protein produc-

tion under different conditions to drawing a protocol for chitinase production by *T. harzianum* and *T. viride*.

MATERIALS AND METHODS

Culture maintenance and inoculum preparation. *T. harzianum, T. viride* and fusant *Trichoderma* HF9 were used from CAS102 lab Collection Center, Chennai, India. They were maintained on PDA medium (g/l): potato – 200; dextrose – 20 and agar – 20, pH 6.5. The inoculum was prepared by adding 5 ml of sterile distilled water to 5-days old scrapped slant culture. The suspension was transferred into 100 ml of molten PDA, mixed thoroughly, poured into sterile Petri plates and incubated at room temperature (28°C). Mycelial discs of 9 mm were cut randomly from 5 days old cultures with sterile cork borer and used throughout the investigation and were subcultured at regular intervals to prepare conidial suspension.

Preparation of cell free culture filtrates. Basal medium containing (g/l): $(NH_4)_2SO_4$ – 2.8, urea – 0.6, KH_2PO_4 – 4.0, $CaCl_2 \cdot 2H_2O$ – 0.6, glucose – 40, $MgSO_4$ – 0.2, $FeSO_4$ – 0.01, $ZnSO_4$ – 0.028, $MnSO_4$ – 0.032, $CoCl_2 \cdot 6H_2O$ – 0.04 g and colloidal chitin – 5 (pH 6.5) was used. Conidial suspension (10^6 conidia/ml) served as inoculum. The fungus was grown at 200 rpm and 28°C. The culture medium was collected by passing it through 0.22 μm filter paper (Millipore, USA) and its pH was monitored using pH meter (Orion Research, USA) at 28°C. The culture filtrate was used as an enzyme source.

Preparation of fungal cell wall. *Rhizoctonia solani* cell wall was prepared according to the procedure of Sivan and Chet [19]. *R. solani* culture was grown at 200 rpm and 28°C for 6 days in 200 ml PDB (g/l): potato – 200, and dextrose – 20, pH 6.5. The mycelia were collected, thoroughly washed with sterile distilled water and homogenized using a homogenizer (Braun, Germany) for 5 min with maximum speed. Mycelial suspension was centrifuged at 1600 g (Sigma, USA) for 20 min at 4°C. The pellet was resuspended in distilled water and sonicated on ice (30 s × 3 cycles) using a sonicator (Braun, Germany) at maximum amplitude. The suspension was centrifuged again at 200 g (Sigma, USA) for 10 min at 4°C to precipitate the coarse debris. The pellet was frozen and lyophilized until powder (Virtex Company Inc., USA) sealed and stored at 4°C until use.

Plate assay for lytic activity. Five days old cultures of *T. harzianum, T. viride* and fusant HF9 on 1.0% colloidal chitin media with 2% agar were flooded with an aqueous solution of 1.0% Congo red in water followed by 1 N NaCl with intermittent shaking at 50 rpm for 15 min. The fungal growth was stopped by flooding the chitin agar plates with 1 N HCl which changed the dye colour (blue to violet) and the enzymatic activity further was inhibited with 1 N NaOH which slightly changed the dye colour (brown to red colour). Lytic activity was revealed by the size of clear zone near mycelium on plate with colloidal chitin.

Effect of carbon source on chitinase and protein production. The basal medium with different carbon source of 1% glucose, sucrose, fungal cell wall, 0.5, 1.0 and 1.5% of colloidal chitin were used as a substrate for the production of chitinase and protein under shaken conditions at 200 rpm for 10 days.

Effect of pH, temperature and incubation period on chitinase and protein production. Colloidal chitin medium with *Trichoderma* strains at different pH of 5.5, 6.5, 7.5, 8.5 and temperature of 24, 28, 32, 36°C and incubation at 200 rpm for 10 days was used to observe the chitinase and protein production periodically.

Chitinase assay. Colorimetric assay of chitinase was followed by Reissing et al. [20]. Briefly, the reaction mixture contained 1 ml of 0.1% colloidal chitin in 0.05 M sodium acetate buffer, pH 5.2, and 1 ml of culture filtrate (enzyme) incubated at 37°C for 2 h. Respective substrate and enzyme blanks were included. Heat denaturated enzyme was also used as control. The reaction was terminated by adding 0.1 ml of 0.08 M potassium tetraborate, pH 9.2, to 0.5 ml of reaction mixture and boiling in a water bath for 3 min. Obtained GlcNAc was determined spectrophotometrically at 585 nm. Specific activity of chitinase was expressed as amount of GlcNAc per mg of protein per h (U).

Protein estimation. Protein concentration in the culture filtrate was estimated by the dye binding method of Bradford [21] using bovine serum albumin as standard protein. One ml of culture filtrate was used with 5 ml of dye binding reagent (Thermo Scientific, USA) and mixed well. The absorbance was read at 595 nm after 30 min incubation.

SDS-PAGE. SDS–PAGE analysis was performed with the Bio-Rad System (USA) using 12% slab gels according to Laemmli [22]. Proteins of culture filtrate were precipitated by 30% trichloroacetic acid in acetone at –20°C for overnight and centrifuged at 13,000 *g* (Sigma, USA) for 10 min. The pellet was washed twice with ice-cold acetone, air dried until acetone evaporated, and then resuspended in 20 μl of 50 mM Tris-HCl buffer, pH 8.0. Equal volume of 2x sample buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue in 125 mM Tris-HCl buffer, pH 6.8) was added and proteins were denatured for 3 min in boiling water bath. The gels were subjected to electrophoresis at 120 V for 2 h and stained with Coomassie blue (R250) to visualize protein bands. Medium range protein markers were used (Bio-Rad, USA).

Amino acids analysis from extra cellular protein using HPLC. The total amino acid content of the protein in cultivation filtrate was analyzed in parental strains of *Trichoderma* as well as fusant HF9 strain. The parent and fusant extracellular proteins (70 μg/ml) were hydrolysed with the addition of 5 ml of 6 N HCl. After hydrolysis HCl was removed in a rotary evaporator and the residue dissolved in water. It was made up to a known

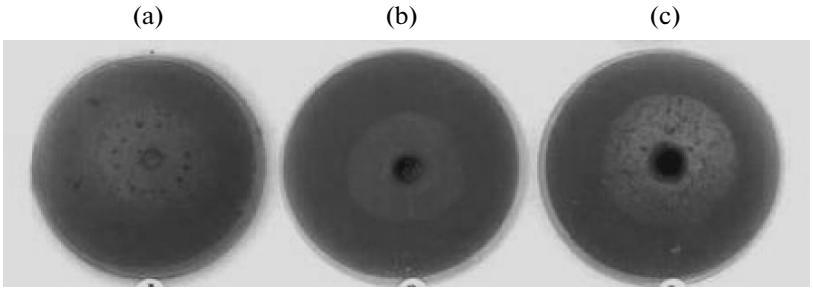

Fig. 1. Plate assay for lytic activity of chitin revealed by staining with Congo red for *T. harzianum* (a), *T. viride* (b) and fusant HF9 (c) grown on 1% colloidal chitin medium.

volume and used for amino acid analysis. The HPLC (Shimadzu, Japan) and column C18 (4.6 × 250 mm) with 5 μm packing size was used. Individual amino acids were separated by gradient 0.1 M acetate buffer, pH 6.8 (mobile phase A) and 3% tetrahydrofuran in methanol (mobile phase B) at a flow rate of 1.5 ml/min. Resolution of amino acid derivatives were monitored through fluorescence detector with excitation and emission set at 330 and 450 nm, respectively.

Peak identification and quantification. Amino acids were detected based on the retention time established for the individual amino acid under defined experimental conditions. Linearity of the peak areas for different concentrations from 20 to 200 pM, of individual amino acids was determined. Calculation was based on the area under peak established for a definite acid of known concentration. NIST amino acid mixture (USA) was used as a control.

RESULTS

Plate assay for lytic activity. Lysis of colloidal chitin in the growth medium was revealed by staining with Congo red and determined as the clear zone around the mycelium. It differed distinctly between the parent strains (*T. harzianum* and *T. viride*) and the fusant HF9 and was prominent and larger in interfusant HF9 than the parent strains (Fig. 1). The fusant HF9 larger clear zone indicated enhanced production of chitinase and it could be directly related to strain improvement in *Trichoderma*.

Effect of glucose, sucrose and fungal cell wall on chitinase and protein production by parental strains of *Trichoderma*. 1.0% glucose, sucrose and fungal cell wall were added in growth medium of *Trichoderma* as a carbon source. The results obtained revealed decrease of chitinase and protein production in the presence of glucose and sucrose in comparison with usage of the *R. solani* cell wall. It could be explained by the fact that the *R. solani* cell wall containing chitin and it could induce secretion of more chitinase and protein (Table 1).

Effect of chitin concentration on chitinase and protein production by parental strains of *Trichoderma*. Significant chitinase activity was observed when the fungus was grown in basal medium containing chitin. *T. harzianum* and *T. viride* were tested in different concentrations of colloidal chitin (0.5, 1.0 and 1.5%) for their ability to produce chitinase and protein under shaken conditions. Increased concentrations of substrate more than 5 mg/ml, decreased the enzyme and protein activity. Maximum total chitinase (Fig. 2a), specific chitinase (Fig. 2b) and protein content (Fig. 2c) recorded on 0.5% colloidal chitin amended medium under shaken conditions was obtained after 6 days of fungal growth. Total chitinase activity was estimated as the amount of enzyme required to produce 1 μmole of GlcNAc in 1 ml of reaction mixture under standard

Table 1. Effect of adding glucose, sucrose, and fungal cell wall in growth medium on chitinase and protein production by parental strains of *Trichoderma*

Organism	Carbon source	Total chitinase, U/ml	Specific chitinase, U/mg	Protein, μg/ml
T. harzianum	Glucose	3.86 ± 1.09*	1.02 ± 0.56**	3.6 ± 1.07*
	Sucrose	5.11 ± 1.95*	1.21 ± 0.93**	5.8 ± 1.86*
	Fungal cell wall	7.25 ± 0.87**	1.97 ± 0.17**	7.8 ± 1.65*
T. viride	Glucose	3.21 ± 0.94**	0.69 ± 0.64**	3.5 ± 1.84*
	Sucrose	4.90 ± 1.28*	0.82 ± 0.82**	4.8 ± 1.95*
	Fungal cell wall	6.86 ± 1.95*	1.48 ± 0.55**	6.5 ± 2.13*

* $P < 0.05$ is significant; ** $P < 0.01$ is highly significant. Values are mean of triplicates with ± standard error.

assay conditions. Specific chitinase activity was estimated as the units of enzyme/mg protein.

Effect of pH, temperature and incubation on chitinase and protein production by parental strains of *Trichoderma*. The optimum parameters for maximum chitinase and protein production by *T. harzianum* and *T. viride* were revealed at pH 6.5 (Table 2), temperature of 28°C (Table 3) and incubation for 6 days (Fig. 2). The aerated conditions were optimum for higher growth of fungi produced more enzyme and protein.

Chitinase and protein production by fusant *Trichoderma* HF9. After standardization of parent strains we used fusant *Trichoderma* HF9 in growth medium supplemented with 0.5% colloidal chitin, at pH 6.5 and temperature of 28°C under shaken conditions for the chitinase and protein production. In these conditions fusant *Trichoderma* HF9 in comparison with parent strains had 3-, 2.5- and 1.5-fold increase of total chitinase, specific chitinase and protein, respectively (Fig. 2).

SDS-PAGE. The denatured SDS-PAGE analysis showed different protein patterns for culture filtrate of *T. harzianum* and *T. viride* and their fusant HF9 (Fig. 3). Among these three stains, the fusant HF9 clearly showed additional protein bands appeared in comparison with the both parent strains. Nine major protein bands with up regulation were identified in the fusant strain HF9, suggesting the recombination of two parental strains after protoplast fusion.

Amino acids analysis from extracellular protein. The total amino acids of protein secreted by *T. harzianum* and *T. viride* and their fusant HF9 were identified and quantified based on the standard amino acids peak detection (mentioned in materials and methods). Eight, six and ten amino acids were identified for *T. harzianum* and *T. viride* and fusant HF9 respectively (Table 4). Interestingly, we observed that 3 amino acids were additionally appeared in fusant *Trichoderma* HF9 protein in comparison with the parent strains. These results indicated that genetic recombination might have taken place during protoplast fusion of parental strains, which could give additional amino acids found in fusant *Trichoderma* HF9.

Table 2. Effect of pH on extra cellular chitinase production by *T. harzianum* and *T. viride* under shaken conditions

Organism	pH	Total chitinase, U/ml	Specific chitinase, U/mg	Protein, μg/ml
T. harzianum	5.5	132 ± 1.98*	4.8 ± 0.53**	14.2 ± 1.12*
	6.5	195.4 ± 2.07*	7.5 ± 0.85**	24.5 ± 1.26*
	7.5	187.5 ± 1.55*	6.1 ± 0.65**	21.7 ± 1.74*
	8.5	163.2 ± 1.75*	5.0 ± 0.23**	17.4 ± 1.27*
T. viride	5.5	98.7 ± 1.07*	3.6 ± 0.73**	11.2 ± 1.86*
	6.5	162.7 ± 1.87*	6.8 ± 0.82**	17.4 ± 1.92*
	7.5	146.2 ± 1.56*	5.2 ± 0.65**	14.1 ± 1.55*
	8.5	132.7 ± 1.93*	4.5 ± 0.73**	13.3 ± 1.42*

* $P < 0.05$ is significant; ** $P < 0.01$ is highly significant. Values are mean of triplicates with ± standard error.

Table 3. Effect of temperature on extra cellular chitinase production by *T. harzianum* and *T. viride* under shaken conditions

Organism	Temperature, °C	Total chitinase, U/ml	Specific chitinase, U/mg	Protein, μg/ml
T. harzianum	24	172.2 ± 1.08*	5.4 ± 0.76**	21.4 ± 1.45*
	28	194.2 ± 1.36*	7.9 ± 0.56**	26.0 ± 1.66*
	32	174.5 ± 1.35*	6.0 ± 0.70**	23.2 ± 1.54*
	36	162.5 ± 1.74*	5.2 ± 0.65**	21.1 ± 1.90*
T. viride	24	132.6 ± 1.54*	3.7 ± 0.47**	20.2 ± 1.72*
	28	161.5 ± 1.75*	5.8 ± 0.60**	23.5 ± 1.55*
	32	145.7 ± 1.08*	4.8 ± 0.72**	19.4 ± 1.40*
	36	132.8 ± 1.82*	4.0 ± 0.55**	17.2 ± 1.43*

Values are mean of triplicates with ± standard error. * $P < 0.05$ is significant; ** $P < 0.01$ is highly significant.

DISCUSSION

Chitin degradation depends on many environmental factors. pH, temperature, chitin concentration in the medium and incubation time with fungus degrading chitin seem to be the most important of them. In the present study, different carbon source like glucose, sucrose, *R. solani* cell wall and colloidal chitin in cultivation medium, were tested for chitinase and protein production by *T. harzianum* and *T. viride* and their fusant HF9. Colloidal chitin showed maximum chitinase and protein production. High chitinase production using colloidal chitin has been reported for *Fusarium solani* [23] and *Trichoderma pseudokoningii* [24]. It was shown that chitin or its oligomers induce the secretion of chitinase by *T. harzianum* [25].

Similarly, chitinase, 1,3-β-D-glucanase and extracellular proteins were involved in the degradation of *R. solani* cell walls thereby found to induce chitinase production [26]. We found that optimal conditions for the production of enzyme and protein by *Trichoderma* parent strains were at 0.5% concentration of colloidal chitin at 28°C, pH 6.5 and 6 days of incubation time. This result is supported by Prabavathy et al. [27] who described that 0.5% colloidal chitin with pH 6.5 at 28°C under aerated conditions were optimum for chitinase production by *T. harzianum*. On the other hand, the chitinase could be excreted by *T. harzianum* at pH 5.0, however, the enzyme was stable at pH 7.0–9.0 [6]. Chitinase excretion by *T. harzianum* with chitin occurred at 28°C in pH 5.0, but 45 and 65%

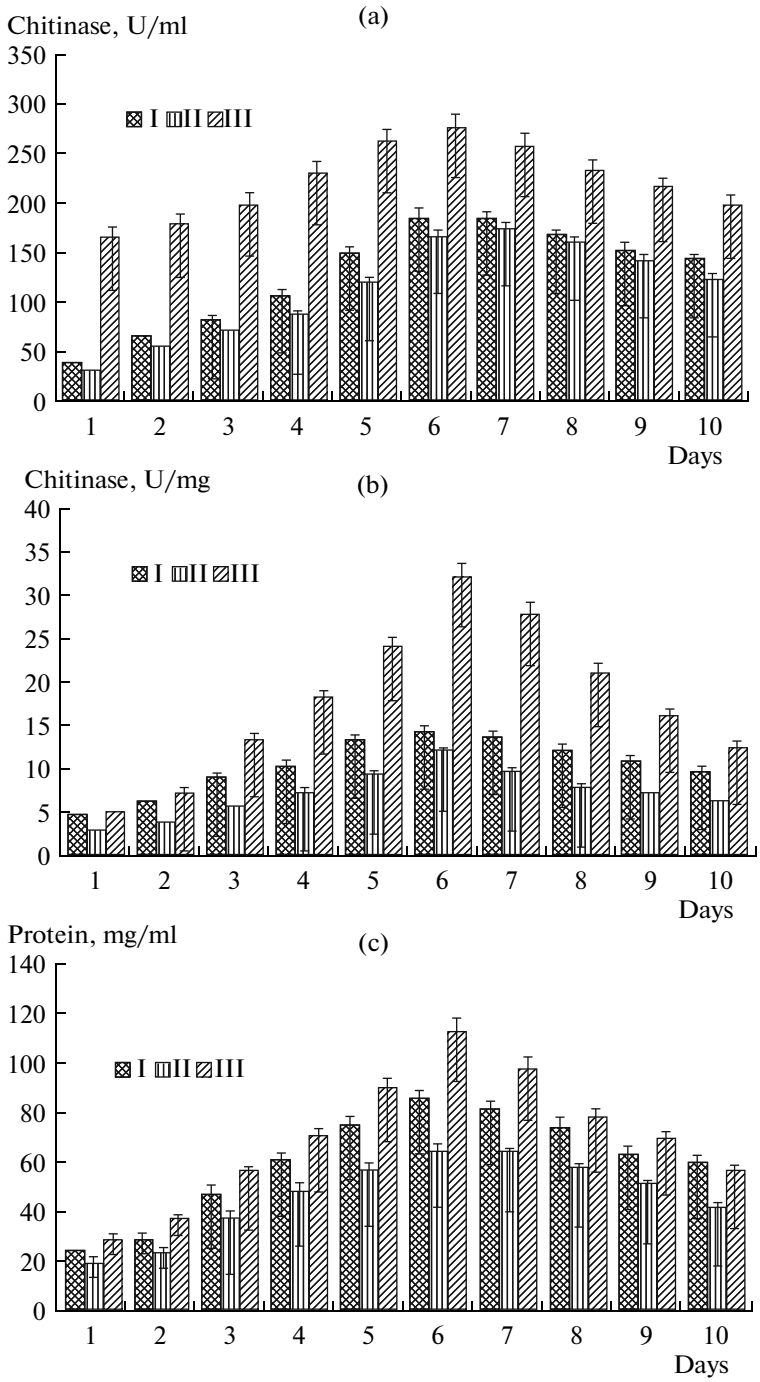

Fig. 2. Total (a) and specific chitinase (b) and protein content (c) of the cultivation filtrate for *T. harzianum* (I), *T. viride* (II) and their fusant (III) grown medium containing 0.5% colloidal chitin medium.

chitinase activity was lost at 40 and 60°C, respectively [28].

We observed that the production of chitinase and protein by *Trichoderma* parent strains was started after 24 h and reached the maximum on the 6-th day of incubation in medium containing colloidal chitin and gradually declined with further incubation. Similar findings were reported by Mathivanan et al. [29] for *T. viride*. On the other hand, Katragadda and Murugesan [24] have described release of chitinase by *T. harzianum* on the second day of incubation, with maximum production on the 8-th day and subsequent decline thereafter. It is important to note that chitin is a hard-decomposable compound and organisms need a longer time to adapt with this substrate in comparison

with the other high-molecular compounds to start the production of necessary enzymes [30].

T. harzianum produced more chitinase and protein when compared to *T. viride*, in the presence of glucose, sucrose, fungal cell wall and colloidal chitin in cultivation media. Furthermore, fusant *Trichoderma* HF9 produced more chitinase and protein than *T. harzianum*. However, self-fusant of *T. harzianum* had increased 2-fold chitinase activity with 0.5% chitin in medium in comparison with parent strain of *T. harzianum* [27]. Similarly, the fusant (R1) from *Geotrichum candidium* and *Phanerochaete chrysosporium* increased protein from 48 to 67 g/kg in corn stover [31]. Seven fusants exhibited higher extracellular glucose oxidase activities than their parent strain of *A. niger* [32]. Interestingly, 3 fusant strains AT 11-2-3, AT 11-2-10, and AT 11-2-14 produce 19.2, 6.1, and 10.5 g/l citric acid, respectively, compared with non-fusant strains *A. niger* and *T. viride* in semisolid culture containing cellulose [33]. Furthermore, the fusant MMFu3 showed increase in the production of protease (1.90 U/ml), peroxidase (1100 U/ml) and laccase (200 U/ml) when compared to the two mutant strains *Mucor mucedo* [34]. The enhanced production of extracellular chitinase and protein found in our study could be directly related to improved strains of *Trichoderma*.

It is interesting to note that the plate bioassay for chitinase produced by parent strains of *Trichoderma* and their fusant HF9 revealed that the fusant always secreted more enzyme that was evident from lysis of chitin around the colonies. These results clearly suggested that the superior performance of the improved strains by over production of both the parent strains.

We could observe protein variations among the parent strains of *Trichoderma* and their fusant HF9 by SDS-PAGE analysis. Fusant *Trichoderma* HF9 clearly showed 9 major protein bands with up regulation than both parent strains. Following protein analysis, we used proteins of cultivation filtrate for amino acids analysis. Fusant strain showed mix of amino acids observed in either of parents. His, Cys and Tyr were present in the protein produced by *T. harzianum* and fusant while Ser and Gly were found in the *T. viride* and fusant. Besides, Asp and Pro were common to both parental strains and fusants. Phe, Glu and Asn were additional amino acids recorded in fusant *Trichoderma* HF9 and, thus, the above results further confirmed that it was a hybrid of *T. harzianum* and *T. viride*. Interestingly, additional amino acids and their increased quantity observed in fusant HF9 strain. The alteration in extra cellular chitinase and protein followed by additional amino acids with their increased content in fusant *Trichoderma* HF9 added weight to the more probability of enhanced chitinase and protein production.

Enhanced antibiotic or enzyme production in hybrid strains of microorganisms by protoplast fusants alter the genetics of strains [35]. Increased chitinase, protein and amino acid contents produced by fusant *Trichoderma* HF9 demonstrated integration of both parental strains. Eventually, this fungus could be used for chitinase and protein production in large scale.

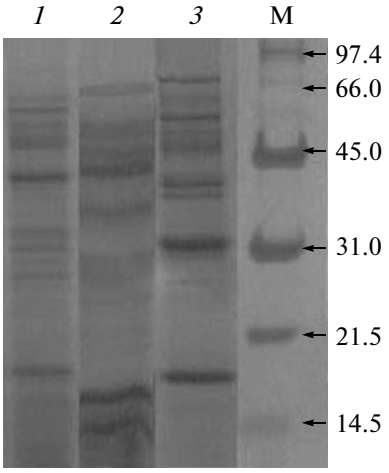

Fig. 3. Protein profile after SDS-PAGE of culture filtrate for *T. harzianum* (*1*), *T. viride* (*2*) and their fusant HF9 (*3*). M – protein markers.

Table 4. Amino acid analysis of protein secreted by parental strains of *Trichoderma* and their fusant

Organism	Amino acid	Concentration, mM/70 μg protein
T. harzianum	Asp	78.0
	Glu	405
	Thr	127
	Pro	2952.5
	Lys	1082.5
	His	335.0
	Cys	87.7
	Tyr	14.1
T. viride	Asp	43.4
	Thr	1102.5
	Pro	827.5
	Gly	855.0
	Ser	194.5
	Ala	275.0
Interfusant HF9	Asp	35.2
	Pro	1899.5
	Gly	186.5
	His	517.5
	Ser	552.5
	Cys	302.5
	Tyr	132.5
	Glu	26.2
	Asn	183.5
	Phe	42.5

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