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PLANT PATHOLOGY

Isolation and Selection of Fungal Strains for Multienzyme Production from Western Ghats

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ABSTRACT

Western Ghats is one of the eccentric biodiversity niches, with varied flora and fauna, in which fungi are one of the dominant group of microbes present in soil which strongly impact ecosystem structure. Hence there is a growing interest in assessing soil fungal biodiversity in Western Ghats and its biological functioning in production of various significant biomolecules. With the advent of new frontiers in the field of biotechnology the spectrum of cellulase and amylase has expanded in various industries, including food, fermentation, textiles, laundry, pulp, paper, agriculture as well as in research and development. The present study deals with the screening of fungal strains that amylase and cellulase which are industrially indispensable. The fungal strains were collected from the Western Ghats and were screened for initial multienzymes production. Thirty-two fungal strains were isolated among which eight were found to have both cellulolytic and amylolytic activity. Three fungal strains (TBG – 14, 5 and 4) were selected for further studies due to their capability in multienzyme production.

Highlights

- Multienzyme production was successfully attained from eight species of fungal strains isolated from different regions of Western Ghats and can be exploited for different industrial progression.

Keywords: Western Ghats, isolation, screening, fungal strains, multienzyme production

The Western Ghats originating from southern tip of Gujarat, extending from Satpura Range in the north traverses through the States of Maharashtra, Goa, Karnataka and Kerala and concludes at the southern tip of India and encopass 30% of all plant, fish, herpeto-fauna, bird, and mammal species found in India. Because of this superabundance in overall species diversity, India is recognized as one of the 17-mega diversity regions of the world. India's contribution to the global diversity is around 8%. Among various assortments of organisms in Western Ghats, vast array of novel and unidentified microbes could be explored for potential relavance, especially in enzyme production. Usually enzymes are very important for industrial, pharmaceutical and biotechnological process. The total market of industrial enzymes is expected to reach $7billion by 2018 with a compound annual rate (CAGR) of 8.2% from 2013 to 2018 (Anonymous – BBC research, 2015). India has a marginal share in the global market for industrial enzymes, which is estimated to be about US $3387.30 million and the segment is forecasted to grow at a CAGR of 15 percent till 2015 (Anonymous, 2013). Now india imports 70% of total enzyme consumed by its market which indicates need of indigenous manufacturers and technologies. Commercially availabile enzymes are derived from plants, animals and microorganisms. The enzymes derived from plant include papain, bromelin, lipooxygenase, among several others, and those derived from animal sources include pepsin and renin (Prakash *et al.* 2013). However, a major fraction of commercially available enzymes are derived from microbes due to their ease of growth, nutritional requirements and downstream processing. An important criterion for enzymes

derived from microbes remains that the source microbe should have a GRAS (Generally Regarded as Safe) status. Many microbes are currently employed for the production of various industrial enzymes (Souza and Magalhaes, 2010). Among the industrial enzymes hydrolases (used for the degradation of various natural substances) and proteases (used in detergent and dairy industries) remains the dominant enzyme types. Various carbohydrases, primarily amylases and cellulases, used in industries such as the starch, textile, detergent and baking industries, represents the second largest group (Gurung *et al*. 2013). There has been extensive research on microbial production of carbohydrases, potent fungal genera able to produce carbohydrases include *Bulgaria, Chaetomium and Trichoderma*, *Coriolus, Phaenerochaete, Coriolus, Schizophyllum*, *Aspergillus, Geotrichum and Penicillium* (Lynd *et al*. 2002).

Submerged Fermentation (SmF) and Solid State Fermentation (SSF) are used for the production of enzymes. SSF system appears promising especially to fungi because it resembles the natural habitat of microorganism (Laderman *et al*. 1993; Kunamneni *et al*. 2005). The enzyme activities were increased about 30-80% when produced by SSF in comparison with conventional SmF enzyme production. The microbial activities, soil microbial biomass and enzyme release are sensitive to soil biochemical characters like pH, temperature, electrical conductivity, total organic matter etc. In addition, it can also be used as active measures of soil productivity and effect of pollutants (Tate 1995). Production of multienzymes (pectinase, pectate lyase, cellulose, xylanase, β-xylosidase and invertase) through SSF have been reported in several instances through utilization *Aspergillus, Fusarium, Neurospora* and *Penicillium* sp using orange peel as a substrate (Mamma *et al.* 2008).

In another study by Delabonsa *et al*. 2013 multienzyme preparations containing pectinase, cellulase and xylanase enzymes have been produced using six fungal isolates. Among the six fungal isolates tested, *Aspergillus niger* proved to be the most potent and produced highly active multienzyme systems. Hence, in our present study we have made an attempt to isolate and identify different fungal isolates with their ability to produce multienzymes (cellulase and amylase) through fermentation technologies and corelated their enzyme production with different soil parameters.

MATERIALS AND METHODS

Sample collection and Isolation of Fungal Culture

Soil samples were randomly collected from 4-5 cm depth with help of sterile spatula from the various locations in Western Ghats. Isolation of fungal colony was performed by serial dilution and spread plate method. One gram of soil sample was serially diluted in sterilized distilled water to get a concentration range from 10^{-1} to 10^{-6}. A volume of 0.1 ml of each dilution was transferred aseptically to Potato dextrose agar plates. The sample was spreaded uniformly using a glass rod. The plates were incubated at 28 °C for 72 hr.

Screening of potent amylase producing fungi

The selected fungal isolates were screened for amylolytic activity by starch hydrolysis test on starch agar plate. The selected fungal isolates were streaked on the starch agar plate and incubated at 37°C for 2-3 days. After incubation iodine solution was flooded with dropper for 30 seconds on the starch agar plate (Sujeeta *et al*. 2017).

Screening of potent cellulase producing fungi

The isolated fungus was grown on carboxy-methylcellulose agar medium. The pure cultures were inoculated in the centre with almost equal amounts and incubated at 30 ± 2°C until substantial growth was recorded. The Petri plates were flooded with Congo red solution (0.1%), and after 5min the Congo red solution was discarded, and the plates were washed with 1N NaCl solution, allowed to stand for 15 - 20 minutes (Reddy *et al*. 2014).

Determination of Soil pH and Electrical Conductivity

Soil pH and electrical conductivity (EC) were determined in 1: 3.0 soil/water ratio by a combination glass electrode HI98129, Hanna Instruments.

Determination of Soil Organic Carbon and Soil Organic Matter

The soil organic C (SOC) content was estimated

by dichromate oxidation method in which the oxidation of $K_2Cr_2O_7$ in a concentrated H_2SO_4 medium and the excess dichromate was measured using $(NH_4)^2Fe(SO_4)^2$ (Yeomans and Bremner, 1989). Soil organic matter (SOM) were determined according to Pribyl, 2010.

Quantitative Enzyme Assays

Amylase assay

Enzyme production medium

Production medium contained (g/l) peptone- 26.7g; dipottasiumorthophosphate – 2.7g; tween 80 – 7.3ml; soluble starch10%. 100 ml of medium was taken in a 250 ml conical flask. The flasks were sterilized in autoclave at 121 °C for 15 min and after cooling the flask was inoculated with fungal cultures. The inoculated medium was incubated at 27 °C in shaker incubator for different incubation time. At the end of the fermentation period, the culture medium was centrifuged at 5000 rpm for 15 min to obtain the crude extract, which served as enzyme source. The enzyme activity was assayed following the method of using 3, 5- dinitrosalicylic acid.

Amylase activity was determined by measuring the release of reducing sugar from starch by DNS method (Miller 1959). The reaction mixture contains 0.2ml of crude enzyme and 0.8ml of 100mM phosphate buffer (p^H 7) containing 1% (W/V) of soluble starch. The mixture was indubated at 55 °C for the reaction was stopped by adding 2ml of DNSA (3, 5dinitrosalicyclic acid). The content were boiled exactly for 5 minute in water bath and cooled for 20- 25 minutes after which 1ml of 40% Rochelle salt (sodium pottassium tartarate) was added. Finally the colour developed was read at 540nm in a spectrophotometer. The amount of reducing sugar released in the mixture was determined.

Cellulase assay

The positive fungal strains were used to know their potential for cellulase production and activities. A volume of 100 ml of Czapek-Dox broth medium amended with 1% cellulose was distributed into separate 250 ml conical flasks. The pH of the medium was adjusted to 5. After autoclaving at 121°C and 15 lb. pressure, the fungal spore suspensions were inoculated into the conical flasks. The flasks were incubated at 27 °C on a rotary shaker at 120 rpm for 3 days. After 3 days, culture filtrate was collected, centrifuged at 6000 rpm for 15 min and supernatant was used to the estimation of cellulase source.

Activity of Cellulase in the culture filtrates was determined and quantified by carboxy- methyl cellulase method (Ghosh 1987). The reaction mixture with 1.0 ml of 1% carboxymethyl cellulose in 0.2 M acetate buffer (pH 5.0) was pre-incubated at 50°C in a water bath for 20 minutes. An aliquot of 0.5 ml of culture filtrate with appropriate dilution was added to the reaction mixture and incubated at 50 °C in water bath for one h. Appropriate control without enzyme was simultaneously run. The reducing sugar produced in the reaction mixture was determined by dinitro- salicylic acid (DNS) method (Miller 1959). 3, 5-dinitro-salicylic acid reagent was added to aliquots of the reaction mixture and the color developed was read at wavelength 510 nm.

Morphological & Microscopic Identification of selected fungal strain

Morphological characters were studied by inoculating the fungal isolates onto Czapek Solution Agar (CZA) which contained (g/L): Sucrose, 30.0; $NaNO_3$, 2.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.01; Agar, 15.0; pH 7.3 ± 0.2 and incubated for 5 to 7 days. Every 24 h plates were examined and the colony characteristics like surface and reverse colony colour, colony margins, elevations, growth rate etc were noted.

Micro-morphological characters were studied by staining the 5 day old fungal colonies with lactophenol cotton blue. One loopful of culture was aseptically transferred onto a clean glass slide with the help of sterile inoculating needle. The slide was placed on a staining tray, flooded with lactophenol cotton blue and left it for 1 min. A clean cover slip was placed onto it with the help of a needle and excess stain was blotted with bibulous paper and examined under low, high & oil immersion objectives. Only those isolates which were morphologically similar to Aspergillus were selected. The selected isolates were maintained on Sabouraud Dextrose Agar (SDA) slants and stored at 4 °C for further study.

Molecular characterization

The genomic DNA of the fungi was extracted using modified CTAB method (Moller *et al*. 1992) and ITS1-5.8S-ITS2 rDNA fragment region was amplified with primers, ITS1 (5'- TCCGTAGGTGAAC CTGCGG -3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'. Bio-Rad S1000 thermal cycler was used for amplification with the following PCR profile using Emerald master mix: an initial denaturation for 1 min at 98°C, followed by 38 cycles of 10 sec at 97 °C, 30 sec at 48°C and 2 min at 72 °C and a final extension at 72 °C for 10 min. The amplified product was eluted using Wizard® SV gel and PCR clean up system and sequenced in ABI 3500 DNA Analyzer using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol. The sequences were searched against those already known at NCBI GenBank using BLAST search option. The sequences of ITS region were aligned with sequences of similar fungi retrieved from databases using CLUSTAL W (Thompson *et al.* 1997) and a phylogenetic tree was constructed using the neighbour- joining method using MEGA 7.0 with a bootstrap analysis of 1000 replicates (Kumar *et al.* 2004). The fungal ITS ribosomal region gene sequences determined in this study were deposited in GenBank and accession numbers obtained.

RESULTS AND DISCUSSION

In this investigation, we have navigated amylolytic and cellulolytic fungi from different locations of south Western Ghat mountain rain forest (Ponmudi, Kallar, Kulathupuzha, Menmutty and Marayoor) and the evergreen forest of Wayanad (Fig. 1), which marks the transition zone between the north and southern ecological region of Western Ghats.

Fig. 1: Sampling regions

A total of thirty two fungal isolates were scraped up from different regions of Western Ghats. The southern ecological regions are more wetter and species rich and that's why we have select these south Western Ghat regions. pH and EC are the most significant parameters for measuring soil quality and soil microbial biomass. Soil samples collected from six high land areas were showed slight variations in pH ranging from 5.6 to 8 (Table 1). Strains from different land areas were showed significantly wide ranges in cellulase and amylase activity and were more prominent in regions having neutral pH and electrical conductivity in the range of 43 - 49µs. Highest EC and pH was recorded in Ponmudi and Wayanad, fungal strains isolated from Ponmudi and wayanad were not able to produce both cellulase and amylase. However the number of organisms isolated from both wayanad and Ponmudi were competable with other sites. Strains isolated from locations with neutral pH and less electrical conductivity (Kallar, Meenmutty, Kulathupuzha and Marayoor) were able to produce both cellulase and amylase but the microbial biomass was less.

All the isolates were primary screened for production of amylase and cellulase using starch agar plate method and carboxymethyl cellulose plate method. Freshly prepared single spore cultures of fungal strains were point inoculated on the centre of the plates and incubated at 30 °C for 3 days. In case of amylase producing strains hydrolysis of starch around the colonies were visualized by flooding the plates with Gram's iodine solution (Fig. 9). The zone formation around the colony was due to the hydrolysis of starch by amylolytic enzymes produced by the strains. For detecting cellulolytic activity CMC agar plates were flooded with 0.1% (w/v) Congo red solution for 15 minutes followed by destaining with 1M NaCl solution for 15 minutes. Congo red clearing zone assay is suitable for qualitative display of cellulase activity (Fig. 10). The clearing zone of enzymatic activity will be visible around the batch of growth. The NaCl solution elutes the dye in the clearing zone where the cellulose has been degraded into simple sugars by the enzymatic activity. Only eight fungal isolates (Fig. 2 – 9) were found to be positive for both amylase and cellulase production, as determined by measuring the width of the clear zone (zone of hydrolysis) formed around the fungal colonies

Table 1: Soil physiological characters

Sl. No.	Sample collection sites	Number of fungal strains collected	Number and name of cellulase and amylase positive strains	PH	Electrical conductivity	Organic carbon	Soil organic matter
1	Wayanad	7	0	5.6	65µs	3.09%	5.32
2	Ponmudi	6	0	8	86µs	1.68%	2.88
3	Kallar	4	3 (TBG -5 14 & 16)	6.9	43µs	10.02%	17.23
4	Kulathupuzha	4	1 (TBG-1)	7	45µs	3%	5.16
5	Meenmutty	5	2 (TBG- 2 & 4)	7.11	53µs	0.99%	1.70
6	Marayoor	6	2 (TBG - 7 and 2)	7.20	49µs	2%	3.44

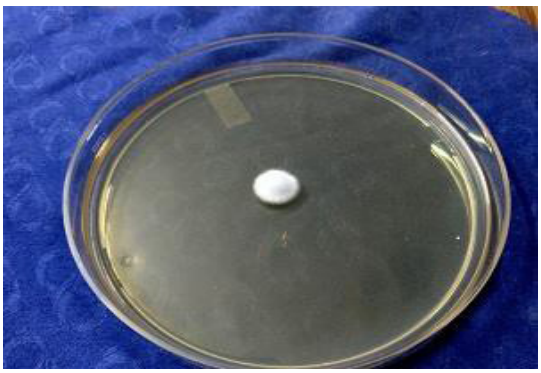

Fig. 2: TBG - 1

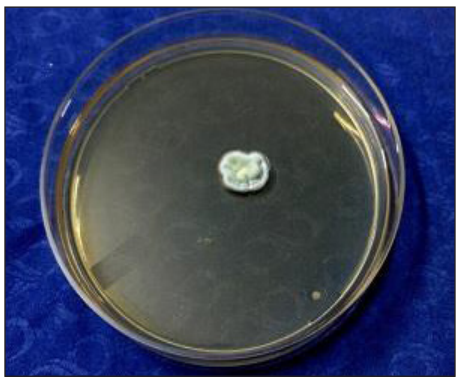

Fig. 3: TBG - 2

Fig. 4: TBG - 3

Fig. 5: TBG- 4

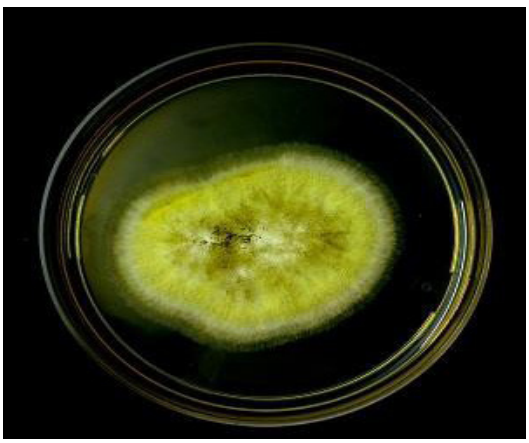

Fig. 6: TBG- 5

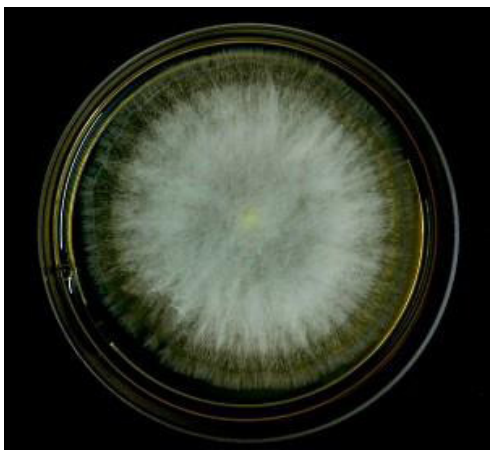

Fig. 7: TBG- 6

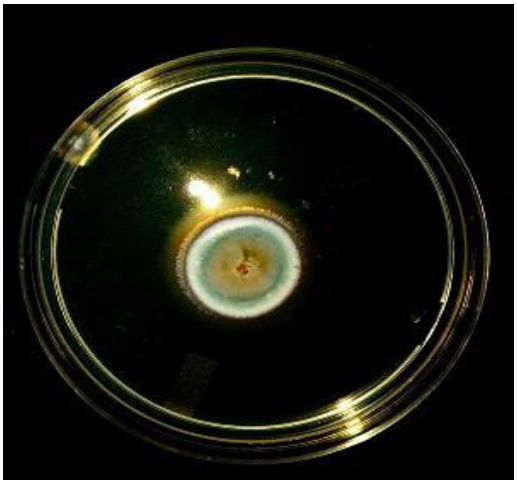

Fig. 8: TBG - 7

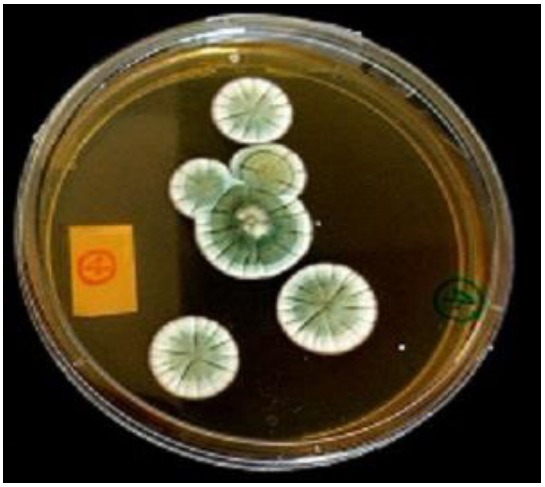

Fig. 9: TBG - 8

Fig. 10: Amylolytic activity in starch agar medium

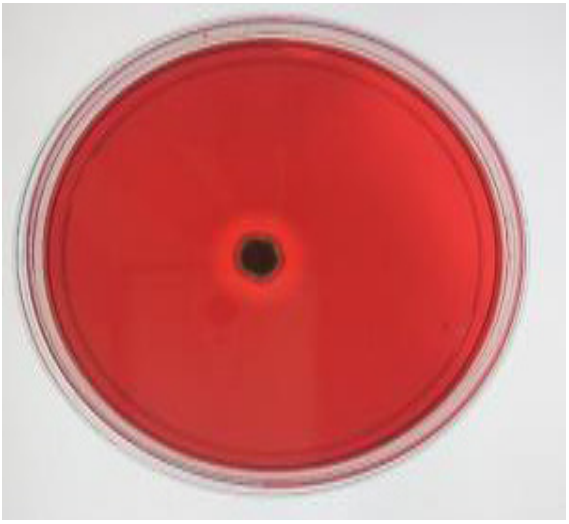

Fig. 11: Cellulolytic activity in CMC medium

on starch agar (SA) medium and carboxymethyl cellulose (CMC) agar medium. The fungal isolate TBGRI-7 isolated from Marayoor showed maximum zone of hydrolysis i.e. 1.7cm and 1.0 cm in starch agar and carboxymethyl cellulose agar medium respectively. Followed by TBGRI- 4 (1.2 cm, 1.0 cm), TBGRI -1 (1.1 cm, 0.8 cm), TBGRI- 2, 5 & 16 (1.0 cm, 0.72 cm), TBGRI – 12 (0.8 cm, 1.2 cm) and TBGRI - 14 (0.5 cm, 0.8 cm), respectively in SA and CMC agar plates respectively.

Studies on fungal amylases, especially the developing countries are concentrated mainly on *Rhizobium* sp. probably because of their ubiquitous nature and non-fastidious nutritional requirements (Abe *et al.* 1998). Hence there is an increasing worldwide interest in the screening of new microorganisms producing amylases suitable for industrial application (Burhan *et al.* 2003; Gupta *et al.* 2003). In our study the isolates were screened for production of amylase using starch plate method, positive strains showed clear zone of starch hydrolysis in the Petri dishes after iodine treatment. These isolates were selected for divination of enzyme activity. Kumar and Duhan, 2011 studied about the amylase production in five different strains of *Aspergillus sp.* All the five strains which produced the clear zone of varying size and were selected for amylase production. Out of the five strains *Aspergillus niger* MTCC-104 was found to be the supreme (i.e. 1249 U/mL) amylase producer while *A. flavus* was least (i.e. 338 U/ml) producer. Tripathi *et al.* 2011 isolated several fungal strains for amylase production in which 18% from selected isolates showed high potential of enzyme production whereas 36% resulted moderate enzyme

production and the rest 45 % showed low potential for amylase.

Kathiresan *et al.* (2006) reported that maximum activity was detected in 96h (136 U/ ml) by *Penicillium fellutanum* under sub merged fermentation. Sharma and Shukla, 2008 reported the maximum amylase production (185U/ml) was found with *Aspergillus fumigatus* for 6 days of incubation at 30 °C. The fungal strains which are commercially exploited for cellulase preparations were *T.viride (*Benkun *et al.* 2007; Shafique *et al.* 2009), *T. harzianum* (Shafique *et al.* 2009) *T.reesei* (Shafique *et al.* 2009) *Aspergillus niger* (Milala *et al.* 2005; Sharada *et al.* 2012) *Penicillium chrysogenum* (Nwodo *et al.* 2007), *Acrophialophora nainiana* and *Ceratocystis parodoxa* (Barros *et al.* 2010) and *A.japonicus* (Sanyal *et al.* 1988). It was found that cellulase activity was maximum on the 4th day for all strains and decreases gradually as incubation time increases. It might be due to the depletion of nutrients in the medium which stressed the fungal physiology resulting in the inactivation of secretary machinery of the enzymes. Similar results was observed by (Kuhad *et al.* 2013) 45 cellulase producing fungal strains were isolated from compost samples, out of which 36 fungal isolates were purified and 23 isolates showed the cellulase activity. The isolates were identified as *Trichoderma, Aspergillus, Rhizopus* and *Penicillium* species. In which maximum fungal growth and enzyme production by *Aspergillus* sp. was obtained after 4 days of incubation period whereas *Trichoderma, Penicillium* and *Rhizopus* sp exhibited maximum fungal growth and enzyme production after 5 days of incubation. Liming *et al.* (2014) reported cellulose production in *Trichoderma resei* ZU – 02 using corn cob residues. Cellulase activity reached 5.25IU/ml after seven days of incubation. It might be due to the depletion of nutrients in the medium which stressed the fungal physiology resulting in the inactivation of secretary machinery of the enzymes. In comparison with the above references the fungal strains isolated from Western Ghats showed exorbitant quantity of cellulase production and can be used for industrial purposes.

Batch fermentation was carried out in triplicate flasks and enzymatic activity was estimated at regular intervals. The differences among the mean values data to the activity obtained at different hours were statistically tested using one way ANOVA. Pre-induced fungal spores (1×107 spores/mL) were inoculated onto sterilized media and incubated. The culture filtrate was centrifuged at 12,000 rpm for 30 min at 4 °C and assayed for enzyme activity. The isolate TBG - 14 showed maximum amylase activity of about 56.43 U/mL on 5th day of incubation followed by TBG – 4 (51.25 U/mL), TBG -5 (50.69 U/mL), TBG – 7 (46.5 U/mL), TBG – 2 (45.35 U/mL), TBG – 16 (40.02 U/mL), TBG – 12 (37.47 U/mL) and TBG - 1 (35.30 U/mL) (Fig. 10). TBG – 1and 14 were the strains showing maximum activity at 5th day the other six strains showing their maximum activity at 4th day. In case of cellulase production maximum activity (380.19 U/mL) was showed by TBG - 5 on 4th day of incubation followed by TBG – 4 (363.05 U/mL), TBG-16 (343.44 U/mL), TBG – 14 (336.71 U/mL), TBG – 7 (335 U/mL), TBG – 1 (300 U/mL), TBG – 12 (273.72 U/mL) and TBG - 2 (266.93 U/mL) (Fig. 11). All the strains showed maximum cellulase production on 4th day. TBG – 14 was showed momentous production of both cellulase and amylase so the strain was preferred for further studies.

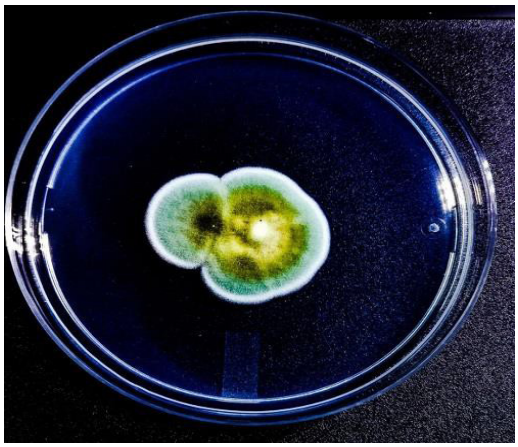

Fig. 12. Colony morphology of selected fungus

TBG - 14 on Czapek solution agar attaining 50 mm diameter after seven days, colony color was initially white becoming deep green to black with conidial production, reverse mostly pale brown with entire margins and slow growth (Fig. 13). Conidia were subglobose, smooth, with length 2.8µm and 2.5µm width. Stipes was smooth with 258µm length and 3.4 µm width. Phiallides were in flask shaped and following quarte verticillate pattern (Fig. 13&14). Molecular characterization of TBG 14 was done by extracting genomic DNA followed by amplification of ITS regions and sequencing. DNA was extracted

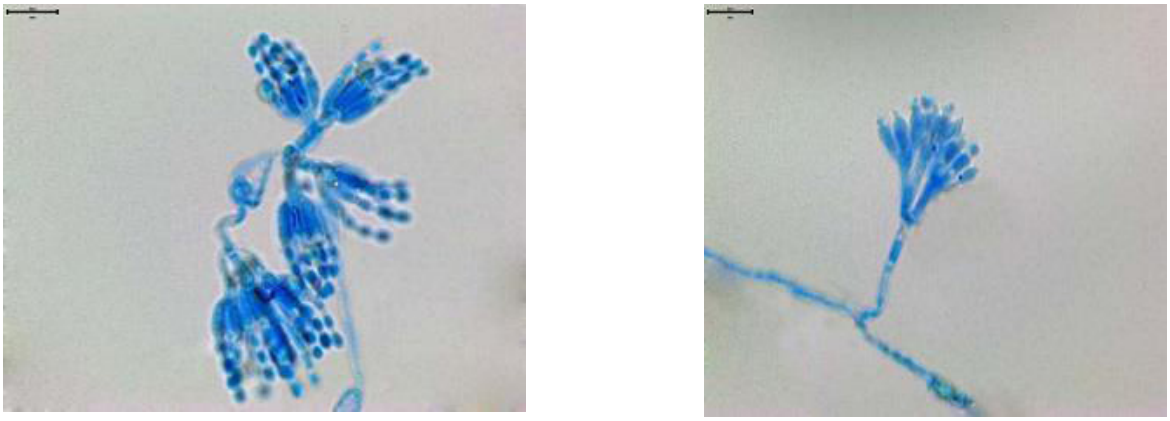

Fig. 13 &14: Microscopic characteristic

by modified CTAB method and the extracted genomic DNA was resolved in 0.8% agarose gel containing 0.5 mg/mL ethidium bromide. Amplification of ITS1-5.8S-ITS2 rDNA fragments was done using the primer pair ITS4 and ITS5 and the molecular size of the product were found to be 821 bp (Fig. 15).

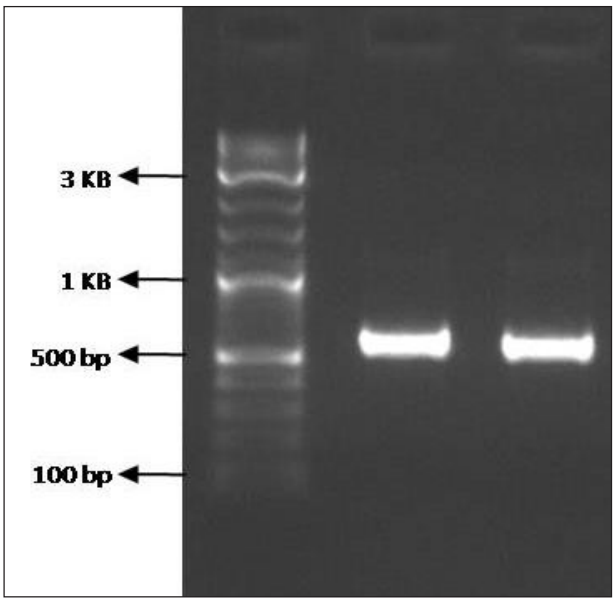

Fig. 15: Agarose gel pic showing DNA bands of the isolates. Lane1-100bp ladder

The amplified fragment was sequenced and compared with sequences in the BLAST alignment program of the Genbank database. The phylogenetic tree was constructed using MEGA 7.0 (Fig. 16). Based on the results of similarity comparison of the ITS-5.8S gene sequences it is concluded that the isolate TBG 14 is closely related to *pencillium chrysogenum* (Accession no.MH 201392).

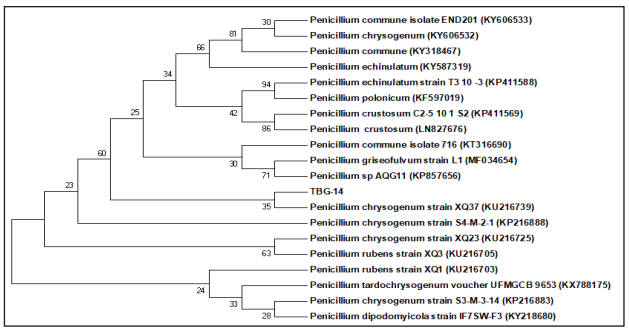

Fig. 16: Phylogenetic tree showing evolutionary relationships of 30 taxa based on the similarities of ITS1-5.8S-ITS2 Sequences. The evolutionary history was inferred using the Neighbour joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Phylogenetic analyses were conducted in MEGA 7

Several microorganisms can be obtained to enzyme production. However, filamentous fungi are important organisms for production of useful enzymes and biological active secondary metabolites. Studies on fungal enzymes especially in the developing countries are of great concern, probably because of the ubiquitous nature and non fastidious nutritional requirements of these organisms (Abe *et al.* 1988). *Penicillium* is particularly interesting for industrial enzymes due to their easy cultivation, and high production of extracellular enzymes of large industrial potential. These enzymes are applied in many industries. Khokhar *et al.* 2011 isolated four *Penicillium* species viz. *P. janthinellum* (IK-48), *P. melinii* (IK-49), *P. velutinum* (IK- 51) and *P. waskmanii* (IK- 50) having clever to produce both cellulase and amylase. The strains

were hyperactive in the presences starch and cellulose as compared to control. In which *P. waskmanii* (IK- 50) showed the highest growth in cellulose and starch medium. *Penicillium* species with the ability to produce multienzymes have been described by Brown *et al.* 1987; Adeleke *et al.* 2012. In comparison with *Trichoderma* and *Aspergillus* the production of cellulase and amylase by *Penicillium* species is less.

CONCLUSION

The role of many enzymes has been known for a long time. Their existence was associated with the history of ancient Greece, where they were using enzymes from microorganisms in brewing, alcohol production, cheese making etc. Multi enzyme production was succesfuly attained from eight species of isolated fungal strains. From which one prominent strains (*pencillium chyrsogenum*) isolated from Kallar was selected for the greatest production of enzymes. This study confirms the potentiality of the strain for multi-enzyme production. Future work of the present study includes production and purification of enzymes and may help us decipher the characteristics of this strain for potential biotechnological and industrial utilizations.

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