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Hunt for Cellulase Producing Fungi from Soil Samples

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Large number of bioprocesses in industrial, environmental and food biotechnology make use of enzymes which can be procured from microbial sources.Cellulase, a complex of enzymes which catalyze the hydrolysis of cellulose and derived oligosaccharidesis potent enzymatic tool for industrial scarification of cellulosic biomass.Fungi are preferred due to their ease of cultivation and large production of extracellular enzymes of industrial importance. The present study aimed for screening and production of cellulose (Carboxy Methyl cellulase) producing fungi from soil samples of different ecological habitats.In the secondary screening, the specific activity of cellulose from strain FGCC/BLS 1, *Penicilliumsp*(2.22 U/mg) was found to be significantly higher than the reference strain (1.91U/mg). Further it was observed that specific activity was more in *Penicillium*and *Aspergillus* isolates as compared to the isolates of other genera.

Key words: Cellulases, Enzyme activity, Fungi, Soil.

Enzymes being one of the essential products required for human needs. Large number of bioprocesses in industrial, environmental and food biotechnology make use of enzymes which can be procured from microbial sources (Pandey*et al*.1999, Sharada *et al*. 2013).Cellulose is a branched glucose polymer made up of an -1, 4 glucose units linked by β –1, 4- D- glycosidic bond. Cellulose can be breakdown into sugars byeither acid hydrolysis or by enzymatic hydrolysis. But hydrolysis through enzymatic reactions is ideal because it produces fewer by-products and proceeds under milder condition (Gielkens *et al*. 1999,Acharya *et al* 2008).Cellulase, a complex of enzymes which catalyze the hydrolysis of cellulose and derived oligosaccharides is potent enzymatic tool for industrial scarification of cellulosic biomass.The cellulase complex used in simultaneous scarification and fermentation systems (SSFS) generally includes C1 [(EC 3.2.1.9), (exo-1, 4-β-D- glucanase, cotton lyase), Cx (EC 3.2.1.4), (endo- 1, 4 - β-D- glucanase, carboxymethylcellulase, or CMC ase) andCb (EC 3.2.1.21), (β- glucosidase)] (Chinedu *et al*. 2007, Jing *et al*. 2007, Sukumaran *et al*. 2009).

Fungi are microorganisms which are well known for their broad range of enzymes they produce and enzymes of fungal origin are financially viable.Fungi are preferred due to their ease of cultivation and large production of extracellular enzymes of industrial importance.The adequate enzyme production was reported by most of the fungi like *Aspergillus* and *Trichoderma sp.* Soil provides a varied and intricate environment for soil microbial population like fungi (Mandels and Sternberg 1976, Mukunda *et al*. 2012).Keeping in view the above situations, the present study was therefore undertaken with the aim of screening and production of cellulases producing fungi from soil samples.

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MATERIALS AND METHODS

Sample collection and Isolation of fungi

Soil samples from different environmental source within Jaipur region (Farm, uphill, and garden soil) were collected aseptically from 6 to 10 cm depth in sterile zip lock plastic bags. One gram of each Soil sample will be serially diluted ten times in sterile physiological conditions.From suspension 100 microliter will be spread on to potato dextrose agar (PDA) and incubated at 28°C for 7-10 days. The media will be amended with Streptomycin to prevent the growth of bacteria. Fungi will be isolated from each plate and sub-cultured on PDA to maintain the pure strain. Stock cultures will be maintained on PDA at 4°C(Sasi *et al.* 2010,Mukunda *et al*. 2012)

Primary Screening for Cellulase Production

Preliminary screening was done by inoculating the fungal isolates on CMC agar media with 1% (w/v) Carboxy Methyl Cellualse as a carbon source. After four days of incubation, the plates were kept at 50°C for enzyme activation and then flooded with 1% Congo red solution for 10 minutes followed by washing with 1N NaOH. The plates were observed for the clear zone of hydrolysis surrounding the colony. *Aspergillus niger* (Provided by Dr. B. Lal Clinical Laboratory Pvt. Ltd.)was used as a reference strain for cellulse production (Bakri *et al*. 2009, Mishra *et al*. 2010).

Identification of fungal isolates

The isolates which were found positive during plate hydrolysis were identified on the basis of cultural/macroscopic and microscopic characteristics using lacto phenol cotton blue (LCB) staining method and confirmed as per standard manuals, reference slides and available literature. In LCB staining fungal culture was placed with the help of adhesive tape on a clean glass slide, a drop of lactophenol cotton blue stain was mixed with culture. A clean cover slip was placed over the culture and viewed under the microscope (100X)(Alexopoulos1952).

Carboxy Methyl CellulaseProduction by Submerged Fermentation

Fungal isolates showing positive detection during primary screening were selected for the quantitative determination of cellulase production by submerged fermentation.

Inoculum Preparation and cellulose production

Spore suspension was prepared by adding 5 ml of sterilized distilled water on slants containing fungal spores and then scraped to loosen the spores. A uniform soil suspension was prepared by mixing spores vigorously and standard amount of inoculums (1ml) containing approximately 10^5 spores per ml counted by heamocytometer was transferred into a flask containing 50 ml of production medium.Submerged fermentation was carried out in Erlenmeyer flask by taking 50 ml of cellulase production medium and pH adjusted 5.5. The flasks were autoclaved, cooled at room temperature and inoculum was incubated for 4 days at 28ºC±2ºC on a shaker at 120 rpm(Jahangeer *et al*. 2005)

Determination of Carboxy Methyl Cellulase (endoglucanase) Activity

The cellulase activity of the crude enzyme was determined using 1% carboxy methyl cellulase as substrate, prepared in sodium acetate buffer (0.2 M, pH 4.8). The reaction mixture containing 0.5 ml of enzyme and 0.5 ml of substrate (1% Carboxy Methyl Cellulose) along with an appropriate blank, were incubated at 50°C for 15mins. The DNS method was followed for the incubated samples and optical density was read at 575 nm against blank. A standard curve of glucose (1mg/ml) was developed under identical conditions to determine the reducing sugars formed. One unit of cellulose activity of filtrate was expressed as Unit per ml (U/ml), which is defined as the amount of enzyme which liberates 1 µM of reducing sugar per ml per minute under assay conditions. Enzyme activity is expressed as specific activity which is represented as U/mg of protein (Sethi *et al*. 2013).

Protein Estimation

The total protein content in cell free filtrate (extracted from flask) was determined by Lowry's methods of protein estimation in which crude enzyme extract was reacted with the Lowry's reagents and the absorbance obtained was compared with a standard graph plotted by reacting a standard protein bovine serum albumin (1mg/ml) with Lowry's reagents. Then a graph was plotted between concentration of standard protein (Bovine Serum Albumin) on X axis and absorbance at 750nm on Y axis. Enzyme activity is expressed as specific activity which is represented as U/mg of protein.

The experiments were carried out in triplicates and were statistically analysed (Lowry *et al*. 1951).

RESULTS

Isolation of Fungi from different soil sample

Isolation of fungi was carried out by Serial dilution method using Potato Dextrose Agar media. Individual colonies of Fungi obtained were shown in Fig. 1.

Subculturing and Preservation

Thirteen isolates (four from farm soil, four from uphill soil and five from garden soil) were randomly picked up on the basis of difference in their plate morphology**.** The pure fungal isolates were subcultured and also preserved on slants and petriplates containing potato dextrose agar media.

Table 1. Showing results of Primary screening for cellulase production of all the 13 fungal isolates

Isolates	Primary Screening of cellulose
FGCC/BLS1	Positive
FGCC/BLS2	Negative
FGCC/BLS3	Positive
FGCC/BLS4	Negative
FGCC/BLS5	Positive
FGCC/BLS6	Negative
FGCC/BLS7	Positive
FGCC/BLS8	Positive
FGCC/BLS9	Positive
FGCC/BLS10	Positive
FGCC/BLS11	Positive
FGCC/BLS12	Negative
FGCC/BLS13	Positive

Primary screening of isolates for CellulaseProduction

The fungal isolates were screened for their cellulytic activity by visual detection of the clear zone around the fungal isolates formed on plates containing CMC agar media in presence of Congo red dye (Fig. 2). On the basis of appearance of the area of clearance, it was found that nine fungal isolates (Table 1 and Fig. 3) were showing positive test for cellulytic activity to variable extent and therefore further selected for cellulase production**.**

Identification of Fungal Isolates

Positive fungal isolates detected for cellulytic activity during primary screening (n=9) were identified (Fig. 3) up to genus and few up to species level based on their morphological (macroscopic and microscopic features) Table 2.

Secondary Screening forcellulaseProduction by Submerged Fermentation

Fungal isolates (n=9) were inoculated into production media with uniform spore suspension incubated under controlled conditions for four days. On the fourth day, growth of fungal biomass was observed.The cell free filtrate was processed for determination of enzyme specific activity. A variable pattern of activity was observed from the enzymes of cellulytic soil mycotic flora from different environment .The specific activity of FGCC/BLS1was significantly higher than reference strain. (Fig. 4)

DISCUSSION

Cellulases are hydrolytic enzymes which cleave cellulose into sugar subunits like glucose. There is a great demand of cellulases in food,

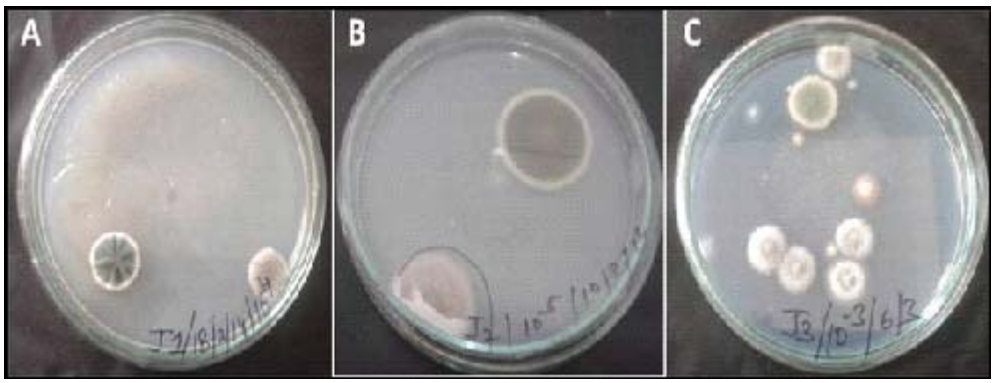

Fig. 1. Plates showing individual colonies of fungi isolated from soil of farm (A), uphill (B) and garden(C) area

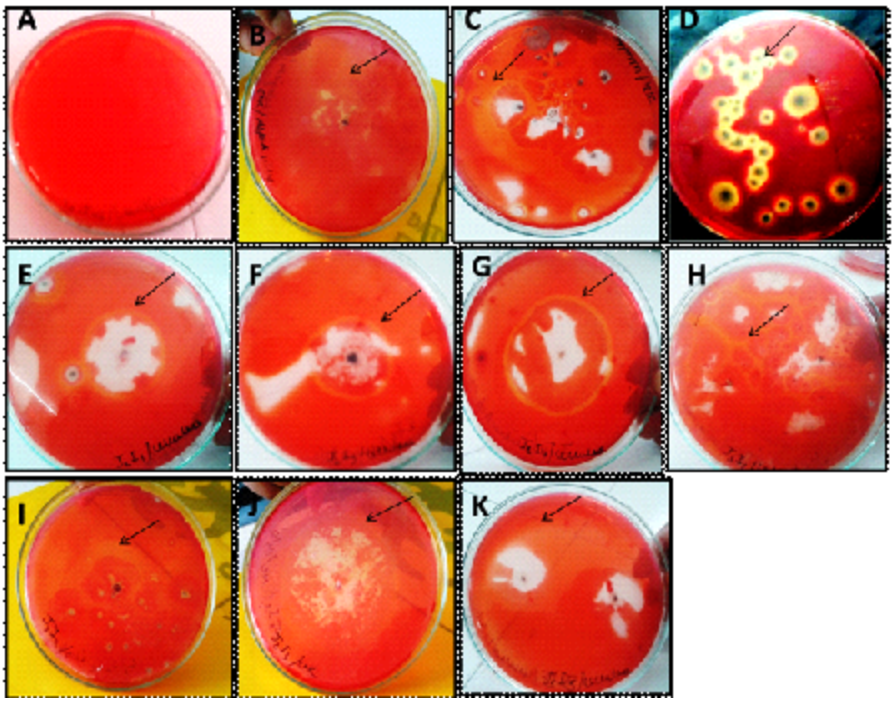

Fig. 2. Yellow halo zones or Clear areas (marked with arrow) showing positive detection of fungal isolates (n=9) for cellulase activity by chromogenic reaction in media containing CM-cellulose and congo red dye. A – uninoculated, B- Reference strain : *A. niger*, C – FGCC/BLS1, D – FGCC/BLS 3, E – FGCC/BLS 5, F – FGCC/BLS 7, G – FGCC/BLS 8, H – FGCC/BLS 9, I – FGCC/BLS 10, FGCC/BLS 11, K – FGCC/BLS 13

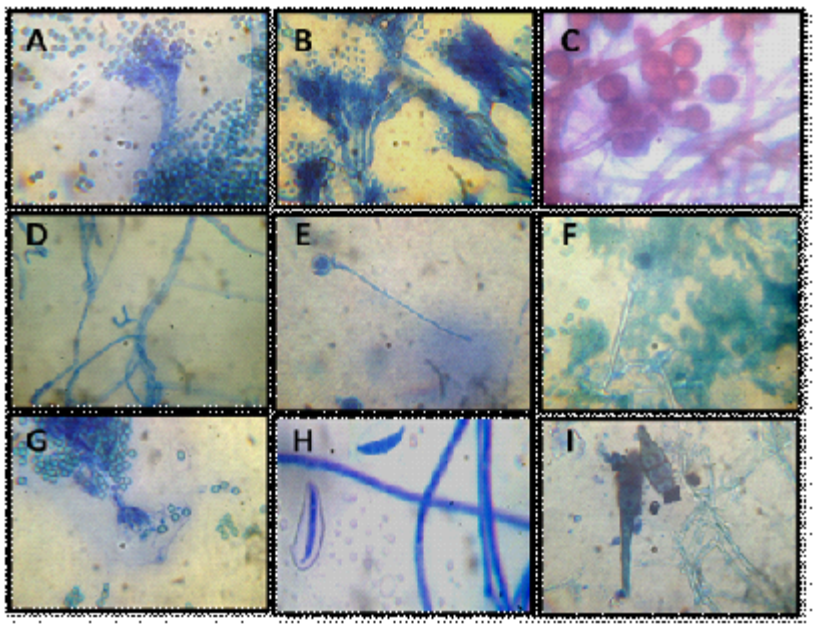

Fig. 3. Microscopic view of lacto phenol cotton blue staining of isolates at 100 X A-FGCC/BLS1(*Penicillum* sp),FGCC/BLS3(*Penicillum* sp.)FGCC/BLS5(*Phoma* sp.),FGCC/BLS7(*Sterilemycelia*),FGCC/BLS8(*Absidia* sp.) FGCC/BLS9 (*Aspergillus flavus*), FGCC/BLS10 (*Penicillium* sp.), FGCC/BLS11 (*Fusarium* sp.), FGCC/BLS 13 (*Microsporum* sp.)

Table 2. Identification of fungal isolates on the basis of Morphological (macroscopic and microscopic) features

S.No.	Strain code	Macroscopic characteristics	Microscopic characteristics	Probable strain
1	FGCC/BLS1	Velvety green colonies, spreading with white margin and yellow bottom	Short conidiophores with Chains of single called conidia are produced in besipetal succession specialisedconidiogenous cell called a phailide produced singly in groups or branched metulae, giving a brush like appearance (a Penicillus)	*Penicilliumsp*
2	FGCC/BLS 3	Green colonies with narrow white margin and pale yellow brown bottom	Chains of single called conidia on long conidiophores, are produced in besipetal succession specialisedconidiogenous cell called a phailideproduced singly in groups or branched metulae, giving a brush like appearance (a penicillin)	*Penicilliumsp*
3	FGCC/BLS 5	White to pink velvety colonies become olivaceous green with dark reddish brown reverse	Sub-hyaline to hyaline (dark pigmented/brown), septate hyphae, and produces rather large pyriform to globose shaped pycnidiaPhialides line the interior of each pycnidium, which produce single celled conidia. and also produces chlamydospores (chamydoconidia) in branched or un-branched chain	*Phomasp*
4	FGCC/BLS 7	Green with white surrounding circular colony with reversed plate showed dark green coloured due to pigmentation.	Septate hyphae	*Sterile mycelia*
5	FGCC/BLS 8	Colonies are fast growing, floccose, white at first becoming pale brown with age with dark reverse pigmentation.	The sporangia are relatively small, globose, pyriform or pear-shaped and are supported by a characteristic funnel-shaped apophysisoften with a short projection at the top Sporangiophores are hyaline to faintly pigmented, simple or some-times branched, arising solitary from the stolons, in groups of three, or in whorls of up to seven. Rhizoids are very sparingly produced.	*Absidiasp*
6	FGCC/BLS 9	spreading yellow-green colonies	Vesicles bearing phialides over their en-tire surface, biseriate or uniseriate. Conidiophore stipes are hyaline and coarsely roughened, Conidia are globose to subglobose and pale green in color.	*Aspergillus flavus*
7	FGCC/BLS 10	White colonies and White Bottom with reddish	Chains of single-celled conidia are produced in basipetal	*Penicilliumsp*

		yellow spot at the center.	succession from a specialisedconidiogenous cell called a phialide produced singly in groups orfrom branched metulae, giving a brush-like appearance (a Penicillus)	
8	FGCC/BLS 11	The colonies are whitish to brownish, pink, reddish shades with dark pink to brown reverse pigmentation	Macro conidia are hyaline, two- to several-celled, fusiform- to sickle-shaped, mostly with an elongated apical cell and pedicellate basal cell. Micro conidiaare 1- to 2-celled, hyaline, pyriform, fusiform toovoid, straight or curved. Chlamydoconidia may be present or absent	*Fusariumsp*
9	FGCC/BLS 13	Slightly raised Grey white colonies with reddish brown reverse on agar plate	Septate hyphae Thick walled Macro conidia spindle or fusiform, beaked and often have a septae at irregular intervals and a narrowed portion usually 5-15 celled	*Microsporumsp*

beverages, and textile, laundry, paper and pulp industries. The present study was aimed to screen the cellulytic fungi from natural environmental niche. For the similar effort thirteen different fungi were isolatedby serial dilution techniques **(Figure 1)**from soilmycotic flora of farm, uphill and garden area of Jaipur region. The isolates were randomly selected based on the difference in the plate morphology and were subjected to routine subculturing and preservation**.**All the pure isolates were primarily screened for cellulaseproduction out of which nine isolates shows positive detection (Table 1)for cellulase production during primary screening by plate assay(Fig 2)Further these isolates were identified(Table 2) and selected for secondary screening by submerged fermentation. *Aspergillusniger* was selected as a reference strainfor evaluating cellulase production and specific activity of isolated fungi. *A. niger* found to be good producer of cellulases (CMCases) which is well documented (Acharya *et al*. 2008).

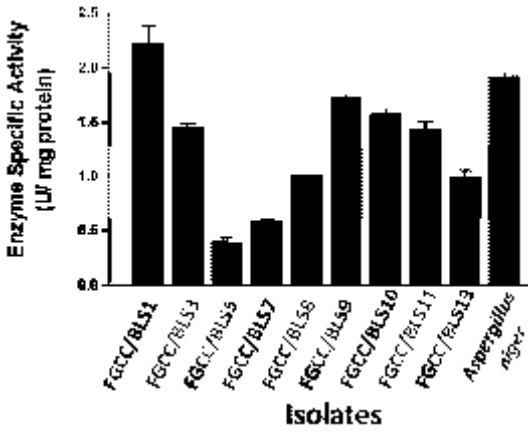

Fig. 4. Bar diagram represents the specific activity of cellulase as Unit per mg of protein in different fungal isolates (n=9) of soil samples compared with reference strain *Aspergillusniger*. The data is representation of 3 independent experiments

The positive isolates were characterized on the basis of colony morphology (macroscopic features) and microscopic mount using LCB staining method by referring standard manuals (Fig 3)**.**The isolates showing cellulose production during primary screeningwere from the genera*Aspergillus sp, Penicillium sp, Phoma sp, Fusarium sp, Absidia sp* and *Mycelia sterilia*. Among fungal isolates it was observed that the members of genera*Penicilliumsp*were most prevalent(Varalakshmi *et al*. 2009).

For submerged fermentation under controlled conditions, the production media was prepared and inoculated with uniform fungal spore suspension of respective isolates (n=9) and incubated for continuousfour days under shaking conditions. During growth, fungal hyphae consume nutrients present in the production media and then produced biomass as observed on the fourth day.

For enzymatic reactions,measurement of enzyme purity is most important whichcan be assessed by calculating enzyme activity and specific activity (activity per unit mass). In the present study for detection of enzyme activity, cell free filtrate was harvested on the fourth day and processed under optimum conditions followed by DNS method for enzyme assay(Varalakshmi *et al*. 2009). The specific activities (U/mg) of cellulase for reference strain and respective isolates were statistically calculated, are shown in the Figure 4.In the secondary screening, the specific activity of strain FGCC/BLS 1, *Penicilliumsp*(2.22 U/mg) was found to be significantly higher than the refrence strain (1.91U/mg). Further it was observed that specific activity was more in *Penicillium*and *Aspergillus* isolates as compared to the isolates of other genera. This was in agreement with Salgado *et al*. 2014 and Reddy *et al*. 2015.

Among all the isolates, the isolate FGCC/BLS1 which shows higher enzyme specific activity, need to be further investigated under optimum conditions for enhanced enzyme production as well as enzyme specific activity so that it can be used for scale up of cellulase production. Physico-chemical parameters which include media constituents, temperature and the pH of production medium,influences enzyme secretion as well as production which is very well documented. Therefore, molecular characterization of fungal isolate FGCC/BLS1 along withprocess optimization need to be carried out foridentification and enhanced enzyme production respectively.

CONCLUSION

Fungal cellulases have excellent industrial applications. The present study was carried out to evaluate the cellulytic activity of fungi isolated from the mycotic flora of soil samples. Screening and determination of cellulase enzyme activity revealed that isolate FGCC/BLS 1 is promising candidate for large scale cellulase production. Molecular Characterization of fungal isolate and process optimization for physico-chemical parameters needs further experimentation in order to improve enzyme secretion, purity and activity.

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REFERENCES

1. Acharya, P.B., Acharya, D.K., Modi, H.A. Optimization for cellulase production by *Aspergillus niger* using saw dust as substrate. *Afr J Biotechnol.,* 2008; **7**(22): 4147-4152.
2. Alexopoulos, C.J. Introductory mycology. Soil Sci., 1952; **74**(6): 481.
3. Bakri, Y., Magali, M., Thonart, P. Isolation and identification of a new fungal strain for amylase biosynthesis. *Pol J Microbiol.,* 2009; **58**(3): 269-273
4. Chinedu, S.N., Okochi, V.I., Smith, H.A., Okafor, U.A, Onyegeme-Okerenta, B.M, Omidiji, O. Effect of carbon sources on cellulase (EC 3. 2. 1. 4) production by *Penicillium chrysogenum* PCL501. *Afr. J. Biochem Res.*, 2007; **1**(1): 006-010
5. Gielkens, M.M., Dekkers, E., Visser, J., de Graaff, L.H. Two cellobiohydrolase-encoding genes from *Aspergillus niger* require D-xylose and the xylanolytic transcriptional activator XlnR for their expression. *Appl. Environ Microbiol.,* 1999; **65**(10): 4340-434
6. Jahangeer, S., Khan, N., Jahangeer, S., Sohail, M., Shahzad, S., Ahmad, A., Khan, S.A. Screening and characterization of fungal cellulases isolated from the native environmental source. *Pak J Bot.,* 2005; **37**(3): 739-748
7. Jing, D., Li, P., Xiong, X.Z., Wang, L. Optimization of cellulase complex formulation for peashrub biomass hydrolysis. *Appl Microbiol Biotechnol*., 2007; **75**(4):793-800
8. Lowry, O.H., Rosebrough, N.J., Farr. A.L., Randall, R.J. Protein measurement with the Folin phenol reagent. *J biol Chem.,* 1951; **193**(1):265-275
9. Mishra, B.K., Dadhich, S.K. Production of amylase and xylanase enzymes from soil fungi of Rajasthan. *J Adv Dev Res.,* 2010; **1**(1):21-23

10. Mukunda, S., Onkarappa, R., Prashith, K.T.R. Isolation and Screening of Industrially Important Fungi from the Soils of Western Ghats of Agumbe and Koppa, Karnataka, India. *Sci Technol* Arts *Res J.,* 2013; **1**(4): 27-32
11. Pandey, A., Selvakumar, P., Soccol. C.R., Nigam, P. Solid state fermentation for the production of industrial enzymes. *Curr sci.,* 1999; **77**(1):149-162
12. Reddy, G.P.K., Narasimha, G., Kumar, K.D., Ramanjaneyulu, G., Ramya, A., Kumari, B.S., Reddy, B.R. Cellulase production by *Aspergillus niger* on different natural lignocellulosic substrates. *Int J Curr Microbiol App Sci.,* 2015; **4**(4):835-845.
13. Sasi, A., Kani, M., Panneerselvam, A., Jegadeesh, G., Muthu, K., Kumar, R.M. Optimizing the conditions of á-amylase by an Esturian strain of Aspergillus spp. *Afr. J. Microbiol. Res.,* 2010; **4**(8):581-586
14. Sethi, S., Datta, A., Gupta, B.L., Gupta, S. Optimization of cellulase production from bacteria isolated from soil. *ISRN Biotechol.,* 2103; 2013.
15. Sharada, R., Venkateswarlu, G., Venkateshwar, S., Rao, M.A. Production of cellulase–a review. *Int J Pharmaceut Chem Biol Sci.,* 2103; **3**(4):1070-1090.
16. Salgado, J.M., Abrunhosa, L., Domínguez, J.M., Venâncio, A., Belo, I. Optimization of initial moisture and temperature in solid-state fermentation to produce cellulases by Aspergillus uvarum on olive mill and winery wastes. *Biomass Conv. Bioref.,* 2014; **4**: 201–209
17. Sukumaran, R.K., Singhania, R.R., Mathew, G.M., Pandey, A. Cellulase production using biomass feed stock and its application in lignocellulose saccharification for bio-ethanol production. *Renew Energ.,* 2009; **34**(2):421-424
18. Varalakshmi, K.N., Kumudini, B.S., Nandini, B.N., Solomon, J., Suhas, R., Mahesh, B., Kavitha, A.P. Production and Characterization of a-Amylase from *Aspergillus niger* JGI 24 Isolated in Bangalore. *Pol J Microbio.,* 2009; **58**(1):29-36.