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To isolate & characterized properties of cellulolytic fungi from different soil sample

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Abstract: *Soil contain a wide assortment of living matter in it so by using that property of soil isolation of fungus having cellulolytic activity has been isolated by using PDA. From 21 soil samples, 40 fungus were isolated. Morphological identification of isolated fungus was carried out among them four genus were identified as Penicillium spp., Fusarium spp., Trichoderma spp. and Aspergillus sp. Celluloytic activity of isolated fungus were carried by using Solid and liquid medium contain cellulose.*

Key words: *Cellulolytic fungi, Cellulase, soil.*

1. INTRODUCTION:

Soil is a characteristic body comprising of layers (soil skylines) of mineral constituents of variable thicknesses, which contrast from the parent materials in their morphological, physical, substance, and mineralogical attributes. It is made out of broken particles of rock that have been modified either by chemical or environmental processes because of erosion or weathering. Plants, animals, fungi, bacteria and humans affect soil formation. Mix soils to frame burrows and pores permitting moisture and gases to saturate further layers. Similarly, plant establishes open directs in the dirts, particularly plants with profound taproots which can enter numerous meters through the distinctive soil layers to raise supplements from more profound in the dirt. Plants with fibrous roots that spread out close to the dirt surface; have roots that are handily disintegrated, adding natural issue. Microorganism, including parasites and microscopic organisms, influence compound trade among roots and soil and go about as a save of supplements. Cellulolytic compounds assume a significant job in normal biodegradation measures in which plant lignocelluloses materials are productively corrupted by cellulolytic organisms. In industry, these compounds have discovered novel application in the creation of fermentable sugars and ethanol, organic acid, detergents and other chemicals. They have been used in pulp and paper industry, in textile industry, animal feed and even in the food industry, as well as biotransformation of waste cellulose to fermentable sugars. Fungal genera like *Trichoderma* and *Aspergillus* having capacity to produce cellulase and crude enzymes which are commercially available for agricultural use. As a rule, bacterial cellulases are constitutively created, while contagious cellulase is delivered uniquely within the sight of cellulose. Filamentous organisms especially Aspergillus and Trichoderma spp. are notable effective makers of cellulases.

2. MATERIALS & METHODS:

2.1. Sampling: Different sampling spots were selected such as sugarcane field, banana field, river soil, gulf soil and decaying wood soil. Soils were collected from various areas of Anand district, Rajkot district, Baroda district, Valsad district and from gulf of Khambhat, Gujarat, India (Table 2). About 40 to 50 gms soil samples were collected from 6-10cm depth into sterile plastic bag from different spots and region respectively. Collected Soil sample were transferred at 4°C for storage.

Table 1 : sample spots with respective areas

Sample no	Soil sample	Place
1	Banana field	Lambhwel Road, Anand
2	Banana waste	Lambhwel Road, Anand
3	Banana field	Vadod, Anana
4	Coconut leaf waste	Gulf of Khambhat
5	Sea grasses	Gulf of Khambhat
6	Guinea bags	Gulf of Khambhat
7	Leaves wastes	Gulf of Khambhat

8	Bhadar river I	Jetpur
9	Bhadar river II	Jetpur
10	Sugarcane field I	Jetpur
11	Sugarcane field II	Jetpur
12	Coconut waste (par river)	Umarsadi
13	Sugarcane field	Umarsadi
14	Degrading wood	Umarsadi
15	Rice field	Umarsadi
16	Banana field	Umarsadi
17	Mango field	Umarsadi
18	Decay wood (powder)	Umarsadi
19	Banana waste	Walasan
20	Banana field	Sarsa
21	Mahisagar river	Vadodara

2.2. Isolation: 1gram of soil sample was resuspended in 10ml of sterile distilled water and was allowed to stand for 10-15 minutes. About 200µl of supernatant was spreaded on the PDA plates using spreader. The plates were incubated for 7 days at 28^0C temperature and observed Daily observations were made for checking the growth and contamination. For isolating a single fungi from the master plate containing many fungal colony, single colony was selected and stabbed on PDA plate. The plates were incubated for 7 days at 28°C temperature.

2.3. Identification of Isolates: The tentative identification of the isolates was carried out by studying their colony characteristics, carried out by observing the colony on the plates and microscopic examinations (Morphology). It was carried out by using Lacto phenol cotton blue dye, colony were selected and by using needle it was put on to the slide, 2 drops of dyes were put on the colony and then it was covered by coverslip after that it was observed under Light microscope under 10x/45x objective lens.

2.4. Preservation of isolates: Isolates were streaked / stabbed on the PDA slant and incubated at 28°C for 7days. Slants were preserved on PDA at 4°C in refrigerator.

2.5. Cellulolytic activity: Cellulolytic activity was done for verifying the cellulolytic properties of fungus.
Solid Medium: Fungal spores from the preserved PDA slants were selected and stabbed on the Modified Czapex-Dox agar medium by using stab. Plates were incubated for 5 days at 28°C. Plates were sprayed with iodine solution and allowed to stand at room temperature for 5 minutes. The organisms producing cellulose showed clear zone on plates (Naoto Yoshida et al., 1989). Further confirmation of cellulose degradation was carried out in liquid medium.
Liquid medium About 100 ml of the liquid medium was taken in 250 ml Erlenmeyer flask and sterilized by autoclaving at 121°C for 15 min. This was cooled and inoculated with $1x10^7$ spores of the organism from PDA culture slants and after inoculation the flasks were incubated in orbital shaker-incubator (Yogi environmental shaker) at 28 ± 2°C at 120 rpm. Cultures were harvested at 24 h intervals by centrifugation at 5000 rpm for 10 min at 4°C using refrigerated ultracentrifuge (REMI, C-24 BL) over a period of 144 h. After centrifugation supernatant was used for the enzyme assay and for the protein estimation.
Enzyme assay (filter paper assay) : Enzyme assays for cellulases have been performed using substrates as a Whatman No.1 filter paper (50mg/1cm×6cm). Add 0.5ml of culture supernatant to 1ml of 0.05 M citrate buffer (pH 4.8) and filter paper strip was put in it. Incubate it for 1 hour at 50°C. After incubation reaction was terminated by adding 3 ml DNS reagent. Tubes were incubated in boiling water bath for 15 minutes, and 10ml of distilled water was added for dilution O.D. was taken at 540nm. Standard reading of 1mg/ml of glucose solution is used as substrate for calculating enzyme activity. Enzyme activity was assayed according to the methods recommended by the International Union of Pure and Applied Chemistry (IUPAC) commission of biotechnology
Protein Estimation:
Protein estimation was carried out by Folin Lowry's method. Different aliquots of sample were taken and final volume was made up to 1ml. About 5ml of Folin Lowry's reagent was added and tubes were incubated for 30 minutes in dark. About 0.5 ml of phenol ciocalteau (1:1) was added and mixed well by vortexing. Tubes were incubated for 10 minutes and then O.D. was taken at 630nm. Bovine serum albumin was used as a control.

3. RESULT:

3.1. Isolation: Many fungal colonies were obtained on the master plate (Potato Dextrose Agar medium). A single colony of one fungus were selected and spread on a fresh Potato Dextrose Agar plate. From all 21 soil samples, 40 isolate were obtained. Among those isolates 13 fungi were of banana field, 7 were of river soil and degrading and decay wood soil, 6 were of sugarcane soil and Gulf soil and 1 was of mango field respectively.

3.2. Identification: Primary identification by colonial characteristic, morphological characteristic and microscopic observation, only four genus were identified as *Penicillium spp., Fusarium spp., Trichoderma spp*. and *Aspergillus spp* whereas, for rest of the fungi were not identified.

3.3. Cellulolytic activity on solid: Modified Czapex Dox agar medium with cellulose as a carbon source was used for cellulose degrading property. When it is treated with iodine solution all of the 40 isolates were given zone of clearance which marked as a cellulase positive.

3.4. Cellulolytic activity on liquid: Cellulase assay was performed using DNSA method cellulolytic properties of the isolated fungus were checked on 5 different batches as numbers of isolates were 40 and having limited amount of space for flask in the shaker incubator. On liquid medium out of 40 isolates around 9 isolates were highly cellulolytic, which were further used for physico-chemical analysis.

3.5. Batch 1 Fungus of banana field: By using fungus isolates from the soil of banana field and carried out cellulase assay, out of 9 fungus, 2 (20.1, 20.4) were shown more cellulolytic activity as compare to other. Shown in graph 1 and table 3.

3.6. Batch 2 Fungus of Sugar Cane Field: By using fungus isolate from the soil of sugarcane field and carried out cellulase assay, out of 6 fungus 4 (10.1, 10.2, 11.1 13.2), were shown high cellulolytic as compare to other. Shown in Graph 2 and table 4.

3.7. Batch 3 Fungus of Gulf Soil By using fungus isolate from the soil of gulf and carried out cellulase assay, out of 6 fungus, 1 (4.1) were shown highly cellulolytic activity as compare to other. Shown in Graph 3 and table 5.

3.8. Batch 4 Fungus of River Bank Soil By using fungus isolate from the soil of river and carried out cellulase assay, out of 5 fungus, 4 (8.1, 8.2, 9.1, 21.1) were shown highly cellulolytic activity. Shown in Graph 4 and table 6.

3.9. Batch 5 Fungus of mango field soil, and decaying wood By using fungus isolate from the soil of mango field, degrading and decay wood and carried out cellulase assay, out of 7 fungus, 4 (14.2, 17.1, 18.1, 18.3) were shown high cellulolytic activity as compare to other. Shown in graph 5 and Table 7.

Table 2 : Primary identification of fungus.

Sample no.	Lab. Code	Color	Colony characteristics	Genus
1	**MKH 1.1**	Black	Dusty, flat and circular	*Aspergillus spp.*
2	**MKH 1.2**	White	Wooly and pluffy	Not identified
3	**MKH 2.1**	Brown	flat and circular	Not identified
4	**MKH 2.2**	Yellow	Small, flat and circular	Not identified
5	**MKH 2.3**	Green	Compact, Smooth and velvety	*Penicillum spp*
6	**MKH 4.1**	Gray	Wooly and compact	Not identified
7	**MKH 4.2**	White	Cottony and compact	Not identified
8	**MKH 4.3**	Orange	Wooly and loose	Not identified
9	**MKH 4.4**	Black	Dusty, flat and circular	*Aspergillus spp.*
10	**MKH 5.1**	Green	Compact, Smooth and velvety	*Penicillum spp*
11	**MKH 5.2**	Yellow	Small, flat and circular	Not identified
12	**MKH 6.1**	Green	Compact, Smooth and velvety	*Penicillum spp*
13	**MKH 7.1**	Black	Dusty, flat and circular	*Aspergillus spp.*
14	**MKH 7.2**	Brown	Dusty, flat and circular	*Aspergillus spp.*
15	**MKH 8.1**	White	Wooly and flower shaped	Not identified
16	**MKH 8.2**	White	Wooly and pluffy	Not identified
17	**MKH 9.1**	Brown	Dusty, flat and circular	*Aspergillus spp.*
18	**MKH 9.2**	White	Wooly and pluffy	Not identified
19	**MKH 10.1**	White	Wooly and pluffy	Not identified
20	**MKH 10.2**	Black	Dusty, flat and circular	*Aspergillus spp.*
21	**MKH 11.1**	Black	Powdery and circular	*Aspergillus spp.*
22	**MKH 11.2**	Brown	Dusty, flat and circular	*Aspergillus spp.*
23	**MKH 13.1**	White	Wooly and pluffy	Not identified

24	**MKH 13.2**	Black	Dusty, flat and circular	*Aspergillus spp.*
25	**MKH 14.1**	Black	Dusty, flat and circular	*Aspergillus spp.*
26	**MKH 14.2**	Green	flat and compact	*Trichoderma spp*
27	**MKH 16.1**	Gray	Wooly and compact	Not identified
28	**MKH 16.2**	Brown	Dusty, flat and circular	*Aspergillus spp.*
29	**MKH 16.3**	Green	Loose and circular	Not identified
30	**MKH 16.4**	Orange	Woolly velvety and compact	Not identified
31	**MKH 17.1**	Green	Cottony flat and compact	Not identified
32	**MKH 18.1**	Green	Fluffy and loose	Not identified
33	**MKH 18.2**	White	Compact Smooth and velvety	*Penicillum spp*
34	**MKH 18.3**	Yellow	Dusty and circular	*Aspergillus spp.*
35	**MKH 18.4**	Black	Dusty, flat and circular	*Aspergillus spp.*
36	**MKH 20.1**	Green	Compact Smooth and velvety	*Penicillum spp*
37	**MKH 20.2**	Greenish	Dusty, flat and circular	*Aspergillus spp.*
38	**MKH 20.3**	Black	Dusty, flat and circular	*Aspergillus spp.*
39	**MKH 20.4**	Pink	Wooly, Loose and grow very rapidly	*Fusarium spp.*
40	**MKH 21.1**	Black	Dusty, flat and circular	*Aspergillus spp.*

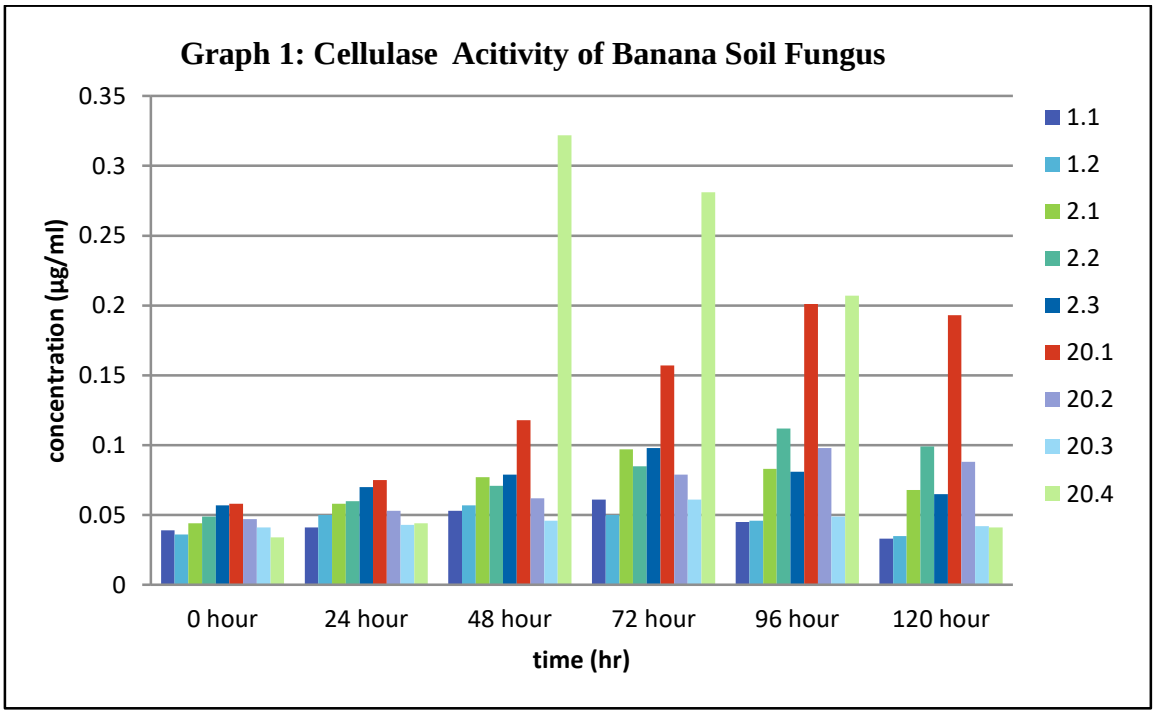

Table 3: Cellulase Acitivity of Banana Soil Fungus

Hours / Sample	0 hour	24 hour	48 hour	72 hour	96 hour	120 hour
1.1	0.039	0.041	0.053	0.061	0.045	0.033
1.2	0.036	0.050	0.057	0.050	0.046	0.035
2.1	0.044	0.058	0.077	0.097	0.083	0.068
2.2	0.049	0.060	0.071	0.085	0.112	0.099
2.3	0.057	0.070	0.079	0.098	0.081	0.065
20.1	0.058	0.075	0.118	0.157	0.201	0.193
20.2	0.047	0.053	0.062	0.079	0.098	0.088
20.3	0.041	0.043	0.046	0.061	0.049	0.042
20.4	0.034	0.044	0.322	0.281	0.207	0.041

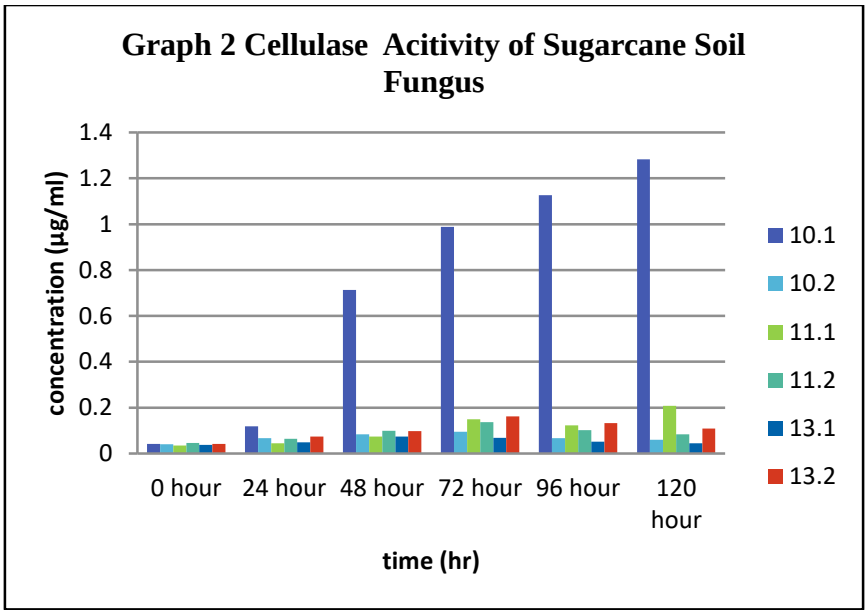

Table 4 : Cellulase Activity of Sugarcane Soil Fungus

Hours/ samples	0 hour	24 hour	48 hour	72 hour	96 hour	120 hour
10.1	0.042	0.119	0.713	0.988	1.127	1.283
10.2	0.040	0.067	0.084	0.095	0.067	0.060
11.1	0.034	0.045	0.074	0.149	0.123	0.208
11.2	0.046	0.064	0.099	0.136	0.102	0.083
13.1	0.037	0.049	0.074	0.068	0.051	0.045
13.2	0.042	0.074	0.098	0.162	0.132	0.109

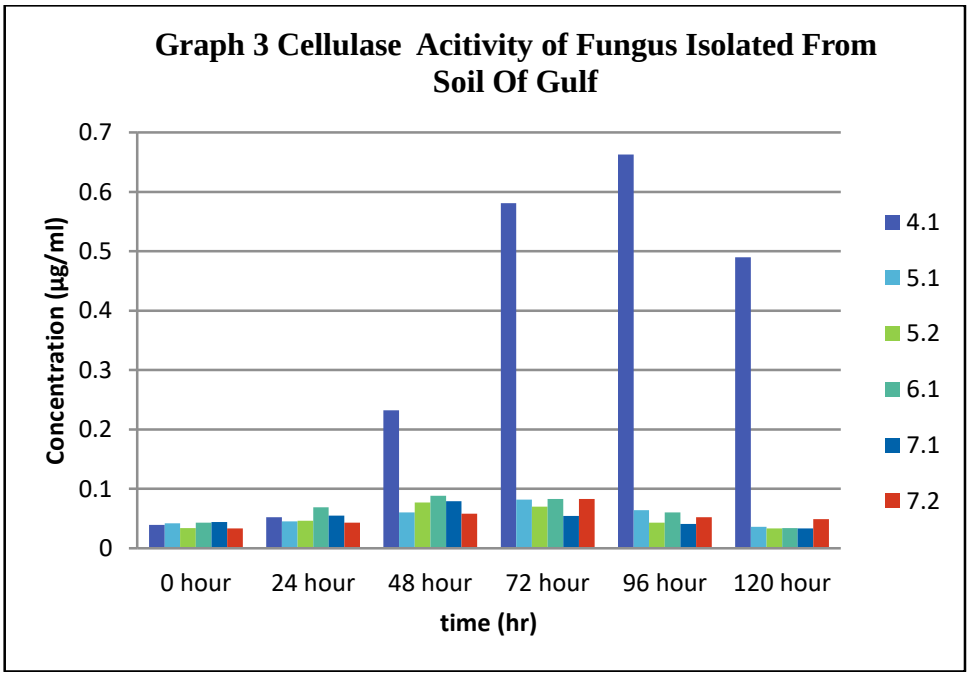

Table 5 : Cellulase acitivity of fungus isolated from soil of gulf

Hours/ samples	0 hour	24 hour	48 hour	72 hour	96 hour	120 hour
4.1	0.039	0.052	0.232	0.581	0.663	0.490
5.1	0.042	0.045	0.060	0.082	0.064	0.036
5.2	0.034	0.046	0.077	0.070	0.043	0.033
6.1	0.043	0.069	0.088	0.083	0.060	0.034

7.1	0.044	0.055	0.079	0.054	0.041	0.033
7.2	0.033	0.043	0.058	0.083	0.052	0.049

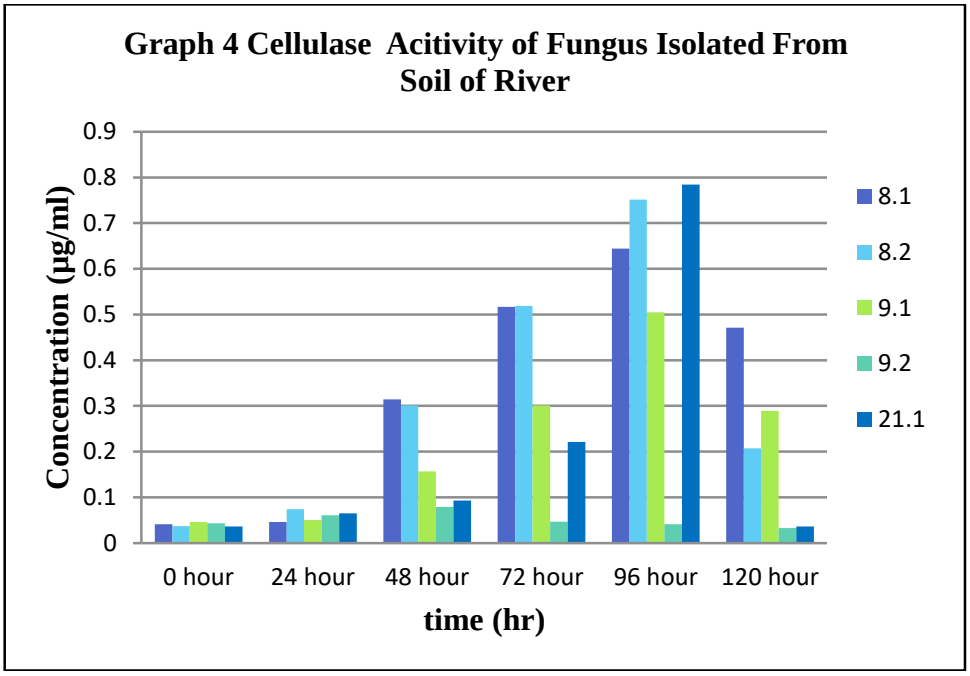

Table 6 : Cellulase acitivity of fungus isolated from soil of river

Hours/ samples	0 hour	24 hour	48 hour	72 hour	96 hour	120 hour
8.1	0.041	0.046	0.314	0.517	0.644	0.471
8.2	0.037	0.074	0.301	0.519	0.751	0.207
9.1	0.046	0.050	0.157	0.301	0.505	0.289
9.2	0.043	0.061	0.079	0.047	0.041	0.033
21.1	0.036	0.065	0.093	0.221	0.784	0.036

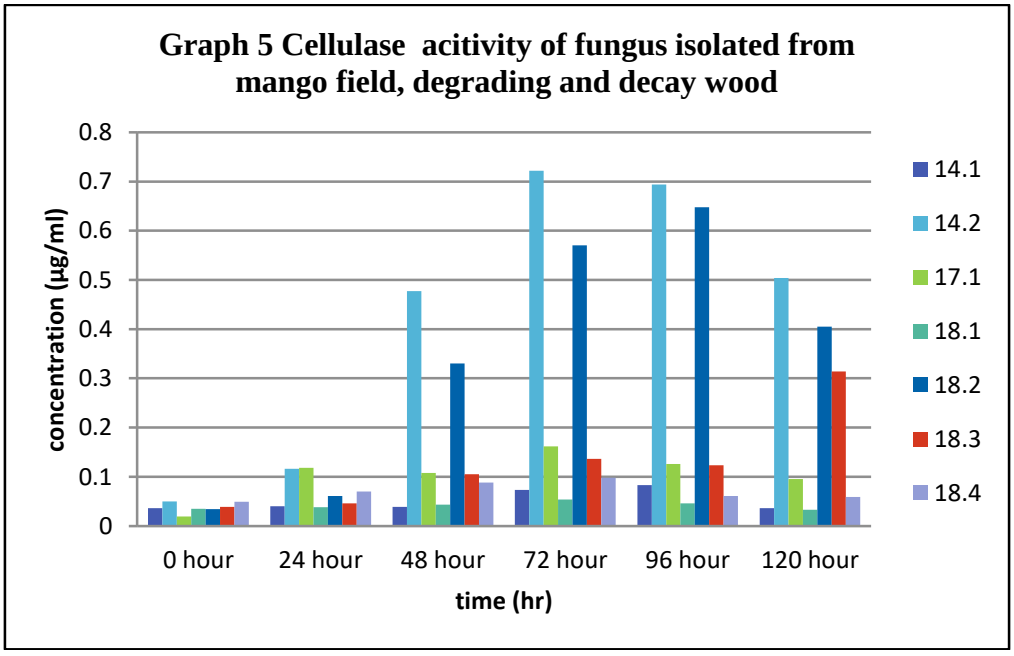

Table 7 : Cellulase acitivity of fungus isolated from mango field, degrading and decay wood

Hours/ samples	0 hour	24 hour	48 hour	72 hour	96 hour	120 hour
14.1	0.036	0.040	0.039	0.073	0.083	0.036
14.2	0.050	0.116	0.477	0.722	0.694	0.504
17.1	0.019	0.118	0.108	0.162	0.126	0.095
18.1	0.035	0.038	0.043	0.054	0.046	0.033
18.2	0.034	0.061	0.330	0.570	0.648	0.405
18.3	0.039	0.046	0.105	0.136	0.123	0.314
18.4	0.049	0.070	0.088	0.098	0.061	0.059

4. DISCUSSION:

Sampling spots were selected from different locations of Gujarat as soil characteristics mostly depends upon many factors like parental material, climate, biological factors and time. Soil formation mostly depends on the climate. Temperature and moisture affects the weathering and leaching. Wind plays major role in sand as well as other particles movement in arid region. The type and amount of precipitation also influences soil formation. Soil content affects soil behavior, including the retention capacity for nutrients and water. Thus characteristics of soil changes soil texture and its capacity (Breure & Bilthovan; KE Giller *et al* 1997). Torsvik and his team had stated that single gram of soil contains several thousand species of bacteria. Among the soil fauna, some 100000 species of protozoa, 500,000 species of nematodes (Hawksworth and Mound, 1991), 1500000 species of fungi (Hawksworth, 1991) and 3000 species of earthworms (Lee, 1985) are estimated to exist worldwide (Voroney RP, 2006). Change in environmental condition causes change in the genomic content of the biological factors present in the soil, thus results in evolution of new organism. Therefore, it is essential to collect samples from different locations which can be helpful in studying the diversity. (Soil Biodiversity, Agriculture ecosystem Biodiversity Strategy Action Plan)

There are many media's available commercially and many new were developed for fungus isolation as well as for further analysis like biochemical, cellulose hydrolyzing, etc. (Khalid Mahmood *et al.*, 2006*)* Amongst them potato dextrose agar is the medium, which is easy to prepare as well as cheaper in cost therefore, it was used to isolate fungus from the soil sample. Apart from soil there are reports of cellulolytic fungus isolation from waste paper gradual recycling material and many other sources. (Del Peciulyte, 2007) All isolates were identified based on their colony characteristics as well as microscopic identification. Out of 40 fungi about 17 fungus are unidentified as their colonial characteristic was novel to us. Whereas from remaining 23 fungus, 15 fungus features are matching with *Aspergillus spp,* 1 fungi feature is matching with *Fusarium spp,* 1 with *Trichoderma spp and remaining six fungus matches with Penicillium spp* (Alexopoulus CJ). Although the colonial characteristic cannot be considered as final for identification of fungi, but there are reports on predicting genus of each isolates based on their colonial characteristics, morphology, etc (Lidia, *et al.*, 2006).

In microscopic identification lacto phenol cotton blue dye was used. It has three important functions. Phenol molecules destroys contaminant compounds that fungi has, whereas the lactic acid conserves the fungi structure by provoking osmotic gradient change with relation of its internal structure, thus making a protective layer. Last but not the least, the cotton blue is the compound which adheres to the chitin of the fungi walls of hyphae and conidia

Cellulose degrading properties can be identified on solid as well as liquid medium. On solid medium the cellulose present in the media will react with potassium iodide in the presence of chloride molecule (i.e HCl) and thus give dark brown color. The area where cellulose have been utilized remains colorless as reaction do not take place (Naoto Yoshida *et al.*, 1989). In liquid medium the fungi produces cellulase enzyme to get carbon source for their growth from cellulose present in the medium. Cellulose degrading reaction takes place to degrade cellulose present in filter paper, which is further terminated by DNS reagent giving brown color. At 540nm absorbance indicates the total cellulase present in the medium which is produced by the fungus. In the year 1959, Miller and his team has developed the above mentioned protocol for checking cellulolytic activity and our isolates had also shown cellulolytic activity of the fungi by same protocol. Out of 40 isolates around 18 fungi were highly cellulolytic where as amongst them 9 were from sugarcane, Banana and decaying wood. Tan TK *et al.,* (1987) made studies of cellulolytic activities of some filamentous fungi. In respect to above mentioned work most of our isolates are filamentous and among forty the nine isolates were highly cellulolytic and structure was also filamentous. In the year 1998, Shrinivasan and Laxman had studied that microorganism produces cellulase enzyme. Their reports supports cellulolytic activity of soil fungal isolates, as they are also producing cellulase enzyme. Several reports on enzymatic hydrolysis of cellulose as well as cellulase production by microorganism/fungi strengthen the cellulolytic properties of our isolates. (G Immanuel *et al.*, Ojuma TV *et al.*, 2003; Reece ET, 1956; Milala MA, *et al.*,2005;) Kathiresan and Manivannan had also reported that *Penicillium fellutanum* isolated from coastal mangrove rhizosphere rich soil produces cellulase.

5. CONCLUSION:

Out of 40 isolates from various spots 18 isolates were highly cellulolytic. The origin of isolates were different sources like banana field, sugarcane field, mango field, rice soil, mangrove soil from gulf of Khambhat, river bank from Pardi, Jetpur and Vasad and decaying wood. 9 isolates out of 18 were from banana field, sugarcane field and decaying wood.

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