

Original Research Article

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Influence of Sampling Sites on the Occurrence and Distribution of Cellulose Degrading Microbial Communities

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ABSTRACT

An experiment was conducted for isolation of cellulolytic microorganisms from diverse environments. Samplings were done from 8 locations representing 27 samples viz. forest ecosystem, composting units, incorporation experiments and agricultural fields. A total of 653 microorganisms including bacteria, actinomycetes and fungi were isolated using serial dilution plate assay. All the cultures were initially screened for cellulolytic potential using carboxy methyl cellulose medium on the basis of zone of hydrolysis obtained. Among these cultures, 44 bacteria, 2 actinomycetes and 2 fungi were found to be efficient cellulose degraders. A variation in morphotypes obtained from different samples was found with maximum number of morphotypes were obtained from long term straw incorporation experiments. Among all the 48 cultures highest number of cellulolytic cultures were obtained from forest samples i.e. 35.4% followed by 29.2% from incorporation of crop residues, 25% from compost samples, 6.3% from field soil and 4.2 % from cow dung. Among 48 cultures 21 cultures each produced zones ≤ 10 mm and ≤ 20 mm, 4 cultures exhibited zones ≤ 30 mm and 2 cultures produced highest zones of ≤ 40 mm. So overall highest number of efficient cultures was obtained from forest followed by incorporation and compost samples. In nutshell, this study revealed that these systems represent a rich source of cellulolytic microbial biodiversity and needs further research for better understanding synergism between different microorganisms and decomposition process in nature.

Keywords

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Cellulose,
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Introduction

The microbes are considered as pillars of life on earth as they play a major role in the nutrient cycling. Plant residues are generated in huge quantities throughout the world. Under natural conditions the degradation of plant residues is carried out by specific group of microorganisms which are involved in C-

cycle. Addition of compost or wood residuals to topsoil results in an increase in carbon availability for beneficial soil microorganisms as well as maintains physical, chemical and biological properties of soil (Munten, 2005; Larney and Angers, 2012). Forest ecosystem represents a niche with enriched biodiversity of both microbes and plants which grows on relatively nutrient-poor soils. In order to fulfill

the nutrient requirements, the microorganisms present in these systems have developed enzymatic systems for the rapid turnover of organic matter and hence these processes results in rich plant diversity in the forest (Parton *et al.*, 2007). Plant residues consist of variable fractions of cellulose, lignin and hemicelluloses. Cellulolytic microorganisms are an important part of carbon cycle and they produce enzymes, which are responsible for the hydrolysis of cellulose. Enzymatic hydrolysis involves a series of enzymes, which act in a synergistic way to completely hydrolyze cellulose to simple sugars (Yu, 2007). The product of one enzyme acts as a substrate for another enzyme and in this way cellulase system completely degrades cellulose to simple sugars. The goal was to isolate cellulolytic microorganisms from diverse climatic conditions. Exploring the cellulolytic enzymatic potential in the residue degradation may lead to the production of improved end product that could be useful in enhancing the *ex-situ* composting

Materials and Methods

Site of sampling

The samples were collected to assess the performance of different agricultural systems from different sites of Punjab, Haryana and Himachal Pradesh using wide range of indicators (crop residues, composting sites, forest, crop establishment, crop rotation, rotten wooden logs, termite mounds in forest, cow dung piles).

Sampling procedure and preservation

The sampling sites were selected from hot spots of cellulolytic getting maximum population of cellulolytic microorganisms. Samples were collected randomly from different spots using auger. The collected samples were packed in polythene bags and

either processed on the same day or kept in deep freezer at -20°C till further use.

Isolation and maintenance of cultures

The isolation of microbes was done using two methods. In first method 10 grams of the respective samples was transferred to Erlenmeyer flask (150 ml) with 90 ml sterilized water and 5% CMC followed by shaking at 120 rpm for 7 days to encourage the growth of cellulolytic microbes whereas, in the other method direct serial dilution of the samples was prepared. In both the methods, Nutrient agar, rose Bengal agar and Kenknight's medium was used for selective isolation of bacteria, fungi and actinomycetes and the media was autoclaved at 15 psi and 121°C for 20 minutes. Serial dilution of the sample was made up to 10⁻⁶ dilution and appropriate dilutions (100µl) were pour plated or spread plated using Rose Bengal medium (Papaic digest of soybean meal: 5.0, Dextrose: 10.0, Monopotassium phosphate: 1.0, Magnesium sulphate: 0.5, Rose Bengal: 0.05, Agar: 15.0 and Final pH 7.2±0.2) for isolation of fungi, nutrient agar medium (Animal tissue peptic digest: 5.0, Sodium chloride: 5.0, Beef extract: 1.5, Yeast extract: 1.5, Agar: 15.0, pH 7.4±0.2) for isolation of bacteria and Kenknight's medium (Dextrose: 1.0, monopotassium dihydrogen phosphate: 0.1, sodium nitrate: 0.1, potassium chloride: 0.1, Magnesium sulphate: 0.1 and agar: 15.0) for actinomycetes then were incubated at 30°C for 4-7 days for the isolation of microbial colonies. Morphologically different colonies were selected for isolation and sub-culturing. Microbial colonies were picked carefully and every single colony was maintained on slants.

***In vitro* screening of cellulolytic activity**

Qualitative assay for cellulase

The presence of cellulose degrading capability was tested by using cellulolytic basal medium

(Minamiyama *et al.*, 2003), with the following ingredients: CMC agar medium I was used for the isolation of bacteria and actinomycetes. The media consisted of (g/L) NaNO₃: 2.0, K₂HPO₄: 1.0, MgSO₄: 0.5, KCL: 0.5, CMC Na Salt: 2.0, Peptone: 0.2 and Agar: 20.0. Cellulolytic basal medium II was used for isolation of fungi. The components of medium are as (g/L): KH₂PO₄: 0.5 Ammonium tartarate: 0.5 MgSO₄:0.1, CaCl₂.2H₂O: 0.01, Yeast extract: 0.001, CuSO₄: 0.001, Fe(SO₄)₃: 0.001, MnSO₄: 0.001 CMC: 20.0 and Agar: 20.0. The various components were dispensed in 1000 ml sterilized water and media was autoclaved at 121°C at a pressure of 15 lbs psi for 20 minutes.

Screening of cultures for cellulolytic potential

The plates were inoculated by spotting for bacteria and actinomycetes on CMC media (I) and 6 mm agar discs cut from 5 day old fungal culture on CMC media (II) of each strain separately and were incubated at 30°C in darkness. After 4-7 days the plates were flooded with Congo red dye (2% w/v) for 15 min. Then, the petriplates containing medium were rinsed with distilled water followed by flooding with 1 M sodium chloride for 1 min. Yellow opaque area around the colonies represented cellulase activity of microorganisms. The diameter of bacterial, fungal or actinomycetes colony and zone diameter were recorded to determine zone of hydrolysis obtained.

Results and Discussion

A total of 27 samples representing forest ecosystem, composting units, rotten wooden logs, termite mounds in forest, incorporation experiments, cow dung piles *etc.* were collected from different locations (Table 1). All the samples were processed using serial dilution plating on different media such as Nutrient agar, Kenknight medium and Rose

Bengal medium for isolation of bacteria, actinomycetes and fungi, respectively (Figure 1). Based on different morphotypes obtained on respective media, the colonies of bacteria, actinomycetes and fungi were isolated. A total of 520 bacterial cultures, 33 actinomycetes and 100 fungi were isolated. All the cultures were purified, sub cultured and preserved under 4°C.

Gunathilake *et al.*, (2013) isolated cellulose degrading bacteria and fungi from soil, compost and leaf litter. Sahni and Phutela (2013) isolated several cellulose degrading fungi from soil, compost, digested slums and plant debris using paddy straw agar medium containing chloramphenicol. Mahanta *et al.*, (2014) also isolated and evaluated native cellulose degrading microorganisms for efficient bioconversion of weed biomass and rice straw. Padaria *et al.*, (2014) isolated a total of 144 microbes from soils of Darjeeling hills of India, among which only few isolates were found to be cellulose degraders under *in vitro* conditions using CMC agar plate method.

The morphotypes of bacteria, actinomycetes and fungi were selected to get maximum number of diverse groups. Among all the samples maximum number of bacteria were isolated from long term straw incorporation fields, actinomycetes from compost experiment and fungi from rotten wood logs in forest. Overall maximum number of morphotypes was obtained from long term straw incorporation followed by rotten wood logs (Table 1 and Figure 3).

Qualitative assay for screening of cellulolytic microorganisms

All the bacterial, actinomycetes and fungal cultures were screened for cellulolytic activity using qualitative assay on CMC agar medium. The bacterial and actinomycetes cultures were spot inoculated on the centre of

petriplates, while for fungal cultures a 6mm disk on centre of solid medium was placed followed by incubation for 7days at 30°C. Among all the 653 cultures screened, 44 bacterial cultures, 2 actinomycetes cultures and two fungal culture exhibited formation of zone using congo red dye. The diameter of hydrolysis zone varied from 2 mm to 32 mm. Largest zones were obtained from the culture CDM35 followed by CDM5, CDM10, CDM2 and CDM23 while, it was minimum for CDM25, CDM29, CDM33 and CDM34 (Table 2). Cellulases can convert cellulose into monomeric or dimeric structures hence carboxymethyl cellulose (CMC) was used as a carbon source, it is a soluble form of cellulose (Angsana 2009; Sissons *et al.*, 1987; Sazci and Erenler 1986). Kasana *et al.*, (2008) has also used four dyes to determine cellulase activity in different cultures.

The reason for zone formation in the plates treated with dyes is that it binds with the polysaccharide and forms a visible complex. Cellulase produced by cellulolytic microorganisms in the plate breaks down the polysaccharide as a result of which area surrounded by colony were exhausted with polysaccharides and replaced with smaller monosaccharides and disaccharides. These mono and disaccharide dyes cannot be bind efficiently to the dye hence results in the formation of a visible clear zone. Padaria *et al.*, (2014) used congo red dye and NaCl solution for screening cellulose degrading bacteria. The zone of clearance ranged from 8.16 mm to 26.83 mm. Sharma *et al.*, (2013) isolated 43 potential cellulolytic and xylanolytic bacteria from compost. Gomashe *et al.*, (2013) also screened bacterial and fungal cultures for cellulase activity on CMC agar plates but with 1% congo red dye. Ponnambalam *et al.*, (2011) performed Congo red clearing zone assay for qualitative display of cellulase activity. Gaur and Tiwari (2015) isolated fifty bacterial cultures among which only twenty nine bacterial isolates exhibited

good cellulase activity. These cultures were reassessed for cellulolytic potential by loading their culture broth in the wells on CMC agar plates and staining them with Congo-red and NaOH solution.

Among all the 48 cultures highest number of cellulolytic cultures were obtained from forest samples i.e. 35.4% followed by 29.2% from incorporation of crop residues followed by 25% from compost samples, 6.3% from field soil and 4.2 % from cow dung (Figure 4). On the basis of zone of hydrolysis obtained on CMC agar media with congo red dye, the cultures were divided into four groups (zones $\leq 10\text{mm}$, $\leq 20\text{mm}$, $\leq 30\text{mm}$ and $\leq 40\text{mm}$). Among 48 cultures 21 cultures produced zones $\leq 10\text{mm}$ and among these 14.6% cultures were from incorporation samples, 10.4% cultures were from forest, 8.3% from compost, 6.3% from field soil and 4.2 % from cow dung. Twenty one cultures produced zones $\leq 20\text{mm}$, among which 20.8% cultures were from forest followed by 10.4% from incorporation and 12.5% from compost however, no culture was obtained from cow dung and field soil. Four cultures exhibited formation of $\leq 30\text{mm}$ zones among which 4.2% cultures were from incorporation and 4.2 % cultures were from compost. Highest zones of $\leq 40\text{mm}$ were obtained from 4.2% cultures from forest ecosystem (Figure 5). So overall highest number of efficient cultures was obtained from forest followed by incorporation and compost samples. Lopez-Mondejar *et al.*, (2016) performed the screening and identification of bacteria showing potential cellulolytic activity from litter and organic soil of a temperate oak forest. Stursova *et al.*, (2012) concluded that the cellulose represents a preferred substrate for bacteria, whereas fungi are able to utilize a wider range of litter constituents. These findings are in accordance to our study as among all the potential cultures maximum cellulolytic cultures were bacteria (Fig. 2).

Table.1 Sampling sites, source and number of microorganisms isolated from different sites

S.No.	Place	Sample number	Source	Number of morphotypes isolated			Total
				Bacteria	Actinomycetes	Fungus	
1	PAU	S1	Paddy incorporation	25	2	2	29
		S2	Poplar cropping system	12	1	2	15
		S3	Crop retention	13	3	0	16
		S4	Compost	20	4	1	25
		S5	Termite mounds	10	2	3	15
2	Faridkot	S6	Cow dung	15	1	1	17
		S7	Cowdung+straw	15	1	5	21
		S8	Decomposed straw	35	2	6	43
		S9	Open compost trial	15	1	0	16
3	Palampur	S10	Argemone compost	25	1	4	30
		S11	Rice-wheat	26	0	6	32
		S12	Forest soil	30	2	8	40
		S13	Him compost	2	0	0	2
		S14	Steria compost	3	0	0	3
		S15	Lantana compost	4	0	0	4
4	Harike	S16	Rotten wood logs in forest	33	3	12	48
		S17	Undisturbed forest	27	0	7	34
		S18	Forest soil with residues	20	0	6	26
5	Moga	S19	Paddy incorporated	20	1	3	24
		S20	Rotten wood	24	2	4	30
6	Bathinda	S21	Rotten wood log forest	36	3	5	44
		S22	Wheat straw	24	0	6	30
		S23	Forest soil	16	0	3	19
		S24	Paddy straw	2	0	4	6
7	Hisar	S25	Wheat field 1	8	1	3	12
		S26	Wheat field 2	12	0	1	13
		S27	Long term straw incorporation field	44	1	4	49
8	Ladhowal	S28	Rice-Wheat incorporation	4	2	4	10
			Total	520	33	100	653

Table.2 In vitro screening of microbial cultures for cellulolytic activity

S. No.	Microbial cultures	Isolates	Source of isolation	Zone of hydrolysis (mm)
1	CDM 1	Bacteria	Field soil	5.0
2	CDM 2	Bacteria	Compost	27.0
3	CDM 3	Bacteria	Cow dung	5.0
4	CDM 4	Bacteria	Incorporation	13.0
5	CDM 5	Bacteria	Forest	31.0
6	CDM 6	Bacteria	Field soil	10.0
7	CDM 7	Bacteria	Incorporation	13.0
8	CDM 8	Bacteria	Incorporation	26.0
9	CDM 9	Bacteria	Compost	13.0
10	CDM 10	Bacteria	Compost	29.0
11	CDM 11	Bacteria	Compost	13.0
12	CDM 12	Bacteria	Incorporation	7.0
13	CDM 13	Bacteria	Forest	9.0
14	CDM 14	Bacteria	Field soil	5.0
15	CDM 15	Bacteria	Forest	12.0
16	CDM 16	Bacteria	Cow dung	7.0
17	CDM 17	Bacteria	Incorporation	6.0
18	CDM 18	Bacteria	Incorporation	12.0
19	CDM 19	Bacteria	Incorporation	12.0
20	CDM 20	Bacteria	Forest	13.0
21	CDM 21	Bacteria	Incorporation	11.0
22	CDM 22	Bacteria	Forest	16.0
23	CDM 23	Bacteria	Incorporation	27.0
24	CDM 24	Bacteria	Forest	11.0
25	CDM 25	Bacteria	Incorporation	3.0
26	CDM 26	Actinomycetes	Forest	16.0
27	CDM 27	Bacteria	Compost	10.0
28	CDM 28	Bacteria	Incorporation	6.0
29	CDM 29	Bacteria	Forest	3.0
30	CDM 30	Bacteria	Forest	6.0
31	CDM 31	Bacteria	Forest	5.0
32	CDM 32	Bacteria	Forest	7.0
33	CDM 33	Bacteria	Compost	2.0
34	CDM 34	Bacteria	Incorporation	5.0
35	CDM 35	Bacteria	Forest	32.0
36	CDM 36	Bacteria	Forest	15.0
37	CDM 37	Bacteria	Forest	12.0
38	CDM 38	Bacteria	Forest	4.0
39	CDM 39	Bacteria	Compost	5.0
40	CDM 40	Bacteria	Incorporation	5.0
41	CDM 41	Bacteria	Forest	13.0
42	CDM 42	Actinomycetes	Compost	18.0
43	CDM 43	Bacteria	Compost	5.0
44	CDM 44	Bacteria	Incorporation	9.0
45	CDM 45	Fungi	Compost	7.0
46	CDM 46	Fungi	Compost	13.0
47	CDM 47	Bacteria	Compost	15.0
48	CDM 48	Bacteria	Forest	11.0

Incubation period = 7 days

Incubation temperature = 30°C

Fig.2 Number of morphotypes of bacteria, actinomycetes, fungi and total morphotypes obtained from each sample

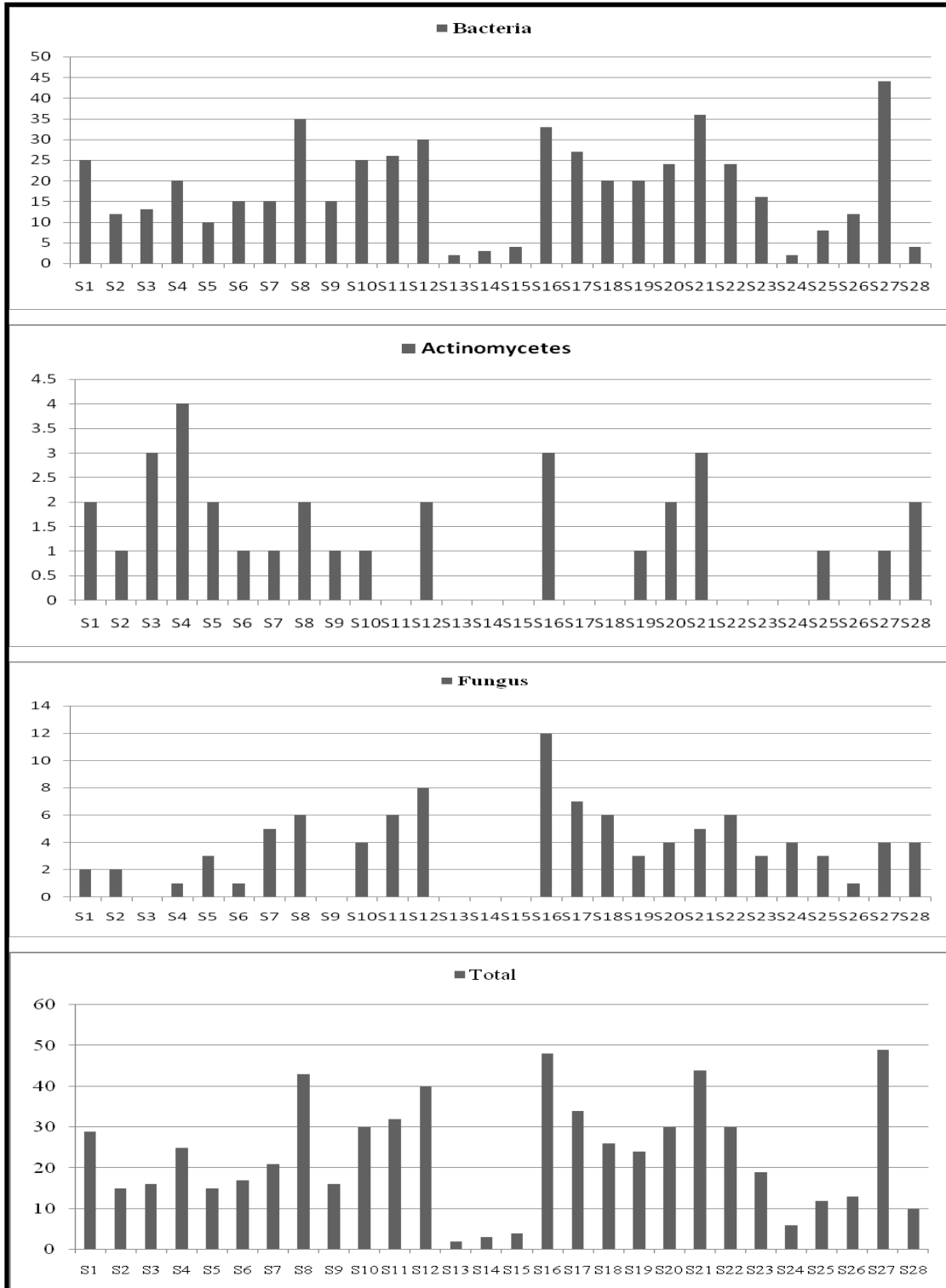


Fig.1 Isolation of (a) actinomycetes, (b) bacterial and (c) fungal cultures on their respective media

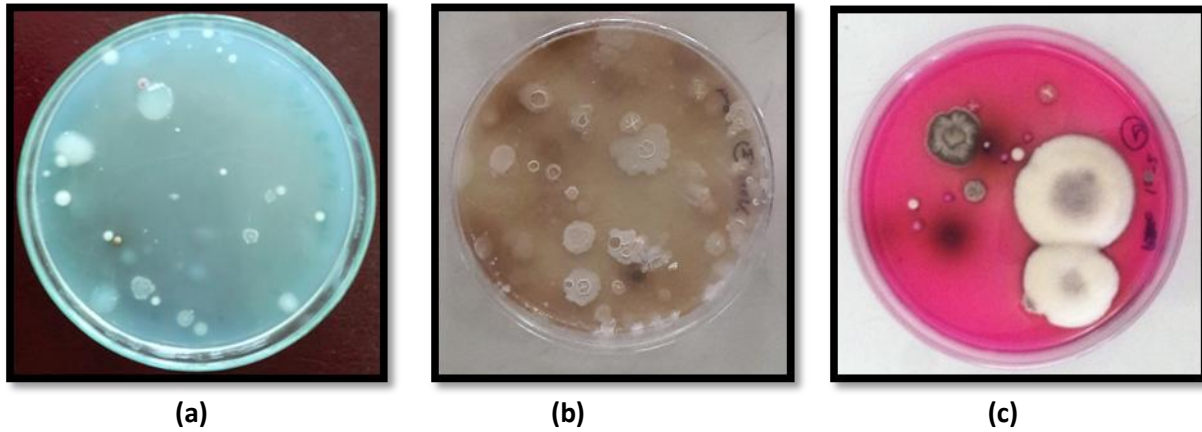


Fig.3 Zone of clearance of a) actinomycete culture b) bacterial culture and c) fungal culture on agar plate after staining with Congo red

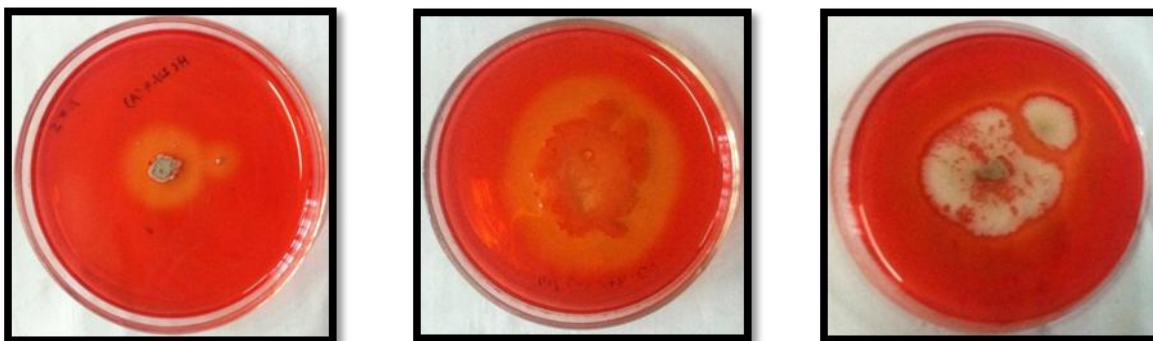


Fig.4 Percentage of efficient cellulolytic cultures obtained from different sources

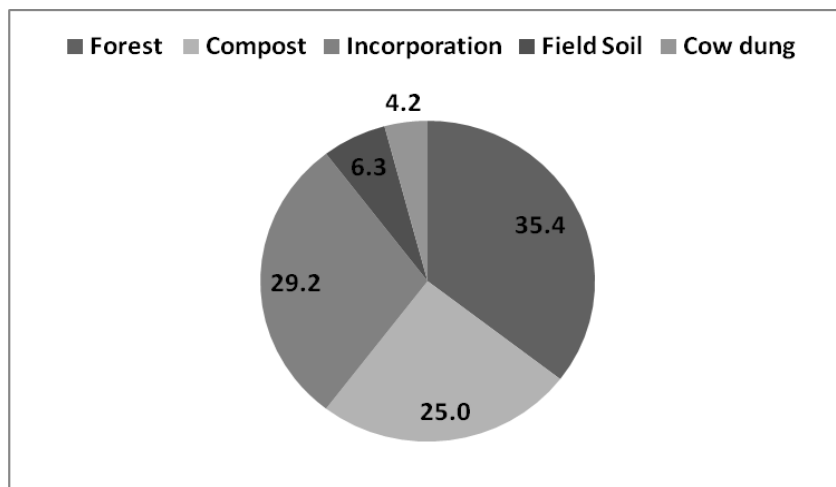
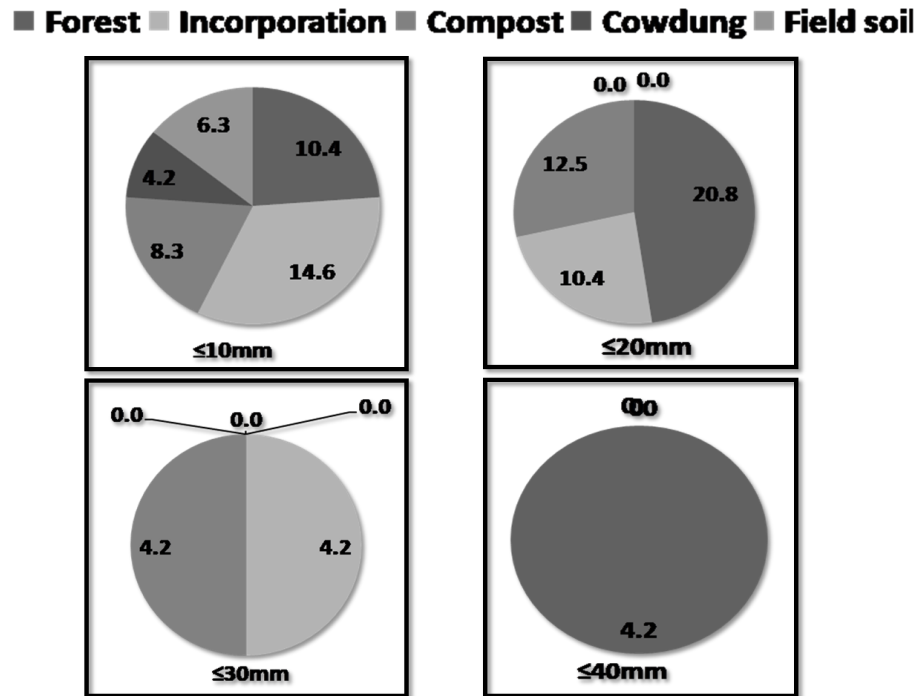


Fig.5 Per cent efficiency of microbial cultures on the basis of zone of hydrolysis



Rawway *et al.*, (2018) also carried out a study in which he found maximum number of cellulolytic cultures i.e. 40.83% were obtained from garden soil followed by 26.67% from gut of ruminant, 15.83% from compost, 10.83% from agricultural soil and 5.83% from River Nile sediment.

The possible reason for obtaining highest number of cultures from forest could be that forest represents an ecosystem where the plant litter, both above and below ground is decomposed by the microbial succession so a large number of microorganisms are involved in this decomposition process. In case of incorporation of crop residues, the plant material is incorporated in the field and then specific groups of microbes acts on the residue components resulting in its decomposition. In case of composting, different types of microbes appear at different stages of composting which includes mesophilic and thermophilic organisms. These systems represents an environment

where we can get maximum diversity and abundance of lignocellulolytic microbes. However, direct isolations of microorganisms from cow dung or field soil comparatively represents less abundance of cellulolytic microbes.

So from the observations made in this study we can conclude that forests represents a rich source of microbial biodiversity. Further understanding of these systems with respect to microbial biodiversity will be helpful to study decomposition processes, synergism between different microorganisms and the biochemical basis of degradation of residues in forest.

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