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Microbial Diversity from Solid Wastes Disposal of Paper Industry, Panchgram, South Assam

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Microbial diversity constitutes the most extraordinary reservoir of life in the biosphere that we have only just begun to explore and understand. A preliminary investigation on the microbial diversity of paper mill effluents organic wastes from the landfill site of Cachar Paper mill, Panchgram, South Assam, the isolation and characterization of native microbes on different media may generate information on the nature, characteristics, and degrading efficiency of various hazardous wastes by the micro-organisms. Sludge samples were collected randomly from paper mill solid wastes dumping sites and their physico-chemical characteristics such as pH, moisture content, NPK and Cellulose content were determined. The qualitative analysis of species in microbial community was undertaken. The isolated bacteria and fungi were screened for their cellulase activity, characterized and identified. The study revealed that bacterial isolates showing higher zone of cellulolytic activity belong to *Bacillus, Pseudomonas and Serratia spp,* while fungal isolates mostly belong to *Aspergillus* and *Penicillium spp* .The isolates have also shown a wide range of pH and temperature tolerance. The study has suggested that the paper mill waste site harbors various microorganisms that are active in cellulose breakdown.

Key words: Microbial diversity, organic wastes, biological degradation, bacterial isolation, characterization, cellulose degradation.

Microorganisms constitute a huge and almost unexplained reservoir of resources likely to provide innovative applications useful to man and are capable of exploiting a vast range of energy sources and thriving in almost every habitat.

Microbial diversity offers an immense field of environment friendly options for mineralization of contaminants or their transformation into less harmful non-hazardous compounds. Thus there is a general interest in studying the diversity of indigenous microorganisms capable of degrading different pollutants because of their varied effects on the environment. Efforts to convert waste in to useful products can be achieved and maintained by biotechnological measures that include action of microorganisms, enzymes and technologies[1]. Paper industry is one among various industries that produces large amount of cellulosic (cellulose, hemicelluloses and lignin) along with other toxic organic wastes generally disposed off as landfills that accumulate in nature leads to significant environmental impacts[2]. Cellulosic biomass has

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attracted worldwide attention as renewable resource and now several federal agencies including National Science foundation, the US department of Energy (DOE) and US department of agriculture (USDA) are strongly expanding the role of biomass (all plants and plants derived materials) as an alternative energy source that can be converted into bio based products and bioenergy[3]. The microbial communities of the site are responsible for pollutant degradation and transformation.

Glucose, an appropriate hydrolysis product of cellulosic biomass can be used in different applications such as production of fuel, ethanol, single cell protein, feed stock, industrially important chemicals and so on[4-6]. The role of fungi, bacteria and actinomycetes in the natural biodegradation process of cellulosic wastes from various environments has well being documented[7-11]. Lignin degrading fungi and their enzymes also have their ability to degrade highly toxic organic compounds such as dioxins and polychlorinated biphenyls and could have an important role to play in the remediation of contaminated soils. Bacteria also have their ability to detoxify the heavy metals (Lead, chromium, cadmium) along with degrading organic wastes of the contaminated site[12].

Microbial communities however are subjected to various perturbations, such as variation of pH, temperature, organic loading rates, the toxicant level, and seasonal variations[13.] Ecological studies on microbial communities may provide useful information on their capability of degradation of wastes by native microbes.

The Cachar paper mill(CPM), Panchgram is the only major industrial undertaking in south Assam and the adjoining states of Mizoram, Meghalaya and Tripura. This pulp and paper mill has an annual capacity of 1,00000 tonnes of products and is utilizing bamboo as a raw material for paper production. The production process produced large amount of effluents, both liquid wastes generally discharged into water bodies and solid wastes such as wastewater treatment sludge, unused bamboo chips, Lime sludge and coal ash generally disposed as landfills. Land fillings are relatively cheap, so the industry takes little efforts for making more efficient use of its materials. The paper mill sludge consumes large percentage of local landfill space each year and also cause several hazards including – increased alkalinity of the soil, fires in waste materials, increase in the population of disease vectors, offensive odors, methane leakage, leaching of toxic and corrosive compounds to surface and ground waters etc. Moreover due to slow degradation of the wastes the aesthetic value of that area is lost[14]. Worst yet, burning of sludge in incinerators, contribute to serious air pollution problems. Sludge from pulp and paper mills mainly contains cellulose fiber and are recyclable organic solids.

Microorganisms in the site use the waste constituents as nutrients, thus detoxifying the materials as their digestive processes breakdown complex organic molecules into simpler less toxic molecules[15].

The present work aims to study the diversity of bacteria and fungi and their relative occurrence from waste mixed soil of paper mill, South Assam. Isolation and characterization of cellulose degrading microorganisms i.e. bacteria and fungi were studied on different media. Cellulolytic microorganisms from this site are not explored to a large extent and there is scanty information available on cellulolytic enzymes associated with it.

MATERIAL AND METHODS

Description of the site

The solid wastes dumping sites of Cachar Paper mill, Panchgram, located in Barak valley, South Assam, India is selected for study purpose. This zone is geographically located between 24° 152 and 25° 92 N latitude and between 90° 162 and 93°152 E longitude.

The climate of the area is subtropical, warm and humid. The average annual rainfall is 3180mm with an average of 146 rainy days per annum. The period from Dec- Feb is dry while the period May-Sept is usually featured by heavy rainfall with occasionally floods. The minimum and maximum annual temperature varies from 12.2°C-24°C in Dec-Jan to 28°C- 38°C in June-July. The relative humidity varies from 92 to 98 % in the morning and 43 to 78% in the evening. The texture of the soil varies from sandy to clay type with pH of 4.7 to 5.7 (acidic range).

Collection of samples

Samples (sludge mixed with soil) were collected randomly from four different locations of the paper mill solid wastes dumping sites at bimonthly interval. At each sites, the soil dug to a 20cm was scooped into sterilized polythene bag, labeled and brought to laboratory for analysis. The study comprises of the following:

Study of the Physiological characteristics of wastes

The physico-chemical characteristics of the samples such as moisture content, pH, NPK and organic % carbon content, cellulose content were determined[16]. Microbial respiration rates were also determined to estimate the microbial activity in the site.

Total microbial community study of the Waste

For enumeration of total bacterial and fungal load in the samples, 100µl of each dilution was spread on pre-sterilized agar plates. For bacterial isolation, the aliquots were plated on nutrient agar (NA) and fungi plated on Rose Bengal Agar (RBA) media and Czapex Dox Agar (CDA) media with added antibiotics. The mixed colonies that appeared on the plates are noted and counted.

Identification of the isolates

The isolated bacterial colonies showing different morphological features were picked, restreaked several times on NA plates to get pure cultures of isolates. The isolates were identified following various morphological and biochemical methods.[17] The parameters for biochemical investigation included colony morphology, color, size, nature of the growth, Gram staining, catalase test, citrate utilization test, motility test, methyl red test, voges-proskauer test, starch hydrolysis test, indole test, gelatinase production test, and growth in different pH, different mediums, temperatures and NaCl concentrations.[18]

The isolated fungi from the mixed culture plates were subcultured repeatedly to get pure cultures. The isolated pure cultures of fungi were subjected to taxonomic studies by comparing with the "A manual of soil fungi" by Gilman (1971)[19]. All the isolates were maintained at 4°C for future use.

Screening of isolates for cellulase production

To screen for cellulolytic organisms, the isolated bacterial and fungal isolates were grown on NA and RBA supplemented with 1% (w/v) carboxymethylcellulose. For primary screening, the isolates that grow on the above media were inoculated on fresh CMC agar plates, containing KH_2PO_4 (0.1%), $(NH4)_2SO_4$ (0.4%), NaCl (0.6%), $MgSO_4$ (0.05%), $CaCl_2$ (0.01%), and Carboxy Methyl Cellulose (0.5%) as carbon source, incubated at 28-30°C for 2-3 days. The plates were observed for clear zone formation around the colonies produced by cellulose degraders after staining with 1% Congo red dye and destaining with 1M NaCl[20]. The diameter of the clear zone and the colony was measured and the ratio of clear zone was calculated. Bacterial and fungal isolates showing clear zones were taken for further characterization.

Effect of Temperature and pH on the growth of the isolates

The bacterial and fungal isolates showing cellulolytic activity on plate screening were tested for their ability to tolerate and grow at different pH and temperature.

Antibiotic sensitivity tests of the bacterial isolates

The bacterial isolates were grown overnight in nutrient broth at 30°C and following disc diffusion method of Bauer *et.al* (1966)[21], the test organisms were spreaded over the surface on Muller Hinton agar plates to make a uniform lawn culture of the isolates. Selective antibiotics discs were put on the plates and incubated at 30°C for 24hrs. The antibiotics were selected depending on their use and their mode of action. The test antibiotics used were Ampicillin, Tetracycline, Penicillin, Vancomycin, and Erythromycin. The zone of inhibition was measured with the help of scale and categorized as sensitive and resistant.

Statistical analysis of data

The correlation coefficient study between microbial population and soil physico-chemical properties was undertaken to study the effects of those factors on the growth of microbes.[22]

RESULTS AND DISCUSSIONS

The physico-chemical characteristics of waste soil and their microbial diversity are presented (Table 1) and their correlation coefficients are also presented (Table 2). The present study revealed that moisture content and

Table 1. Physico-chemical characteristics and microbial diversity of wastes mixed in soil.

Sampling period	Bacterial population/g of soil CFU/ml×10^3	Fungal population/g of soil CFU/ml×10^3	pH	Moisture content (%)	Nitrogen (P_2O_5) (%)	Phosphorus (K_2O) Kg/ha	Potassium respiration rate Kg/ha	Microbial mg/C g^{-1}
June,07	76.5	12.3	5.6	66.0	1.15	35.0	320	57.0
Sept,07	34.3	17.4	6.2	48.4	1.70	23.8	245	45.0
Dec,07	22.2	26.8	5.9	25.5	2.25	22.5	275	22.0
Mar,08	60.8	6.40	5.7	40.8	1.88	33.0	272	34.0
June,08	65.5	14.7	5.6	54.0	3.13	28.5	330	55.0
Sept,08	26.3	17.2	6.1	42.3	2.75	32.5	352	49.0
Dec, 08	25.8	19.6	6.0	29.7	1.98	19.9	229	32.0

soil pH played significant role on the microbial population of the waste dumping site. The microbial community and their activity in the waste soil increased with decreased in pH and increased in moisture content. The negative correlation between soil organic nitrogen content and microbial population is that the nitrogen is utilized by the microbes during their growth [23]. Fungal population seemed to decrease when bacterial population increased, thereby showing that they respond differently to seasonal influence. Bacteria and fungi compete for simple plant-derived substrates and might have developed antagonistic relationship.

However, for more recalcitrant organic substrates, e.g. cellulose and lignin, both competitive and mutualistic strategies between bacteria and fungi appear to have evolved.[24] A total of 33 (designated as D1-D33) bacterial colonies were isolated from the samples at different period of time. The study of morphological, physical and biochemical characteristics of bacteria on pseudo selective media showed that 61% were gram –ve, 23% hydrolyzed starch, 92% produced the enzyme catalase, 30% produced gelatinase, 61% utilized citrate, 15% were indole positive and 23% were methyl red positive. The isolated bacteria are most commonly aerobes or facultative anaerobes. The bacterial species belong to genera *Bacillus, Streptococcus, Kleibsiella, Staphylococcus, Pseudomonas, Serratia, Proteus, E. coli, Corynebacterium* and *Salmonella spp.* The isolated fungal species were identified as *Mucor, Rhizopus, Aspergillus, Penicillium, Fusarium, Gliocladium, Alternaria, Geotrichium* spp, *etc*. (Gilman, 1957).[19] Relative occurrence and isolation of the more species of genus *Bacillus* (39.3 %), *Pseudomonas* (33.3%), *Streptomyces* (18.1 %) among the bacteria and of genus *Aspergillus* (36.3%), *Penicillium* (22.7%) are probably due to their diverse and extensive enzyme system that protect them from other soil organisms.[25] (Fig. 1 A and B). Based on the preliminary screening studies, 14 (42.4%) bacterial isolates shows signs of growth on CMC-NA agar, of which 6 isolates shows higher clear zone on CMC agar plates by Congo red test (Table 3). Of the 22 fungal species isolated, 13(59.09%) fungal isolates grows on the CMC-RBA, of which only 4 responds to Congo red test (Table 3). Based

on the screening results on CMCase plates, these isolates with higher zone of activity were selected for further morphological, biochemical and physiological identification and characterization studies (Table 4 & 5). Studies revealed that the isolated bacterial strain D2 and D3 are rod shaped, gram negative, non spore forming bacteria which imparts green coloration to the medium characteristic of *P.auruginosa*[18] Moreover, the morphological characteristics having circular, raised, entire, creamish, opaque colony formation is in agreement with Carson *et. al.* (1972)[26] who also described the good growth of *P. cepacia* on citrate agar slants along with other positive tests for catalase, and negative tests for indole production and starch hydrolysis. Also no hydrogen sulfide production and rapid gelatinase activity[18] which is also true in case of these two strain. Like Pseudomonas spp. D2 and D3 also shows the optimum growth around 40°C and no tolerance to acidic pH. From table 4, the D4, D6 and D11 was found to be rod shaped, gram positive bacteria, optimal growth temperature found to be around 40°C and pH tolerance at pH 5, like *Bacillus* strains[27]. Like *Bacillus* it showed non-pigmented circular colony showing no growth at pH 4. The characteristic biochemical tests for those bacteria were found to be in agreement with *Bacillus cereus and Bacillus licheniformis.* e.g., positive results for catalase, gelatin, citrate utilization, hydrogen sulfide production and starch utilization [18] and the isolates D7 produced pink-red pigment, Gram –ve rods, positive for catalase, indole, methyl red and motile, thus showed the characteristics with *Serratia sp*. Moreover, the fungal isolates showing higher zone of CMCase activity belong to genus *Aspergillus* spp and *Penicillium* sp. (Table 5). The fungal

Table 2. Correlation coefficient between microbial populations with soil Physico-chemical characteristics

Parameter	Correlation Coefficient	
	Bacterial population	Fungal population
pH	-0.17	-0.67*
Moisture content (%)	0.66*	0.48
Nitrogen %	-0.1	-0.42
(P_2O_5)	0.63*	0.89**
K_2O	0.33	0.57

*Significant at P> **0.05**, ** significant at P>0.01

Table 3. Screening of bacterial and fungal isolates by Congo red test for cellulolytic activity

Designated bacterial strain no.	Colony diameter (cm)	Clear zone diameter (cm)	Clear zone ratio
D11	1.3	2.9	2.3
D6	1.5	3.4	2.2
D3	1.6	3.1	1.9
D2	1.2	3.2	1.8
D4	0.8	1.1	1.3
F14	2.2	1.5	1.46
F3	2.1	1.6	1.31
F2	2.7	2.2	1.22
F8	2.2	2.0	1.1

*D11, D6, D3, D2, D4, and D7—reffered to the designated bacterial isolates and
*F14, F3, F2 and F8 are referred to designated fungal isolates

genera of *Aspergillus sp*. and *Penicillium sp*. have been extensively studied due to their ability to secrete cellulose-degrading enzymes which help in industrial applications[28]. They had the ability to produce cellulolytic enzymes which act synergistically in the conversion of cellulose to glucose[29]. Also the characterized bacterial and fungal isolates showed extra cellular endoglucanase activity, having a wide range of tolerance to pH and temperature. Moreover the

Table 4. Biochemical and physiological characterization for identification of cellulose degrading bacteria (CDB)

Characterization parameters		Bacterial isolates					
pH	Range	D11	D3	D6	D2	D4	D7
	4	_	-_	_	_	_	_
	5	+	±	+	_	+	+
	6	++	+	++	+	++	++
	8	+	±	+	_	±	±
	9	±	_	_	_	±	_
Temperature(ºC)	20	+	+	+	_	+	_
	25	++	+	++	++	++	+
	40	++	±	++	+	+	+
	50	+	±	++	±	_	_
	55	_	_	_	_	_	_
Salt conc.(%)	0.5	++	+	++	+	+	+
	1.5	++	+	++	++	++	+
	2.5	++	±	++	+	+	+
	5	+	_	+	_	_	_
	7.5	+	_	±	_	±	_
	10	_	_	_	_	_	_
Biochemical tests	H_2S	_ve	_ ve	Ve +	_ ve	_ ve	_ ve
	Catalase	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
	Iodole	_ ve	_ ve	_ ve	_ ve	_ ve	+ ve
	MR	_- ve	_ ve	+ ve	_ ve	_ ve	+ ve
	VP	+ ve	_ ve	+ ve	_ ve	+ ve	_ ve
	Citrate	+ ve	+ ve	+ ve	+ ve	+ ve	_ ve
	Starch	+ ve	_ ve	+ ve	_ ve	+ ve	_ ve
	Gram's	+ ve	-– ve	+ ve	_ ve	+ ve	_ ve
	reaction	Rod	Rod	Rod	Rod	rod	rods

-no growth, ± slight growth, + moderate growth, ++ abundant growth
-ve negative, +ve positive, for the biochemical tests

Table 5. Characterization of isolated cellulose degrading fungi

Fungal Isolates	Identification	Physiological parameters									
		pH					Temperature (ºC)				
		5.0	6.0	7.0	8.0	9.0	20	30	40	50	60
F8	*Aspergillus niger*	-	2+	3+	3+	-	-	3+	3+	2+	-
F3	*Pencillium funiculosum*	-	2+	3+	3+	-	-	3+	3+	2+	-
F2	*Fusarium oxysporium*	-	2+	3+	3+	-	-	3+	3+	2+	-
F14	*Aspergillus fumigatus*	-	2+	3+	3+	-	-	3+	3+	2+	-

* - means no growth, 2+ means moderate growth, 3+ means abundant growth

antibiotic sensitivity tests pattern showed that nearly 68% of the isolated bacteria were resistant to most commonly used antibiotics which are of major concern regarding to the use of antibiotics.

The present study also revealed that the bacteria produced larger clear zone than that of fungi indicating the active role of bacteria in cellulosic waste degradation. Though fungal

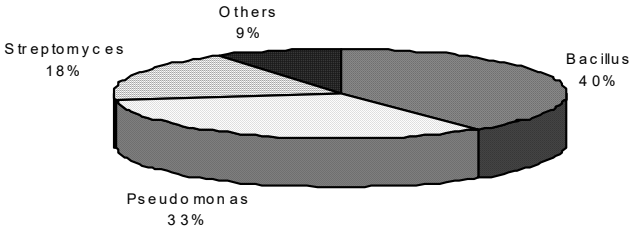

Fig. 1a. Relative occurrence of the Bacterial species found in effluents mixed soil

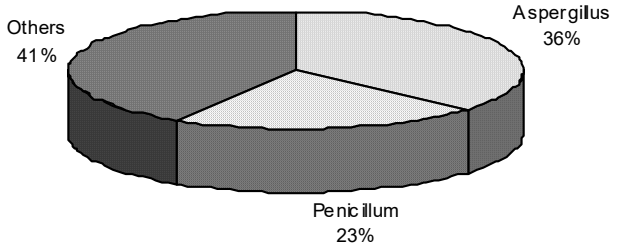

Fig. 1b. Relative occurrence of the fungal species found in effluents mixed soil

cellulases have been widely studied but cellulase production from bacteria can be an advantage as the enzyme production rate is normally higher due to high bacterial rate compared to fungi. [30] Among bacteria *Pseudomonas aeruginosa, P. fluorescens, Cellulomonas spp., Clostridium spp., Bacillus spp*. etc. are found to have cellulolytic activity and well documented. Therefore the solid waste disposals sites of paper industry harbored the rich diversity of microbes which may prove to be the source of different enzymes of industrial importance.

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