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Prevalence of Keratinophilic fungi from Sewage Sludge at Some Wastewater out lets along the coast of Visakhapatnam: A case study

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ABSTRACT

Now a day, the use of treated sewage sludge in fertilization of agricultural and a forest area is very common. Several studies have shown that the sewage sludge contains different types of fungi, which can be transferred and distributed from these areas. The present study was reported on the isolation of keratinophilic fungi in sewage sludge from wastewater out lets in the Visakhapatnam is first of its kind. It has also identified some cycloheximide-resistant saprophytic fungi were grown in SDA medium. Using the hair-baiting technique, however, M. gypseum, a pathogenic geophilic dermatophyte, was isolated from wastewater treatment plant (Appugarh) samples. In addition, Chrysosporium spp., a keratinophilic and saprophytic fungus, was isolated by this technique Vanbreuseghem's Hair Bait Technique.

Keywords: Keratinophilic Fungi, Sewage Sludge and Wastewater outlet.

INTRODUCTION

Sewage sludge contains high quantities of keratin remnants which have particular physicochemical and microbiological characteristics. It can be expected, therefore, that keratinophilic fungi occur abundantly in the sludge environment and the influence of environmental factors on their qualitative and quantitative composition can be observed more easily than in other habitats. Besides, sewage sludge is increasingly being used to fertilize agricultural and forest areas and to reclaim devastated terrains. Hence, the recognition of the distribution of pathogenic fungal species in such sludge is important from a public health point of view [Hedayati and Mirzakhani 2009].

Keratinophilic fungi are ecologically important group as they play a significant role in the natural degradation of keratin substrate and residues. Occur with the variable distribution patterns, these are dependent on factors like human and animal presence. This group of microorganisms utilize keratin present in the soil (Geophilic Species), the cornified substrata in humans (Anthropophilic Species) and animals (Zoophilic Species). This is thus considered to be a natural evolution in keratin utilization and is responsible for organism's pathogenicity Keratinolytic fungi specialize in the decomposition of keratin, being the main component of keratinous substrata. Keratinophilic fungi associate keratinolytic fungi, utilizing non-protein components of the substrata or the products of keratin decomposition [Majchrowicz and Dominik 1969]. Since many Keratinolytic and keratinophilic fungi have been found to be the agents responsible for human mycoses [Hoog et al 2000] and since the fungi occur in abundance in sewage sludge and sludge-amended soils, studies on fungal incidence in these environments are of hygienic, epidemiological and ecological significance. [Ulfig 2006]. Sewage sludge has been surveyed for keratinophilic fungi

a number of times [Majchrowicz and Dominik 1969, Hoog *et al.* 2000, Abdel Hafej , Sharouny 1990 and Ulfig *et al* 1996].

Water pollution is thought to reduce the diversity of sensitive fungi, while increasing the diversity of those that are less sensitive [Cooke 1970]. This could explain the observed increase in numbers of some genera: ***Chrysosporium*** species**,** ***Geotrichum*** species**,** ***Cladosporium*** species**,** ***Gliocladium*** species, ***Paecilomyces*** species and ***Pencillium*** species**,** in sewage receiving soils (as compared to nonpolluted soils), which seem to be less sensitive to soil contamination by organic pollutants of raw city wastewater. In fact some of the fungi in this group ***(Geotrichum* sp.** and ***Chrysosporium*** sp.***)*** were found to be associated with organic wastes [Simard 1971]. On the other hand, the numbers of ***Alternaria*** sp**.** and ***Aspergillus*** *sp.* decreased which may be attributed to their sensitivity to organic pollutants. Some of the recovered fungi did not seem to follow either of the two above-mentioned patterns.

However, there is no published study on the keratinophilic fungi in sewage sludge of Visakhapatnam. The chief objective of this work was to isolate and identify the geophilic keratinophilic fungi from sewage sludge.

MATERIALS AND METHODS

Study area

Visakhapatnam city has a coast length of approximately 30 Km from Dolphin's nose to Bheemili. Many people were attracted more towards locations like Lumbini Park, Tenneti Park, Tourists Resorts located along the Beach and make frequent visits to those places.

The present study was carried out in some selected sampling sites based on the sewage outlets along the coast. The study was conducted by collecting sewage sludge samples from different sewage coast line from Dolphin's nose to Bheemili.

Sludge Collection:

Sludge samples from study areas were collected to screen for keratinophilic by adopting standard method [Baran and Chabasse et al. 2001]. Glassware and spatula were sterilized by keeping them in the Hot air oven at 180^0C for one hour. The sterilized glassware was opened at the field and spatulla were used to collect the sewage sludge. Now carefully the sludge samples were transferred to screw cap test tubes and sterile plastic bags and brought to laboratory in self closed envelops and further processing was done for both samples immediately or after an overnight at room temperature.

Preparation of Medium:

Sabouraud Dextrose Agar (SDA) was used to culture the Keratinophilic fungi of sludge sample. Three grams of Sabouraud's dextrose agar was weighed and mixed with 100 ml distilled water and pH of the medium was adjusted to 7.2 $\pm$ 0.5. The medium was sterilized in an autoclave at 121^0C, 15 lb/inch^2. After cooling the medium, Chloramphenicol was added as antibacterial compound. Then the medium was poured into different Petri plates and were labeled properly.

Isolation of Fungi:

From each Sewage sludge sample, one gram of sludge was taken and dilutions were employed till 10^{-5}. Later, 0.1ml serial diluted sludge solution from each test tube(10^{-2} – 10^{-5}) were inoculated onto the respective culture medium and left at room temperature (25^0C) for the growth of keratinophilic fungi. When growing colonies were observed they were identified by staining [Ulfig and Korcz 1983].

Vanbreuseghem's Hair Bait Technique:

This technique was initially developed by R.Vanbreuseghem, a Belgian Mycologist in 1952. Ten grams of sludge from each sample was taken and placed in Petri dishes of nine cm size. Defatted hair fragments of selected length were scattered over the surface of the sludge in the Petri plates. Then plates were moistened with sterile distilled water to facilitate the germination of fungal spores and Chloramphenicol was added to plates to prevent bacterial growth. Then these plates were put for incubation for a period of 60 days at room temperature. Later baits with fungal growth (inoculums) were taken from the plates and were transferred to Petri plates containing culture medium. The inoculated Petri plates were examined for the growth of fungal colonies.

Identification of fungi:
After the preparation of pure cultures, the cultural and morphological characteristics of fungal colonies and their identification was done by referring Pictorial Atlas of Soil and Seed Fungi Morphologies of cultured fungi and key to species (Tsuneo Watanabe- Second edition) and their morphology was studied by staining them with Lactophenol Cotton Blue Dye. Then the stained organisms were observed under 40x as well as oil immersion 100x objective with the help of Microphotography technique using Labomed Digi-Pro Microscope.

Sludge analysis:
The sludge samples were analyzed for the following physico-chemical parameters like pH, Conductivity, Humus, Moisture content, Organic carbon etc by adopting standard procedures.(APHA)

RESULTS AND DISCUSSION

The collected sewage (effluent) samples were analyzed for the physico-chemical parameters showed the PH ranging from 6.0 to 7.89, Conductivity (mmhos) was 0.21 to 3.76, Humus (%) was 18.8 to 56.4, Organic Carbon (%)1.02 to 2, Moisture content (%)1.08 to 1.35, Chlorides (mg/g) 21.3 to 42.6 and Microbial respiration (mg/100g) 27.94 to 49.5 respectively(Table- 1). Collected Sewage sludge Samples were analyzed for physico-chemical parameters were represented in Table-2, and observed that range of PH was 6.1to 7.2, Conductivity (mmhos) was 84.6 to 538.47, total solids was 170 to 756 mg/l , total suspended solid was 100 to 420 mg/l , total dissolved solids was 35 to 350 mg/l, alkalinity was 70 to 125 mg/l ,Biological oxygen demand was 110 to 590 mg/l and Chemical oxygen demand was 250 to 1000 mg/l.

Keratinophilic Fungi Were Isolated By Feather and Hair Baiting

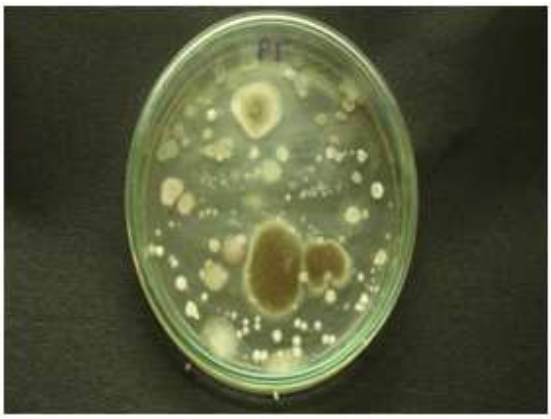


Table – 1: Physico- Chemical Characteristics of Sludge Samples

S.No	Site	Temp. (^{0}C)	pH	Conductivity (mmhos)	Humus (%)	Organic Carbon (%)	Moisture content (%)	Chlorides (mg/g)	Microbial respiration (mg/100g)
1.	S1	27.1	6.30	2.17	18.8	1.08	1.35	21.3	30.14
2.	S2	28.0	6.53	3.76	56.4	2	1.25	35.5	38.94
3.	S3	25.1	7.82	2.50	28.2	1.26	1.08	35.5	27.94
4.	S4	26.1	6.96	1.79	37.6	1.02	1.28	28.4	28.38
5.	S5	28.1	6.0	0.21	56.4	1.38	1.21	35.5	49.5
6.	S6	25.3	6.82	3.56	47.0	1.14	1.17	42.6	36.74
7.	S7	26.3	7.89	1.64	18.8	1.14	1.25	35.5	41.58

Note: Total distance of sampling area is approximately 30 km

Table-2: Physico-Chemical Parameters of Collected Sewage water Samples

Sample code	Physico-Chemical Parameters							
	PH	EC	TS	TSS	TDS	ALKALINITY	BOD	COD
S1	6.5	538.47	756	420	350	70	220	506
S2	6.1	442.55	720	410	310	68	110	250
S3	7	423.07	670	385	275	79	400	1000
S4	7.2	230.76	510	350	150	101	210	480
S5	.7	107.69	350	280	70	114	250	530
S6	6.5	184.6	430	310	120	103	590	810
S7	6.4	123.07	170	100	60	106	320	650
S8	6.6	84.6	185	165	35	125	210	480

**Except PH and EC, all units were in mg/l*

Table-3: Occurrence of Pathogenic Bacteria from Sewage Sludge Sample (MacConkey Agar as culture medium)

S. No		Sample-1		Sample-2		Sample-3		Sample-4		Sample-5		Sample-6	
	Dilution	Coli forms	Others	Coli forms	Others	Coli form	Others	Coli forms	Others	Coli forms	Others	Coli forms	Others
1	10^{-1}	>300	>300	>300	>300	>300	>300	>300	>300	176	>300	172	103
2	10^{-2}	76	65	79	59	60	63	72	78	78	102	23	21
3	10^{3}	14	25	12	15	6	11	9	8	6	13	3	1

Table-4: Occurrence of pathogenic bacteria from Sewage Sludge samples (EMB Agar as culture medium)

S. No		Sample-1		Sample-2		Sample-3		Sample-4		Sample-5		Sample-6	
	Dilution	Coli forms	Others	Coli forms	Others	Coli forms	Others	Coli forms	Others	Coli forms	Others	Coli forms	Others
1	10^{-1}	>300	>300	>300	>300	>300	>300	>300	>300	172	>300	180	105
2	10^{-2}	184	149	72	55	62	58	70	75	69	85	20	22
3	10^{3}	21	30	10	13	8	10	11	10	5	8	4	5

Table-5: Fungi isolated from Sewage sludge samples at different wastewater out lets (SDA as culture medium).

1.

S. No	Genus	Sample-1		Sample-2		Sample-3		Sample-4		Sample-5		Sample-6		Total	
		No	%	No	%	No	%	No	%	No	%	No	%	Total	%
1	***Geotrichum spp***	60	68.2	50	57.5	23	57.1	20	45.5	25	75.8	16	55.2	194	59.5
2	***Cladosporium sp***	11	12.5	16	18.4	16	35.6	-	-	2	6.1	-	-	46	13.8
3	***Alternaria spp***	11	1.1	21	24.1	5	11.1	-	-	-	-	10	34.5	37	11.3
4	***Penicillium spp***	9	10.2	-	-	1	2.2	19	43.2	4	12.1	2	6.9	35	10.7
5	***Yeast spp***	5	5.7	-	-	-	-	5	11.4	2	6.1	1	3.4	13	4.0
6	***Rhizopus spp***	1	1.1	-	-	-	-	-	-	-	-	-	-	1	0.3
7	***Aspergillus spp***	1	1.1	-	-	-	-	-	-	-	-	-	-	1	0.3
	Total	88	100	87	100	45	100	44	100	33	100	29	100	326	100

Table- 6: Keratinophilic Fungi isolated from Sewage Sludge samples at different wastewater out lets by hair-baiting technique

S. No	Genus	Sample Code					
		S1	S2	S3	S4	S5	S6
1	***Chrysosporium spp.***	-	-	+	+	+	-
2	***Geotrichum spp.***	-	-	-	-	-	+
3	***Yeast***	-	-	-	-	-	+
4	***Microsporum gypseum***	+	+	+	-	-	-

The prevalence of fungi in sewage sludge samples from the 7 different waste water outlets (labeled S1-S7).was represented in tables 5 & 6. Out 0f 35 sludge samples cultured on SDA medium, 28 were shown fungal growth. On SDA medium, all 5 samples from location S7 and one sample each from locations S2and S4 were negative for fungal growth. A total of 326 fungal colonies belonging to 7 species were isolated. *Geotrichum* spp. (59.5%), *Cladosporium* spp. (13.8%), *Alternaria* spp. (11.3%) and *Penicillium* (10.7%) spp. were the most prevalent. No growth of keratinophilic fungi was observed in SDA medium. Table 6 shows the keratinophilic fungi isolated from sewage sludge by the hair-baiting technique, viz *Microsporum gypseum*, *Chrysosporium* spp., *Geotrichum* spp. and yeasts were isolated. No keratinophilic fungi were not seen in the culture plats of locations S2 and S7.

Now a day, the use of treated sewage sludge in fertilization of agricultural and a forest area is very common. Several studies have shown that the sewage sludge contains different types of fungi, which can be transferred and distributed from these areas [Ulfig 2006, Majchrowicz and Dominik 1969, Hedayati and .Mirzakhan 2009, Hoog, et al 2000, and Ulfig 2005]. Some of the keratinophilic fungi such as *M. gypseum* are pathogenic for human and animals. *M. gypseum*, a common agent of dermatophytosis (tinea) in human and animals [Dumonet et al 2003], was isolated in our study. Our study report on the isolation of keratinophilic fungi in sewage sludge from wastewater out lets in the Visakhapatnam is first of its kind. We also identified some cycloheximide-resistant saprophytic fungi were grown in SDA medium. Using the hair-baiting technique, however, *M. gypseum*, a pathogenic geophilic dermatophyte, was isolated from wastewater treatment plant (Appugarh) samples. In addition, *Chrysosporium* spp., a keratinophilic and saprophytic fungus, was isolated by this technique. Similar results were reported by Ulfig et al (2006) when they carried out similar study at Poland. Abdul-Hafeez et al 1990 isolated 10 species of keratinophilic fungi in sewage sludge: *Chrysosporium* and *Microsporum.* . Ulfig et al.(2006) opined that sewage sludge treatment technologies, together with sludge structure, humidity and pH, were critical factors in determining the occurrence of keratinophilic fungi in the sludge environment [Abdul-Hafeez et al 1990]. Among the isolated species, only *M.gypseum* was more frequently found in medical laboratories waste. However some opportunistic fungi such as *Cladosporium spp*, *Alternaria spp* , *Penicillium spp* and *Rhizopus* spp. could survive in sludge and be transferred to the environment. These experiments illustrate the possible health risk problems that may arise in the use of sludge for land reclamation and fertilization. An interesting feature of the results was the high percentage of potentially pathogenic fungi (***M.gypseum & C.keratinophilum*)** were either well known agent of human as well as animal mycoses. Sludge with high organic carbon were found to be favorable habitats for ***Chrysosporium spp*** *.and* ***Microsporum gypseum*** [Ulfig and Korcz 1994 and Ulfig 2005b], it was thought that the abundance of keratinous debris favors the growth of keratinophilic fungi in these sludge especially like ***Geotrichum spp., Yeast*** *and* ***M.gypseum*** ***(***Table-1& 6). Hair inoculated sludge samples showed dominant growth of keratinophilic fungi at room temperature. Ascomata of ***Chrysosporium spp*** *.and* ***Microsporum gypseum*** were observed under microscope. ***Microsporum gypseum*** was reported with higher frequency (Table—6).

A health risk evaluation system for pathogenic fungi was proposed by Hoog et al. (1996). The system was included with fungal pathogenic properties, immunological system factors, therapeutic methods etc. for the ill effects caused by these opportunistic mycoses (Table-3 &4).The results however indicated that the use of Hair Bait method and dilution techniques facilitated the recognition of keratinophilic mycoflora.

CONCLUSION

The risk of fungal infections is increasing in the environment contaminated with sewage. Epidemiologists, Hygienists and Waste managers cannot ignore these findings and necessary treatment methods should be taken up periodically in and around the sewage outlets. In view of these results it can be concluded that the tourist places along the coast of Visakhapatnam do not seemed to be an ideal environment for children to play and for people to take dips in the waters. From hygienic point of view, necessary measures should be adopted to control the spread of

pathogenic fungi in this environment.

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