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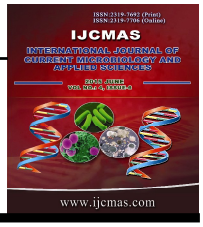

Original Research Article

Bacterial profile and isolation of PHA producing bacteria from Uppal Lake, India

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K e y w o r d s

Probiotics, L. Casei, L. sporogenous, Antagonistic effect, *Staphylococcus, Pseudomonas and Escherichia*

A B S T R A C T

Uppal Polluted lake, which is located in Hyderabad is one of the specialized ecosystems. It is nutritionally rich, due to domestic sewage and industrial effluents as well as pesticides and hydrocarbons. In these unbalanced conditions, it has been found that microorganisms reslake by the production of storage polymers. Bacterial flora of polluted water samples from different sites of lake, when screened for PHA accumulators, 180 isolates scored positive for PHA accumulation, when observed under UV light after staining with Nile Blue A. All isolates accumulating PHA were maintained on Luria Bertani agar slopes as working cultures. The monthly variation in the bacterial flora of the polluted water samples were studied by selecting different sampling sites from August, 2012 – July,2014 for two consecutive years. The number of bacteria isolated was highest (130) from Fourth site sample during June, 2014 and also the least number of isolates (71) were reported from the same site sample during the months of November, 2013 and February, 2014. The highest value of $2.38x10^9$ (per ml) of Colony Forming Units (CFU) was reported during March (2013), with the least of $1.30x10^9$(per ml) of CFU for the sample during May (2013). The number of bacteria isolated was highest (130) from Fourth site sample during June, 2014 and also the least number of isolates (71) were reported from the same site sample during the months of November, 2013 and February, 2014. The CFU values were in the range of $1.06x10^9$ (per ml) being the lowest value and $2.60x10^9$(per ml) being the highest value reported from fourth site sample.

Introduction

Biological wastewater are characterized by the exposure of microorganisms to transient conditions, where biomass is submitted to alternating periods of high and low substrate concentrations, aerobic and anaerobic environments.

In these unbalanced conditions, it has been found that microorganisms reslake by the production of storage polymers. Different types of organic storage polymers have been reported (Zevenhuizen and Ebbink, 1974). Among them, polyhydroxyalkanoates (PHA)

and polyglucose-like substances are the most frequently encountered (Beun *et al*., 2002; Karahan *et al*., 2008). Besides its role as a carbon and energy storage reserve for the microorganisms, PHA may represent an environmental friendly alternative to petrochemical plastics (Xiao *et al*., 2011).

Polluted lake is one of the specialized ecosystems. It is nutritionally rich, due to domestic sewage and industrial effluents as well as pesticides and hydrocarbons. The bacterial flora of Polluted water tends to be physiologically diverse due to the presence of rich nutrients. Microorganisms in such ecosystem utilize detritus matter and other available nutrients including PAH (Polycyclic aromatic hydrocarbon) break down compounds (Lillo and Rodriguez, 1990). Conventional microbiological techniques, based on the isolation of pure cultures and morphological, metabolic, biochemical, and genetic assays, have provided extensive information on the biodiversity of microbial communities in natural systems (Paramjit and Nitika, 2011). To obtain a novel PHA accumulator with features such as wide ranging substrate specification, we attempted to isolate bacteria accumulating PHA from polluted water, the environment of which is different from that of normal waters. In this study PHA accumulating bacteria were successfully isolated from polluted lake water samples.

Materials and Methods

Sampling

Samples were collected from the Uppal lake (Nalla Cheruvu) near Peerzadiguda surrounded by various industries, located at the outskirts of Hyderabad city, beside national (Warangal Road) highway 202. The samples were collected at four different sites of Uppal lake and screened for PHA accumulators. The collection of samples and survey for PHA accumulating bacteria was done during August2012 – July2014, for two consecutive years. The screening was done regularly on monthly intervals to determine the variation in bacterial flora and the PHA accumulators simultaneously.

Bacteriological analysis

The water samples 1 ml**,** were measured, and mixed vigorously for 10 min. Samples were serially diluted ten folds before plating. A 0.1 ml sample of each dilution was surface spread on sterile Luria Bertani agar medium. After incubation of 48 h at room temperature, the colony forming units (CFU) were counted to check the total viable count. After the initial sampling, the incubated plates were used to estimate CFU/ ml sample. The colonies formed on these plates were also checked for pigment production. These pigments have much value due to their natural origin and industrial use.

Staining and microscopic examination

Intracellular lipids: Intracellular lipids were looked for by staining the cells with Sudan black B (Norris and Swain, 1971). Smears of saline suspension of various bacterial isolates were made on glass slide and heat fixed. The slides were then flooded with Sudan black B and replenished as it dried out for 15 min. The excess stain was drained and the slides were blotted to dry. The slides were then counter stained with safranin for 10 s, after which it was washed with tap water, dried and then examined under light microscope. The bacteria accumulating lipids appear as pink cells with black granules in the cytoplasm.

Screening of PHA accumulators

Bacterial flora of polluted water samples from different sites of lake, when screened

for PHA accumulators, 180 isolates scored positive for PHA accumulation, when observed under UV light after staining with Nile Blue A. The yield of PHA accumulated by these isolates was further quantified after growth and extraction, with the method using UV spectrophotometer (Law and Slepecky, 1961).

Maintenance of cultures

All isolates accumulating PHA were maintained on Luria Bertani agar slopes as working cultures. Culture stocks were also maintained on Luria Bertani agar slopes, by sealing the tubes with paraffin wax. Preservation of cultures at 4°C was achieved by growing the isolates in 0.5 ml half strength Luria Bertani broth in sterile capped vials. Glycerol was sterilized and 0.5 ml was added to the grown culture as a cryoprotectant and the vials were preserved at 4°C.

Results and Discussion

Sampling and bacteriological analysis

The isolates obtained from the water samples during the investigation were picked at random and studied for their morphological as well as other characteristics. The monthly variation in the bacterial flora of the polluted water samples were studied by selecting different sampling sites from August, 2012 – July,2014 for two consecutive years.

Accumulation of PHA in various isolates screened from polluted water

During the screening of polluted water samples for PHA accumulating bacterial isolates, the number of bacterial isolates and colony forming units (CFU) were varied from month to month during August, 2012 to April, 2014. On the whole 450 bacterial isolates were screened for PHA accumulation during this period. These isolates were selected on the distinct morphological features from the samples of August, 2012 to July, 2014. 180 isolates were identified as positive isolates for PHA accumulation out of 450 isolates screened. Table.1. shows the results of bacterial screening from different samples during 2012 to 2013. The results indicate that during June, 2013, the number of bacteria isolated were maximum (130), which were isolated from the site 1 sample. During August, 2012 the isolates reported were least (70) from the site 3 sample. The highest value of $2.38x10^9$ (per ml) of Colony Forming Units (CFU) was reported during March (2013), with the least of $1.30x10^9$(per ml) of CFU for the sample during May (2013).

The results of bacterial screening of PHA accumulating isolates during August, 2013 to July, 2014 are reported in table.2. The number of bacteria isolated was highest (130) from Fourth site sample during June, 2014 and also the least number of isolates (71) were reported from the same site sample during the months of November, 2013 and February, 2014. The CFU values were in the range of $1.06x10^9$ (per ml) being the lowest value and $2.60x10^9$(per ml) being the highest value reported from fourth site sample. The pigment producing bacteria were reported from all the samples collected.

Their minimum number was 9 from the sample of August, 2013 and the maximum number was 22 isolates during the month of September, 2013 and January, 2014. The intracellular lipids were reported highest (56) during October, 2013 and the least number (27) of these lipids were reported during the month of November, 2013. The PHA producing bacteria were isolated in large number (50) during the month of

October, 2013 and the least number (24) was reported during the month of November, 2013.

Month wise investigation of all the samples revealed that more number of isolates were present in the month of May. Where as during monsoons and winter seasons the total numbers of organisms isolated from these samples were much lower compared to summer. When compared between different months of the same season some variation in the total number of organisms isolated was often present. This could be due to various factors, such as, environmental conditions like: temperature, rain, pH variation etc.

In and all mostly Gram positive, as well as Gram negative bacteria to some extent were isolated from the samples. All selected isolates were maintained and preserved on LB agar slants (fig.1.) The Gram positive out numbered the Gram negative. Almost all the isolates were rod shaped either long or short, besides few irregular rods and sometimes rods in chains. The cocci forms of bacteria were also found in the samples, but to a very negligible extent. The bacterial intracellular lipids were found in 60% of the sewage sludge samples. The intracellular lipids percentage in samples, were 4.5%, 3.6% and 3.9% and 4.5% for 1.2.3.4 site samples respectively. The accumulation of the Polyhydroxyalkanoates (PHA) were studied both by plate assay and microscopic method (Figure 2and3) as described earlier. The plate assay method was preferred to screen PHA accumulators, as it is a more rapid technique. The isolates showing the characteristic orange color fluorescence under the UV light of plate assay (Figure 2 and 3) and emmitted fluorescence were selected for further studies

The month wise variation of the bacterial flora of polluted water is evident from tables 1 and 2. The heterotrophic bacterial count varied from month to month, which as a part of variation bacterial counts were increased in the summer and decreased in the monsoon months. The study of cultural characteristics of the isolates revealed the presence of significant number of pigmented organisms. Pigments have a great commercial value and are used immensely as a colorant in numerous industries such as plastics, gums, food, dyes and stains etc (Martin and Williams, 2003).

Overall, the Gram positive bacteria tend to dominate the polluted water. Isolates with multiple enzyme activity, though small in number are important in such ecosystems, due to their potentials in industrial applications. Bacteria tend to accumulate reserve materials like intracellular lipids and Polyhydroxyalkanoates, which are generally utilized when the conditions are adverse. Here, 180 isolates of the bacteria from polluted water accumulated PHA as well as intracellular lipids. A large number of organisms accumulated intracellular lipids. Accumulation of PHA occurs in the presence of excess carbon, which is available for the organisms from the degradation products of diverse nutrients, in the water (Elías *et al*., 2010). The high counts of bacteria accumulating PHA during the June signify the availability of necessary nutrients and small molecular weight components for growth of bacteria and accumulation of PHA.

High concentrations of organic and inorganic nutrients at the polluted lake had a clear effect on the composition and diversity of the microbial community compared to the fresh water lake. Growing concern about environmental pollution has renewed interest in the development of PHA, which are completely biodegradable by bacteria present in most environments.

Table .1 Profile of Bacterial flora isolated during August,2012 to July,2013 from polluted pond Numbers 1,2,3,4 showing four samples from 4 different sites

Month	No. of isolates from four sites				CFU /ml sample				Pigment producers				Intracellular lipids				PHA producers			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Aug 2012	106	90	70	98	$2.12X10^9$	$1.80x10^9$	$1.40x10^9$	$1.96x10^9$	5	4	7	1	10	14	12	9	9	14	12	8
Sept 2012	108	81	94	102	$2.16x10^9$	$1.62x10^9$	$1.88x10^9$	$2.04x10^9$	2	5	8	2	8	21	6	12	8	20	5	4
Oct 2012	124	110	103	81	$2.48x10^9$	$2.20x10^9$	$2.06x10^9$	$1.62x10^9$	4	3	1	7	13	12	18	15	10	10	15	11
Nov 2012	115	91	120	95	$2.30x10^9$	$1.82x10^9$	$2.40x10^9$	$1.90x10^9$	3	9	8	7	16	14	10	8	15	12	8	8
Dec 2012	105	82	94	71	$2.10x10^9$	$1.64x10^9$	$1.88x10^9$	$1.42x10^9$	1	3	5	2	12	8	11	7	10	8	10	7
Jan 2013	96	102	120	87	$1.92x10^9$	$2.04x10^9$	$1.40x10^9$	$1.74x10^9$	9	4	2	4	15	13	15	11	12	11	13	10
Feb 2013	106	71	84	96	$2.12x10^9$	$1.42x10^9$	$1.68x10^9$	$1.92x10^9$	7	2	3	3	7	11	12	14	7	11	10	11
Mar 2013	115	119	87	98	$2.30x10^9$	$2.38x10^9$	$1.74x10^9$	$1.96x10^9$	3	1	5	1	6	9	11	15	6	9	10	15
Apr 2013	124	115	108	94	$2.48x10^9$	$2.30x10^9$	$2.16x10^9$	$1.88x10^9$	2	6	7	2	11	12	14	18	11	12	11	11
May 2013	107	94	124	115	$2.14x10^9$	$1.88x10^9$	$2.48x10^9$	$1.30x10^9$	4	5	1	9	16	12	10	14	15	10	9	12
Jun 2013	130	112	121	124	$2.60x10^9$	$2.24x10^9$	$2.42x10^9$	$2.48x10^9$	5	3	8	2	15	18	16	12	14	18	15	12
Jul 2013	94	99	105	71	$1.88x10^9$	$1.98x10^9$	$2.10x10^9$	$1.42x10^9$	2	4	1	7	12	4	8	10	12	4	8	10

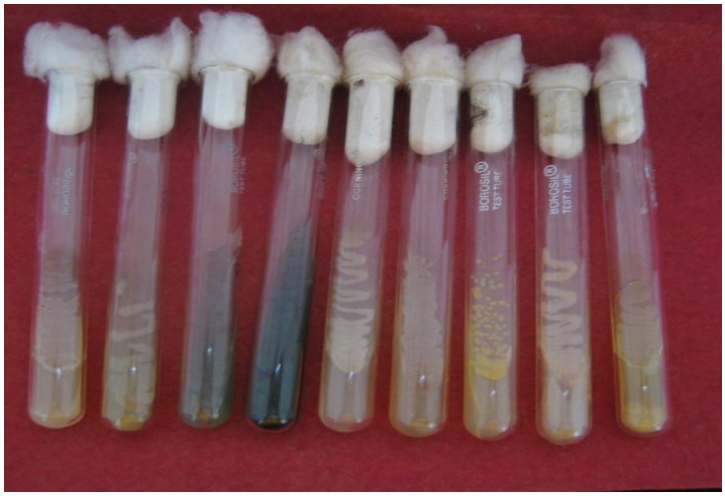
Numbers 1,2,3,4 showing four samples from 4 different sites
CFU= colony forming units

Table.2 Profile of Bacterial flora isolated during August,2013 to July,2014 from polluted pond

Month	No. of isolates from Four sites				CFU/ ml sample				Pigment producers				Intra cellular lipids				PHA producers			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Aug 2013	101	84	74	120	$2.02x10^9$	$1.68x10^9$	$1.48x10^9$	$2.40x10^9$	1	2	4	2	14	7	9	11	12	6	9	11
Sep 2013	105	94	81	72	$2.10x10^9$	$1.88x10^9$	$1.62x10^9$	$1.48x10^9$	6	3	7	6	13	11	10	10	11	8	10	10
Oct 2013	117	90	102	119	$2.34x10^9$	$1.8x10^9$	$2.04x10^9$	$2.38x10^9$	7	2	3	2	10	16	18	12	10	15	15	10
Nov 2013	120	108	107	71	$2.40x10^9$	$2.16x10^9$	$2.14x10^9$	$1.42x10^9$	4	5	7	0	3	5	10	9	3	5	8	8
Dec 2013	96	84	87	94	$1.92x10^9$	$1.68x10^9$	$1.74x10^9$	$1.88x10^9$	7	4	4	3	9	11	8	12	9	10	8	10
Jan 2014	115	105	96	98	$2.30x10^9$	$2.10x10^9$	$1.96x10^9$	$1.06x10^9$	8	5	3	6	7	14	13	9	7	13	10	9
Feb 2014	120	115	118	71	$2.40x10^9$	$2.30x10^9$	$2.36x10^9$	$1.42x10^9$	6	3	2	2	11	8	12	9	10	8	10	9
Mar 2014	125	114	89	90	$2.50x10^9$	$2.28x10^9$	$1.78x10^9$	$1.80x10^9$	4	2	0	5	9	10	13	14	9	10	12	12
Apr 2014	120	108	124	117	$2.40x10^9$	$2.16x10^9$	$2.48x10^9$	$2.34x10^9$	6	4	3	3	10	12	8	11	10	12	7	10
May 2014	116	112	118	108	$2.32.x10^9$	$2.24x10^9$	$2.36x10^9$	$2.16x10^9$	3	3	2	5	7	10	13	15	7	10	12	10
Jun 2014	94	120	121	130	$1.88x10^9$	$2.40x10^9$	$2.42x10^9$	$2.60x10^9$	2	6	4	0	5	15	12	5	5	14	10	5
Jul 2014	98	94	105	120	$1.96x10^9$	$1.88x10^9$	$2.10x10^9$	$2.40x10^9$	1	4	5	7	9	12	7	8	9	12	6	5

Numbers 1,2,3,4 showing four samples from 4 different sites
CFU= colony forming units

Fig.1 Maintanance of Isolated cultures on LB slants



Nile Blue staining

Fig.2 Control Bacterial colonies showing orange florescence

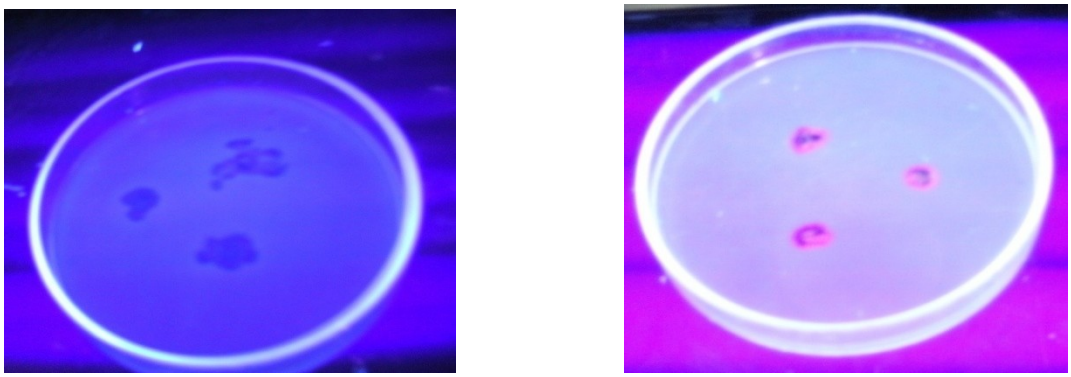
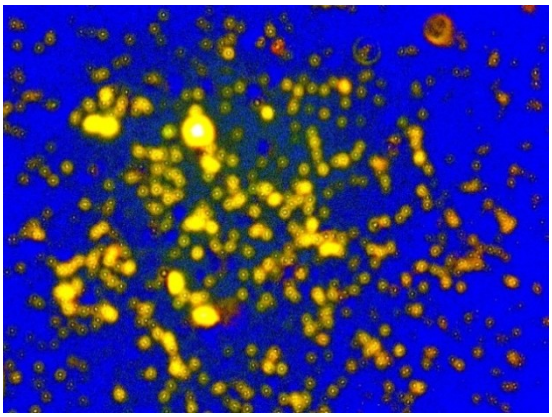


Fig.3 Flourescence microscopy of PHA producing bacteria



High concentrations of ammonia and organic matter, including lipids, produce toxic effects on bacteria communities. Synthesis of PHB has been proposed as a detoxifying mechanism of bacteria in water with high concentrations of fatty acids. Because PHA genesis is linked to lipid metabolism, PHA producers are more competitive in these environments (Kranz *et al*., 1997). Thus, PHA production in the microbial mat probably does not function only as a storage material, but also as a mechanism to cope with stressed and imbalanced nutrient environments, such as the polluted lake.

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