*Available online at*www.scholarsresearchlibrary.com

Scholars Research Library

Archives of Applied Science Research, 2015, 7 (5):32-38
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0975-508X
CODEN (USA) AASRC9

A study on polluted water pond: Water profiling and screening of PHA producing bacteria

K. Chaitanya[1]*, P. Nagamani[2], Rana Kausar[3] and S. K. Mahamood[4]

[1]*JNTUH, Hyderabad, India*
[2]*Environmental Microbiology lab, Osmania University, India*
[3]*Dept of Botany, Osmania University, Hyderabad, India*
[4]*Dept of Botany, Nizam College, Osmania University, Hyderabad, India*

ABSTRACT

Polluted pond 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). The bacterial flora in presence of rich nutrients tends to accumulate certain storage materials like volutin granules, lipids and polyhydroxyalkanoates (Du et al., 2004). Such a rich and diverse sewage ecosystem despite its potentials has not been adequately explored, for bacteria accumulating polyhydroxyalkanoates (PHA), and hence considered to be the potential environment for screening of bacteria accumulating PHA.

Keywords: Polluted water pond, Polyhydoxyalkonates, PHA.

INTRODUCTION

Polyhydroxyalkanoates represents a complex class of bacterial polyesters consisting of various hydroxyalkanoic acids that are synthesized by bacteria as storage materials of energy and carbon or used as anti-stress mechanisms (Lenz and Marchessault, 2005). Their applications as bioplastics, composite materials, bio implant materials, and medicines have been developed due to their biodegradability, biocompatibility, thermal process ability, and chirality (Anupama and Misra, 2011).

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, and aerobic and anaerobic environments. In these unbalanced conditions, it has been found that microorganisms respond 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).

Polyhydroxybutyrate (PHB) has been shown to be naturally produced by several bacteria such as *Alcaligenes, Pseudomonas, Bacillus, Rhodococcus, Staphylococcus* and *Micrococcus.* However, some bacteria accumulate PHA concomitantly during normal growth condition, i.e. they exhibit growth associated PHA accumulation (Son *et al*., 1996; Ibrahim and Steinbuchel, 2010). The most common polymer among these polyesters is poly (3HB), which was first to be discovered in *Bacillus megaterium* by Lemoigne in 1926. Since then, several other bacteria were characterized to produce poly (3HB) or PHA with other hydroxyalkanic acids. More than hundred different hydroxyalkanoic acids have been identified as constituents of PHA (Ibrahim *et al.,* 2010). Poly (3-hydroxybutyrate) is stored as carbon and energy reservoir in the organisms. It is currently being commercially produced by a US-based company, Metabolix, which has further plans of expansion to produce various biopolyesters by microbial fermentation processes at an annual capacity of 50,000 tonnes (Philip *et al*., 2007).

MATERIALS AND METHODS

2.1 Sampling

Samples were collected from the Hussain Sagar Lake situated between Hyderabad and sec-bad twin cities, Telangana region. The samples were collected at four different sites of polluted pond (Figure 1.1) and screened for PHA accumulators. The collection of samples and survey for PHA accumulating bacteria was done during November 2011 – October 2013, for two consecutive years. The screening was done regularly on monthly intervals to determine the variation in bacterial flora and the PHA accumulators simultaneously.

Figure 1.1: Plan of four sampling sites.1, 2, 3, 4 indicates four different sampling sites of Hussain sagar lake at Telagana

2.2 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.

Luria Bertani Agar Medium

Tryptone	10 g
Yeast extracts	5 g
Sodium chloride	5 g
Distilled water	100 ml
Agar	2.0 g
pH	7.2

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.

2.3. Accumulation of PHA in bacteria

Bacteria accumulating PHA were screened by the plate assay method (Shirokitamaru and Doi, 1994), from the water samples of the polluted pond. The bacterial isolates were replica plated on E2 mineral medium (Lageveen *et al.*, 1988), with 2 % (w/v) glucose. The colonies were allowed to grow till a diameter of 5 mm, approximately 2-3 days. The plates were then flooded with ethanol- Nile blue A solution for 20 min with intermittent shaking at room temperature. The staining solution was decanted and the plate allowed to dry. The plates were then exposed to UV light keeping at a distance of 10 cm from the light source. Colonies exhibiting an orange fluorescence were scored as PHA accumulators (figure1.3). For this experiment, *Ralstonia eutropha* MTCC 1285 was used as a positive culture.

E2 Mineral Medium

Basal medium		MT microelement	stock (g/l)
Microcosmic salt (NaNH$_4$ HPO$_4$) K$_2$HPO$_4$.3H$_2$O	3.5 g 7.5 g	FeSO$_4$.7H$_2$O MnCl$_2$.4H$_2$O CoSO$_4$.7H$_2$O	2.78 g 1.98 g 2.81 g
KH$_2$PO$_4$	3.7 g	CaCl$_2$.2H$_2$O	1.47 g
MgSO$_4$. 7H$_2$O (100mM)	10 ml	CuCl$_2$. 2H$_2$O	0.17 g
MT microelement stock	1 ml	ZnSO$_4$.7H$_2$O	0.29g
Yeast extract	0.004 g		
Carbon source (w/v)	2%		
Distilled water	989 ml		
pH	7.2		

Nile blue A Solution for Plate Assay

Nile blueA	0.05 g
Ethanol	100 ml

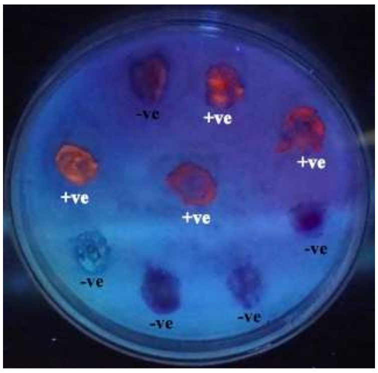

Figure 1.3: Nile blue staining of E$_2$ plate showing positive and negative PHA bacterial colonies (Plate assay method)

RESULTS AND DISCUSSION

3.1 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 November, 2011 – October,2013 for two consecutive years. The pH values were reported around 7.0 for the two consecutive years, the TSS values varied from month to month, which were reported in the range of 120 mg/l - 217 mg/l. The lowest value of TSS was reported during April, 2012 and the highest value was recorded in December, 2012. The BOD values showed a maximum value of 52.0 mg/l during December, 2011 and a minimum value of 30.0 mg/l during April, 2013. The COD values ranges from 180.0 mg/l (Aug, 2013) to 280.0 mg/l (Dec, 2012). Maximum amount of Nitrates were reported in April, 2012 as 7.26 mg/l and minimum recorded in the month of August, 2013 as 4.20 mg/l (Table 1.1).

Table 1.1: A general profile of physicochemical characteristics of polluted water samples collected during various time intervals

Parameter	DEC 2011	APR 2012	AUG 2012	DEC 2012	APR 2013	AUG 2013
PH	7.08	7.46	6.99	7.84	7.10	7.29
TSS(mg/l)	142	118	124	217	156	139
TDS(mg/l)	1086	1468	1152	1227	1143	1043
Nitrates(mg/l)	6.10	7.26	4.20	6.28	4.74	2.80
phosphorus(mg/l)	4.62	3.89	4.11	4.28	2.40	2.26
COD(mg/l)	150	214	180	280	118	146
BOD(mg/l)	52	33	42	46	30	36

3.2 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) (Figure 1.2) were varied from month to month during November, 2011 to October, 2013. On the whole 510 bacterial isolates were screened for PHA accumulation during this period.

Figure 1.2: Screening on LB media

3.3 Selection of the potential PHA accumulating bacteria

The isolates were first grown in E_2 media broth in 50 mL flasks, and were employed to extract PHA after two days of incubation on orbital shaker as described previously. The PHA from the isolates was extracted by the hypochlorite method, developed by Rawte and Marvinkurve (2002). Other extraction methods like chloroform extraction method (Yellore and Desai, 1998) and another method by Sayyed and Chincholkar (2004) were also employed. The efficiency of each of these methods depends upon the circumstances of the extractions and the nature of the isolates. In the present work, mostly hypochlorite method was used, but during large-scale production chloroform- extraction method is most useful as compared to the hypochlorite method.

Table 1.2: Accumulation of PHA (g/l) in various bacterial isolates, screened from Polluted water samples

Isolates	PHA	Isolates	PHA	Isolates	PHA
SKM 1	0.324	SKM 26	0.823	SKM 51	0.355
SKM 2	0.421	SKM 27	0.5769	SKM 52	0.260
SKM 3	0.820	SKM 28	0.795	SKM 53	0.478
SKM 4	0.220	SKM 29	0. 36	SKM 54	0.139
SKM 5	0.236	SKM 30	0.929	SKM 55	0.098
SKM 6	0.546	SKM 31	0. 413	SKM 56	0.029
SKM 7	**1.021**	SKM 32	0. 709	SKM 57	0.684
SKM 8	0.653	**SKM 33**	**1.032**	SKM 58	0.954
SKM 9	0.122	SKM 34	0.564	SKM 59	0.368
SKM 10	0.965	SKM 35	0.341	SKM 60	0.642
SKM 11	**1.078**	SKM 36	0.893	SKM 61	0.453
SKM 12	0.122	SKM 37	0.112	SKM 62	0.834
SKM 13	0.245	SKM 38	0.746	SKM 63	0.086
SKM 14	0.260	SKM 39	0.89	SKM 64	0.785
SKM 15	0.280	SKM 40	0.910	SKM 65	0.136
SKM 16	**1.312**	SKM 41	0.042	SKM 66	0.843

SKM 17	0.428	SKM 42	0.734	**SKM 67**	**1.130**
SKM 18	0.90	SKM 43	0.326	SKM 68	0.195
SKM 19	0.796	SKM 44	0.641	SKM 69	0.094
SKM 20	0.451	SKM 45	0.431	SKM 70	0.542
SKM 21	0.941	SKM 46	0.834	SKM 71	0.82
SKM 22	0.340	SKM 47	0.149	SKM 72	0.152
SKM 23	0.659	SKM 48	0.213	SKM 73	1.211
SKM 24	0.587	SKM 49	0.091	SKM 74	0.812
SKM 25	0.82	**SKM 50**	**0.974**	SKM 75	0.235

Isolates	PHA	Isolates	PHA	Isolates	PHA
SKM 76	0.412	SKM 101	0.117	SKM 126	0.763
SKM 77	0.81	SKM 102	0.094	SKM 127	0.234
SKM 78	0.141	SKM 103	0.064	SKM 128	0.196
SKM 79	0.685	SKM 104	0.783	SKM 129	0.453
SKM 80	**1.211**	SKM 105	0.147	SKM 130	0.639
SKM 81	0.431	SKM 106	0.753	SKM 131	0.621
SKM 82	0.195	SKM 107	0.594	SKM 132	0.973
SKM 83	0.093	SKM 108	0.148	SKM 133	0.540
SKM 84	0.045	SKM 109	0.368	SKM 134	0.099
SKM 85	0.974	SKM 110	0.632	SKM 135	0.641
SKM 86	0.462	SKM 111	0.853	**SKM 136**	**1.171**
SKM 87	0.763	SKM 112	0.941	SKM 137	0.653
SKM 88	0.826	SKM 113	0.741	SKM 138	0.139
SKM 89	0.186	SKM 114	0.630	SKM 139	0.450
SKM 90	0.541	SKM 115	0.147	SKM 140	0.091
SKM 91	0.946	SKM 116	0.090	SKM 141	0.238
SKM 92	0.148	SKM 117	0.178	SKM 142	0.692
SKM 93	0.183	SKM 118	0.874	SKM 143	0.136
SKM 94	0.983	SKM 119	0.118	SKM 144	0.183
SKM 95	0.84	SKM 120	0.093	SKM 145	0.189
SKM 96	0.120	SKM 121	0.79	SKM 146	0.174
SKM 97	0.246	SKM 122	0.919	SKM 147	0.189
SKM 98	0.846	**SKM 123**	**1.087**	SKM 148	0.045
SKM 99	**1.075**	SKM 124	0.181	SKM 149	0.129

Isolates	PHA	Isolates	PHA	Isolates	PHA
SKM 150	0.245	SKM 173	0.620	SKM 196	0.296
SKM 151	0.101	SKM 174	0.113	SKM 197	0.528
SKM 152	0.013	SKM 175	0.011	SKM 198	0.451
SKM 153	0.706	SKM 176	1.011	SKM 199	0.183
SKM 154	0.901	SKM 177	0.146	SKM 200	0.673
SKM 155	**1.021**	SKM 178	0.784	SKM 201	0.921
SKM 156	0.394	SKM 179	0.385	SKM 202	0.215
SKM 157	0.612	SKM 180	0.284	SKM 203	0.391
SKM 158	0.131	SKM 181	0.742	SKM 204	0.467
SKM 159	0.741	SKM 182	0.914	SKM 205	0.472
SKM 160	0.164	SKM 183	0.168	SKM 206	0.328
SKM 161	0.014	SKM 184	0.294	SKM 207	0.834
SKM 162	0.019	SKM 185	0.712	SKM 208	0.063
SKM 163	0.541	SKM 186	0.395	SKM 209	0.118
SKM 164	0.256	SKM 187	0.680	SKM 210	0.193
SKM 165	0.378	SKM 188	0.921	SKM 211	0.911
SKM 166	0.497	SKM 189	0.681	SKM 212	0.623
SKM 167	0.689	SKM 190	0.721	SKM 213	0.063
SKM 168	0.241	SKM 191	0.059		
SKM 169	**1.140**	SKM 192	0.231		
SKM 170	0.385	SKM 193	0.091		
SKM 171	0.397	SKM 194	0.013		
SKM 172	0.362	SKM 195	0.010		

PHA accumulation in bacterial cells increases as the incubation period increases, and reaches maximum at late exponential stage of the growth curve and declines on further incubation. Since, all the cultures were quantitated after a certain period of 48h, it is possible that the low yield of PHA obtained for certain cultures is probably due to the time of selection of harvesting the cells, which was either prior to late exponential stage of the growth curve or after onset of PHA hydrolysis. Twelve such isolates were obtained which accumulated more than 1.0g/l of PHA. These were selected as the potential PHA accumulators for further study.

3.4 Quantitative assay of PHA

All the 213 positive isolates selected from the total 510 isolates from different samples were quantified for the polymer accumulation. The amount of PHA accumulated by these isolates was tabulated in (Table 1.2). The amount of PHA formed from different isolates varied considerably. Among the overall isolates, 12 isolates producing PHA in the highest range were selected for further studies and preserved in LB slants. The selected isolates include SKM7, SKM11, SKM16, SKM33, SKM50, SKM67, SKM80, SKM99, SKM123, SKM136, SKM155 and SKM169.

The presence of PHA, if any, in the polluted water samples were studied, considering each of the water sample showed the presence of bacterial isolates accumulating PHA. Multiple ecological roles have been proposed for PHA metabolism in sewage water system (Comeau *et al.,* 1986; Liu *et al.,* 2000). Small quantities of the polymer were detected in the water samples during some months, which indicate the high rate of the polymer degradation, since non accumulators also possess the enzymes necessary for PHA depolymerization (Barnard and Sanders, 1989; Kaparitchkoff *et al.,* 2005). This could be one of the reasons for the lower content of PHA in the polluted water samples. With a density more than that of water, PHA tends to sink to the bottom of the water layer and remain in the sludge, helping the microflora of the pond during starvation. Since a wide variety of bacteria were scored positive for PHA accumulation, thus proving the potential source of the sewage water system. Under aerobic conditions, these polymers may serve as a source of carbon and energy. However, our understanding of the role of PHA metabolism in the polluted pond system is limited.

CONCLUSION

In the present study, High concentrations of organic and inorganic nutrients at the polluted pond had a clear effect on the composition and diversity of the microbial community compared to the fresh water pond. Growing concern about environmental pollution has renewed interest in the development of PHA, which are completely biodegradable by bacteria present in most environments. 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 pond. In this study, PHA producing bacteria were successfully isolated from polluted water. Accumulation of PHB and its co polymers were identified from various isolates under aerobic conditions. In conclusion, this study contributes to the comprehension of the diversity of PHA producers isolated from polluted pond subject to environmental stress by organic pollution of various industries, which contribute to the imbalance of nutrients.

Acknowledgement

Authors are thankful to JNTUH and Head Dept of Botany, Osmania University.

REFERENCES

[1] Anupama S, Sanjiv KM, Imran P, Deepti J, Sourish B, Sheetal P, Sandhya M. *World J Microbiol Biotechnol*, **2011**, 27, 1531–1541.
[2] Barnard GN and Sander JK. *J .Biol .Chem*. **1989**, 264, 3286–91.
[3] Beun JJ, Dircks K, van Loosdrecht MCM and Heijnen JJ. *Water Res*. **2002,** 36(5), 1167–1180.
[4] Comeau Y, Hall KJ, Hancock REW and Oldham WK, *Water Res*. **1986**, 20, 1511–1521.
[5] Du G,Chen LXL and Yu J, *J. Polym. Environ*. **2004**, 12 (2), 89-94.
[6] Elías R. Olivera, Mario Arcos, Germán Naharro and José M. Luengo. *Microbiology Monographs*. **2010,** 14, 133-185.
[7] Ibrahim, MHA and Steinbuchel, A. *J Appl Microbiol*. **2010**, 108, 214–225.
[8] Kapritchkoff FM, Viotti AP, Alli RCP, Zuccolo M, Pradella JGC, Maiorano AE, Miranda EA and Bonomi A. *J. Biotechnol*. **2006,** 122, 453-462.
[9] Karahan O, Orhon D and van Loosdrecht MCM. *Water Sci Technol.* **2008,** 58(4), 945–951.
[10] Lageveen RG, Huisman GW, Preustig H, Ketelaar P, Eggink G and Witholt B. *Appl. Environ. Microbiol*. **1988,** 54, 2924-2932.
[11] Lenz RW and Marchessault RH.. *Biomacromolecules*. **2005**, 6, 1–8.
[12] Lillo JG and Rodriguez-Valera F. *Appl. Environ. Microbial*. **1990,** 56, 2517-2521.
[13] Liu SJ and Steinbüchel A. *Environ. Microbiol*. **2000**, 66, 739-743.
[14] Madison LL, Huisman GW. *Microbiol. Mol. Biol Rev*. **1999,** 63, 21-53.

[15] Norris JR and Swain H. Staining bacteria, In: Norris JR and Ribbons DW (eds) Methods in Microbiology (Academic press, London). **1971**, 5A, 125-143.
[16] Ostle AG and Holt JG. *Appl. Environ.Microbiol*. **1982,** 44, 238-241.
[17] Paramjit Singh and Nitika Parmar. *African Journal of Biotechnology*. **2011** 10, 4907-4919.
[18] Philip S, Keshaverz T and Roy I. *J.Chem.Technol. Biotechnol*. **2007,** 82, 233–247.
[19] Rawte T and Mavinkurve S. *Indian Journal of Experimental Biology*. **2002,** 40, 924–929.
[20] Sayeed RZ and Chincholkar. *Indian J. Microbiol*. **2004,** 44(4), 269-272.
[21] Shirokitamura and Doi Y. *Biotechnol. Techn*. **1994,** 8, 345-350.
[22] Son HJ, Park, GT and Lee, SJ. *Biotechnol Lett*. **1996**, 18, 1229–1234.
[23] Xiao WS, Zhen YS, Ge S, Zheng JL and Guo Q Chen. *Appl Microbiol Biotechnol*. **2011,** 91, 655–665.
[24] Yellore V and Desai A. *Lett. Appl. Microbiol*. **1998,** 26, 391-394.
[25] Zevenhuizen LPTM and Ebbink AG. *Anton Leeuw Int J G*. **1974,** 40(1), 103–120.