Species

ISSN 2319 – 5746 EISSN 2319 – 5754

ANALYSIS

Isolation and characterization of potent poly – (3-hydroxybutyrate)(PHB) producing bacteria from various environmental samples collected from locality of Bangalore

Mahadevaiah, Shanmugam

Department of Sugar Technology, University of Mysore, Sir MVisvewaraya Post-Graduate Centre, Tubinakere, Mandya; E-mail: prohelix.sms@gmail.com

Publication History

Received: 10 November 2014
Accepted: 27 December 2014
Published: 28 January 2015

Citation

Mahadevaiah, Shanmugam. Isolation and characterization of potent poly – (3-hydroxybutyrate)(PHB) producing bacteria from various environmental samples collected from locality of Bangalore. *Species*, 2015, 12(35), 81-86

ABSTRACT

Bacterial isolates from sludge samples collected at a local area of Bangalore were screened for bacteria producing Polyhydroxyalkanoates (PHA) specifically for Poly – (3-hydroxy butyrate) (PHB) at one month interval between April 2012 to March 2013. Initially Sudan Black B staining was performed to detect lipid cellular inclusions. PHB-positive isolates were then grown in a modified mineral E2 medium containing 1% (v/v) molasses with nitrogen limitation to promote accumulation of PHA before the subsequent staining with Nile blue A. The positive isolates were quantified initially with a UV-VIS spectrophotometer, for a very large number of isolates (364) and among them high PHB-producing isolates (12) were selected. The proportion of the PHB-positive bacterial isolates showed variability in the number of PHB accumulators during various months. Among all the bacterial CDW 63.67% cell dry weight PHB, is the highest report of PHB -accumulating bacteria from Metal plate industrial effluent (MEJN01) and next in Garden soil isolate (GSJN04) of about 45.89%, when grown on the E2 mineral media supplemented with 1% molasses. The correlation of PHB production with the cell dry weight (CDW) was found to be statistically significant. The metabolism of PHB in the selected 12 isolates was studied using the Nile blue A staining, which showed an initial increase in the fluorescence followed by a decline after 72 hours, on further incubation. All the selected 12 isolates were classified to genus level by studying their morphological and biochemical characteristics. There were eight *Bacillus* species, one *Escherichia* species, one *Azotobacter* species, and two *Staphylococcus* species.

Key Words:

PHB, Sudan Black B staining, Nile Blue A, *Bacillus* species, mineral media with molasses.

Mahadevaiah and Shanmugam,
Isolation and characterization of potent poly – (3-hydroxybutyrate)(PHB) producing bacteria from various environmental samples collected from locality of Bangalore, Species, 2015, 12(35), 81-86,

1. INTRODUCTION

The indiscriminate dumping of plastic waste has led to serious threat to the environment, thus causing plastic pollution. These conventional chemically synthesized xenobiotic plastics derived primarily from petroleum are not biodegradable [9] and they cause serious threat to living organisms thereby cause imbalance in the ecosystem. In search of alternative for these problems, current research is directed towards an ecofriendly technology to produce biopolyesters called as bioplastics. They are synthesized by various bacterial community and they are biodegraded by bacteria itself. They utilize metabolite acetyl-coA, an intermediate from main metabolic pathway to produce bioplastics. These biodegradable plastics are used as alternative source for the conventional plastics, and produced by various microbial fermentations [4] or isolated and extracted from natural organisms of different ecosystems.

Polyhydroxyalkanoates (PHA) are common intracellular granules found in many prokaryotes and they are produced under nutritional stress such as excess carbon stress. PHA is a biodegradable bacterial polyester group with varied thermo kinetic properties that may be of use as bulk commodity plastics. They are produced by biochemical transformation and polymerization reaction of acetyl Co-A, a major intermediate metabolite in the living cell under adverse physiological conditions [14]. These biopolymeric inclusion bodies accumulated in higher rate under aerobic conditions. This often occurs when the carbon source is in excess but one or several other nutrients are limited such nitrogen, oxygen, phosphorous or magnesium. The trace elements will have some effect on sporulation and inclusion body formation. A few facultative aerobic bacteria and anaerobic bacteria have been reported to accumulate PHA and other plastics [7]. The most common PHA is Polyhydroxybutyrate (PHB)[14], which was the first to the studied in detail.It is metabolically synthesized via acetyl-CoA or butyrate by β-hydroxybutyryl-CoA. The stored PHB provides bacteriawith a reserve source of carbon and energy that enhances protection to the bacteria under various stresses [1].Some reports on studies of PHBrevealed that it actsas an electron sink that helps to regulate the balance of reducing equivalents when cells are grown under O_2 limited conditions [1] [5] [10].

Most of the aerobic and some anaerobic bacterial flora in the presence of excess carbon rich nutrients tend to accumulate certain storage materials like lipids, polyhyroxyalkanotes and other volutin granules [6]. Such diverse set of microflora will be commonly seen in the agro-industrial wastes, effluents from some metal industries, sewage and its sludge, garden soil etc. Hence we considered such locations as potential environment for the isolation and screening of bacteria accumulating PHB.

2. MATERIALS AND METHODS

Collection of samples

Samples from various locations in Bangalore were collected at monthly intervals between 2012 and 2013. The samples collected included garden soil, soil from plastic dumping sites, sewage water samples collected from Bangalore University, effluent water sample from metal plate industry (industrial estate, Rajajinagar, Bangalore) and water samples from storage water tanks. The soil samples were collected in sterile glass container and water sample in reagent bottle. The samples for the isolation of Polyhydroxybutyrate producing isolates were collected in the second week of every month.

Isolation of bacterial strains

A total of 60 samples (5 samples in 12 months) were collected during sampling period. The isolates were obtained by serial dilution – pour plate technique on modified nutrient agar plates supplemented with 1% cane molasses. Using different dilutions (10^{-5} and 10^{-6}) each sample collected from various locations in Bangalore were subjected to incubation and growth on modified molasses nutrient agar (MMNA) plates. After incubation for 24 hours at room temperature, three to six isolates were taken and streaked onto fresh media (as duplicates). Mother cultures were prepared and stored for further use.

Screening for PHB producing bacteria

The colonies obtained from the second plate were subjected for the detection of presence of intracellular lipids by Sudan black B staining technique. All Sudan Black B positive isolates were selected and cultured on separate modified nutrient mineral minimal medium containing 1% molasses. The Sudan Black B positive bacterial isolates were also checked for PHB production by specific Nile blue staining method[6] [11]. The PHB producing bacteria, after Nile Blue A staining, showed bright orange fluorescence when observed under fluorescence microscope under UV light (by using Labo and Maeyer microscope fitted with UV light). The intensity of orange color increased based on PHB accumulation by the bacterial cells.

Quantitative analysis of PHB

The PHB producing bacterial colonies were grown on modified nutrient mineral minimal medium in 100ml conical flasks and incubated at 28^0C ± 1^0C on thermo-regulated orbital shaker incubator at 150rpm. After 48 hours of incubation, the bacterial cells were harvested and the polymers were extracted by chloroform extraction method [12]. The bacterial cells were centrifuged; the bacterial pellets were used for cell lysis by sodium hypochlorite, followed by a wash with cold diethyl ether, the PHB from the bacterial cells was precipitated after subsequent centrifugation. The PHB thus extracted were subjected for acidic condition to convert from PHB to crotonic acid. The amount of crotonic acid produced was analyzed by UV visible spectrophotometer, calibrated at 235nm [12].

Mahadevaiah and Shanmugam,
Isolation and characterization of potent poly – (3-hydroxybutyrate)(PHB) producing bacteria from various environmental samples collected from locality of Bangalore, Species, 2015, 12(35), 81-86,

Identification of PHB producing bacteria

The PHB producing bacteria isolated, were identified by biochemical tests as described in *Bergey's Manual of Determinative Bacteriology*[8]

Statistical Analysis

The correlation between the production of PHB and dry cell weight of the isolates was determined by correlation coefficient test

$$\rho = 1 - \frac{6\sum d_i^2}{n(n^2-1)}$$

where $d_i = x_i - y_i$, is the difference between the ranks of corresponding values X_i and Y_i and n is the number of values in each data set.

3. RESULTS AND DISCUSSION

The plastic pollution is one of the silent and serious threats to the environment because of their recalcitrant nature [1]. It is the one of the major solid waste pollutant released into the environment along with other organic biomass and waste effluent which is released by various agro-industrial processes [14]. Only few research articles are available about waste management process to address these solid waste released to the environment. To address these problems caused by plastic and other organic agro-industrial waste, some ecofriendly technologies should be searched and employed. Production of eco-friendly biodegradable plastics which mimics the commercially available petrochemical plastics. The major objective in this research work undertaken is to isolate PHB producing bacterial population.

A total of 364 bacteria isolated were identified and screened for PHB accumulation. These bacterial isolates are the count of both the plates isolated from 5 different samples. The bacterial isolates were obtained every month at the second week. The variations in bacterial colonies were seen between the April 2012 to March 2013. The Garden soil sample showed and yielded the highest number of bacterial isolates, about 32.9 % of total isolates. This was followed by sewage water sample (26.6%), storage water tank sample (17.8%) and soil samples from plastic dumping (12.3%) and effluent water sample from metal plate industry (10.1%) (Table 1)

During the month of November 2012 to January 2013, the environmental conditions such as temperature 27 ± 1^0C, humidity 73 – 76% and pH (7.0±0.2) were favorable for the growth of the various bacteria. This signifies the availability of nutrients for the bacteria. The bacterial flora was identified based on gram staining method to divide the bacterial flora into two main groups as gram positive and gram negatives. The gram negative rods (52.4%) dominated over gram positive bacteria (48.6%). The PHB producing bacterial isolates were about 15.6 % of the total 364 bacterial isolates. The Garden soil sample showed maximum PHB accumulators when compared to other samples (Table 1).

Selection of the potential PHB accumulating bacteria

Various reports are available globally for bacteria accumulating PHB. These bacteria are commonly present in soil and water environments. In the present study, 12 PHB positive bacterial isolates were selected by Nile Blue A staining method from the total 364 bacterial isolates screened from 5 different samples. The PHB accumulating bacterial isolates were 15.6 % of the total 364 bacterial isolates. In the five samples taken for the study, Garden soil and sewage water samples showed maximum PHB producing bacterial growth. The PHB accumulation in bacterial cells increased for incubation upto 48 hours and 72 hours. The declines in growth were observed after 72 hours of incubation. The pattern of PHB metabolism in the cells could be observed by the change in intensity of fluorescence under UV light after staining with Nile blue A.

Quantitative assay of PHA

The PHB-positive isolates selected after Nile blue A staining were first grown in modified minimal mineral broth supplemented with 1% Molasses in 50-ml flasks, and were employed to extract PHB after 2 - 3 days of incubation on orbital shaker. The PHB from the isolates was extracted by the hypochlorite method, developed by Rawte and Mavinkurve (2002). After this initial quantitative analysis with UV-VIS spectrophotometer method [2] [3] [15], 12 such isolates were obtained which accumulated more than 0.78 g/l of PHB (Table 3). These were selected as the potential PHA accumulators for further study.

The selection of the final 12 isolates was based on the highest amount of PHB produced by these isolates in modified mineral media with 1% molasses and was measured by UV-VIS spectrophotometer. Initially these isolates produced PHB from 0.79 to 1.80 g/l, amounting to about 17.23 – 63.67% PHB of cell dry weight (Table 3). These 12 isolateswere further studied for their morphological and biochemicalcharacteristics. Since all the cultures were quantified after a defined period of 48 hours, it is possible that the low yield of PHB obtained for certain cultures is 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 PHB hydrolysis.

The highest Cell dry weight (CDW) of 63.67% was from metal plate industrial effluent followed by Garden soil isolate (GSJN04) of 45.89%, when grown on the modified mineral media supplemented with 1% molasses, whereas, Rohini*et al*. (2006) reported 62.10% PHB of CDW from soil bacteria, when grown on the media supplemented with glycerol.

The correlation value between dry weight and PHB production was found to be 0.954, which was significant (Critical value at 13 degree of freedom and significance level at 0.005). All the selected 12 isolates were classified to genus level by studying their morphological and biochemical characteristics. There were eight *Bacillus* species, one *Escherichia* species, one *Azotobacter*species, and two *Staphylococcus* species

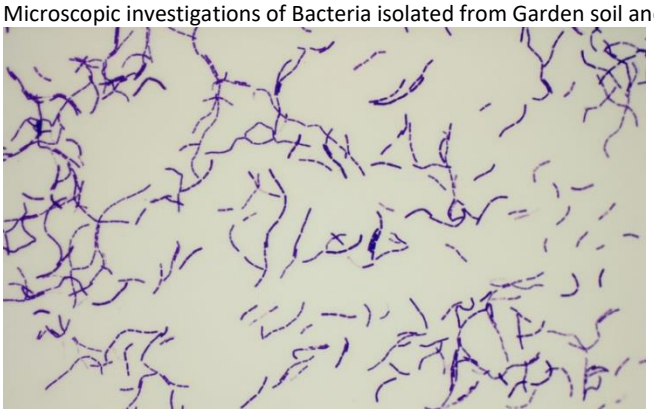
Mahadevaiah and Shanmugam,
Isolation and characterization of potent poly – (3-hydroxybutyrate)(PHB) producing bacteria from various environmental samples collected from locality of Bangalore, Species, 2015, 12(35), 81-86,

4. CONCLUSION

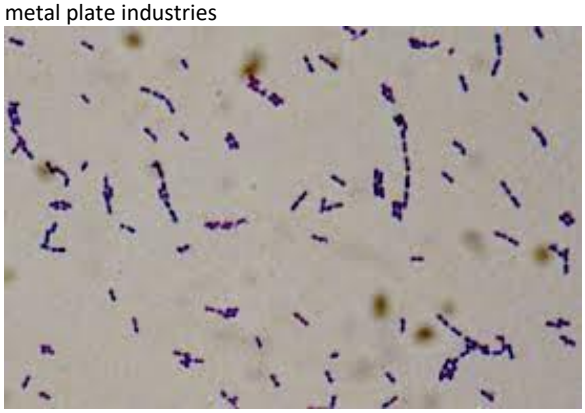
Among the 12 optimum PHB-producing bacteria, which produced PHB greater than 0.79 g/l were selected. The highest amount of PHB produced is 1.64 g/l, corresponding to 63.67 % by the bacterial isolate (MEJN01) isolated by metal plate industrial sample, followed by production of PHB at the rate of 45.89% was produced by bacterial isolate (GSJN04) which was isolated from Garden soil sample. In view of the above findings, it could be concluded that these isolates could be utilized to achieve cost-effective production of biodegradable polymers. Further study involves both the characterization of the selected isolates through molecular methods as well as the characterization of the polymers produced by these selected isolates using advance chromatographic analysis like FTIR. Study on various effect of commercially available carbon and nitrogen effects, optimization of fermentation media using sugar industry byproducts such as molasses and hydrolyzed bagasse are the future research goal and objectives. This will lead the way for effective use of sugar industry by products such as molasses and bagasse for the cost effective industrial production of PHB.

Images

Microscopic investigations of Bacteria isolated from Garden soil and metal plate industries



MEJN01



GSJN01

Table 1

Bacterial profile of various samples isolated during 2012 and 2013 in and around locality of Bangalore

MONTH	No. of isolated					No .of Gram positives					Intracellular lipids					PHB producers				
	GS	SW	STW	PDS	EM	GS	SW	STW	PDS	EM	GS	SW	STW	PDS	EM	GS	SW	STW	PDS	EM
APR-12	5	4	5	3	2	3	2	3	2	1	2	1	2	1	1	2	1	1	0	1
MAY-12	4	4	5	4	3	2	2	3	3	2	2	2	2	1	2	1	1	2	1	1
JUN-12	6	5	6	6	5	3	3	4	4	3	3	2	2	1	1	2	2	2	1	1
JUL-12	6	6	5	4	3	4	4	3	3	1	4	2	2	2	1	2	2	1	0	0
AUG-12	6	7	5	5	4	3	4	3	4	3	3	2	2	3	2	2	1	1	1	1
SEP-12	8	6	4	3	2	4	3	2	2	1	2	1	1	1	1	1	0	1	0	0
OCT-12	13	11	10	8	6	4	5	4	4	4	2	2	3	3	2	1	1	2	1	2
NOV-12	16	13	6	4	3	5	4	3	3	2	3	2	2	2	3	2	1	1	1	2
DEC-12	16	10	4	3	4	4	2	2	2	3	1	1	1	1	1	1	0	0	0	0
JAN-13	18	12	8	2	2	6	2	4	1	1	5	3	3	1	1	3	1	0	0	0
FEB-13	12	11	6	1	2	6	4	4	0	1	5	3	3	0	1	2	1	0	0	0
MAR-13	10	8	1	2	1	5	5	1	1	1	4	1	1	1	0	3	0	0	0	0
TOTAL	120	97	65	45	37	49	40	36	29	23	36	22	24	17	16	22	11	11	5	8

Mahadevaiah and Shanmugam,
Isolation and characterization of potent poly – (3-hydroxybutyrate)(PHB) producing bacteria from various environmental samples collected from locality of Bangalore, Species, 2015, 12(35), 81-86,

Table 2

Intensity of fluorescence exhibited by different isolates with increasing incubation periods after staining with Nile Blue A

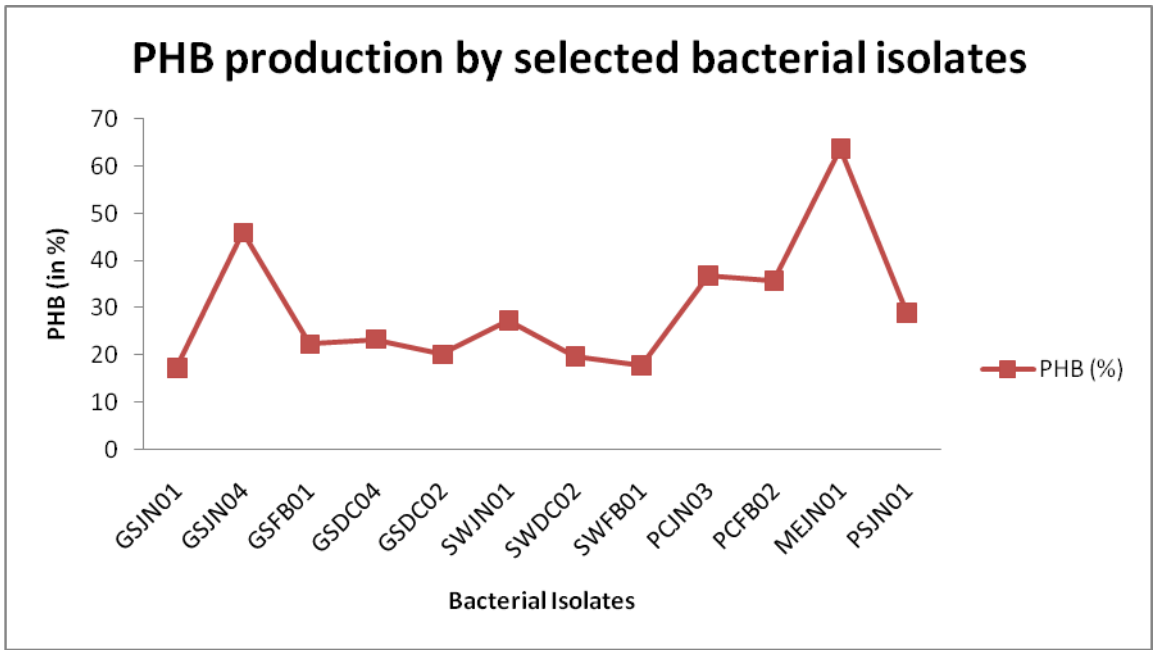
sl. No.	isolate number	24 h	48 h	72h	96h	120hr
1	GSJN01	+	++	+++	++	+
2	GSJN04	++	+++	++	+/-	+
3	GSFB01	++	++	++	+/-	-
4	GSDC04	+	++	+++	++	+
5	GSDC02	++	+++	++	-	-
6	SWJN01	++	++	+++	++	-
7	SWDC02	++	+++	++++	++	-
8	SWFB01	+	++	++	+	+
9	PCJN03	++	++	+++	++	+
10	PCFB02	+	++	+++	++	+
11	MEJN01	++	+++	++++	++	+
12	PSJN01	+	+	++	+	+

Table 3

PHB accumulated by 12 selected bacterial isolates

Sl. No.	isolate number	$A_{420\ nm}$	DCW (g/l)	wet weight (g/l)	PHB (g/l)	PHB (%)
1	GSJN01	0.105	4.78	39.85	0.79	17.23
2	GSJN04	0.170	2.79	31.42	1.28	45.89
3	GSFB01	0.138	4.65	38.46	1.03	22.34
4	GSDC04	0.121	3.92	46.23	0.91	23.33
5	GSDC02	0.124	4.67	34.78	0.94	20.13
6	SWJN01	0.009	2.64	37.50	0.71	27.23
7	SWDC02	0.119	4.56	43.69	0.90	19.74
8	SWFB01	0.116	4.91	39.03	0.87	17.87
9	PCJN03	0.240	4.91	39.02	1.80	36.86
10	PCFB02	0.115	2.44	30.16	0.86	35.65
11	MEJN01	0.219	2.59	30.50	1.64	63.67
12	PSJN01	0.136	3.55	33.18	1.02	28.97

Mahadevaiah and Shanmugam,
Isolation and characterization of potent poly – (3-hydroxybutyrate)(PHB) producing bacteria from various environmental samples collected from locality of Bangalore, Species, 2015, 12(35), 81-86,



REFERENCES

1. J.Anderson andE. A. Dawes, "Occurrence, metabolism, metabolic role and industrial uses of bacterial Polyhydroxyalkanoates",*Microbiol Rev* 54:450–472, 1990.
2. B.Aslim, F.Caliskan, Y.Beyatli and U. Gunduz,"Poly-β-hydroxybutyrate production by lactic acid bacteria", *FEMS MicrobiolLett* 159:293–297, 1998.
3. G. A.Bonartseva and V. L. Myskina, Fluorescence intensity of strains of nodule bacteria (*Rhizobium melliloti, R. phaseoli*) differing in activity, grown in the presence of the lipophilic vital stain phosphine 3R. Microbiology 54(4):535–541, 1985.
4. H.Chau, Yu PHF, SXing andL. Y.Ho Potential of biodegradable plastics as environmentally friendly substitute for conventional plastics in Hong Kong. Presented in the 17th Symposium on Biotechnology for Fuels and Chemicals, May 1995, Colorado, USA
5. E. A.Dawes andP. J.Senior,"The role and regulation of energy reserve polymers in micro-organisms",*AdvMicrobPhysiol* 10:135–266, 1973.
6. G.Du, L. X. L.Chenand andJ. Yu, "High-efficiency production of bioplastics from biodegradable organic solids", *J Polym Environ* 12(2):89–94, 2004.
7. L.Girbal, J.Orlygsson, B. J. Reinders and J. C. Gottschal, "Why does Clostridium acetireducens not use interspecies hydrogen transfer for growth on leucine?, *CurrMicrobiol.,* 35:155–160, 1997.
8. J. G.Holt, N. R.Krieg, Sneath, J. T.Stanley andS. T.Williams, Bergey's manual of determinative bacteriology, 9th ed.Lippincot, Williams and Wilkins, Baltimore. 2000.
9. T.Huang, J. Q.Zhao andJ. R.Shen,"The progress in microbiodegradable plastics", *Plast. Ind.,* 4:23–27, 1991.
10. E. Hustede, A.Steinbuchel and H. G. Schlegel "Relationship between the photoproduction of hydrogen and accumulation of PHB in non-sulphur purple bacteria", *ApplMicrobiol. Biotechnol.,* 39: 87–93, 1993.
11. G.Ostle and J. G. Holt "Nile blue as a fluorescent stain for PHB",*Appl. Environ.Microbiol.,* 44:238–241, 1982.
12. T.Rawte andS.Mavinkurve,"A rapid hypochlorite method for the extraction of polyhydroxyalkanoates from bacterial cells", *Indian J. Exp. Biol.* 40:924–929, 2002.
13. D.Rohini, S.Phadnis and S. K. Rawal, "Synthesis and characterization of poly-β-hydroxybutyrate from *Bacillus thuringiensis* R1", *Indian J.Biotechnol.* 5:276–283, 2006.
14. R. A. J. Verlinden, D. J. Hill, M. A. Kenward, C. D. Williams and I. Redecka, "Bacterial synthesis of biodegradable Polyhydroxyalkanoates",*J. Appl.Microbiol*. 102:1437–1449, 2007.
15. M.Yilmaz,H.Soran andY.Beyatli, "Determination of poly-β-hydroxybutyrate (PHB) production by some *Bacillus species"*. *World J.Microbiol.Biotechnol.* 21:565–566, 2005.

Mahadevaiah and Shanmugam,
Isolation and characterization of potent poly – (3-hydroxybutyrate)(PHB) producing bacteria from various environmental samples collected from locality of Bangalore, Species, 2015, 12(35), 81-86,