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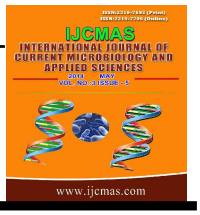

Original Research Article

Phylogenetic heterogeneity of the rhizospheric soil bacterial isolates producing PHAs revealed by comparative analysis of 16s-rRNA

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A B S T R A C T

Polyhydroxyalkanoates (PHAs) are gaining more importance over the world due to their structural diversity, close analogy to plastics and their biodegradability which makes them extremely desirable substitutes for synthetic plastics. PHAs are produced by various microorganisms under certain stress conditions. In this study, 78 bacteria were isolated from rhizospheric soil region of different plants growing in Odisha and screened for presence of PHAas hydrophobic inclusions in the cytoplasm. Out of all, 16 bacterial isolate showed presence of PHAs granule. All the PHA producing bacterial isolates were identified by molecular characterisation to study the evolutionary relationships. These 16 strains were characterized by sequencing partial 16s rRNA gene and belong to genus *Bacillus* such as *Bacillus flexus* strain MRK13, *Bacillus* sp.S4(2013b), *Bacillus* sp. P1(2013b), *Bacillus* sp. S1(2013b), Bacillus sp. S6(2013b), *Bacillus* sp. O1, *Bacillus* sp. P2(2013), *Bacillus* sp. P3(2013), *Bacillus* sp. P4(2013c), *Bacillus* sp. B2(2013c), *Bacillus* sp. B5(2013b), *Bacillus* sp. C1(2013), *Bacillus* sp. C3(2013), *Bacillus* sp. O6, *Bacillus* sp. S8, *Bacillus thuringiensis* strain RKD12. The nucleotide sequence submitted have been appear in the GenBank database under accession numbers KF626466 to KF626481. Under optimized condition *Bacillus* sp. S1 2013b possesses the potential for the production PHA (80.94%) in vitro.

Keywords

Rhizospheric; Hydrophobic inclusions; Bacillus sp.

Introduction

Growth in the human population has lead to the accumulation of huge amounts of non-degradable waste materials across our planet. The accumulation of plastic wastes has become a major concern in terms of the environment (Guillet, 2002; Derraik, 2002; Thompson et al., 2004). Conventional plastics not only take many decades to be decomposed in nature, but also produce toxins during the process of degradation. For this reason, there is special interest in producing biopolymer that can be readily eliminated from our biosphere in an "environmentally friendly" fashion (Gross and Kalra, 2002). Poly-hydroxyalkanoates (PHAs) are the boipolymer produced and accumulated in prokaryotes as carbon and energy storage materials (Rehm, 2003). They are water insoluble polymers and stored in the cell

cytoplasm as granules. Chemically, it consists of poly-ß-hydroxybutyrate (PHB) and poly-ß-hydroxyvalerate or copolymers (poly-ß-hydroxy- butyrateco -3- hydroxy valerate; PHBV). In recent years, PHAs has gained attention due to their biodegradable, biocompatible and thermoplastic features. (Jamil et al., 2007; Sangkharak and Prasertsan, 2008; Shrivastav et al., 2010). Thus it could be substitutes for petrochemical derived plastics and be used as packaging and biomedical materials (Madison and Huisman, 1999).

Many researchers have explained that soil bacteria generally produce PHB which is the best known PHA. Among PHB producer, members belong to genus *Bacillus* have been reported and extensively studied (Valappil et al., 2008; Adwitiya et al., 2009; Reddy and Mahmood, 2009; Gurunathan et al., 2010). The genus *Bacillus* was identified as one of the first Gram positive bacteria capable of producing PHB (Lemoigne, 1926). This genus has been widely used for a long time in industry and academia, due to the stability of its replication and maintenance of plasmids (Biedendieck et al., 2007). Up to now, many species of PHA producing bacilli have been isolated from various environments (Singh et al., 2009).This paper reports genotypic characterization and PHA production by *Bacillus* sp. isolated from rhizospheric soil region of different plants.

Materials and Methods

Sample collection

Representative soil samples were collected from the rizhosperic soil region of different plants growing in Odisha. Samples were collected aseptically from *Saccharum officinarum*, *Ficus benghalensis*, *Bambusa vulgaris*, *Calotropis procera* and *Ipomoea batatas* in sterile plastic bags. Then the samples were processed for physico-chemical parameters and bacteriological analysis. Physico-chemical parameters viz., temperature, pH and moisture content were analysed by using (Okatone) digital thermometer, (Systronics 361) pH meter and oven drying method respectively.

Isolation and preservation of bacterial isolates

Soil samples were processed for isolation of bacterial cultures using standard procedures of serial dilution and spread plating. Colonies of distinguished morphologies were individually picked and sub-cultured and preserved at 4°C for further use.

Screening of PHA producing bacterial isolates

Sudan Black B staining was used for detection of the existence of PHA in cytoplasm of bacterial cells by Schlegel et al., (1970). However, before screening, the isolates were induced to accumulate PHA by growing in GM medium that contained L-glutamic acid (3.8 g L^{-1}), malic acid (2.7 g L^{-1}) ,yeast extract (2.0 g L^{-1}), KH_2PO_4 (0.5 g L^{-1}), K_2HPO_4 (0.5 g L^{-1}), $(NH_4)_2SO_4$ (0.8 g L^{-1}), $MgSO_4.7H_2O$ (0.2 g L^{-1}), $CaCl_2.2H_2O$ (0.053 g L^{-1}), $MnSO_4.5H_2O$ (0.001 g L^{-1}), NaCl (5.0 g L^{-1}), nicotinic acid (1.0 mg L^{-1}), thiamine (1.0 mg L^{-1}), biotine (0.01 mg L^{-1}), glucose (10.0 g L^{-1}) medium for 24 h. Smears of cells deposited on a glass slide were heat fixed and stained with a 3% (w/v in 70% ethanol) solution of Sudan Black B (Hi-Media) for 10 min, followed by immersion of the slides in xylene until complete

decolorization. Then the samples were counterstained with safranin (Hi-Media; 5% w/v in de-ionized water) for 10 s, washed with water and air dried and examined using oil immersion microscope (1000X, Leica DM5000B). Simultaneously Nile red (Spiekermann et al., 1999) staining was also used for detection of PHA granule in the cytosol of bacterial cells.

Morphological and Physiological characterization of the isolates

Preliminary identification of bacterial isolates were done on the basis distinguished observable colony morphology on the Minimal salt agar that contained NaCl (3.0 g L^{-1}), KH_2PO_4 (1.5 g L^{-1}), K_2HPO_4 (1.5 g L^{-1}), $MgSO_4.5H_2O$ (1.0 g L^{-1}), glucose (10.0 g L^{-1}), ammonium nitrate (0.5 g L^{-1}), agar agar (5.0 g L^{-1}), Gram's reaction followed by observation under oil immersion (1000X) and biochemical tests.

Molecular identification of bacterial isolates

Molecular identification of the bacterial isolates was carried out by sequencing of 16S rRNA gene followed by submission of sequence to NCBI GenBank. Genomic DNA was extracted from the isolates (Sambrook et al., 1989) followed by amplification of 16S rRNA gene using universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-ACG GCTACCTTGTTACGA-3') and sequencing at Xcelris Genomics, India. Raw sequences were analysed by Bio-Edit software (7.0.5.3) and identification of the isolates by BLASTN in NCBI database (www.ncbi.nlm.nih.gov/nucleotide). Multiple alignments of sequences were performed with the ClustalX (1.83) (Thompson et al., 1994). A phylogenetic tree was constructed using the neighbour-joining DNA distance algorithm (Saitou and Nei, 1987) using Mega 5. The resultant tree topologies were evaluated by bootstrap analysis of neighbour-joining data sets based on 1000 resamplings. The final sequences were submitted to NCBI GenBank to obtain the accession number of each isolates.

Optimization of PHA production

For optimum biomass production by the isolates bioparameters such as pH, temperature, inoculum size; nutrient conditions such as different medium, combination of carbon and nitrogen source were analysed. Briefly, over-night broth culture of the individual isolates (0.5 McFarland standards) was inoculated to 1,000 ml of both MSM and GM medium and incubated at 37°C for 96 h with 120 rpm. Biomass production was compared by taking the average weight of the pellets obtained after centrifugation at 10,000 rpm for 15 min.

The optimized medium was used subsequently for further studies. Similarly, overnight grown culture was used to study the effect of other parameters on bacterial growth by varying one parameter at a time keeping all other parameters to be constant. The parameter studied includes pH (6-10), temperature (16-51°C), glucose concentration (5-25 g L^{-1}), combination of carbon (glucose, sucrose, fructose and lactose) and nitrogen sources (beef extract, urea, peptone and yeast extract) in 6:1 ratio. Comparative growth of the isolates in the various parameters was studied by measuring the OD_{600} in UV-Vis spectrophotometer (λ35, Perkin-Elmer) after 24h of incubation at 37°C with 120 rpm shaking.

Production and Extraction of PHA by the isolates

Bacterial isolates were grown in GM for 96 h at 37°C with 120 rpm and the cell mass was harvested by centrifugation at 6500 g, 10 min and kept at -20°C for 12 h (Narayanan and Ramana, 2012). The dried weight of the pellet was taken to know the biomass weight and the predominant biomass producing isolate was subjected to PHA production using optimized parameters. Subsequently, the cell pellet was re-suspended with sodium hypochlorite followed by incubation at 37°C for 2 h for digestion of non PHA material. The mixture was centrifuged with the same condition to harvest the PHA with twice washing with 10 ml distilled water. Similarly, the cell biomass was washed twice with acetone, methanol and diethyl ether (1:1:1). Finally, the PHA was dissolved with boiling chloroform and subsequent evaporation by air drying to yield dried form of extracted PHA (Preethi et al., 2012). PHA production was also quantified using the formula: % of PHA production = (Weight of PHA/ Weight of biomass) × 100 (Abinaya et al., 2012).

Characterization of PHA by FTIR analysis

The functional group present in the extracted PHA was determined by FTIR spectroscopy. PHA sample was mixed with 2 % KBr. The mixtures were compressed into translucent sample discs and fixed in the FTIR spectrometer (Perkin-Elmer RX I) for analysis (Dash et al., 2013).

Statistical analysis

Obtained data were analysed using one-way ANOVA, where, $p < 0.05$ was considered to be significant statistically using statistical package for social science (SPSS 12.0) software.

Results and Discussion

Physico-chemical parameter analysis

Physico-chemical parameters of a study site always impose a high impact on the bacterial population. Various parameters such as temperature, pH and moisture content of rhizospheric samples were analysed (Table 1). The pH of water, soil is almost neutral in nature and the temperature ranges from 34 to 37^0C.

Isolation, screening and characterization of PHA producing bacterial isolates

Total heterotrophic bacterial count in the study site ranged from 6.8×10^4 to 1.75×10^7 CFU/g on Nutrient agar plates. A total of 72 nos. of bacteria were selected for the screening of PHA production based upon their distinguished colony morphology on agar plates. Screening of PHA production by Nile red and Sudan black staining showed positive for 16 isolates to accumulate PHA granules in their cytosol. All of the isolates were found to be Gram positive rods under oil immersion microscope and the corresponding biochemical tests (Table 2) confirmed as *Bacillus*.

Molecular identification of the isolates

Sequencing of 16S rRNA gene confirms the molecular identification of the isolates. All of them were found to be within the genus *Bacillus* and out of the sixteen isolates fourteen were identified to be *Bacillus* sp., however other two were *Bacillus flexus* MRK13 and *Bacillus*

thuringiensis RKD 12. After submission at NCBI GenBank the accession number were obtained to be from KF626466-KF626481. The phylogenetic relationship among the isolates has been provided in Figure 1.

Optimization of PHA production

Biomass production of all the isolates was preliminarily optimized for growth medium in compared to minimal salt medium (MSM). The result showed the higher level of biomass production with formulated growth medium in contrast to the minimal salt medium used (data not shown here). Similarly, after statistical analysis the growth parameters were optimized for pH 7.0, temperature 37˚C, glucose concentration for 10% and inoculum size to be 10%. However, C: N combination did not found to affect the biomass production of the isolates at 95% ($P< 0.05$) significant level (Figure 2a, 2b, 2c, 2d and 2e). When the isolates were subjected for PHA production assay at the optimised conditions, *Bacillus* sp. S1 was found to produce maximum amount of PHA (80.94%). Thus, PHA produced by the isolate was characterized in subsequent experiments.

Characterization of PHA by FTIR analysis

The functional groups of the extracted PHA samples from the potent isolate *Bacillus* sp. P1 showed the characteristic peaks at 3434 cm^{-1} (H-bonded O-H stretch), 2956 cm^{-1} (O-H stretch), 2924 cm^{-1} (C-H stretch), 1714 cm^{-1} (C=O stretch), 1276 and 1223 cm^{-1} (C-O stretch), 1131 and 1053 cm^{-1} (C-N stretch), 981 cm^{-1} (=C-H bend) (Table 3). High intense peaks were obtained at 3434 cm^{-1}, 2956 cm^{-1}, 1714 and 1276 cm^{-1} (Figure 2f) however less intense peaks found at 2924 cm^{-1}, 1223 cm^{-1}, 1131 cm^{-1}, 1053 cm^{-1} and 981 cm^{-1}.

Table.1 Physico-chemical and microbiological parameters of the rhizospheric soil samples

Sl. No.	Plant	pH	Temp. (°C)	Moisture (%)	Total heterotrophic bacteria (CFU/g)
1	*Saccharum officinarum*	7.28	37	63	11×10^5
2	*Ficus benghalensis*	7.04	36	21	6.8×10^4
3	*Bambusa vulgaris*	7.76	35	12	7.2×10^6
4	*Calotropis procera*	8.08	36	14	1.75×10^7
5	*Ipomoea batatas*	7.91	34	72	7.4×10^5

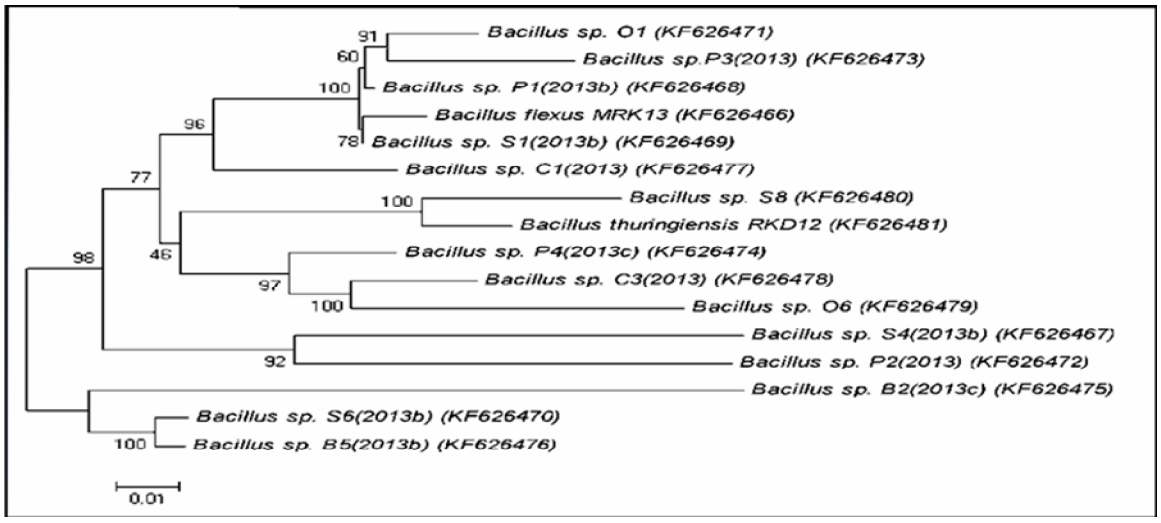
Table.2 Biochemical characteristics of PHA producing bacterial isolates

Sl. No	Biochemical test	B1	B2	B3	B5	C1	C3	O1	O6	P1	P2	P3	P4	S1	S4	S6	S8
1	Growth at 10% NaCl	-	+	+	+	+	+	+	+	+	+	-	+	-	-	+	+
2	Hippurate hydrolysis	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
3	Anaerobic growth	-	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-
4	MR-VP test	-	-	-	-	-	+	+	-	-	-	-	-	-	+	+	-
5	Citrate reductase	+	+	-	+	+	-	-	-	-	+	-	-	-	-	-	-
6	Starch hydrolysis	-	+	-	+	-	-	-	-	-	-	-	+	-	+	-	-
7	Oxidase reductase	-	+	+	+	-	+	+	+	+	-	+	-	+	+	+	+
8	Casein hydrolysis	+	-	+	+	-	+	+	+	+	+	+	-	+	+	+	-
9	Uerase hydrolysis	-	+	+	+	-	+	+	-	-	-	-	-	+	+	+	+
10	Nitrate hydrolysis	+	+	-	+	+	+	+	+	-	+	-	+	+	+	+	+
11	Esculin hydrolysis	+	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+
12	Growth at 50ºC	+	+	-	+	+	+	-	+	+	+	+	+	-	+	-	-

Table.3 Peaks obtained by FTIR and their corresponding annotations

Peaks (cm^{-1})	3434	2956	1714	1276
Bonds	O-H stretch, H-bonded	O-H stretch	C=O stretch	C-O stretch
Corresponding functional groups	Alcohols, Phenols	Carboxylic acids	Alpha, beta-unsaturated esters	Alcohols, carboxylic acids, esters, ethers

Fig.1 Phylogenetic relationship among the isolates characterized in this study drawn by MEGA5 with 1000 resamplings



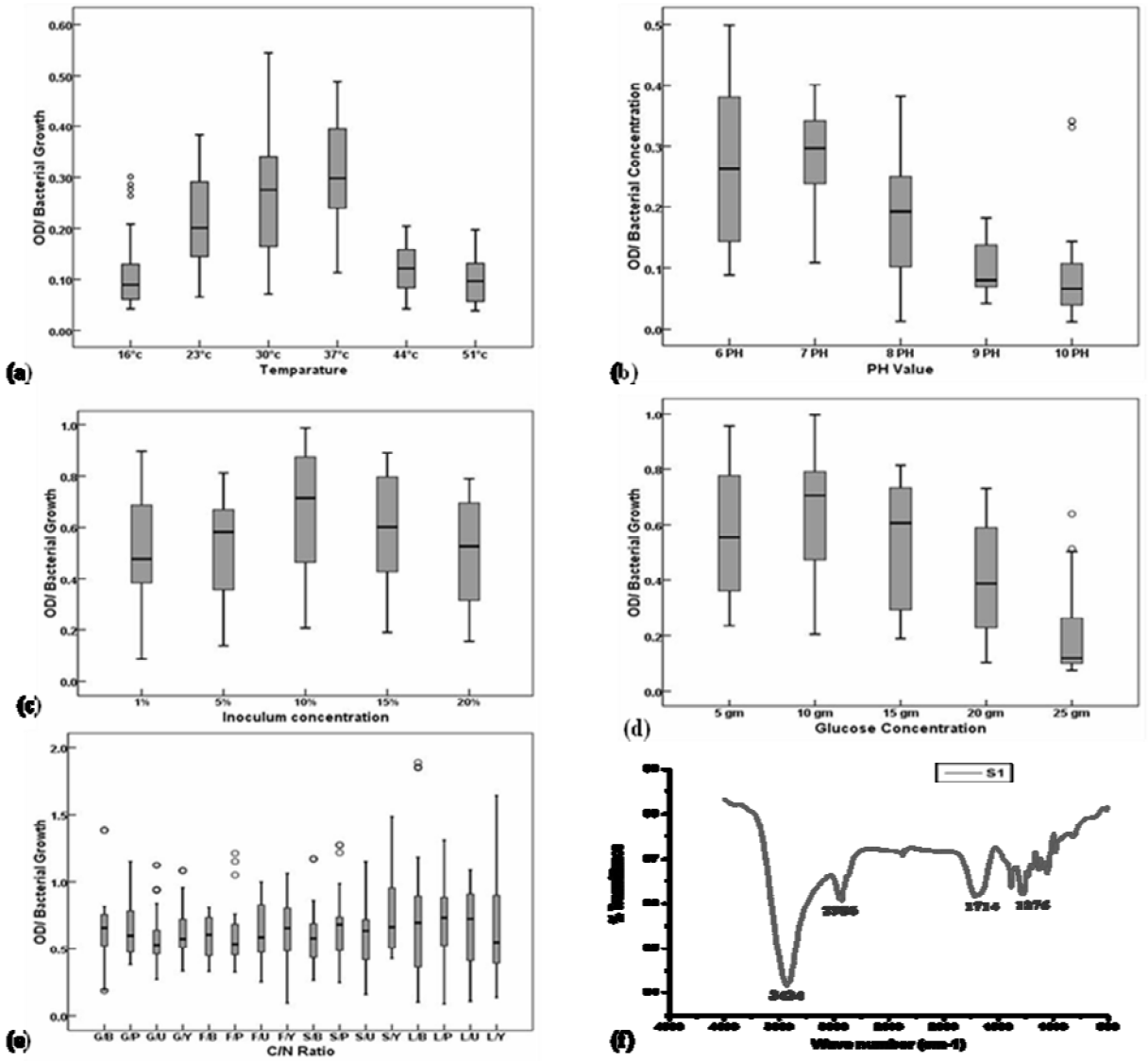


Fig. 2. Optimization of the growth parameters used in this study. The data represented here for the maximum biomass production at the corresponding variations of growth parameters (a) temperature, (b) pH, (c) concentrations of glucose (d) inoculum concentrations and (e) various combinations of C: N sources (f) FTIR analysis of the PHA sample extracted from *Bacillus* sp. S1 showing characteristic peak for PHA

PHA accumulation in bacteria is highly related to the limiting nutritional factors such as low nitrogen, sulphur and phosphorus content with excess of carbon content. In this regard, rhizospheric region of soil provides the optimum environmental condition by providing these desired parameters, for which the sample has been collected for the present investigation. Soil is the heterogeneously composed environmental condition providing the microhabitats for the diverse group of microorganisms. The bacteria inhabiting in those environment are highly adapted to the rapidly changing conditions by accumulating certain substances or by secreting their extracellular metabolites to overcome the noxious environmental

condition. In this case, bacterial population inhabiting in the rhizospheric region of the plants overcome the limiting nitrogen and phosphorus content by accumulating PHA reserve materials in their cytosol which may provide as the carbon source for its survival during limiting environments in future and can utilize it by β-oxidation.

Hence, out of 72 isolates screened, 16 of them were found to accumulate PHA in their cytosol which has been confirmed by Nile red and Sudan black staining. The PHA production by bacteria can be correlated to the fact that, suppression of citrate synthase and isocitrate dehydrogenase at nutrient limiting conditions favours the PHA biosynthetic pathway. Enzymatic activities of the isolates were fund to be in the trend of negative amylase and cellulase production. It can be correlated to the fact; presence of high concentration of simple sugars limits the utilization of complex sugars by the isolates in the environmental conditions. Thus the bacterial isolates are highly adapted for the production and accumulation of PHA de novo.

The screened isolates have been identified to be under *Bacillus* sp. by 16S rRNA gene sequencing. The result corroborated with the fact of their abundance in soil and high adaptability to the rapidly changing environmental conditions. In addition to that, use of *Bacillus* sp. for the production of PHA has much advantage over other bacterial species due to their absence of lipopolysaccharides layer which makes the extraction much easier, its capability of growing in cheap raw materials and high growth rate in comparison to other bacteria (Khiyami et al., 2011). In this regard, many studies involve the use of *Bacillus* sp. for the production of PHA such as *B. megaterium* (Otari and Ghosh, 2009), *B. cereus* (Valappil et al., 2007), *B. thuringiensis* (Rohini et al., 2006), *B. subtilis* (Singh et al., 2009) as *Bacillus* sp. are considered to be the most suitable candidates for economic production of PHAs. *Bacillus* sp. are capable of producing co-polymers of PHAs utilizing the relatively simple, inexpensive and structurally unrelated carbon sources. Additionally, the isolates possess the ability to produce a variety of hydrolytic enzymes that can be exploited for cost-effective production of PHAs such as by utilization of agro-industrial waste materials (Israni and Shivakumar, 2013).

The environmental parameters play a vital role for the biomass production and synthesis of PHA in vitro. The optimized condition for the biomass production of the isolates was pH-7.0, temperature-37˚C, carbon source- glucose, inoculum size-10% and sugar concentration-10%. The obtained result falls within the results obtained by Hungund et al., (2013), Reddy and Thirumala (2012) and Boyandin et al., (2012). In addition to that, Khiyami et al., (2011) reported that, the PHA production in *Bacillus* sp. was found to be optimum with 0.5 g/l of nitrogen source, 120 rpm of agitation and 6.4 g/l of biomass inoculum which in accordance to the result obtained during the present study. The C: N ratio during the study has been maintained at 6:1 as reports suggested the highest dry cell weight at this ratio for many bacterial species (Sangkharak and Prasertsan, 2008).

The FTIR analysis of the extracted PHA from the isolate *Bacillus* sp. S1 showed the distinct peaks corresponding to C=O groups which has been shown in Figure 2f. The spectroscopic analysis gave proper insight for the chemical structure of PHB by reflecting the monomeric units. As the

functional group of C=O has been confirmed by FTIR in the present study, which is predominantly present in the PHA polymer, the production of PHA is validated in the studied isolate. The result obtained in this study is well within the result obtained by the previous workers (Shah, 2012; Jeyaseelan et al., 2012; Preethi et al., 2012; Otari and Ghosh, 2009). The IR spectrum indicates the presence of monomeric units with a strong absorption band at 1714 cm^{-1} corresponding to C=O valence vibration of the thio-ester bond.

PHA is the biopolymer that can be degraded completely to form CO_2 and water and if optimized properly it can be synthesized from the sustainable raw material reducing its cost of production and high productivity. However, the researches till now have not come close enough to decrease its cost of production and mainstream application. The isolate characterized in this study *Bacillus* sp. S1 2013b (KF626469) possesses the potential for the production of higher amount of PHA (80.94%) in vitro which is much higher level to any report available so far. Being *Bacillus*, the isolate possess many advantages such as rapid growth, secretion of hydrolytic enzymes and production of co-polymers from structurally unrelated sources and can be used as an ideal candidate for PHA production in industrial scale. This study exploits a new insight for unexplored *Bacillus* sp. from rhizosphere region of soil which is the most suitable environmental conditions for harbouring PHB producing bacterial isolates for further use.

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