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Characterization of poly-β-hydroxybutyrate producing halophilic bacteria and nutrient optimization for its maximum production

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ABSTRACT

An experiment was conducted at the Indian Agricultural Research Institute, New Delhi (2017-18) to report the identification of halophilic poly-ß-hydroxybutyrate (PHB) producing bacteria and optimization of process parameters for maximum PHB recovery. The phylogenetic analysis classified the isolates into bacterial phylum α, γ-Proteobacteria, and Firmicutes. *Halomonas* sp. KB7 and *Stenotrophomonas maltophila* B11 produced maximum PHB in mineral salt media having optimized C and N concentration with 10% NaCl. Optimizing media increased PHB production from 23.80–73.7% w/w for *Halomonas* sp and 29.5–78.3 % w/w for *S. maltophila*.

Keywords: Growth kinetics, Halophilic bacteria, Nutrient optimization, Poly-ß-hydroxybutyrate

The recalcitrant nature of plastics has created long-term problems of plastic accumulation in nature and research on finding biodegradable plastic sources is required (Moharir *et al.* 2019). A polyester, poly-β-hydroxybutyrate (PHB), which is synthesized by bacteria under low nitrogen and excess carbon in growth medium resembles petroleum-derived plastics but completely degrades into CO_2 and water (Li *et al*. 2016). Besides, it has applications in medicine, veterinary, and agriculture due to its biocompatibility (Li *et al*. 2016). To produce PHB economically and competitively, a wide range of carbon sources, novel bacterial strains, fermentation conditions, and recovery methods have been proposed (Zulfiqar *et al*. 2018). Recent studies attempt to solve the costliest factors by investigating the use of cheaper carbon sources and different fermentation strategies by using novel microorganisms (Chee *et al*. 2010). PHB production could become cheaper if there is a way to make bacteria produce larger amounts of polymer within shorter periods, without involving much fermentation cost. Halophilic bacteria are of much interest because at a high salt concentration of >8%, the non-halophilic growth is prevented, hence allowing the fermentation process without strict sterile conditions and thereby reducing the inherent costs for sterilizing the culture media and equipment (Quillaguaman *et al*. 2010). Cell walls can also easily lyse in the absence of salt, especially in distilled water thus, enabling the recovery of PHB from extreme halophiles much easier in an economically viable manner (Quillaguaman *et al*. 2010). The current study was carried out to report the identification of PHB producing halophilic bacteria and the optimization of their growth media for maximum PHB recovery.

MATERIALS AND METHODS

Sixty bacterial cultures previously isolated from hypersaline soils of Rann of Kutch, Gujarat, India (Yadav *et al.* 2019) were screened for their ability to grow at 10% NaCl in mineral salt (MS-NaCL) medium (Schlegel *et al*. 1970) at 30°C by estimating total protein after 72 h of incubation by Bradford's method. Halophilic isolates were screened qualitatively for PHB production in the MS-NaCl medium at 30 °C by Sudan Black staining (Schlegel *et al*. 1970). The yield of PHB in positively stained isolates was quantified at 48, 72, 96, and 120 h of incubation in MS-NaCl broth. PHB was extracted from the cells (Hahn *et al*. 1994) and quantified by HPLC (Karr *et al*. 1983). Samples (10µl) were eluted with 5mM H_2SO_4 at a flow rate of 0.6 ml/min from an Aminex HPX-87H column (300 mm × 7.8 mm) using HPLC (Waters India limited model 515) and the absorbance was measured at 210 nm. The amount of PHB was calculated based on chromatograms of standard PHB and expressed as (%) gm PHB/g Cell Dry Weight (CDW).

For identification of selected PHB producing isolates, the 16S rRNA gene sequences were amplified by PCR from their genomic DNA using universal primers pA and pH (Edwards *et al*. 1989) and outsourced to Agrigenome Private Limited, Kochi, India for Sanger sequencing. A similarity search of all the sequences was done using the

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nBLAST at the NCBI GenBank database and assigned accession numbers were assigned (KJ874354 to KJ874382). Nitrogen source and its concentration were optimized for maximum PHB production. Seven N sources, viz. KNO_2, KNO_3, $(NH_4)_2HPO_4$, $(NH_4)\ H_2PO_4$, $(NH_4)_2SO_4$, NH_4Cl, and L-glutamate, were tested by replacing regular N source in MS-NaCl media with 4 different concentrations (0.1%, 0.25%, 0.50%, and 0.75%). The 1% inoculum (10^8 cells/ml) of two best PHB producing was inoculated in modified MS-NaCl broth and shake incubated for 72 h at 30°C. For C source optimization, 7 compounds, viz. fructose, lactose, mannitol, sodium acetate, xylose, starch, and glucose were used to modify MS-NaCl media having optimized N in 4 concentrations (1.0, 1.5, 2.0, 2.5%). The PHB production was estimated by HPLC.

The optimized MS-NaCl media was made by replacing 1% glucose and 0.1% NH_4Cl in the MS-NaCl medium with the optimized concentration of N and C source. Growth kinetics of both the selected isolates using optimized MS-NaCl media was studied by plotting the bacterial growth curve up to 96 h of incubation at 30°C using Microbial Growth Kinetic Analyser (BIOSCREEN C System, OY Growth Curves Ab Ltd, Finland). From the growth curve the doubling time (t) of isolates between two-time interval, t_1, and t_0, was calculated and used to calculate the specific growth rate (μ) using the following equation:

$$\mu = \frac{2.303}{t} \times \log_{10} N1 - N0$$

where $t = (t_1 - t_0)$ and N1-N0 = population at time t_1 – population at time t_0. The generation time (g) of the isolates was calculated by the following formula:

$$g = \frac{0.693}{\mu}$$

The PHB from both the cultures grown on optimized MS-NaCl media quantified by HPLC initially at the interval of 5 h till 20 h and then at the interval of 10 h till 96 h of incubation in triplicate. The % PHB was plotted on a time scale along with the growth curve.

Statistical analyses of the data were performed using STATISTICA 10. Unless indicated otherwise, differences were considered only when significant at P=0.05.

RESULTS AND DISCUSSION

Characterization of bacteria for salinity tolerance and PHB production: Forty-one isolates showed significant growth in MS-NaCl media and their total protein ranged from 14.60– 307.84 μg/mL (S Table 1). Subsequently, only 29 isolates showed PHB accumulation as indicated by dark pigmentation after Sudan Black staining (S Fig 1). The PHB in these isolates ranged from 0.20–29.50% of CDW (Table 1). After 48 h, 12 isolates produced PHB ranging from 0.20–16.40% of CDW and maximum production was observed by B11 (16.40%) followed by B13 (15.70%) and KB7 (11.58%). After 72 h, (only 9 isolates accumulated PHB) B11 (29.5%) and KB7 (23.80%) produced significantly higher PHB. Singh and Parmar (2011) reported an increase in PHB yield in a time-dependent manner and the highest yield, 5.31 g/L, was obtained after 72 h of growth in *Bacillus* sp. With the

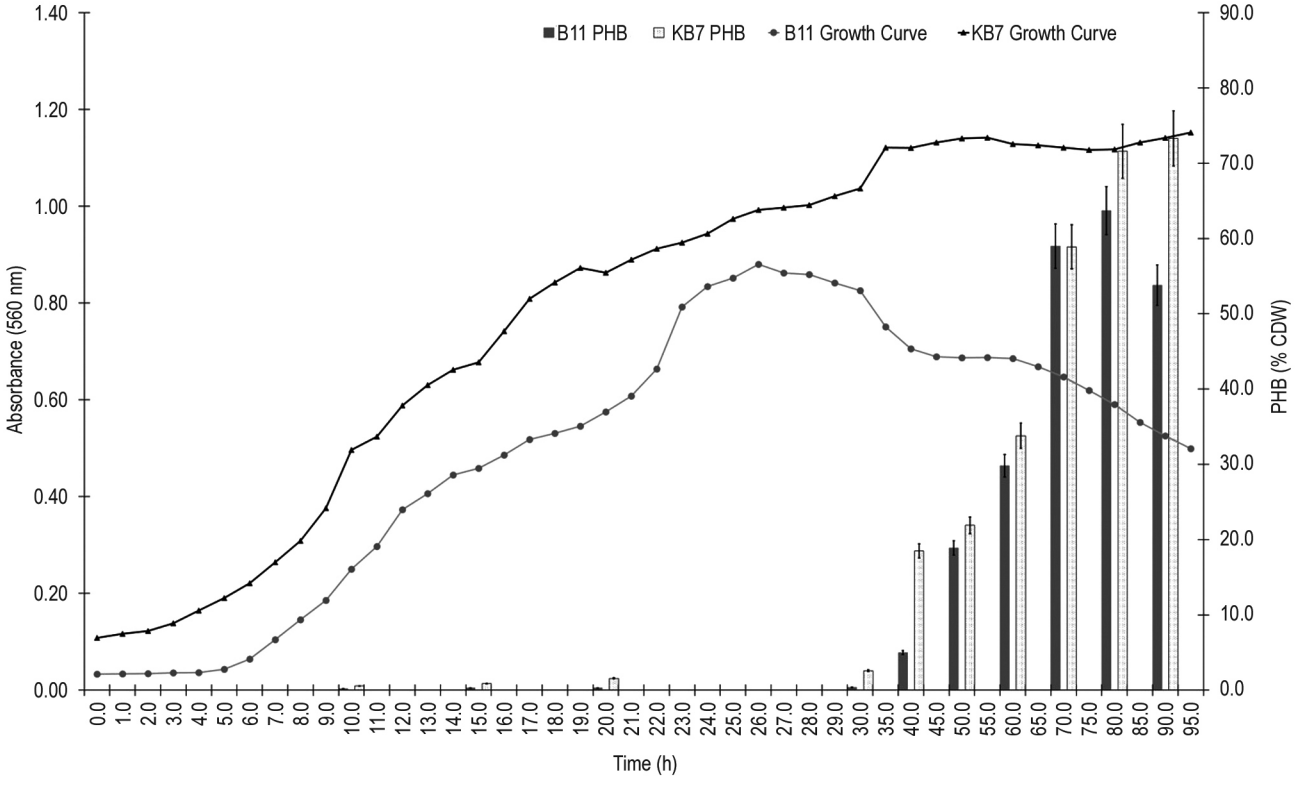

Fig 1 Growth curve and PHB accumulation (% CDW) of *Halomonas sp.*KB7 and *S. maltophila* B11 in 95 h of incubation

Table 1 PHB production by selected bacterial isolates at a different time interval

Isolate No.	PHB (% of CDW)			
	48 h	72 h	96 h	120 h
1S1	0.00	19.70 (±2.33)	0.00	0.00
1S4	0.00	15.30 (±1.69)	0.00	0.00
2S3	4.00 (±0.51)	15.70 (±2.18)	9.20 (±0.21)	1.80 (±0.03)
4S4	8.00 (±1.20)	12.90 (±2.19)	15.00 (±1.36)	1.40 (±0.01)
KB1	5.00 (±0.98)	19.37 (±3.38)	8.00 (±1.59)	8.10 (±1.36)
KB3	2.00 (±0.0.35)	15.30 (±1.38)	11.00 (±1.32)	3.10 (±0.07)
KB7	11.58 (±0.3.67)	23.80 (±3.67)	17.60 (±2.39)	14.90 (±2.31)
B1	0.00	0.00	2.20 (±0.36)	3.70 (±0.05)
B2	0.00	0.00	1.50 (±0.08)	0.00
B3	4.00 (±0.38)	18.60 (±2.35)	10.60 (±1.06)	6.90 (±0.91)
B4	0.00	3.60 (±0.39)	13.80 (±1.09)	0.00
B5	0.00	5.10 (±0.69)	4.80 (±0.06)	0.00
B6	11.00 (±2.69)	17.60 (±1.27)	10.60 (±1.51)	3.40 (±0.29)
B7	6.80 (±1.68)	0.00	0.00	0.00
B9	3.00 (±0.34)	0.00	0.00	0.00
B10	4.80 (±0.89)	11.30 (±2.68)	0.00	0.00
B11	16.40 (±2.69)	29.50 (±3.51)	18.90 (±2.36)	13.43 (±1.61)
B13	15.70 (±1.97)	12.70 (±1.03)	5.40 (±0.53)	0.00
B14	0.20 (±0.06)	2.90 (±0.05)	3.79 (±0.16)	0.50 (±0.07)
B15	10.30 (±2.19)	11.40 (±0.67)	0.00	0.00
B16	0.00	3.40 (±0.21)	0.90 (±0.06)	0.00
B17	9.80 (±2.36)	8.40 (±0.83)	1.10 (±0.08)	0.30 (±0.09)
B18	0.00	6.10 (±0.69)	1.90 (±0.03)	0.00
B22	0.00	2.90 (±0.06)	16.40 (±1.06)	3.30 (±0.94)
B24	0.00	11.60 (±1.16)	6.90 (±0.36)	0.00
B27	0.00	0.00	19.50 (±2.59)	12.89 (±3.64)
B28	0.00	11.30 (±0.62)	4.80 (±0.45)	0.40 (±0.03)
B29	0.20 (±0.06)	2.60 (±0.09)	3.26 (±0.07)	0.00
B30	9.20 (±3.98)	10.10 (±1.11)	0.00	0.00

$LSD_{(P=0.05)}$: Between isolates: 4.86 and Between Time: 4.32
(*Figures in parenthesis are standard deviation)

increase in time, from 72 to 96 h, a significant reduction in PHB was observed, however, isolates B11 (18.90%) and KB7 (17.60%) still produced significantly higher PHB. A further significant decline in PHB accumulation was observed at 120 h of incubation. The decrease in PHB accumulation with time varies between bacteria and depends upon the growth rate of an isolate, lack of micronutrients, viscosity of the medium, and inhibitory effect of secondary metabolites produced during the stationary phase (Flora *et al*. 2010, Belal 2013).

Phylogenetic characterization of PHB producing isolates: Upon identification, the 29 isolates were grouped into 3 phyla, viz. α-Proteobacteria, γ-Proteobacteria, and Firmicutes. Alpha-proteobacteria was represented only by *Paracoccus* sp, which has also been previously reported by Kalaiyezhini and Ramachandran (2014). Among Firmicutes, 6 species of 16 *Bacillus* isolates, i.e. *B. megaterium*, *B. aryabhattai*, *B. cereus*, *B. subtilis, Bacillus* sp. and *B. pumilus* were identified. PHB producing halophilic *Bacillus* was also reported earlier from the marine ecosystem (Singh *et al*. 2009; Thuoc *et al*. 2012, Antony *et al*. 2012). The rest of the 12 isolates of the genera *Enterobacter, Halomonas,* and *Stenotrophomonas* belonged to γ-Proteobacteria. Ceyhan and Ozdemir (2011) reported PHB production by *Enterobacter aerogenes* by using domestic wastewater. Maximum PHB producing isolate KB7 and B11 were identified as *Halomonas* sp. and *Stenotrophomonas maltophila*, respectively. PHB production by the species of halophilic bacterium *Halomonas* was first reported by Quillaguaman *et al*. (2007). Kawata *et al.* (2012) reported PHB production by *Halomonas* sp. KM1 by using 10% glycerol and sodium nitrate (2.0 g/L). PHB production by *Stenotrophomonas maltophilia* was earlier reported by Singh and Parmar (2011).

Optimization of N and C source: Both the isolates, *Halomonas sp.* KB 7 and *S. maltophila* B11, accumulated PHB on all the N sources except for the NO_2-N (Table 2). PHB production by KB7 (57.3% of CDW) and B11 (65.5% of CDW) was significantly higher in MS-NaCl media having 0.25% L-Glutamate over other N sources, whereas, growth was significantly higher on NH_4Cl. Li *et al* (2013) reported glutamate as a preferred N source for PHB production, whereas, maximum reports indicated the preference for NH_4-N (Sangkharak and Parsertsan 2008, Belal 2013). *S. maltophila* B11 accumulated PHB (48.7% CDW) by using 0.25% $(NH_4)_2SO_4$, however, it was significantly lower than that produced by using 0.25% L- glutamate. With increasing nitrogen concentration, from 0.1–0.75%, CDW increased significantly but %PHB accumulation decreased. The N concentrations of 0.75% did not support PHB accumulation except for the L-Glutamate. It was reported that the presence of higher N concentration has an inhibitory effect on PHB accumulation (Belal 2013). It was concluded that both the isolates produced maximum PHB using 0.25% L-Glutamate as the N source.

Halomonas sp. KB7 showed significantly higher cell growth (9.0±0.28 g/L) and PHB production (69.4% of CDW) in the MS-NaCl medium having 2% glucose in 72

Table 2 Effect of different nitrogen and carbon and their concentration on growth and PHB accumulation by *Halomonas* sp. KB7 and *S. maltophila* B11

N and C Sources		Concentration[1]	*Halomonas* sp. KB7		*Stenotrophomonas maltophila* B11	
			CDW (g/L)	PHB (% CDW)	CDW (g/L)	PHB (% CDW)
N-Source	KNO_3	0.25%	4.38	12.30	4.76	37.90
	$NH_4H_2PO_4$	0.1%	4.80	36.80	2.94	34.30
	NH_4Cl	0.1%	3.26	28.90	4.30	42.10
	$(NH_4)_2HPO_4$	0.1%	4.30	23.70	4.66	12.40
	L-Glutamate	0.25%	4.36	57.30	4.12	65.50
	$(NH_4)_2SO_4$	0.25%	4.22	39.90	4.86	48.70
$LSD_{(p=0.05)}$ for N Source			0.49	5.71	0.43	6.98
C-Source	Glucose	2.0%	9.00	69.40	7.80	36.90
	Fructose	1.5%	4.70	53.00	5.60	19.00
	Lactose	1.0%	4.10	35.70	4.60	37.70
	Mannitol	1.5%	3.50	38.60	8.20	61.70
	Na-Acetate	1.5%	5.00	47.20	4.70	55.20
	Xylose	1.5%	4.40	16.70	5.20	14.80
	Starch	2.0%	4.30	27.10	5.30	12.00
$LSD_{(P=0.05)}$ for C Source			0.68	0.32	0.57	0.31

[1]Concentration of N and C source at which significantly higher %PHB production was observed

h (Table 2). Besides glucose, fructose (53% of CDW) and sodium acetate (47.2% of CDW) at 1.5% also produced significantly higher PHB than lactose, mannitol, xylose, and starch. Xylose was found to be the least preferred carbon source and it could be because pentose catabolism affects the growth kinetics of various Gram-negative bacteria. *S. maltophila* B11 produced significantly higher PHB (61.7% of CDW) in MS-NaCl media having 1.5% mannitol as compared to all other carbon sources. Belal (2013) also reported 1-2% of mannitol as the best C source for PHB production.

Based on the optimization studies, for *Halomonas* sp KB7 the MS-NaCl media was modified by replacing 1% glucose and 0.5% $(NH_4)_2SO_4$ with 2% glucose and 0.25% L-glutamate. Similarly for *S. maltophila* B11, 1% glucose and 0.5% $(NH_4)_2SO_4$ was replaced with 1.5% mannitol and 0.25% L-glutamate. L-Glutamate is 10% cheaper than $(NH_4)_2SO_4$, hence it will help in reducing the cost. The concentration of carbon and nitrogen in both the medium was 38:1 and 28:1, respectively. The increase in carbon from 1–2.5% at a fixed N (0.25%) showed a corresponding increase in PHB in isolates (Table 2). Pieja *et al.* (2011) too observed a linear correlation ($R^2 = 0.96$) between %PHB in cells versus C:N.

Growth kinetics and PHB production: The final quantity of PHB yield depends on the growth kinetics of the bacteria (Divyashree *et al.* 2009). The growth curve (Fig 1) of both the isolates revealed that the *S. maltophila* reached stationary phase at 24 h with a specific growth rate (μ) of 0.196/h and generation time (GT) of 3.52 h, whereas, *Halomonas* sp. reached stationary phase at 36 h with μ of 0.187/h and GT of 3.7 h. Similar growth kinetics were demonstrated for *S. maltophila* ATCC 51331, reaching the stationary phase by 24 h (Anderson *et al.* 2006). Burch *et al* (2013) reported doubling time of *Halomonas* sp. about 1.5 h in media containing 3% NaCl at 30–41°C. Although, both KB7 and B11, reached the stationary phase at different time interval but started accumulating PHB at 30 h of incubation and increased significantly up to 70 h.

PHB accumulation in isolate *Halomonas* sp. and *Stenotrophomonas maltophila* increased from 2.59–78.30% of CDW from 30–80 h of incubation, whereas, PHB accumulation by the isolate *Halomonas* sp increased from 0.4–73.7% of CDW from 30 to 80 h of incubation. Similar results for PHB accumulation were obtained by *Bacillus subtilis* in the stationary phase at 72 h (5.191 g/L) using the sugar industry wastewater (Singh *et al*. 2013). The study provided useful data about the diversity of PHB producing halophilic bacteria and optimizes growth conditions for maximum PHB production. Optimization of concentration and source of C and N in MS-NaCl media significantly increased PHB accumulation by *Halomonas* sp KB7 and *Stenotrophomonas maltophila* B11. These bacteria can be utilized for upscaling PHB production industrially, as a fast-emerging alternative to the non-biodegradable plastics.

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REFERENCES

Antony R, Krishnan K P, Laluraj C M, Thamban M, Dhakephalkar P K and Engineer A. 2012. Diversity and physiology of culturable bacteria associated with a coastal Antarctic ice core. *Microbiological Research* **167**: 372–80.

Belal E B. 2013. Production of Poly-β-hydroxybutyric acid by *Rhizobium elti* and *Pseudomonas stutzeri*. *Current Research Journal of Biological Sciences* **5**(6): 273–84.

Burch A Y, Finkel O M, Cho J K, Belkin S and Lindow S E. 2013. Diverse microhabitats experienced by *Halomonas variabilis* on salt secreting leaves. *Applied Environmental Microbiology* **79**(3): 845–52.

Ceyhan N and Ozdemir G. 2011. Poly-β-hydroxybutyrate (PHB) production from domestic wastewater using *Enterobacter aerogenes* 12Bi strain. *African Journal of Microbiological Research* **5**(6): 690–70.

Chee J Y, Yoga S S, Lau N S, Ling S C, Abed R M M and Sudesh K. 2010. Bacterially produced polyhydroxyalkanoate: Converting renewable resources into bioplastics. *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*, pp 1395–04. Mendez Vilas A (Ed). FORMATEX.

Divyashree M, Rastogi N and Shamala T. 2009. A simple kinetic model for growth and biosynthesis of polyhydroxyalkanoate in *Bacillus flexus*. *New Biotechnology* **26**: 92–98.

Edwards U, Rogall T, Blocker H, Emde M and Bottger E C. 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acid Research* **17**: 7843–53.

Flora G D, Bhatt K and Tuteja U. 2010. Optimization of culture conditions for PHB production from *Bacillus* sp. *Journal of Cell and Tissue Research* **10**(2): 2235–42.

Hahn S K, Chang Y K, Kim B S and Chang H N. 1994. Optimization of microbial poly-(3)-hydroxybutyrate recovery using dispersions of sodium hypochlorite solution and chloroform. *Biotechnology and Bioengineering* **44**: 256–61

Kalaiyezhini D and Ramachandran K B. 2014. Biosynthesis of poly-3-hydroxybutyrate from glycerol by *Paracoccus denitrificans* in a batch bioreactor – effect of process variables. *Preparative Biochemistry and Biotechnology* **45**: 69–83

Karr D B, Waters J K and Emerich W. 1993. Analysis of poly-3-hydroxybutyrate in *Rhizobium japonicum* bacteroides by ion-exclusion HPLC and UV detection. *Applied and Environmental Microbiology* **46**: 1339–44.

Kawata Y, Shi L H, Kawasaki K and Shigeri Y. 2012. Taxonomic characterization and metabolic analysis of the *Halomonas* sp. KM-1, a highly bioplastic PHB producing bacterium. *Journal of Bioscience and Bioengineering* **113**: 456–60.

Li Z, Yang J and Loh X J. (2016). Polyhydroxyalkanoates: opening doors for a sustainable future. *NPG Asia Materials* **8**(4):e265.

Li R, Wang X, Yang J, Gao Y, Zi X, Zhang X, Gao H and Hu N. 2013. Psychrotrophic *Pseudomonas mandelii* CBS-1 produces high levels of poly-β-hydroxybutyrate. *Springer Plus* **2**: 335.

Moharir R V and Kumar S. 2019. Challenges associated with plastic waste disposal and allied microbial routes for its effective degradation: a comprehensive review. *Journal of Cleaner Production* **208**: 65–76.

Pieja A J, Sundstrom ER and Criddle C S. 2011. Poly-3-hydroxybutyrate metabolism in the Type II methanotroph *Methylocystis parvus* OBBP. *Applied and Environmental Microbiology* **77**(17): 6012–19

Quillaguaman J, Munoz, M, Mattiasson B and Hatti-Kaul R. 2007. Optimizing conditions for poly(b-hydroxybutyrate) production by *Halomonas boliviensis* LC1 in batch culture with sucrose as carbon source. *Applied Microbiology and Biotechnology* **74**: 981–86.

Quillaguaman J, Guzman H, Van-Thuoc D and Hatti-Kaul R. 2010. Synthesis and production of polyhydroxyalkanoates by halophiles: current potential and future prospects. *Applied Microbiology and Biotechnology* **85**: 1687–96.

Sangkharak K and Prasertsan P. 2008. Nutrient optimization for production of polyhydroxybutyrate from halotolerant photosynthetic bacteria cultivated under aerobic-dark condition. *Electronic Journal of Biotechnology* **11**(3): 83–94

Schlegel H G, Lafferty R and Krauss I. 1970. The isolation of mutants not accumulating poly-ß-hydroxybutyric acid. *Archives of Microbiology* **71**: 283–94.

Singh G, Kumari A, Mittal A, Yadav A and Aggarwal A K. 2013. Poly-β-hydroxybutyrate production by *Bacillus subtilis* NG220 using sugar industry waste water. *BioMed Research International* **2013**(4): 952641.

Singh P and Parmar N. 2011. Isolation and characterization of two novel PHB producing bacteria. *African Journal of Biotechnology* **10**(24): 4907–19.

Singh M, Patel S K S and Kalia V C. 2009. *Bacillus subtilis* as potential producer for polyhydroxyalkanoates. *Microbial Cell Factories* **8**: 38–49.

Thuoc D V, Phong T H, Binh N T, Tho N T, Lam D M and Quillaguaman J. 2012. Polyester production by halophilic and halotolerant bacterial strains obtained from mangrove soil samples located in Northern Vietnam. *Microbiology Open* **1**(4): 395–406.

Yadav A N, Gulati S, Sharma D, Singh R N, Rajawat M V, Kumar R, Dey R, Pal K K, Kaushik R and Saxena A K. 2019. Seasonal variations in culturable archaea and their plant growth promoting attributes to predict their role in establishment of vegetation in Rann of Kutch. *Biologia* **2019**: 1–3.

Zulfiqar A R, Sharbeel A and Ibrahim M B. 2018. Polyhydroxyalkanoates: Characteristics, production, recent developments and applications. *International Biodeterioration and Biodegradation* **126**: 45–56.