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Research Paper

Isolation, Screening and Quantification of Poly-β-hydroxybutyrate (PHB) from extreme halophilic Archaea.

Microbiology

KEYWORDS : Extremely halophilic Archaea, Poly-β-hydroxybutyrate (PHB), bioplastic, hypersaline environments

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ABSTRACT

The Archaea remain the most enigmatic of life's three domains and halophiles constitute a very heterogeneous group of extremophiles in this domain. Due to their special characteristics, they have been suggested to hold potential for a variety of biotechnological applications such as production of enzymes, compatible solutes, degradation of toxic compounds and polymer production. The study aimed at efficient production and screening of thirteen haloarchael strains isolated from saltpans at Newport and Nari for their ability to produce PHB. The production by these strains was determined by the spectrophotometric method and results suggest that all the thirteen haloarchaeal isolates exhibited the ability to produce PHB, though to a varying extent. It was found that PHB yield ranged from 0.01-1.17%. All the thirteen isolates were then subjected to secondary screening for examining DCW, PHB production and yield as a function of incubation time. The maximum PHB production was by four haloarchaeal isolates viz., Haloarcula sp. 1 (3.77%), Halorubrum sp. 2 (2.07%), Halobaculum sp. (2.02%) and Halobacterium salinarum (0.75%). The growth almost became constant after its optimum period whereas, PHB yield declined.

Introduction

Halophiles can be found in each of the three domains of life: *Archaea*, *Bacteria* and *Eukarya*. They are salt-loving organisms that well adapted to saturating NaCl concentrations and have a number of novel molecular characteristic. They are found distributed all over the world in hypersaline environments, many in natural hypersaline brines in arid, coastal, and even deep-sea locations, as well as in artificial salterns used to mine salts from the sea. Halophiles can be classified into following groups on the basis of their response to NaCl (Küshner, 1978; Margesin and Schinner, 2001; Kerkar *et al.,* 2003): Slight halophiles that grow optimally at 0.2-0.85 mol L^{-1} (2-5%) NaCl; Moderate halophiles that grow optimally at 0.85-3.4 mol L^{-1} (5-20%) NaCl and Extreme halophiles that grow optimally above 3.4-5.1 mol L^{-1} (20-30%) NaCl. Extreme halophilic organisms are mainly represented by members of domain *Archaea*, which accumulate inorganic ions (commonly K^+) intracellularly to provide osmotic balance with the high salt concentration present in their environments (Quillaguaman *et al*., 2005).

Modern society has become highly dependent on the use of plastic in various forms because of their many desirable properties including durability and resistance to degradation. They are synthetic polymers that are derived from petroleum and the major problem associated with the use is that, much of it is discarded as waste on landfills. They persist in the environment without getting degraded, thus giving rise to a variety of ecological and environmental problems. On the other hand, problems concerning global environment and solid waste management have generated much interest in the development of novel biodegradable polymers that still retain the desired physical and chemical properties of conventional synthetic plastics and are biodegradable. Among the various biodegradable plastics available, there is growing interest in one of the known biopolymer i.e., poly (3- hydroxybutyrate) (PHB) as a candidate for biocompatible plastics (Lee and Chang, 1995; Mahishi, 2001). PHB is an intracellular carbon and energy storage material accumulated by a variety of microorganism usually under unfavorable growth conditions, such as limitation of nitrogen, phosphorus and/or oxygen (Du *et al*., 2000).

The aim of the present work was screening the thirteen haloarchael strains isolated from saltpans at Newport and Nari for their ability to produce PHB and selection of the most potential PHB producing isolate for further studies on PHB production.

Materials and Methods

Archaeal strains and cultural conditions

The thirteen haloarchael strains isolated form salt pans around Bhavnagar Cost (Gujarat, India) were used throughout this study. The isolates were grown and maintained on enrichment media Tryptone yeast extract salt (TYES) with 50% glycerol (Krieg and Holt, 1984), containing 100µg/ml each of penicillin G, erythromycin and cycloheximide to inhibit growth of bacteria and fungi (Purdy *et al*., 2004; Bae and Roh, 2009) . The slants were stored at 4°C until use. The same medium, with minor modification was used for PHB production

Primary screening for PHB production

All the thirteen isolates obtained were screened for their ability to produce PHB. 10^8 cells / ml of actively growing culture was inoculated into 100ml TYES medium and incubated at 37°C on a rotary shaker at 180 rpm for 20 days. PHB production after 24th day's growth was assayed by the method of Law and Slepecky as below:

Determination of PHB (Law and Slepecky, 1961)

The cultures were centrifuged at 6000 rpm for 45 min. The pellets were incubated at 37°C for 1 h with 5ml of 0.2% (v/v) sodium hypochlorite for cell lysis. PHB granules were collected by centrifugation, washed with water and then with acetone and alcohol for removal of cell lipids and other molecules (except PHB). The final pellet was dissolved in 3 small portions of boiling chloroform and filtered. The chloroform extract was dried by evaporation and the residue was hydrolyzed and dehydrogenated with 10 ml of concentrated sulfuric acid by heating at 100°C in a water bath for 20min to obtain crotonic acid which was quantified by measuring at A_{235} against sulfuric acid as blank. The amount of crotonic acid produced was extrapolated from the standard curve which was obtained by using PHB (Sigma chemicals).

Secondary screening

10^8 cells / ml of actively growing cultures were inoculated into 100ml TYES medium dispended in 250 ml flask and incubated at 37°C on rotary shaker at 180 rpm. PHB production as a function of incubation time was measured at every 4 days interval upto 30 days considering the day of inoculation as day "0".

Growth was noted as dry cell weight (DCW-mg/100ml) and determined by centrifugation of culture at 6000 rpm for 15 min. The pellets were washed with deionized pyrogen free water (Millipore) and dried at 75°C in an oven until constant weight was attained. Growth was expressed as mg difference in weight and denoted as DCW mg/100 ml.

PHB production was quantitated according to the method of Law and Slepecky, 1961 and expressed as mg / 100ml.

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PHB yield (%) was obtained as the percentage of the ratio of PHB to DCW as defined by Lee *et al*., 2000. The experiments were conducted in triplicates.

Statistical analysis

The data of secondary screening were analyzed for examining correlation between growth, PHB production and PHB yield. The correlation was determined by Pearson bivarate correlation coefficient test using SPSS.17.

Results and Discussion

A total of thirteen haloarchael strains were examined for the production of PHB. The dry cell weight-DCW (mg/100ml), PHB production (mg/100ml) and PHB yield (%) by the isolates after 24th day of growth are represented in table. The results suggest that all the thirteen haloarchaeal isolates exhibited the ability to produce PHB, though to a varying extent. Maximum PHB produced in terms of % yield was by isolate *Haloarcula* sp. 1 (1.17%) followed by *Halorubrum* sp. 2 (1.15%), *Halobacterium salinarum* (0.86%) and *Halobaculum* sp. (0.45%). Least PHB production was by *Halorubrum saccharovorum* (0.01%) (Table 1).

As all the thirteen isolates exhibited the ability to produce PHB, they were subjected to secondary screening (Table 2). *Haloarcula* sp. 1 exhibited maximum DCW (104 mg/100ml) on 32nd day, PHB production (2.34 mg/100ml) and yield (3.77%) on 20th day, followed by *Halorubrum* sp. 2 that exhibited maximum DCW (167 mg/100ml) on 32nd day, whereas PHB production (3.43 mg/100ml) and PHB yield (2.07%) on 24th day, *Halobacterium salinarum* that exhibited maximum DCW (157 mg/100 ml) on 32nd day and PHB yield (0.86%) on 16th day and *Halobaculum* sp. exhibited maximum DCW (107 mg/100ml) on 32nd day and PHB yield (0.02%) on 24th day. Table also suggests that growth almost became constant after its optimum period whereas, PHB yield declined. Figs. 1 – 4 indicates growth (DCW-mg/100ml), PHB yield (%) by *Haloarcula* sp. 1, *Halorubrum* sp. 2, *Halobaculum* sp. and *Halobacterium salinarum* the maximum PHB producing haloarchaeal isolates. The results indicate that for *Haloarcula* sp. 1 and *Halobaculum* sp., PHB yield initiated during logarithmic phase, attend its maxima and decline at late logarithmic phase (Figs. 1 and 3) for *Halorubrum* sp.2 and *Halobacterium salinarum* both growth and PHB yield go hand in hand, it attained its maxima during end of logarithmic phase and declined during stationary phase (Figs. 2 and 4). The results thus indicate that as maximum PHB is accumulated during end of log phase or the beginning of stationary phase, it is synthesized when nutrients become the limiting factor.

Table 3 shows Pearson bivariate correlation between DCW (mg/100ml), PHB production (mg/100ml) and PHB yield (%) by the thirteen isolates. There was a positive significant correlation for all the thirteen isolates between DCW (mg/100ml), PHB production (mg/100ml) and PHB yield (%). However, for isolates *Halorubrum sistributum* and *Halogeometricum* sp. there was a non – significant positive correlation between PHB production and PHB yield. .

The thermoplastic properties of the polymer and its biodegradability determine its importance as a substitute for petrochemical plastics (Celik *et al*., 2005). Amongst the thirteen isolates examined, four *viz., Haloarcula* sp. 1, *Halorubrum* sp. 2, *Halobacterium salinarum* and *Halobaculum* sp. produced better amount of PHB compared to other isolates.

Statistical analysis showed that all the three parameters examined (DCW, PHB production and yield) showed significant positive relationship. Mercan *et al*., 2001, 2002 and Yilmaz *et al*., 2005 have reported significant relationship between dry cell weight, PHB production and yield; in support to the present observation of haloarchaea but are contradictory to Yuksekdag and Beyatli, 2008 where they reported negative correlation between DCW, PHB production and yield in *Streptococcus thermophiles* BA21S strain.

Investigations on PHB production by a variety of microorganisms, including moderate halophiles employing different metabolic routes have been initiated with the studies on *Halomonas boliviensis* LC1, which is able to accumulate significant amounts of the polymer (50 – 88 wt. %) when grown on different carbon sources (Quillaguaman *et al*., 2007). It has been reported to be produced by very few haloarchaea, notably species of *Haloferax*, *Haloarcula* and *Halobacterium* only (Ventosa and Neto, 1995). Hence, the present study gains importance in which a variety of haloarcheal genera have been screened for their ability to produce a biodegradable plastic which may prove to be have better biotechnological applications compared to rest of the microorganisms. This may be due to their unique features as it may facilitate many industrial procedures as no sterilization procedures due to their extremely high NaCl concentration preventing contamination by other organisms and thus owing to simple cultivation requirements (Hezayen *et al*., 2000). In addition, no cell – disrupting devices are required, as cells lyse spontaneously in fresh water, an extremely simple production system can be developed such as open ponds, and as they have versatility in the choice of a broad range of substrates and simple carbon sources such as sugars, acetate or succinate that favors the yield of PHB and growth directly reducing production cost (Kauri *et al*., 1990; Calo *et al*., 1995; Asker and Ohta, 1999).

Table 1 Dry cell weight (DCW-mg/100ml), PHB production (mg/100ml) and yield (%) by the isolates

Sr. No.	Isolates	Dry cell weight (mg/100ml)	PHB production (mg/100ml)	Yield of PHB (%)
1	***Haloarcula* sp. 1**	177.5±0.02	2.07±0.01	1.17±0.05
2	***Halorubrum* sp. 2**	13.6±0.07	0.16±0.03	1.15±0.03
3	***Halobaculum* sp.**	161.8±0.12	1.39±0.13	0.86±0.04
4	Halorubrum saccharovorum	270.9±0.16	0.03±0.23	0.01±0.14
5	Halobacterium salinarum	305.1±0.02	1.24±0.09	0.41±0.06
6	Halorubrum sistributum	863.6±0.08	2.30±0.06	0.27±0.04
7	*Halogeometricum* sp.	228.5±0.05	0.23±0.04	0.10±0.01
8	*Haloarcula* sp.	298.3±0.03	0.27±0.04	0.09±0.00
9	*Halococcus* sp.	145.4±0.06	0.03±0.11	0.20±0.10
10	Haloterrigena turkmenica	136.2±0.12	0.19±0.16	0.14±0.05
11	*Halobacterium* sp.	121.4±0.18	0.16±0.12	0.13±0.01
12	Halococcus saccharolyticus	134.5±0.01	0.54±0.08	0.40±0.01
13	*Natrialba* sp.	113.6±0.05	0.29±0.06	0.21±0.02

(± SD) The values represent means of triplicates

Table 2 Dry cell weight (DCW-mg/100ml), PHB production (mg/100ml) and PHB yield (%) by the haloarchael isolates

Sr. No.	Days Isolates		4	8	12	16	20	24	28	32
1	***Haloarcula* sp. 1**	DCW	-	13	31	45	62	78	103	104
		PHB Production	-	-	0.01	1.10	2.34	2.21	1.94	1.90
		PHB yield	-	-	0.03	2.44	3.77	2.83	1.88	1.82

Sr. No.	Days Isolates		4	8	12	16	20	24	28	32
2	***Halorubrum* sp. 2**	DCW	-	92	74	107	156	165	167	167
		PHB Production	-	-	0.02	1.24	2.07	3.43	3.07	2.78
		PHB yield	-	-	0.03	1.16	1.32	2.07	1.83	1.66
3	***Halobaculum* sp.**	DCW	-	55	76	89	101	105	106	107
		PHB Production	-	-	0	0.06	1.05	2.13	2.10	2.03
		PHB yield	-	-	0	0.06	1.04	2.02	1.98	1.89
4	Halorubrum saccharovorum	DCW	-	24	50	63	100	124	126	136
		PHB Production	-	0.03	0.13	0.21	0.34	0.31	0.30	0.29
		PHB yield	-	0.14	0.27	0.33	0.34	0.24	0.23	0.21
5	Halobacterium salinarum	DCW	-	76	89	112	145	156	156	157
		PHB Production	-	0.13	0.76	0.97	1.10	0.94	0.87	0.87
		PHB yield	-	0.17	0.85	0.86	0.75	0.60	0.55	0.55
6	Halorubrum sistributum	DCW	-	57	167	234	342	435	437	438
		PHB Production	-	0.02	0.05	0.06	0.08	0.07	0.06	0.06
		PHB yield	-	0.04	0.03	0.03	0.02	0.02	0.01	0.01
7	*Halogeometricum* sp.	DCW	-	23	44	67	85	114	118	119
		PHB Production	-	0.12	0.25	0.44	0.51	0.51	0.51	0.51
		PHB yield	-	0.53	0.56	0.65	0.59	0.44	0.43	0.42
8	*Haloarcula* sp.	DCW	-	34	48	67	75	103	132	134
		PHB Production	-	0.21	0.32	0.45	0.53	1.02	1.34	1.34
		PHB yield	-	0.6	0.66	0.67	0.70	0.99	1.01	1.00
9	*Halococcus* sp.	DCW	-	58	106	145	176	150	151	152
		PHB Production	-	0.24	0.47	0.78	1.23	1.14	0.97	0.94
		PHB yield	-	0.41	0.44	0.53	0.69	0.76	0.64	0.61
10	Haloterrigena turkmenica	DCW	-	35	56	89	136	142	143	144
		PHB Production	-	0.16	0.23	0.34	0.46	0.59	0.60	0.61
		PHB yield	-	0.45	0.41	0.38	0.33	0.41	0.41	0.42
11	*Halobacterium* sp.	DCW	-	23	49	61	78	81	82	82
		PHB Production	-	0.02	0.07	0.01	0.08	0.08	0.09	0.09
		PHB yield	-	0.08	0.14	0.02	0.10	0.10	0.10	0.10
12	Halococcus saccharolyticus	DCW	-	24	50	63	100	124	126	136
		PHB Production	-	0.03	0.13	0.21	0.34	0.31	0.30	0.29
		PHB yield	-	0.14	0.27	0.33	0.34	0.24	0.23	0.21
13	*Natrialba* sp.	DCW	-	15	36	59	83	100	101	102
		PHB Production	-	0.09	0.18	0.23	0.49	0.71	0.73	0.75
		PHB yield	-	0.6	0.5	0.38	0.59	0.71	0.72	0.73

Table 3 Correlation between Dry cell weight-DCW (mg/100ml), PHB production (mg/100ml) and PHB yield (%) by the isolates

Sr. No.	Isolates		DCW (mg/ 100ml)	PHB production (mg/ 100ml)	PHB yield (%)
1	*Haloarcula* sp.1	DCW	---	.880(**)	.883(**)
		PHB production	.880(**)	---	.988(**)
		PHB yield	.883(**)	.988(**)	---
2	*Halorubrum* sp.	DCW	---	.881(**)	.693(*)
		PHB production	.881(**)	---	.927(**)
		PHB yield	.693(*)	.927(**)	---
3	*Halobaculum* sp.	DCW	---	.714(*)	.717(*)
		PHB production	.714(*)	---	1.000(**)
		PHB yield	.717(*)	1.000(**)	---
4	Halorubrum saccharovorum	DCW	---	.950(**)	.676(*)
		PHB production	.950(**)	---	.808(**)
		PHB yield	.676(*)	.808(**)	---
5	Halobacterium salinarum	DCW	---	.904(**)	.751(*)
		PHB production	.904(**)	---	.926(**)
		PHB yield	.751(*)	.926(**)	---
6	Halorubrum sistributum	DCW	---	.871(**)	.072
		PHB production	.871(**)	---	.409
		PHB yield	.072	.409	---
7	*Halogeometricum* sp.	DCW	---	.963(**)	.553
		PHB production	.963(**)	---	.696(*)
		PHB yield	.553	.696(*)	---
8	*Haloarcula* sp.	DCW	---	.982(**)	.938(**)
		PHB production	.982(**)	---	.881(**)
		PHB yield	.938(**)	.881(**)	---
9	*Halococcus* sp.	DCW	---	.968(**)	.960(**)
		PHB production	.968(**)	---	.945(**)
		PHB yield	.960(**)	.945(**)	---
10	Haloterrigena turkmenica	DCW	---	.990(**)	.699(*)
		PHB production	.990(**)	---	.722(*)
		PHB yield	.699(*)	.722(*)	---

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Sr. No.	Isolates		DCW (mg/ 100ml)	PHB production (mg/ 100ml)	PHB yield (%)
11	*Halobacterium* sp.	DCW	---	.869(**)	.680(*)
		PHB production	.869(**)	---	.873(**)
		PHB yield	.680(*)	.873(**)	---
12	Halococcus saccharolyticus	DCW	---	.950(**)	.676(*)
		PHB production	.950(**)	---	.808(**)
		PHB yield	.676(*)	.808(**)	---
13	*Natrialba* sp.	DCW	---	.975(**)	.828(**)
		PHB production	.975(**)	---	.819(**)
		PHB yield	.828(**)	.819(**)	---

** **Correlation is significant at the 0.01 level (2-tailed) (** - P ≤ 0.01)**

* **Correlation is significant at the 0.05 level (2-tailed) (* - P ≤ 0.05)**

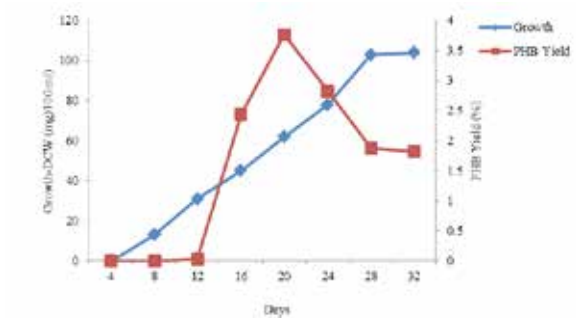

Fig. 1 Growth (mg/100ml) and PHB yield (%) as a function of incubation time by *Haloarcula* sp. 1

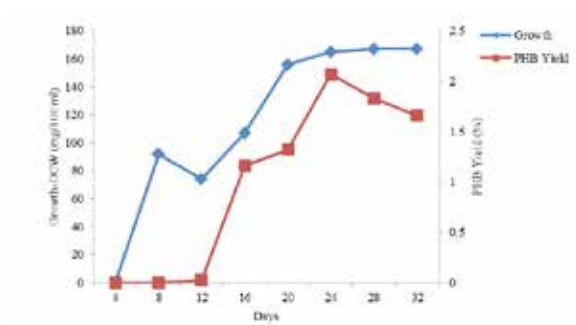

Fig. 2 Growth (mg/100ml) and PHB yield (%) as a function of incubation time by *Halorubrum* sp. 2

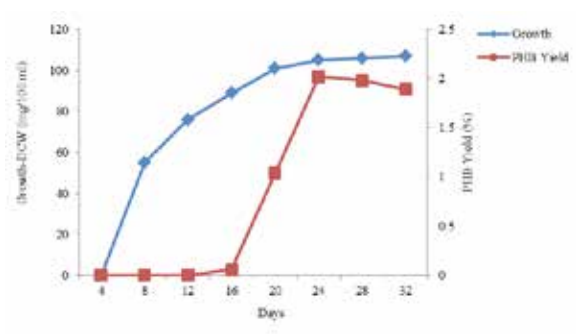

Fig. 3 Growth (mg/100ml) and PHB yield (%) as a function of incubation time by *Halobaculum* sp.

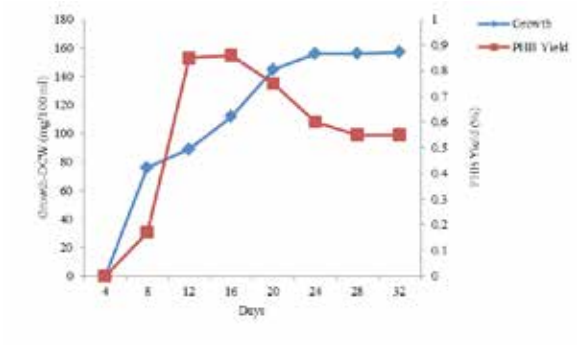

Fig. 4 Growth (mg/100ml) and PHB yield (%) as a function of incubation time by *Halobacterium salinarum*

Fig. 5 Crude film of bioplastic (PHB) by *Haloarcula* sp.1obtained with optimized conditions

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