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Extraction of Environment-Friendly Biodegradable Poly-Hydroxy Butyrate Using Novel Hydrodynamic Cavitation Method

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ABSTRACT

Polyhydroxy butyrate (PHB) is one of the best environment-friendly bioplastic alternatives for petroleum-based plastic due to its biodegradability. However, it has less commercial popularity owing to the high cost of downstream processing that involves repeated centrifugation and the use of costly harmful solvents, as well as a labor-intensive process. Hydrodynamic Cavitation (HC) offers easy and simple mechanisms for downstream processing. Also, biopolymer extracted for haloarchea show an advantage of least contamination under the halophilic condition on an industrial level. In this paper, a haloarchaeal consortium producing biopolymer isolated from commercial rock salt has been subjected to HC as well as distilled water lysis. A maximum of 23 $g.L^{-1}$ PHB was extracted in 40 min run with 50 passes and 0.10 cavitation number at 3.9 bar pressure. The extracted biopolymer was characterized and was found to be PHB. Comparative analysis shows that HC results in a substantial reduction in the downstream processing time. Moreover, it has double the efficiency of PHB extraction as compared to the distilled water lysis method. This paper reports the HC process as a techno-commercial alternative to industrial PHB extraction.

INTRODUCTION

The management of solid waste, especially when it consists of non-biodegradable plastic, is a major cause of environmental pollution. The accumulation of plastic waste has led to overflowing landfills, clogged waterways, health hazards to human life, and endangered terrestrial as well as marine habitats. A cost-effective alternative to petroleum plastic production is the need of the hour. Therefore, accelerating the development of biodegradable polymers needs utmost attention. Polyhydroxy butyrate (PHB), under the class of polyhydroxy alkanoates (PHA), is one such example of a biodegradable polymer with multiple applications (Niti Aayog 2022).

Under nutrient-deficient situations, several types of bacteria accumulate PHAs intracellularly as carbon and energy reserves (Kanekar et al. 2014). Amongst them, haloarchaea are one of the abundantly available species known to produce PHB (Simó-Cabrera et al. 2021). These haloarchaea can be isolated from rock salt available in different regions of the world (Jaakkola et al. 2014, Kondo 2015).

Extraction of PHB from these cells is popularly done with the help of organic solvents such as acetone, chloroform, methylene chloride, and methanol. However, these solvents are both expensive and harmful to the environment. The typical solvent extraction technique necessitates the storage, management, transportation, and recovery of the solvent. It necessitates substantial capital expenditures for the equipment as well as substantial running expenditures throughout the process. Additionally, trash disposal requires treatment. Moreover, obstacles such as poor substrate availability, cost of the carbon sources, low yield, microbe instability under changing circumstances, and greater energy requirements of downstream processing leading to high production costs have restricted the large-scale production of PHB ((Niti Aayog 2022, BIRAC 2021).

One way to reduce the downstream processing cost is using hydrodynamic cavitation. Hydrodynamic cavitation (HC) is an environmentally friendly process in which a great deal of energy is discharged in a flowing liquid as a result of the disintegration of a bubble caused by a decrease and concurrent increase in local pressure. The process consists of passing the liquid through a constriction like an aperture

or venturi or by rotating an item in a liquid (Chen et al. 2023). This method has been used for wastewater treatment, synthesis of chemicals using various reactions like oxidation reactions, emulsion generation, sludge treatment, biodiesel synthesis, degradation of residues from pharmaceutical or dye industries, food processing, and component extraction (Chen et al. 2023, Patil et al. 2021a, Zheng et al. 2022, Wang et al. 2021, Sun et al. 2022, Randhavane & Khambete 2017). Wang et al. (2021) reviewed the advantages of cavitation-based processes, such as cost-effectiveness in operation, higher energy efficiencies, and large-scale operation for treating industrial effluents (Wang et al. 2021). Because of its great energy efficiency, cost-effective operation, ability to generate chemical reactions and scale-up capabilities, HC has been regarded as a viable technology for process intensification (Zheng et al. 2022, Wang et al. 2021).

On similar lines, this approach can be utilized efficiently for industrial cell disruption to extract PHB on an industrial scale. This method will reduce all these expenses, rendering the method cost-effective. Several workers have studied the use of hydrodynamic cavitation for the extraction of metabolites and enzymes. Recent research by Wu et al. (2019) examined the extraction of bioactive chemicals from plants, lipids from algal biomass, and the delignification of lignocellulosic biomass via hydrodynamic cavitation (Wu et al. 2019). Verdini and co-workers evaluated the effectiveness of cavitation as a pretreatment strategy for cellulose recovery by disrupting the resistant lignocellulosic matrix (Verdini et al. 2021). This was accomplished in temperatures as low as 35 to 50°C. Setyawan et al. (2018) extracted the lipids from the microalgae *Nannochloropsis* sp. Using a hydrodynamic cavitation setup of the venturi type and compared it to traditional solvent extraction. Based on the volumetric mass transfer coefficient data, it was determined that hydrodynamic cavitation is more effective than its counterpart (Setyawan et al. 2018). Thus, the utilization of hydrodynamic cavitation for cell disruption on a wide scale with high energy efficiency possesses enormous promise.

Using the concept of location factor, Balasundaram & Pandit (2001b) measured the dependence of enzyme release on their position within the cell. The location factor was defined as the rate of enzyme released to the rate of total protein released. Typically, the location factor for periplasmic enzymes was more than 1, whereas it was less than 1 for cytoplasmic enzymes (Balasundaram & Pandit 2001b). Due to the importance of the enzyme's cellular location, pretreatment techniques can be used to modify the enzyme's cellular location prior to the actual cell disruption procedure. By pretreatment, the translocation of enzymes from the cytoplasm to the periplasm can be used to improve the efficacy of cell disruption and reduce energy needs (Balasundaram & Pandit 2001a). Literature reports that heat stress, period of culture in the fermentation process (Balasundaram & Harrison 2006), variable pH operation, and chemical pre-treatment (Anand et al. 2007) are among the approaches employed for translocation. Compared to the standard solvent extraction approach, this process has been found to be economical, energy efficient, and environmentally friendly (Wu et al. 2019, Gaikwad et al. 2016, Patil et al. 2021a, Patil et al. 2021b).

The purpose of this paper is to investigate the efficacy of the hydrodynamic cavitation method for the extraction of PHB with respect to the osmotic pressure method using distilled water, which further involves the use of solvents.

MATERIALS AND METHODS

Source of Haloarchaea

The haloarchaeal consortium was developed using commercial rock salt procured from the local market in Pune, Maharashtra, India. The haloarchaea were enriched in a modified Sehgal-Gibbons (SG) medium with 20 g% NaCl (Sehgal & Gibbons 1960) and were further grown in PHB Accumulation Medium (PAM) (Kanekar 2014).

Qualitative Characterization of Poly Hydroxy Butyrate (PHB) Producing Haloarchaea Using Sudan Black B Staining

The haloarchaea were grown in PHB Accumulation Medium (PAM), and after 96 hours, Sudan Black B staining was done according to standard protocol (Sheehan & Story 1977).

PHB Production by Haloarchaea

The haloarchaea were inoculated (10% v/v) in PAM with glucose (1%) as a carbon source and 20 g% NaCl in open 3 L in a conical flask. Aeration was provided with the help of an aerator, and the flask was incubated at 37°C in the incubator.

Extraction of PHB Using Hydrodynamic Cavitation Setup

The hydrodynamic cavitation experimentation setup was procured from Vivira Process Technologies, Pune. The schematic in Fig. 1 represents the actual setup. The working volume of the holding tank was 3 L. The line size was 0.25 inches, and a pump of 1.5 hp was used. The valve V1 was provided to regulate the flow rate. A bypass valve (V2) was provided, which was used to vary the pressure from 0 to 4 bar. The culture medium was passed through the cavitation device for cell lysis and collected in the holding tank. This

was recirculated through the device for several runs, which were conducted for up to 30-60 min and translated to 50 passes. The cavitation number was found to be 0.1, as per the methodology described by Marjadi & Dharaiya (2014). The flow rate was between 320-430 $L.h^{-1}$. Inoculum density was monitored and maintained by taking absorbance at 600 nm and CFU (Colony Forming Unit)/mL. Samples were collected at intervals of every 10 min and analyzed for temperature, density, and pH. The experiment was done in duplicate and analyzed for variance. These samples were used further for the extraction of PHB.

The liquid media obtained after hydrodynamic cavitation was subjected to centrifugation for 20 min at a speed of 8000 rpm. The white-colored PHB pellet was dissolved in chloroform and weighed after drying at ambient temperature (37±2°C). PHB dissolved in chloroform was cast in a petri dish and was allowed to dry at 65°C in the oven. After complete drying, PHB film was subjected to characterization by various methods. PHB film was precipitated using methanol, and a methanol precipitate of PHB was obtained.

Extraction Using Distilled Water Lysis

For comparison, liquid growth was subjected to centrifugation for 20 min at 8000 rpm, and the product was resuspended in distilled water. The suspension was allowed to stand for 30 min at ambient temperature to ensure proper lysis. The suspension was centrifuged at 2500 rpm repetitively till the red-orange color of the cells disappeared, and the white PHB pellet remained. The white PHB pellet was dissolved in chloroform and weighed after drying at ambient temperature (37±2°C). The dry cell weight of PHB by HC and distilled water lysis was compared.

Characterization of Biopolymer Using Spectrophotometric Analysis, FTIR

The confirmation and characterization of the PHB were characterized by crotonic acid assay, spectrophotometric analysis, and FTIR.

Estimation of PHB by Crotonic Acid Assay

The amount of PHBs in a sample taken was determined by spectrophotometric assay (Selvakumar et al. 2011). This assay was aided by the transformation of PHBs to crotonic acid via sulfuric acid treatment. Crotonic acid standard solutions of increasing concentrations (10 to 40 g) were developed. Crotonic acid absorbance was determined at 230 nm, and a standard curve was generated. The sample was then placed in a clean test tube containing 5 to 50 g of polymer in chloroform, and the solvent was evaporated by heating in a boiling water bath. The tube was then filled with 10 mL of concentrated H_2SO_4 and heated in a water bath for 10 min at 100°C. After that, the solution was chilled and well mixed. After that, the sample was transferred to a quartz cuvette, and the absorbance at 230 nm was measured against a sulfuric acid blank. A graph was then plotted to calculate the amount of crotonic acid.

UV-Vis Spectroscopic Analysis of PHB

The principle of this assay is to dissolve the samples isolated

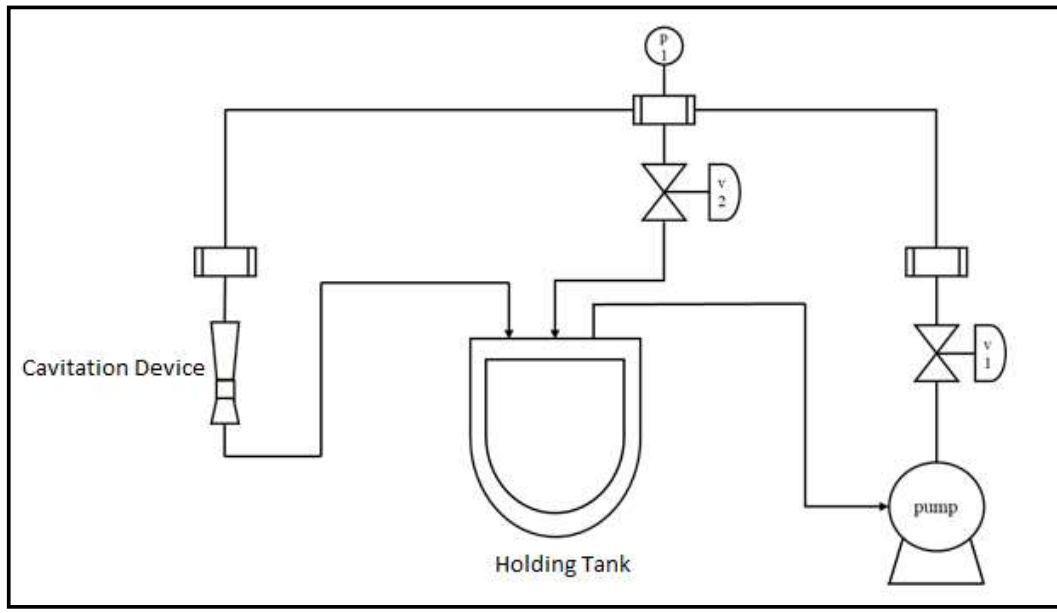


Fig. 1: Schematic representation of hydrodynamic cavitation setup.

from haloarchaeal culture in a suitable solvent and subject them to scanning in a UV-Vis spectrophotometer under a prescribed wavelength range (Selvakumar et al. 2011). The spectrum was then analyzed after the PHB compound was dissolved in chloroform and scanned with a UV-Vis spectrophotometer (Shimadzu) in the range of 800-200 nm against a chloroform blank.

Analysis of PHB Using FTIR (Fourier Transform Infra-Red) Spectrophotometer

A PHB extract sample weighing around 1 mg was dissolved in 5 cubic cm (cc) of chloroform. After the addition of KBr produced a pellet, spectra were captured using the Spectrum 65 FTIR (PerkinElmer) in the 4000-400 cm^{-1} range. The extracted PHB was dissolved in chloroform and scanned in the range of 200–320 nm (UV/Vis spectrophotometer RS-290) against chloroform blank, and the spectrum was analyzed for a sharp peak at 240 nm (Selvakumar et al. 2011).

RESULTS AND DISCUSSION

Source of Haloarchaea

The haloarchaeal consortium was red-orange colored when grown in SG liquid medium and required an optimum 22 g% NaCl for growth. The well-grown haloarchaeal consortium was further grown in nutrient-deficient (C: N – 10:1) PHB Accumulation Medium (PAM) for the production of PHB. Haloarchaea has been isolated from different rock salt samples from different regions of the world (Jaakkola et al. 2014, Kondo 2015). Still, there has not been a study about the extraction of PHB from haloarchaea isolated from rock salt.

Qualitative Characterization of PHB-Producing Haloarchaea Using Sudan Black B Staining

Culture from PHB accumulation medium, when stained with Sudan Black B, black-colored cells, indicate accumulation of PHB inside the cells while pink-colored cells indicate non-PHB producing cells (Fig. 2). This indicates that in PHB accumulation medium, PHB is produced inside the haloarchaeal cells and can be qualitatively confirmed using the method of staining by Sudan Black B (Selvakumar et al. 2011). Marjadi and Dharaiya (2014) stained the haloarchaeal cells of *H. marismurti* with Sudan Black B and have been found to produce PHB intracellularly, in agreement with our findings (Marjadi & Dharaiya 2014).

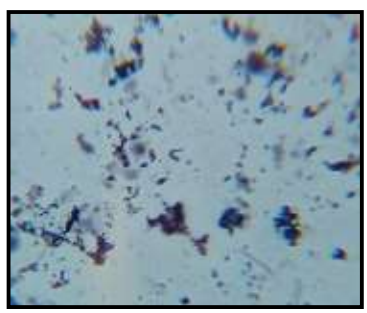


Fig. 2: Sudan Black B staining of PHB producing haloarchaeal culture.

PHB Production by Haloarchaea

As the haloarchaea could grow at a high salt concentration of 20 g%, contamination from other microorganisms was minimized. The culture could grow in an open 3 L flask and was used further for the HC experiment. This proved to be a major advantage of haloarchaea over other microorganisms on a scale-up level (Sheehan & Story 1977). 10% inoculum from haloarchaeal consortium grown in SG medium was used in PAM for scale-up studies.

Extraction of PHB Using Hydrodynamic Cavitation Setup

The growth of the culture was monitored at 600 nm, and in the late log phase or early stationary phase, the liquid culture was subjected to HC. This was the best phase when the organism produced maximum PHB, and thus, maximum PHB from cells could be harvested. In HC experiments, the pressure was varied from 2.5 to 3.9 bar, and the yield of PHB in $g.L^{-1}$ was calculated from the samples taken at 10-minute intervals. Table 1 gives the values of various parameters obtained for the different experimental runs. The results are analyzed in Fig. 3. The graph (Fig. 3) shows time in min on the X-axis and PHB $g.L^{-1}$ on the Y-axis. It can be seen that in the first run, at 2.5 bar pressure, 2.9 $g.L^{-1}$ PHB was extracted after 30 min of HC. In the second run, around 6.6 $g.L^{-1}$ PHB was extracted after 30 min at 3.2 bar pressure. In the third run, 3.9 bar pressure was applied, around 9 $g.L^{-1}$ PHB was extracted at 30 min, and a maximum of 27.097 $g.L^{-1}$ PHB was obtained at 40 min. This was the maximum PHB $g.L^{-1}$ obtained from the haloarchaeal consortium from rock salt.

The results indicate that at higher cell density, more cells produce PHB inside their cells, and thus, maximum PHB production can be achieved. In the third run, it was also seen that as pressure increased from 2.5 to 3.9 bar, the increase in PHB extraction increased proportionately. As the pressure increased, density changed too. This was due to an increase in density of the cellular component after the breakage of cells. Initial cell count (Colony Forming Unit – $CFU.mL^{-1}$)

Table 1: Comparative PHB dry cell weight.

HC run	Absorbance 600 nm	Flow rate, $l.h^{-1}$	Pressure, Bar	Time, min	pH	Density	*CFU.mL^{-1}	Max Temp, °C	PHB, $g.L^{-1}$ dry cell weight	Std. Dev.
1	0.89	321.9	2.5	0	6.5-7	1.206	7.5×10^7	30	1.3	0.065
			2.5	10	6.5-7	1.159		38	2.6	0.13
			2.5	20	6.5-7	1.152		36	1.6	0.08
			2.5	30	6.5-7	1.156		38	2.9	0.145
2	1.0	360.6	3.2	0	7.20	1.111	8.4×10^8	35	1.599	0.0799
			3.2	10	7.27	1.090		39	4.399	0.219
			3.2	20	7.23	1.208		44	7.899	0.394
			3.2	30	7.27	1.182		47	6.599	0.329
3	1.8	427.1	3.9	0	7	1.133	1.5×10^{12}	34	0.199	0.009
			3.9	10	7	1.134		38	8.283	0.414
			3.9	20	7	1.151		41	4.732	0.236
			3.9	30	7	1.165		46	9.032	0.451
			3.9	40	7	1.139		50	27.097	1.354
			3.9	50	7	1.169		53	10.965	0.548
			3.9	60	7	1.149		55	3.033	0.151
**D/W lysis									11.0	0.55

*CFU – Colony Forming Unit

**D/W – Distilled Water

also increased as the optical density increased. This means to get maximum PHB production, more initial number of PHB-producing cells is necessary. The results of the third run at 40 min at 3.9 bar pressure seem to be promising but will need further evaluation and analysis. HC method has been useful in synthesis of chemicals using oxidation reactions (Zheng et al. 2022), sludge and wastewater treatment (Chen et al. 2023, Patil et al. 2021a, Randhavane & Khambete 2017), synthesis of petrochemical products like biodiesel, degradation of pharmaceutical or dye industries residue, extraction of food processing products and cellular component extraction like enzymes, lipids (Wang et al. 2021, Wu et al. 2019, Sun et al. 2022). However, there is no report regarding PHB extraction using HC.

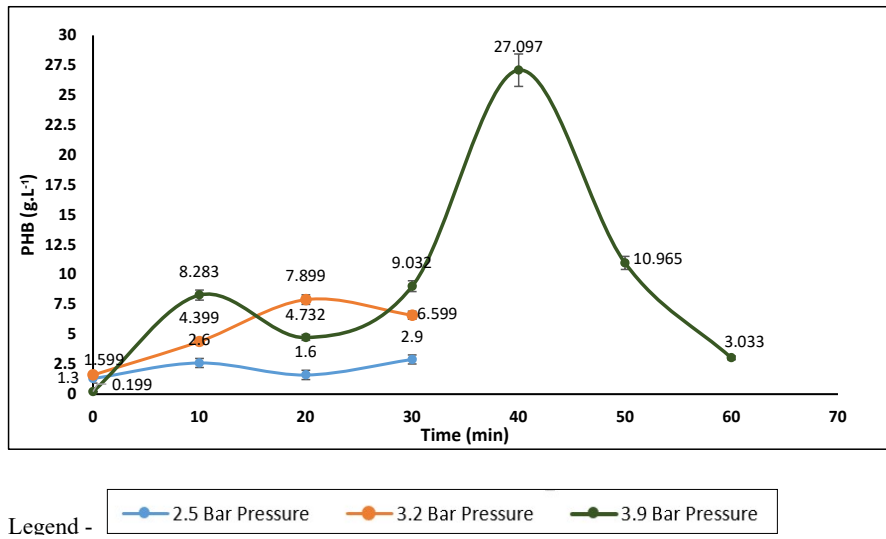


Fig. 3: Graphical representation of HC run for PHB extraction.

Fig. 4a: Casting of PHB film.

Fig. 4b: Methanol precipitate of PHB film.

Table 1 shows that as the initial cell count and pressure increased, PHB extraction increased proportionately. The comparative method of distilled water analysis showed PHB production of 11 $g.L^{-1}$, which is comparable to all the runs at 30 min. In the third run, as the time was increased further to 40 min, PHB extraction doubled, reaching a maximum of 27 $g.L^{-1}$. This indicated further optimization and analysis should be done considering 3.9 bar pressure and 60 60 min run to maximize PHB production.

The PHB film was cast using chloroform and dried in an oven at 65°C (Fig. 4a). This film was milky white and translucent. The film was precipitated using methanol, and a methanol precipitate of PHB film was done (Fig. 4b). It was found to be brittle. Selvakumar et al. (2011) extracted PHB from *Haloarcula marismurti* using the same distilled water lysis method, similar to our results (Selvakumar et al. 2011).

Extraction Using Distilled Water Lysis

For comparison, extraction of PHB using distilled water lysis was done, and it was found that 11 $g.L^{-1}$ PHB could be extracted. As compared to HC, this result is comparable with 30 min of HC at 3.9 bar pressure (9.032 PHB $g.L^{-1}$) but is half when compared to 40 min of HC at 3.9 bar pressure. This supports that HC could be a better alternative for the extraction of haloarchaeal PHB than the distilled water lysis method on an industrial level.

Characterization of PHB Using Crotonic Acid Assay, Spectrophotometric Analysis, and FTIR

Estimation of PHB by crotonic acid assay: For standard crotonic acid assay, 10 to 40 $\mu g.mL^{-1}$ of standard crotonic acid was subjected to H_2SO_4 acid treatment. The peaks of the same standard crotonic acid can be seen in the graph (Fig. 5). The PHB extracted after 30 min of HC was purified and dried.

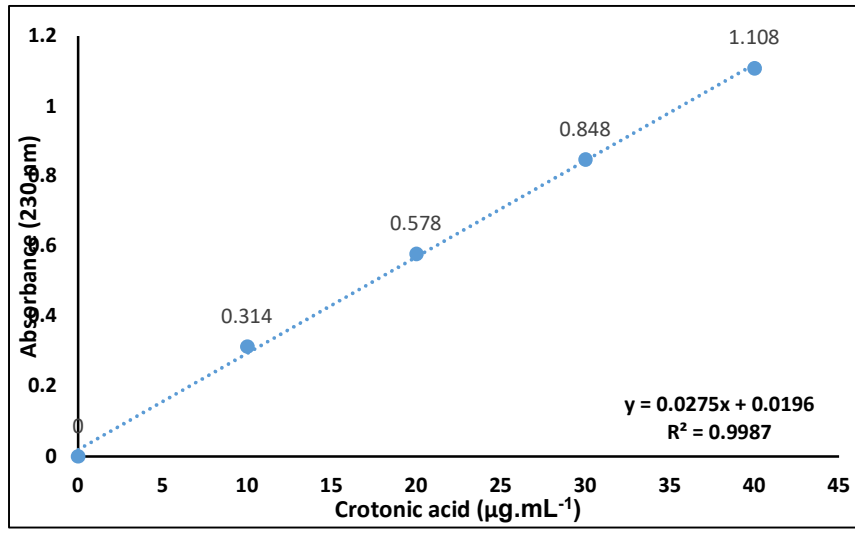


Fig. 5: Standard crotonic acid assay.

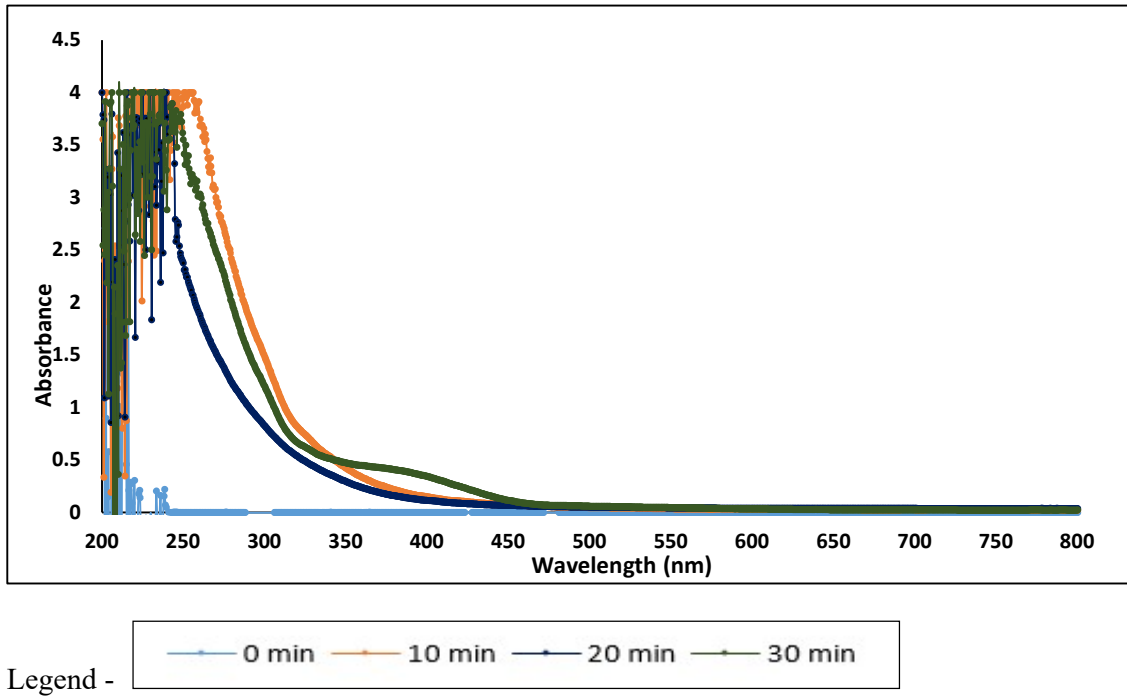
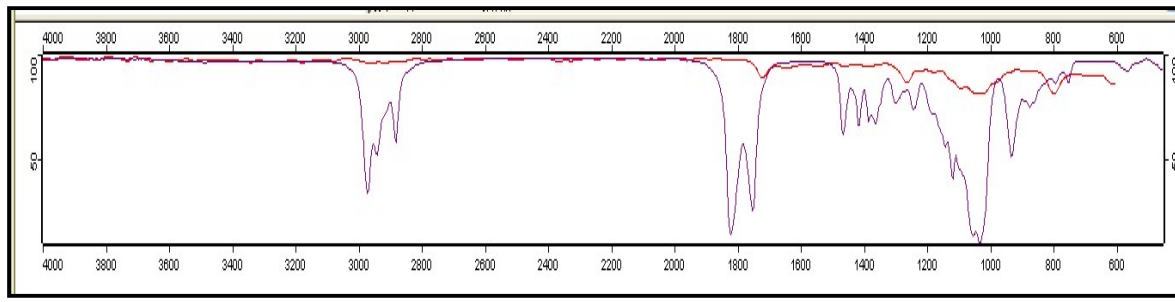


Fig. 6: UV spectrum analysis graph of PHB.



Legend – PHB Test sample (Red line), PHB standard sample (– Purple line)
Fig. 7: FTIR analysis of PHB sample after HC extraction.

This PHB was subjected to acid treatment as previously and was analyzed at 230 nm. The PHB sample showed a peak at 230 nm with more Area Under Curve at 40 µg.mL^{-1} crotonic acid, and it was calculated that the PHB content in the sample after 30 min was found to be 51.19 µg.mL^{-1}. Selvakumar et al. have used Crotonic acid assay to detect PHB and have got similar results (Selvakumar et al. 2011).

UV-Vis spectroscopic analysis of PHB: Samples from the HC experiments at time intervals of 0, 10, 20, and 30 min were dissolved in chloroform and subjected to UV-Vis spectroscopy over the wavelength range of 200-800 nm. The outcome achieved thus far has revealed a prominent peak and absorbance at 240 nm (Fig. 6). In contrast, there was no similar absorption at 240 nm in the control solvent (chloroform). The peak at 240 nm was present in all samples, amply demonstrating the existence of PHB in the extracted material. A similar study by Selvakumar et al. (2011) showed a UV spectrum peak at 240 nm of PHB extracted from *H. marismurti* (Selvakumar et al. 2011).

FTIR spectrophotometer analysis of PHB: It was observed that the FTIR spectrum of the standard and test samples of PHB showed overlapping peaks. The PHB sample (red line) after HC extraction showed a peak overlapping the peaks of PHB (poly-hydroxy butyrate) (butyric anhydride-Purple line) (Fig. 7). This confirms the presence of butyric acid in the test sample.

The spectrum obtained was compared with the spectrum obtained by Marjadi and Dharaiya (2014), as shown in

Table 2: FTIR spectrum of haloarchaeal PHB sample and standard PHB.

Sample	Peak region	Comment
PHB Sample	1650	Carbonyl group (C=O)
	2963	Methine groups (CH)
	1092	Ester group (C-O)
	3578.55	Intramolecular H bond
	3415.06	H bond
PHB (Sigma Aldrich Company)	1673	Carbonyl group (C=O)
	2928	Methine groups (CH)
	1076	Ester group (C-O)
	3330.13	Intramolecular H bond
	3417.70	H bond

Table 2. In that work, the FTIR spectrum of the compounds was recorded in the range of 800-4000 cm^{-1} and showed characteristic bands for the groups CH, C=O, and C-O. Strong bands in the ranges of 1325.99-1439.32 and 2945.23-3038.73 were produced by the methine groups (CH). Due to polymerization, these frequency values were higher than the typical values. The carbonyl group (C=O) produced a noticeable band between 1610.74 and 1670.24. Due to polymerization, these frequency values were lower than the expected value. The (C-O) group demonstrated robust and widespread absorption in the 1048.32–1100.74 range.

Compared to the literature, our results of PHB extraction indicate that HC could be used efficiently for downstream processing for PHB extraction in the near future. To the authors' best knowledge, HC has not been used for PHB extraction to date. Thus, this could be the first report on the use of HC for PHB extraction.

CONCLUSIONS

The present paper describes hydrodynamic cavitation as one of the best alternatives for biopolymer extraction (PHB). HC has been used in wastewater treatment and enzyme extraction successfully, but there is no research related to biopolymer extraction. This paper significantly describes the optimization of parameters related to HC for biopolymer extraction. The results showed that the haloarchaeal consortium showed the presence of PHB inside the cells using Sudan Black B staining. The culture was scaled up to 3 liters in a PHB accumulation medium, and PHB was extracted using HC. The pressure was varied from 2.5 to 3.9 bar in three runs, and it was found that maximum PHB production was found in the third run with a 3.9 bar run after 40 min. This PHB production was approximately twice as compared to distilled water lysis PHB extraction. UV spectrum analysis, Crotonic acid assay, and FTIR confirmed the presence of PHB.

Individual isolates have been used for PHB production and have been reported, but a haloarchaeal consortium from rock salt for PHB production has not been reported. Furthermore, HC has been used for a variety of intracellular and periplasmic enzymes but has not been used for PHB extraction yet. Compared to other methods of PHB extraction, HC thus offers an advantage of large-scale downstream processing in reduced time. Further standardization of parameters for PHB extraction described in this paper can help in future research related to PHB extraction using hydrodynamic cavitation.

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