Research Article

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The production of Poly (3-Hydroxybutyrate) in the Extremely Halophilic Archaea *Halarchaeum acidiphilum* ASDL78

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Abstract

Purpose and aim: Biodegradable Polyhydroxyalkanoates (PHAs), produced predo-minantly by bacteria, are a solid alternative to native plastic. However, also in the archaea domain, some members of the haloarchaea genera have been identified as PHAs producers. In the present study, we selected *Halarchaeum acidiphilum* strain and optimized it for Poly (3-Hydroxybutyrate) production.

Material and Method: *Halarchaeum acidiphilum* ASDL78 was isolated from Lut desert of Iran and performed Sudan black B stain and UV absorption of crotonic acid.

Results and Conclusion: The results showed that the strain produced PHA in the medium containing 12.5% sodium chloride and pH equal to 7.6 at 37°C at the stationary phase. The PHA was extracted in sterile water, and the physico-chemical analysis of PHA was detected by FTIR and ^{1}HNMR test.FTIR and ^{1}HNMR approved the ester structure and the efficiency was almost 71% of the cell's dry weight. The use of haloarchaea in the production of PHA has advantages such as no need for sterile conditions, simple raw materials, and fast lysis of bacteria in the absence of salt, which help the processes of the economic cycle of PHAs extraction.

What is "already known":	• Fat reserves are present in bacteria during nitrogen starvation • purification and extraction of poly (hydroxy butyrate) inclusion body have been Reported • The production of fat beta-hydroxybutyrate, and properties have been less studied in Archaea.
What this article adds:	• extracting polyhydroxybutyrates from bacteria are expensive and difficult, but lipids are easily extracted by placing the Archaea in sterile water • This extraction is cheap and cost-effective. • Optimized method for production in Archaea is needed to increase effeciency of production.

1. Introduction

Today, plastics are widely used due to their beneficial properties. Plastic pollution is considered a global warning that causes the death of animals and brings environmental decadence. Moreover, recycling plastics is difficult or even impossible in most cases [1]. Renewable resources reduction, greenhouse gas emission, the lack of appropriate recycling technology, and non-degradable materials improper disposal cause direct dangerous effects on our environment's conditions [2].

Plastics have become pervasive in food webs worldwide, posing significant threats to ecosystems and human health. Microplastics have been detected in the digestive tracts of organisms across multiple trophic levels, indicating their widespread distribution throughout the food chain [3]. Environmental weathering and degradation processes fragment larger plastic debris into microplastics, which can adversely affect animal species. These particles, along with the chemicals and additives they contain, have been associated with endocrine disruption and alterations in metabolic functions [4]. In humans, airborne microplastic fibres and associated contaminants can be inhaled through the respiratory system, potentially leading to long-term adverse effects on physiological health [5]. Furthermore, the production of conventional plastics relies heavily on petroleum and other fossil fuel resources, contributing to the depletion of non-renewable energy reserves and increasing environmental burdens. Consequently, growing research efforts have focused on the development of biodegradable bioplastics and environmentally sustainable polymer formulations as promising alternatives to conventional plastics.

In the meantime, bioplastics are derived from renewable resources and are broken down by microorganisms such as bacteria and fungi. These plastics can be substitutes for synthetic plastics and reduce their environmental side effects. Polyhydroxyalkanoates (PHAs) are biological polyesters produced through microbial fermentation [5]. These compounds have received much attention due to their biodegradability, biocompatibility, and high stability. Microorganisms produce PHAs when an excess substrate (carbon source) is available under conditions of oxygen, nitrogen or, phosphorus limitation or pH fluctuations, or as response to other stress factors [6]. The first identified PHA is Poly (3-Hydroxybutyrate) (PHB), a bacterial storage substance. All groups of microorganisms synthesize PHA, such as bacteria, and archaea as a carbon and energy storage material. Recently, extremophiles have been recognized as the most reliable microorganisms for PHA production to reduce production costs [7].

Large scales haloarchaeal cultures in medium do not require difficult sterilization due to the high salt concentration needed for cell growth. This makes the conditions more effortless and more comfortable compared to using non-extremophilic bacteria [8]. Moreover, the walls and cell membranes of haloarchaea disintegrate under hypotonic conditions, and this lysis makes releasing PHA granules from cell biomass easier and simpler. Also, the saltwater waste obtained from other processes can be considered a culture medium for the haloarchaea growth, lowering production costs and helping the biological cycle of the environment [9].

Despite increasing interest in polyhydroxybutyrate (PHB) production, studies focusing on archaeal production systems remain limited. In addition, commercially available PHB produced by archaea is scarce, and comprehensive techno-economic assessments of archaeal PHB production processes are still lacking. Therefore, the improvement of processes on the laboratory scale is needed before the developing of PHB production plants with archaea and determining the potential of archaea in the future of sustainable biotechnology and a healthy environment [10].

In this study, the PHB production by halophilic archaea was investigated, and the conditions for its further production were optimized for the strain *Halarchaeum acidiphilum* ASDL78. Finally, the produced PHB was analyzed by physicochemical methods.

2. Materials and Methods

2.1. Microorganism, cultivation and microscopy analysis of haloarchaea cells

Archaea strain of *Halarchaeum* (*Hla*). *acidiphillum* ASDL78 was isolated From the Lut Desert in the previous studies of Emtiazi and Abbasi (2020) and submitted to NCBI database With access number GenBbank MN900607. [11]. The archaeon was cultivated on Archaea medium (Glucose 5 g/l, yeast extract 1 g/l, peptone 1 g/l, NaCl 125 g/l, $MgCl_2$ 6 H_2O 6 g/l, $CaCl_2$ 0.13 g/l, K_2SO_4 5 g/l) Chloramphenicol antibiotic powder was added to the sterilized medium at an amount of 12 µg/ml.

2.2. Staining of archaea producing PHB

Sudan black was used to identify fat granules for preliminary identification. A slide was prepared using 20% NaCl solution, washed with acetic acid, and then immersed in black Sudan solution (0.3% in 60% ethanol) for 20 minutes. In the next step, it was washed with xylol solution for 3-4 seconds and then stained with safranin background solution for one minute. Then it was observed with Olympus CX21 Biological Microscope.

2.3. Assay of PHB

10 ml of the culture medium of *Hla. acidiphillum* was centrifuged with Hettich centrifuge (4000 rpm for 20 minutes), and the cell pellet was washed with distilled water. 10 ml of sodium hypochlorite alkaline solution (5% active chlorine with pH equal to 10) was added to the cell pellet and incubated for 1 hour at 37 °C. Then centrifugation and re-washing of the white precipitate were done with distilled water. In addition, the white sediment was washed with 96% Ethanol and acetone in equal proportions to remove protein and lipid impurities. Finally, the white powder was completely dried in the oven at 50°C. Then 1 ml of concentrated sulfuric acid was added and incubated at 100 °C for 10 minutes in a hot water bath. Finally, after cooling the above solution, the optical absorption of crotonic acid at a wavelength of 235 nm against concentrated sulfuric acid was examined by spectrophotometer UV.

2.4. Production of PHB

The liquid form of Archaea broth medium (without chloramphenicol), a polymer production medium, was used in Erlenmeyer flasks to measure the amount of PHB polymer and cell dry weight (by chemical method). A fresh culture of screened archaea was prepared on Archaea agar medium and turbidity was prepared equal to half of McFarland and inoculated into the flasks. Sampling was done at different times to determine cell dry weight and PHB production.

2.5. Determination of cell dry weight

In order to measure biomass, 50 ml of the culture medium was centrifuged (4000 rpm for 20 minutes), and the sediment was washed. Then a certain share of the cell pellet was transferred to the falcon and placed at 105°C until a constant weight was reached.

2.6. Determination of the PHB optimal growth and production conditions

PHB production process by *Hla. acidiphillum* strain was optimized by adopting a one-factor-at-a-time approach. All experiments were repeated three times and averaged. Inoculation and cultivation conditions were the same as mentioned in the previous section. The studied variables include the following:

Different carbon sources include glucose, sucrose, lactose, fructose, beet molasses, whey, starch powder, hydrolyzed banana peel, and potato peel with a concentration of 10 g/l and different nitrogen sources include ammonium chloride, potassium nitrate, yeast extract, urea, and ammonium nitrate with a concentration of 1 g/l. Growth of *Hla. acidiphillum* and PHB production at different temperatures (15°C, 25°C, 37°C and, 45°C), pH (3.6, 5.2, 6, 7.6, 8.4, and 10), aeration was in a shaker incubator at 80, 100, 120, 150, 200 and 250 rpm and various salt concentrations (5, 10, 12.5, 15, 20, and 25%) was determined on the Archaea medium for one week.

Inoculation and cultivation conditions were the same as mentioned in the previous section.

All experiments were repeated three times and averaged. Analysis of Variance (ANOVA) of the data with a probability of error less than 5% ($P\text{-value} < 0.05$) was applied in different conditions in order to ensure a significant difference between the results obtained at variables different levels.

2.7. Relationship between PHB production and growth

The active culture medium (2ml) of *Hla. acidiphillum* was transferred to 20 Erlenmeyer flasks containing 250 ml of archaea culture medium and placed in a shaker incubator at a temperature of 37°C and 120 rpm. For 240 hours, at 24-hour intervals, the amount of PHB production and cell biomass was determined.

2.5. Analysis and characterization of PHA

2.5.1. Fourier transform infrared spectrometer (FTIR analysis)

Fourier transform infrared spectroscopy is a very beneficial technique for distinguishing of organic and inorganic chemicals. This technique can be used to determine the amount of a substance in a mixture. We mixed some of the PHB samples with potassium bromide to prepare the sample and turned them into special data for FTIR analysis.

2.5.2. Nuclear magnetic resonance spectroscopy (^{1}H NMR analysis)

^{1}HNMR spectroscopic method gives new information about the biology and chemistry of PHB inside the cell. The ^{1}HNMR method can determine the characteristics and size of PHB in the polymer before it is extracted from the cell. ^{1}HNMR nuclear magnetic resonance was performed by suspending PHB in high-purity chloroform, using a spectrometer at 400 MHz to determine the type of monomer. The ^{1}HNMR content of PHB was obtained.

3. Results

Initial staining by black Sudan identified PHB granules inside haloarchaea cells in the form of prominent black grains. The morphological and biochemical characteristics of the *Hla. acidiphillum* ASDL78 were analyzed. The results are given in Table 1. Colonies of *Hla. acidiphillum* ASDL78 were observed on the archaea medium in orange color with a diameter of approximately 2 mm, circular, smooth and, mucoid. The cells were morphologically triangular, Gram-negative, and mobile (Figure 1) .The phylogenetic tree of the isolated strains is shown in Figure 2.

Figure 3 shows the impact of different process variable on PHB production in *Hla. Acidiphillum*. Figure 3a, shows that the production of PHB in the medium prepared with beet molasses as a carbon source has increased significantly (5.2 g/liter) .Beet molasses showed an increase in PHB production level compared to other carbon sources. The results obtained in Figure 3b from the effect of nitrogen sources showed that the bacteria have the maximum production of PHB in the presence of yeast extract and ammonium chloride, the only nitrogen source. The maximum growth of *Hla. acidiphillum* was observed at 12.5% sodium chloride concentration.

The results presented in Figure 3c show that the isolated strain needs 12.5% sodium chloride in the culture medium as the optimal concentration for maximum PHB production. However, the culture media's concentration of 15-20% sodium chloride supports the production of PHB. It should also be noted that at least 12% sodium chloride is required for growth. The isolated strain of *Hla. acidiphillum* showed maximum growth at 37°C. While from the results presented The optimal temperature for maximum PHB production was 37°C. However, the temperature range for PHB production varies between 25°C and 60°C (Figure 3d) .The growth of the strain was observed in the pH range from 3.6 to 10. As can be seen from the figure, the PHB producing strain showed maximum biomass production at pH 7.6. The results presented in Figure 3e showed that the isolated strain could produce PHB in the medium at different pH values between 3.6- 8.4, although the maximum production of PHB was recorded at pH values 7.6. The data in Figure 3f shows the effect of stirring on the amount of PHB production .The results show that more shaking leads to an increase in biomass. Maximum biomass was recorded at 200 rotations, which may be due to better mixing. More mixing facilitates food access and reduces microbes' adhesion to the surface of Erlenmeyer flasks. In this study, PHB's maximum growth and production were obtained at 120 rpm. As shown in Figure 4, the growth and production of PHB were followed for up to 240 hours. The correlation between the growth curve and PHB production showed that the maximum PHB production was in the stationary phase.

Table 1. Morphological and physiological characteristics of *Halarchaeum acidiphilum* strain

Morphological characteristics	
Colony morphology	Orange round regular edges
Form	Triangular
Gram stain	Negative
Spore	Negative
Motility	Positive
Physiological characteristics	
Anaerobic growth	Positive
Acid from glucose	Positive
Gas from glucose	Positive
Hydrolysis of mannitol	Positive
Hydrolysis of mannose	Positive
Hydrolysis of lactose	Positive
Hydrolysis of xylose	Positive
Hydrolysis of sucrose	Positive
Hydrolysis of gelatin	Negative
Utilization of citrate	Positive
Hydrolysis of Casein	Negative
Indol formation	Negative
H_2S	Negative
Metyl Red test	Positive
VP-test	Negative
Urease	Negative
Nitrate reduction	Positive
Growth temperature	37-60˚C

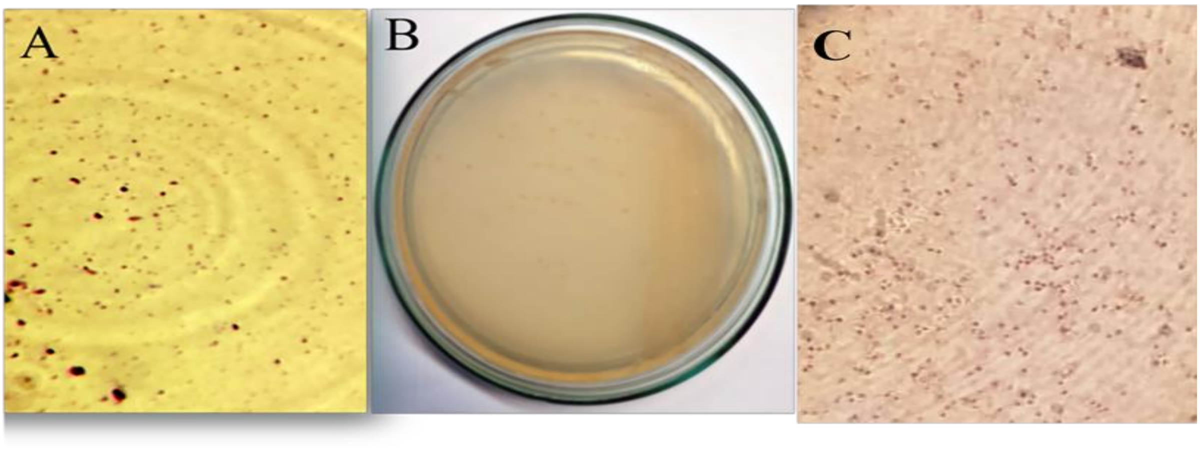

Figure 1 A) Morphology of archaea *Halarchaeum acidiphilum* ASDL78by Gram staining with special method of archaea at light microscopy. B) Colony shape on Archaea agar medium. C) Staining with Sudan black B.

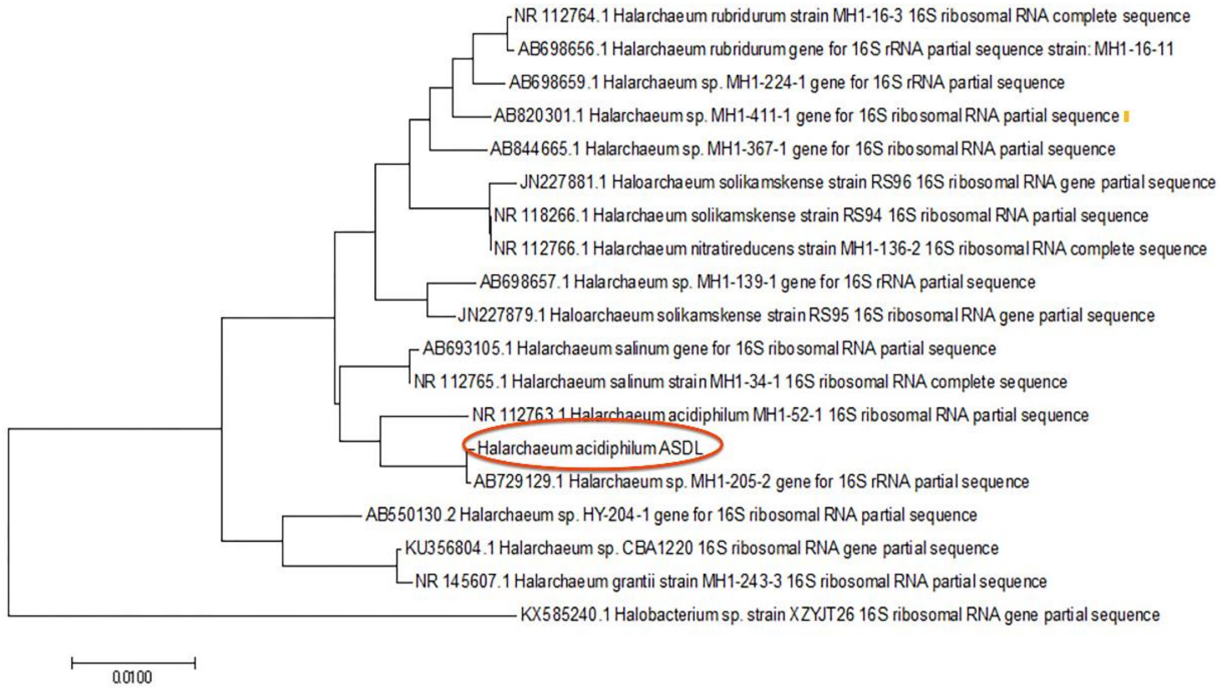

Figure 2 Phylogenetic tree of isolated strain *Halarchaeum acidiphilum* ASdl78 and related haloarchaeal strains. Bootstrap values are shown as percentages of 1000 replicates. GenBank accession numbers are shown in parentheses. Bar, 0.01 changes per nucleotide position.[11].

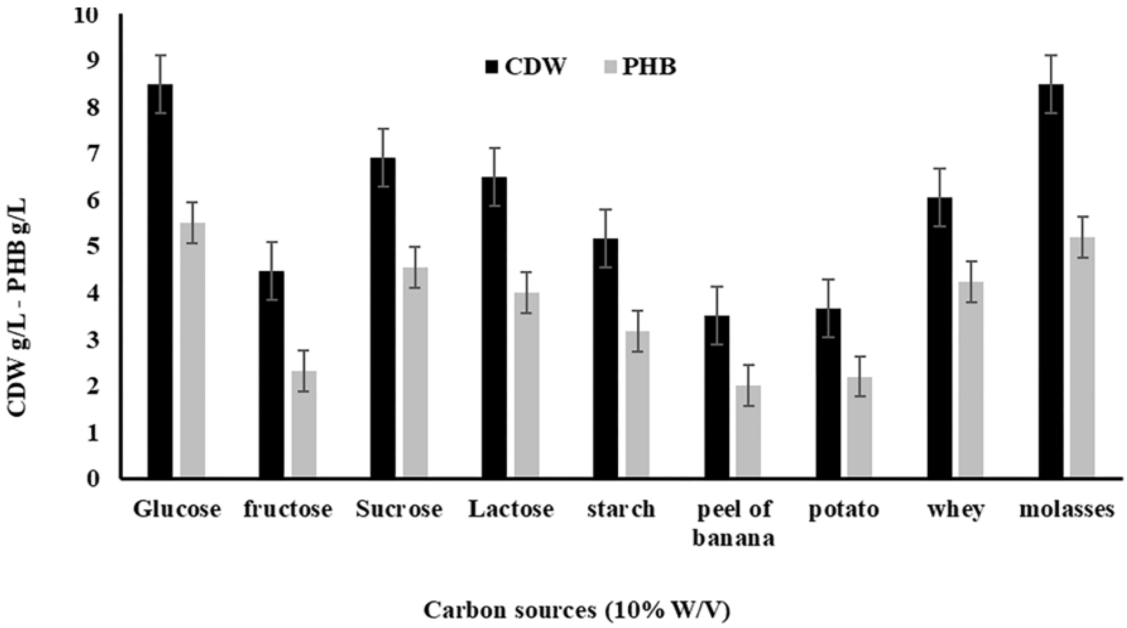

Figure 3a. Effect of different carbon sources on PHB production by *Halarchaeum acidiphilum* ASDL78 in Archaea medium at 37°C for seven days

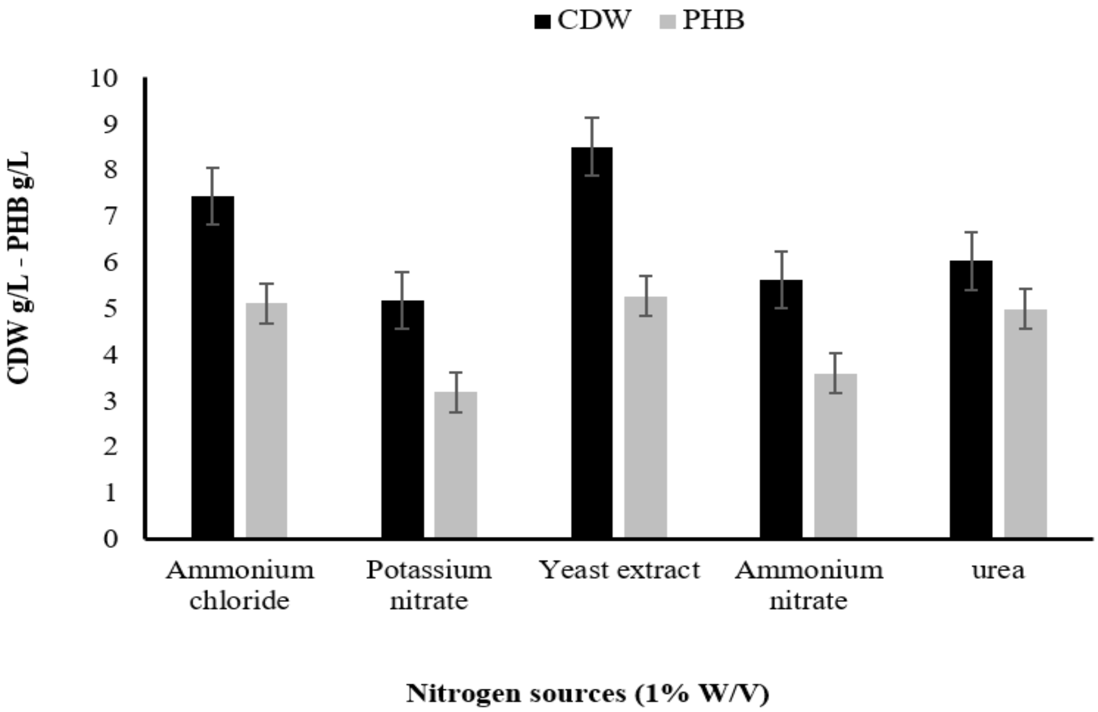

Figure 3b Effect of different nitrogen sources on PHB production by *Halarchaeum acidiphilum* ASDL78 in archaea medium at 37°C for seven days.

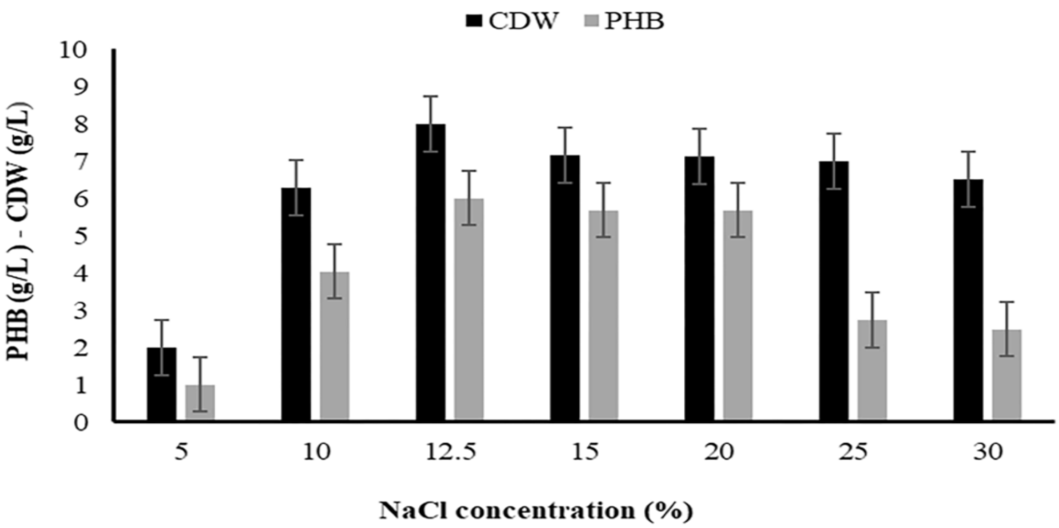

Figure 3c Effect of different concentration of NaCl on the growth and production of PHB by *Halarchaeum acidiphilum* ASDL78

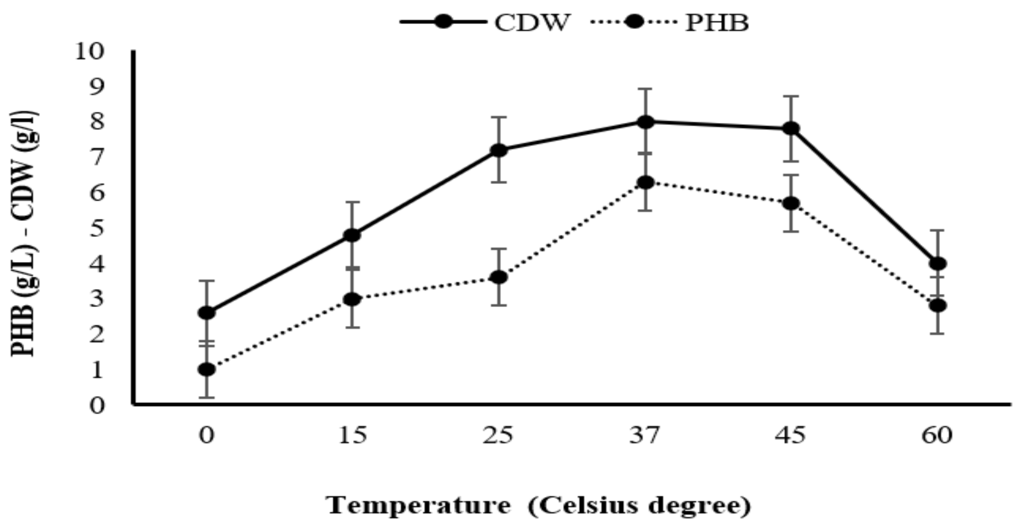

Figure 3d Effect of temperature changes on growth rate and PHB production by *Halarchaeum acidiphilum* ASDL78.

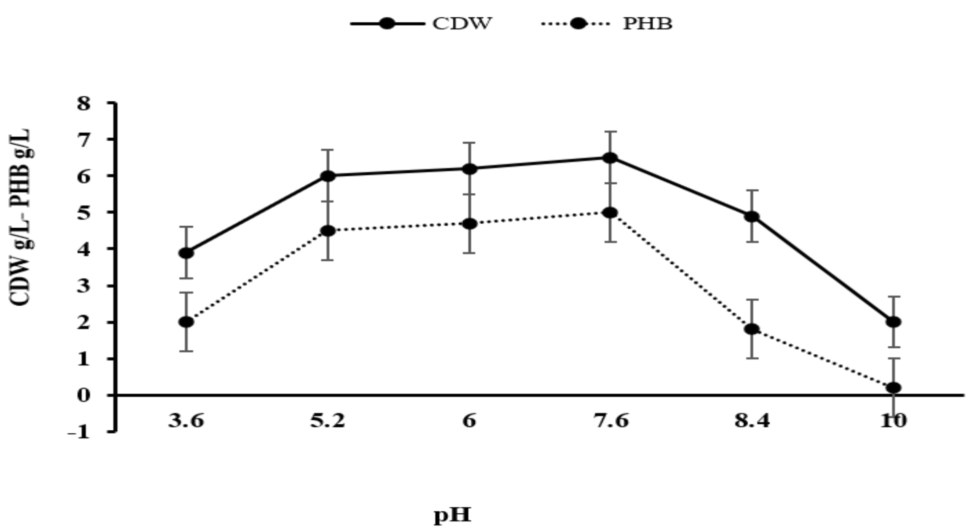

Figure 3e Effect of pH changes on the growth rate and PHB production by *Halarchaeum acidiphilum* ASDL78.

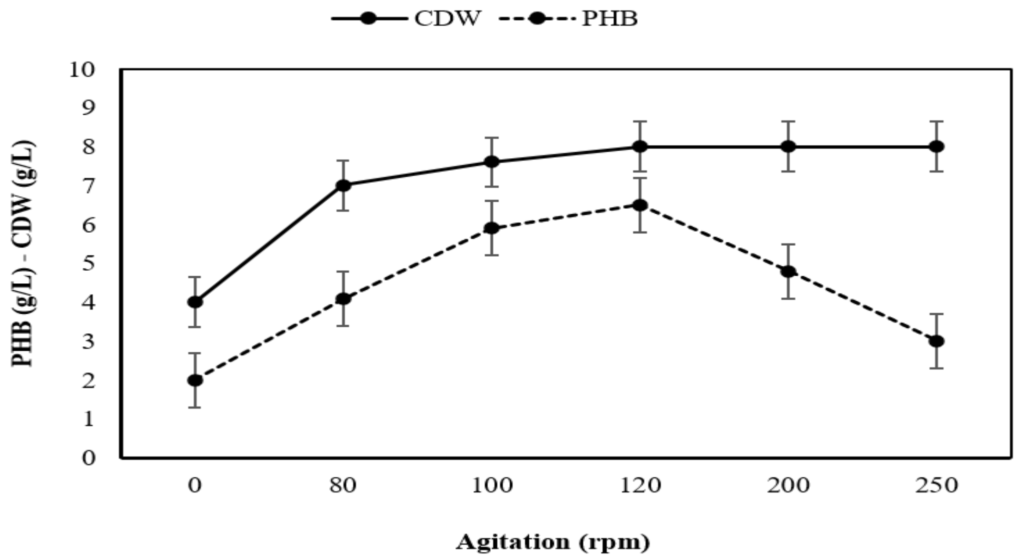

Figure 3f Effect of shaker speed on the production of PHB by *Halarchaeum acidiphilum* ASDL78 in Archaea medium at 40°C for seven days

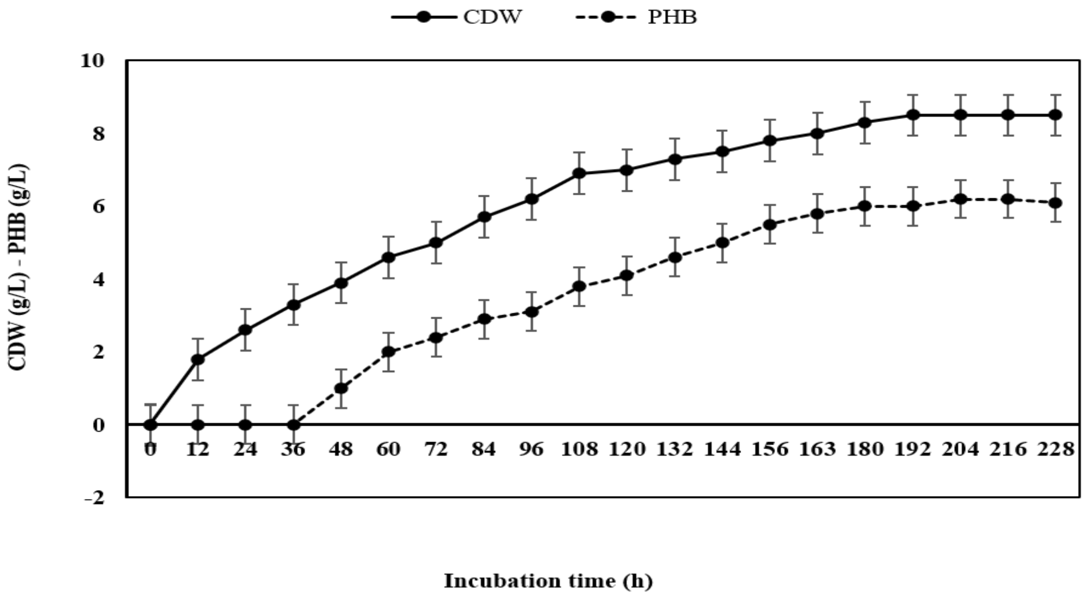

Figure 4 Relationship between the growth curve and the amount of PHB produced in the culture medium by *Halarchaeum acidiphilum* ASDL78.

FTIR analysis is used to detect PHA in the cell suspension. However, it lacks the specificity to distinguish between different monomers; therefore, this method cannot detect copolymers composed of different PHA monomers. Figure 5 shows the FTIR spectrum of polymer extracted from biomass. In this spectrum, the carbonyl ester band can be seen at 1726 cm^{-1} (PHB index band). This band is created by the vibration of the C=O bond from the ester bond of fatty acids. The presence of these bands, together with the

PHB indicator band, is used in identifying this polymer (Figure 5 and table 2).

^{1}HNMR analysis is the most critical in identifying organic compounds' broad structure. The resonance spectrum's entire length for PHB in Figure 6 shows that the methyl group or CH3's signal in ppm is equal to 1.25 and the methylene group or CH2's signal in ppm is between 2.45 and 2.65, and the methine group or CH in ppm is equal to 5.25. Moreover, a peak near 5.17 ppm is characteristic of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV). There is another signal at 7.25 ppm, which is related to chloroform.

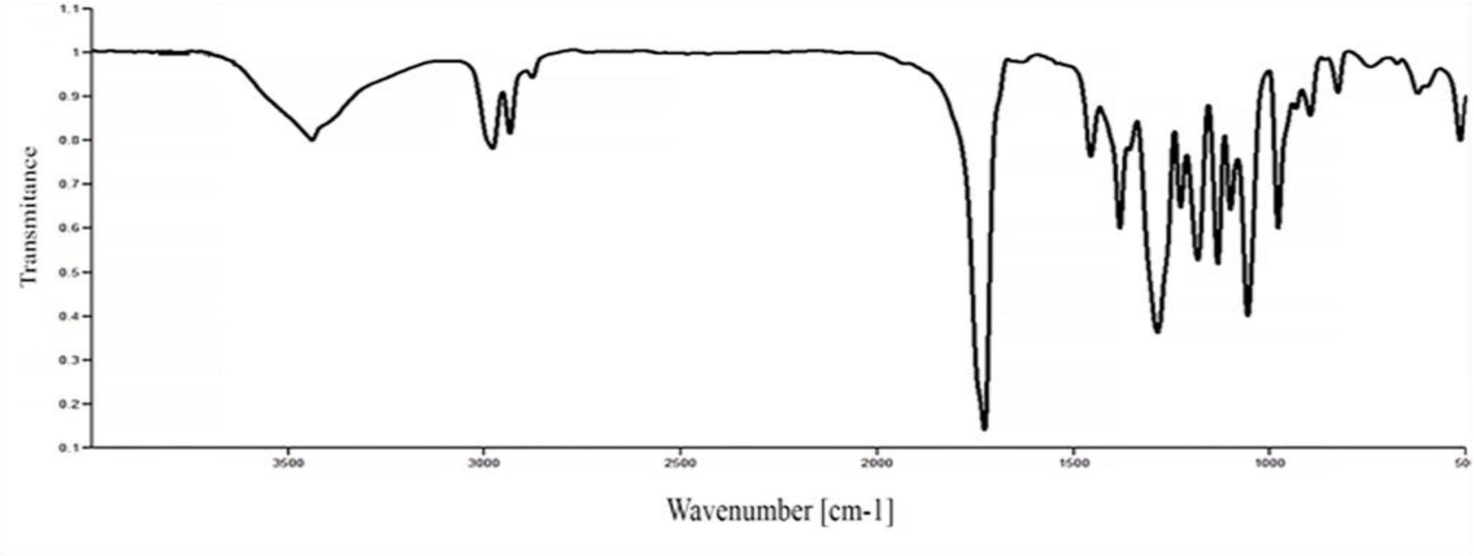

Figure 5. FTIR spectrum of PHB In this spectrum, the carbonyl ester band can be seen at the wave number of 1726 cm^{-1} (PHB index band).

Table 2. FTIR spectrum of polyhydroxybutyrate extracted from *Halarchaeum acidiphilum* strain

Linkage type	wave number
C-O stretch	1050, 1096, 1128, 1180
C-O-C stretch, crystalline	1128, 1263, 1278, 1289
C-O-C stretch, crystalline	1259, 1302
CH3 symmetric deformation	1378
CH3 asymmetric deformation	1449
CH2 deformation	1458
C=O stretch, crystalline	1726
C=O stretch, amorphous	1740
CH2, CH3, symmetric stretch	2875
CH2 asymmetric stretch	2933
CH3 asymmetric stretch, crystalline	2967
CH3 asymmetric stretch, crystalline	2974
CH3 asymmetric stretch, amorphous	2983
CH3 asymmetric stretch, crystalline	2995
CH3 asymmetric stretch, crystalline	3009
C=O overtone, crystalline	3436

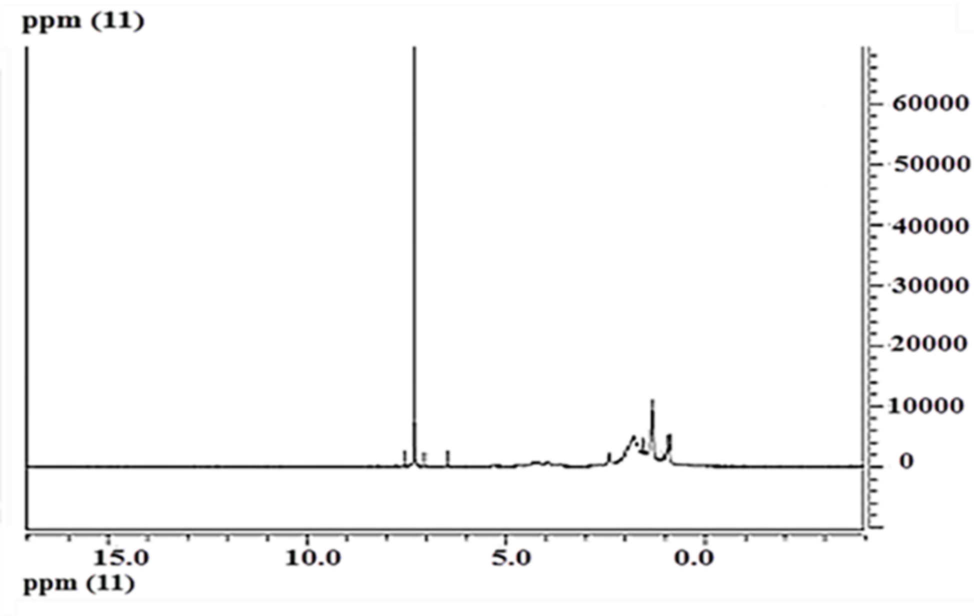

Figure 6 ¹HNMR spectrum of PHB extracted from *Halarchaeum acidiphilum* ASDL78

4. Discussion

In this research, Sudan black staining method, PHB measurement by chemical method, and UV absorption measurement were used to detect preliminary identification for PHB production .Sudan Black B staining is a valuable screening method for the presence of PHAs in haloarchaea cells and observation under the microscope. Staining with the lipophilic dye Sudan Black B as a high-affinity dye for staining PHAs has long been the focus of many scientists, including Murray et al. [12]. Comparative studies by Legat et al in 2010 showed that Nile blue a dye has a greater tendency to bind with PHAs than Sudan black B, but binds to other components non-specifically. Cell-forming lipid sometimes occurs and leads to ambiguous and false positive results; for this reason, it is preferable to use black Sudan for primary screening [13].

In this research, PHB granules were retrieved from sodium hypochlorite, which was used in the studies of Lillo and Rodrigues-Waller, was used to recover the granules [14]. From *Hla. acidiphillum* strain, the amount of PHB was 6.4 g/l in archaea culture medium at 37°C for seven days. Also, the highest efficiency for this isolate was reported as 71.58%. of the cell's dry weight is derived from PHB. The genera are *Haloarcula* and *Haloferax*, from which about 63% [15]. *Halopiger aswanensi* strain also accumulates about 53% of cell dry weight using n-butyric acid and sodium acetate as PHB carbon sources [10]. Our goal was to evaluate the effects of the different culture media formulations in the PHB production period, obtain practical information on this haloarchaeal strain's growth, and improve the PHB's production conditions. The highest amount of PHB produced among the carbon sources was related to the growth on beet molasses, and this shows that different inexpensive substrates, such as beet molasses, can be used to produce PHB. These results are consistent with Obruca et al with *Halomonas halophila* [16]. *Hla. acidiphillum* strain efficiently converted molasses to PHA (6.2 g/L); in this case, no hydrolysis was required before cultivation. In the study of Salgaonkar et al. *Bacillus megaterium* H16 used glucose and starch as the only carbon source [17]. One of the outstanding properties of this bacterial strain was the ability to convert cheap sugars and substrates into PHB, and the bacterial culture was able to use all tested sugars. Using cheap substrates can significantly reduce the cost of PHA production. The maximum PHA production by the strain *Hla. acidiphillum* was in the presence of yeast extract and ammonium chloride as a nitrogen source. Similar conditions for maximum growth rate have been observed by haloarchaeon Sech7a in a culture medium containing glycerol and yeast extract [18]. Some promising PHA producers of halophiles require complex nitrogen sources. An advantageous feature of *Hla. acidiphillum* as a PHA-producing bacterium is the growth of inexpensive minerals without needing complex and expensive nitrogen sources.

NaCl concentration is a critical parameter that affects PHA productivity when using halophilic bacteria and should be optimized for any new PHA-producing strain. Salt concentration plays an essential role in the PHB growth and production by halophiles, which was consistent with the Oren et al.'s study. They showed that extreme halophiles could not grow in a salt concentration below 10%. However, they had optimal growth in a salt concentration of 22% and a salt concentration of 30% [19]. In the study of Rodríguez-Contreras et al., the effect of salinity on the production of PHA by the bacterium *Bacillus megaterium* uyuni S29, the highest amount of PHB (2.22 g/l) was produced, and also the highest amount of biomass was obtained at a concentration of 45 g/l of NaCl, [20]. The studied strain showed its maximum growth at 37°C. While, research has shown that the haloarchaeon Sech7a strain, heat-resistant in terms of physiological properties, has optimal growth at 45°C [21]. Also, Bajpai et al. showed that the optimum temperature for the growth of *Natrinema* sp is 42°C [22].

This study, recorded maximum PHB production at pH 7.6 for the strain *Hla. acidiphillum* ASDL78. In some studies, better growth has been seen in neutral to slightly alkaline pH conditions. *Hfx. larsenii* HA8 is neutral-friendly [23]. It has been reported that the optimum pH for haloarchaeon Sech7a growth is equal to 7.5 [21]. and *Natrinema* sp BTSH10 shows the maximum growth at pH equal to 8 [24].

The result of the stirring effect on the amount of PHB production show that more mixing facilitates access to food and reduces the microbes' adhesion to the Erlenmeyer flasks' surface. In general, the amount of dissolved oxygen is low in water environments with a high concentration of sodium chloride. Since aerobic organisms need to provide enough oxygen to increase electron transfer and subsequently grow, aeration facilitates the penetration of atmospheric air into the culture medium. It provides the necessary oxygen for the growth of archaea [25]. However, oxygen limitation is a factor for PHB storage. Regarding FTIR-studies, the band at 1715 cm^{-1} represents C=O bands. Also, the pure PHB spectrum has dominant peaks at 2975 cm^{-1} and 2935 cm^{-1} corresponding to the C-H bands and a 1054 cm^{-1} peak corresponding to the C-O band. These values are very close to the values reported by Tanase et al., in which the main groups in the PHB spectrum are connected to the C-C bond pair with CH3 oscillation, and C-O-C oscillations in the 978 cm^{-1} and 895 cm^{-1} have been observed [26]. PHB spectra in the study of Chaiane et al. also showed the same results [27]. The ^{1}HNMR spectrum shows a characteristic peak of PHB near 5.25 ppm and another peak near 5.17 ppm, which is characteristic of PHB and valerate. According to Mukhopadhaye et al.'s results in 2005, , the production of some poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in addition to PHB is probable, which has more valuable properties than PHB [28 29].

5. DECLARATIONS

5.1. Acknowledgements

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Technology, Shahid Ashrafi Esfahani University. We would like to thank the officials at this college.

5.2. Conflict of Interest

The authors declare no conflict of interest.

5.3. Authors Contributions

Sheivan Chahianchy participated in conducting the initial experiments and studies and in preparing the main draft of the article. Soheila Abbasi as corresponding author for the study design, methodology, data analysis, participated in data collection, laboratory or experimental procedures, and interpretation of results. Maryam Jalili Tabaii and Martin Koller oparticipated in the critical revision of the article. Giti Emtiazi supervised the study, participated in the development of the conceptual framework, and critically reviewed and edited the article. All authors read and approved the final version of the article.

5.4. Using Artificial Intelligent chatbots

No AI chatbots or tools were used in this research

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