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Characterization of bioplastics produced by haloarchaeon *Haloarcula* sp strain NRS20 using cost-effective carbon sources

Nashwa Hagagy^{1,*} , Amna A N Saddiq², Hend M Tag¹ , Hamada Abdelgawad³ and Samy Selim⁴¹ Department of Biology, College of Science and Arts at Khulis, University of Jeddah, Jeddah, Saudi Arabia² Department of Biology, College of Science, University of Jeddah, Jeddah, Saudi Arabia³ Integrated Molecular Plant Physiology Research, Department of Biology, University of Antwerp, Antwerp, Belgium⁴ Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Jouf University, Sakaka, 72341, Saudi Arabia

* Author to whom any correspondence should be addressed.

E-mail: niibrahem@uj.edu.sa**Keywords:** haloarchaea, bioplastics, polyhydroxybutyrate (PHB), FTIR, HPLC

Abstract

As good models for developing techniques, *Haloarchaea* are using as cell factories to produce a considerable concentration of bioplastics, polyhydroxyalkanoate (PHA), polyhydroxybutyrate (PHB), and polyhydroxyvalerate (PHV). In this study, low-cost carbon sources by Sudan Black staining was applied for screening haloarchaea a hypersaline environment (southern coast of Jeddah, Saudi Arabia). The growth of the selected isolate and PHB-production under different carbon sources, temperature, pH values and NaCl concentrations were investigated. The biopolymer was extracted and quantitatively measured. The biopolymer was qualitatively identified by Fourier-transform infra-red analysis (FTIR) and High Performance Liquid Chromatography (HPLC). The potential *Haloarcula* sp strain NRS20 (MZ520352) could significantly accumulate PHB under nutrient-limiting conditions using different carbon sources including starch, carboxymethyl cellulose (CMC), sucrose, glucose and glycerol with 23.83%, 14%, 11%, 12% and 8% of PHB/CDW respectively under 25% NaCl (w/v), pH 7, at 37 °C. The results of FTIR pattern indicated that the significant peak at 1709.22 cm⁻¹ confirmed the presence of the ester carbonyl-group (C=O) which is typical of PHB. HPLC analysis indicated that produced PHB was detected at 7.5 min with intensity exceeding the standard PHB at 8.0 min. Few potential species of haloarchaea were reported for economical PHB-production, here, *Haloarcula* sp strain NRS20 showed high content of PHB, exhibited a promising PHB-producer using inexpensive sources of carbon.

1. Introduction

Bioplastics are biocompatible and biodegradable polymers that have been suggested as a replacement for oil-based plastics (Luengo *et al* 2003, Thompson *et al* 2009). These biopolymers are produced from a wide-range of *Archaea* and *Eubacteria* by using various sources of carbon. Particularly under stressful condition, biopolymers are used as intracellular storage molecules to support bacteria and archaea survive in imbalanced environments (Karray *et al* 2021). In this regard, *Haloarchaea* that can survive the high salinity condition, are preferred for a variety of these possible applications. For instance, they showed unusual metabolic capabilities including their capability to produce bioplastic. As a result, some haloarchaea can manufacture high levels of commercial bioplastics such polyhydroxybutyrate (PHB), polyhydroxyalkanoate (PHA), and polyhydroxyvalerate (PHV). Furthermore, in terms of sterilization of cultures, growth rate, and other factors, the development of such unique microorganisms at the industrial-scale has significant benefits over other producers of bioplastic (Simó-Cabrera *et al* 2021).

High availability of carbon substrate and deficiencies of essential elements such as phosphorus and nitrogen inducing nutrient-limiting stress conditions that can stimulate the synthesis of bioplastic (Rehm 2007). In this context, synthesis of PHB/PHA by haloarchaea genera such as *Halobacterium*, *Halococcus*, *Halorubrum*,

Haloferax, *Natronococcus*, *Natronobacterium*, *Halopiger* and *Haloarcula* has been documented in several investigations (Legat *et al* 2010, Poli *et al* 2011; Lynch *et al* 2012, Hermann-Krauss *et al* 2013). Giving the high efficiency of bioplastic-production, more studies are needed to investigate the capability of haloarchaeal species for producing bioplastics, particularly in terms of exploiting low-cost carbon sources (Simó-Cabrera *et al* 2021).

The purposes of this study was to screen promising PHB/PHA-producing haloarchaeal strains and to adjust best growing conditions of using the best carbon sources for PHB/PHA synthesis. Then, we extracted and characterized the biopolymer by FTIR from the promising strain *Haloarcula* sp strain NRS20 isolated from a hypersaline environment in Jeddah, Saudi Arabia.

2. Materials and methods

2.1. Sampling and site description

Brine and Sediment samples were collected from a hypersaline environment (southern coast of Jeddah in Saudi Arabia (21°10'16.04"N, 39°11'5.94"E). This collection was in September, 2019. All collected samples were stored in fridges at 4 °C. After arrival to the lab, microbiological examination was carried out within the first day of collection.

2.2. Enrichment, isolation and growth conditions

The samples were cultured in a PHA-accumulating medium described (Han *et al* 2010). A liter of HSM media contained 250 g NaCl, 20 g MgSO₄·7H₂O, 2.0 g KCl, 3.0 g trisodium citrate, 8.0 g Na₂CO₃, 37.5 mg KH₂PO₄, 50 mg FeSO₄·7H₂O, 0.36 mg MnCl₂·4H₂O, and 1 g yeast extract. Media was adjusted at pH of 7.2 and supplemented with 10 g l⁻¹ glucose, as carbon source, incubated at 37 °C for 14 days at 180 rpm. For isolation of halophilic archaeal strains accumulating PHB and/or PHA, samples were employed serial dilution and 1 ml of each dilution were plated onto HSM as described above. Successive cultivation was carried out to obtain pure isolates on HSM.

2.3. Screening for PHB/PHA-producing haloarchaeal isolates

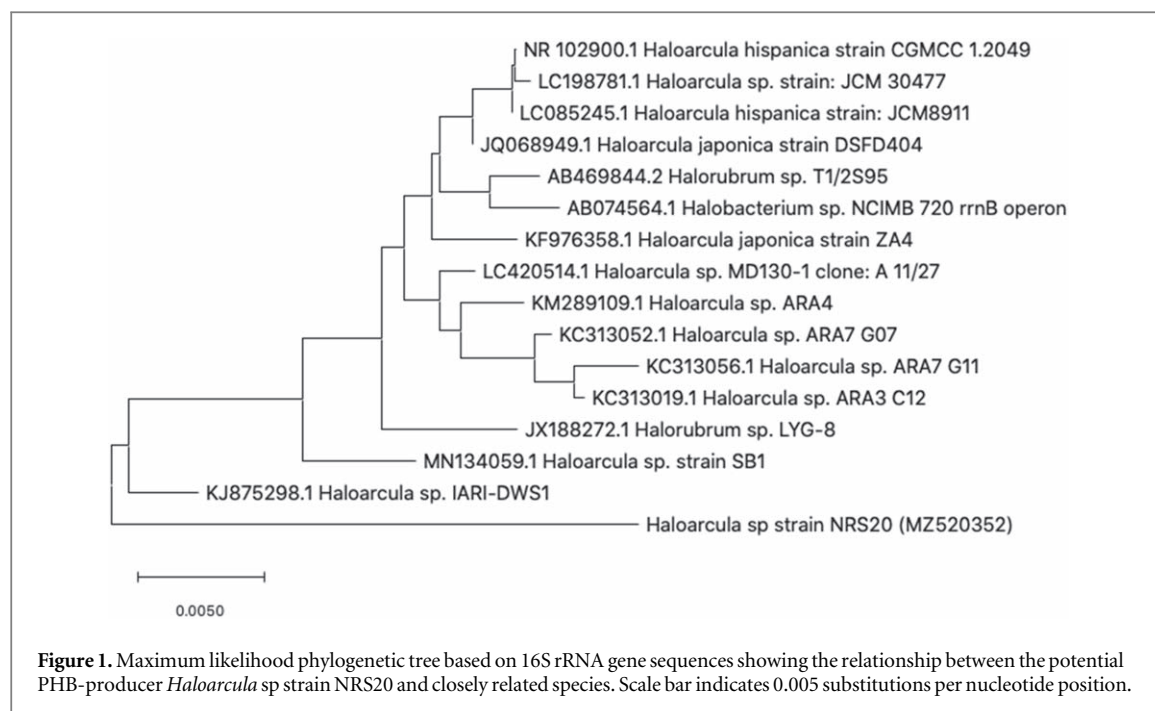
A total of ten distinct halophilic archaeal isolates collected from 2 weeks culture that was incubated at 36.5 °C, were used for screening their potential as PHB/PHA producers. Here staining with Sudan Black B was used (Murray *et al* (1994). The cells, from early stationary growth phase, were smeared on a clean glass slide. The cells heat-fixed to stain them (10 min) with a 3% of Sudan Black B (w/v in 70% ethanol), then they were immersed in xylene until totally decolorized. The sample was counterstained safranin (Sigma; 5% w/v aqueous solution), then they were rinsed and dried. The cells were examined under microscopy (phase contrast, Nikon Eclips E600). The cells that appear as blue-black under microscope were identified as positive strains of PHB/PHA. Moreover, the bacterial type strain, *Escherichia coli* (ATCC35218) are used as negative control.

2.4. Identification of potential strain

DNA was extracted by extraction kit (QIAGEN, Hilden, Germany). Extracted DNA was used for molecular identification. A set of *Archaea*-universal primers (Invitrogen, USA) was used to amplify the 16S rRNA gene i.e., 5'-ATT CCG GTT GAT CCTGCC GG-3' primers (positions 6–25 in *Escherichia coli* numbering) and 5'AGG AGG TGA TCC AGC CGC AG-3' primers (positions 1540–1521) (Ventosa *et al* 2004). The PCR conditions (50 µl of reaction system, 30 reaction cycles, 94° C denaturation 1 min, 60° C annealing 1 min, 72° C extension 1 min 30 s. The samples were sent to MacroGen Company (Seoul, Korea). To get a preliminary identification of the strain, the sequences were examined using BLAST (<http://www.ncbi.nlm.nih.gov/> BLAST) and the cluster analysis was done by The MEGA X software.

2.5. Optimization of growth conditions of the potential PHB/PHA-producing haloarchaeal strain

A total of 100 µl aliquot of selected culture was obtained in exponential phase. The aliquotes were inoculated into 100 ml medium contained glycerol under 37 °C and 180 rpm agitation rate. Up to two weeks, growth in PHB/PHA production medium was monitored by spectrophotometer at 600 nm every 48 h. In PHB/PHA production medium, the effects of pH (5–9), temperature (4, 20, 37, 45, 55 and 65 °C) and NaCl concentration (100–350 g l⁻¹) on the growth of the selected isolate were investigated. Under optimum growth conditions, the effect of starch, carboxy-methyl cellulose (CMC), sucrose, glucose, and glycerol on production of PHB/PHA by the selected strain was investigated. 10 g l⁻¹ of each carbon sources were filtered separately and transferred to the production medium.



2.6. Extraction of the biopolymer

A total of 1 ml aliquot of the selected culture was cultured under optimum conditions in PHB/PHA-producing medium, incubated at 37 °C. Then, at early stationary phase, the culture was pelleted for 25 min at 5000 rpm. The pellet's dry weight was determined, and it was subsequently washed with acetone and ethanol. To recover PHB/PHA, an equal volume of 6% Na hypochlorite was applied to suspend the pellet, which was then incubated for 10 min at 37 °C. The lipid granules were then sedimented by centrifugation for 30 min, at 5000 rpm. The pellet was cleaned in acetone solvent and ethanol (100%) before being treated with hot chloroform. Whatman filter paper (grade 1, Cat No 1001–110) was employed to filter away the cell remnants after the pellet was dissolved in chloroform, leaving only PHB/PHA in the chloroform solution. Evaporation of filtrate at 40 °C, then we calculated the weight of the extracted PHB (Kumar 2017). % of PHB/PHA accumulation was calculated according to Munir *et al* 2015 and Sathiyarayanan *et al* 2017.

2.7. Characterization of the biopolymer by FTIR

FTIR spectroscopy (Perkin Elmer Spectrum GX Range Spectrometer, Bridgeport Avenue, USA) was used for qualitative identification and detection of functional groups such as CH, CH₂, CH₃, C=O, C–O and OH, which are key determinant for the presence of PHB in the extracted biopolymer (Mohapatra *et al* 2017).

2.8. Characterization of polymer by HPLC

Polymer characterization was carried out using HPLC supplied with C18 150 mm × 4.6 mm, 5 cm (Sciex Exion LC HPLC, EquipNet, Inc., US) column at 0.300 ml min^{−1} of flow rate and at wavelength of 306 nm. Mobile phase was a mixture of chloroform: phosphate buffer (0.1 M), pH 4.5 (Duvigneau *et al* 2021) and the chromatographic course was for 9.5 min.

3. Results and discussion

3.1. Isolation, screening and identification of potential strain

In comparison to bacteria, haloarchaea have distinct advantages as bioplastic-producers. Therefore, it is critical to find novel PHB/PHA producers within haloarchaea for cost-effective polymer production (Zhao *et al* 2015). In this study, an attempt has been made to screen PHB/PHA-producing haloarchaea isolated from a Solar Saltern, Jeddah, KSA, using low-cost carbon sources by staining means (Sudan Black B). Out of ten distinct isolates, based on shape and color of colonies, one potential strain NRS20 showed the exist of black granules when stained with the Sudan Black B, which confirming its capability of PHB and/or PHA biosynthesis (Karray *et al* 2021). 16S rRNA gene sequencing revealed that target strain belong to *Haloarcula* (unclassified species) with about 90% sequence similarity. The 16S rRNA gene data was deposited under the accession number **MZ520352** in the NCBI and GenBank nucleotide sequence databases (figure 1). The production of polyhydroxybutyrate

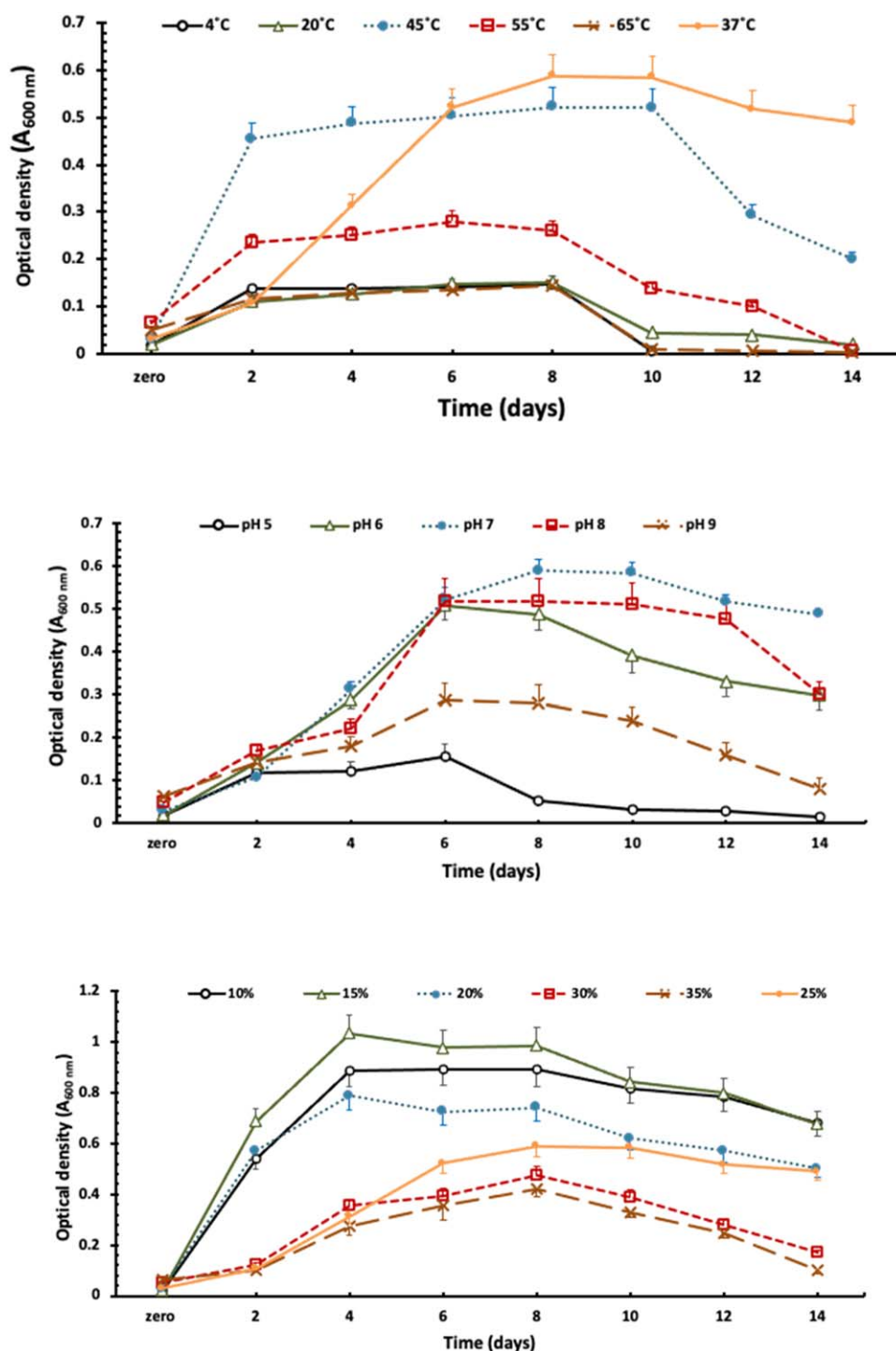


Figure 2. Effect of physical factors on the growth of PHB-producer *Haloarcula* NRS20 grown on PHB production medium with glycerol (10 g l^{-1}) as a carbon source; A. influence of temperature, B. influence of pH, C. influence of different NaCl concentrations.

(PHB) was reported by *Haloarcula marismortui* by Pramanik et al (2012) using vinasse as a carbon source, and also *Haloarcula tradensis* by Karray et al (2021) by using starch as carbon source. Several *Haloarcula* species (e.g., *H. japonica*, *H. amylolytica*, and *H. argentinensis*) can produce PHB (Nicolaus et al 1999, Han et al 2010). Although there are several reports, this is the first report for observation of members of haloarchaea in southern Saudi solar saltern as PHB and/or PHA producers. Moreover, members of *Haloarcula* have great biotechnological significance because they are well understood on genetic level (Han et al 2007, Karray et al 2021). Thus, further studies are needed for exploring of novel species of *Haloarcula* as PHB-producers. *Haloarcula* sp strain NRS20, reported in this study, was pleomorphic in cell shape, non-motile, small colonies (1.5 mm), translucent, convex and showed orange-red pigmentation.

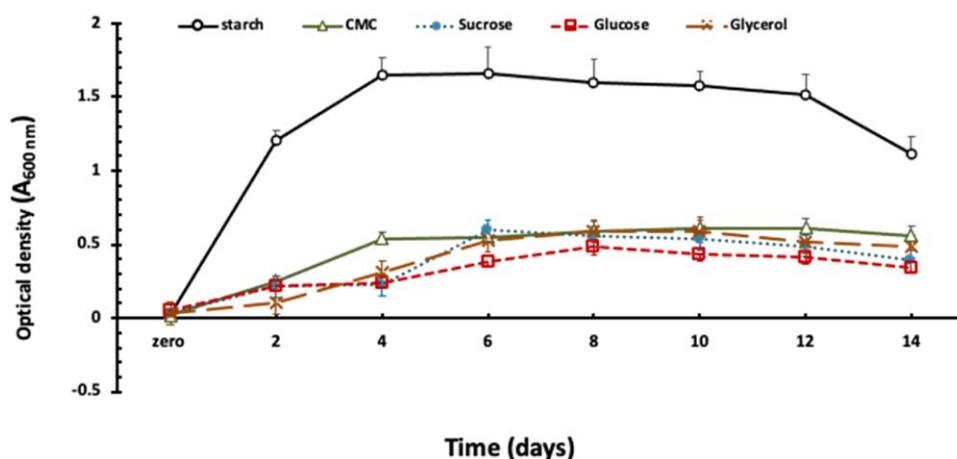


Figure 3. Effect of different carbon sources on the growth of PHB-producers *Haloarcula* sp NRS20 on PHB production medium.

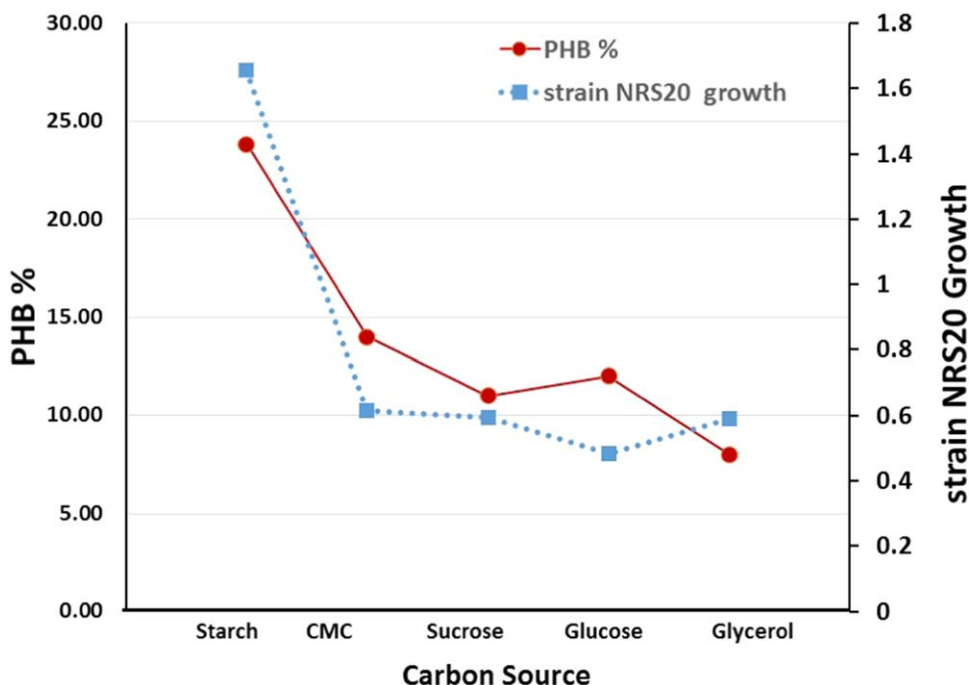


Figure 4. Optimization of different carbon sources for PHB-production by *Haloarcula* sp strain NRS20 grown at 37° C and 25% NaCl (w/v).

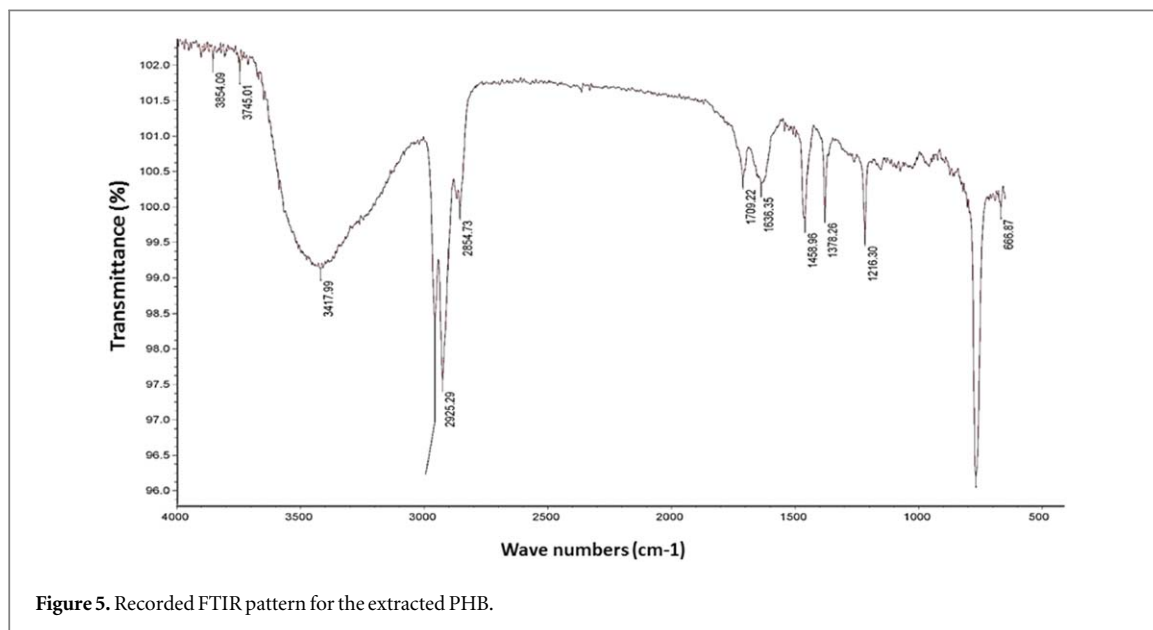
3.2. Optimization of cultural conditions for polymer production

The growth patterns of potential strain on PHB-production medium including glucose as a carbon source were investigated at various temperatures, pH, and salinity (figures 2(A)–(C)). The effects of these variables were investigated in order to increase PHB yield. The strain grew at temperatures (30 to 50° C). The best temperature for growth was 37° C. The rate of growth increased until 37° C, then slowed at higher temperatures (figure 2(A)). Other studies of PHB-production by haloarchaea (Legat *et al* 2010, Poli *et al* 2011, Lynch *et al* 2012, Hermann-Krauss *et al* 2013, Karray *et al* 2021) revealed optimal growth at 25% with the same carbon source, whereas the strain NRS20 survives salinity stress for growth at high concentrations ranging from 10% to 35% (w/v) with an optimal growth at 15% (figure 2(B)). Potential strain NRS20 grew at a pH (5–9), and the optimum pH was 7 (figure 2(C)). This reveals that the maximal specific growth rates of strain NRS20 were slightly different from those of species of *Haloarcula* as described in previous studies (Nicolaus *et al* 1999, Han *et al* 2010, Karray *et al* 2021).

The potential strain showed a considerable growth and PHB-production using different substrates (starch, CMC, glucose, glycerol and sucrose) as shown in figure 3, with an optimal growth and biopolymer-production by using the starch as carbon and energy source after 6 days of growth at 37° C, however, there was a considerable

Table 1. The percentage amount of PHB produced with cell dry weight.

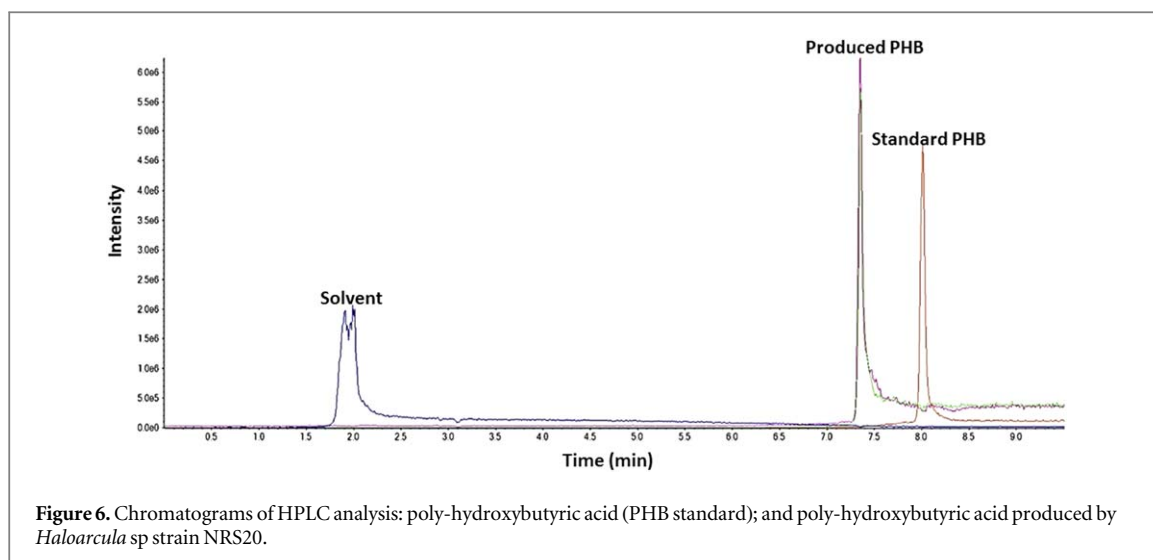
Carbon sources	CDW (g l ⁻¹)	PHB (μg ml ⁻¹)	PHB %
starch	3.44	8.2	23.83
CMC	1.27	3.035	14
Sucrose	1.24	2.946	11
Glucose	1.01	2.397	12
Glycerol	1.22	2.917	8

**Figure 5.** Recorded FTIR pattern for the extracted PHB.

growth after only two days (figure 3), this observation was a remarkable point for using the starch for enhancement of haloarchaeal growth, which known as slow-growers. Table 1 showed the quantitative PHB-production by the potential strain *Haloarcula* sp strain NRSA20. With regards to previous studies of PHB-production by *Haloarcula* sp, only two studies reported using starch (carbon source) for PHB-production including *Haloarcula* sp. IRU1 which produced 57% PHB/CDW (Taran 2011), and study of Karray et al (2021) who reported PHB-accumulation 1.42% PHB/CDW by *Haloarcula* strain CEJ48–10. Other studies (Nicolaus et al 1999, Han et al 2010) reported PHB-production by *Haloarcula japonica*, *Haloarcula amylolytica*, and *Haloarcula argentinensis* with yields obtained from glucose 0.5, 4.4, and 6.5% (of CDW), respectively. In this report, *Haloarcula* sp strain NRSA20 can accumulate PHB 23.83%, 14%, 11%, 12% and 8% of PHB/CDW, by using 10 g l⁻¹ of starch, CMC, sucrose, glucose and glycerol respectively as shown in figure 4, which considered as high PHB content in compared with previous studies of *Haloarcula* species (Han et al 2007, Han et al 2010, , Karray et al 2021).

3.3. FTIR analysis

In the current FTIR pattern (figure 5), the recorded strong band at 3417.99 cm⁻¹ are related to Hydrogen bonding produced by the terminal OH groups (Gumel et al 2012, Ramezani et al 2015), which is characteristic feature of PHB and PHAs (Hedrick et al 1991, Fleming and Williams 2019). The sharp peak around 2933 cm⁻¹ is identified as C–H stretching methyl, meanwhile peak at 2925 cm⁻¹ is assigned to C–H methylene groups (Mostafa et al 2020). The significant peak at 1709.22 cm⁻¹ confirmed the presence of the ester carbonyl group (C = O) typical of PHB (Sabarinathan et al 2018, Mostafa et al 2020). A mutual property in all of the structures of PHB and PHAs (Mostafa et al 2020, Mongili et al 2021). This is interconnect to the findings of Mongili et al (2021) who produced PHB from genetically modified *E. coli* strain and recorded ester carbonyl group at 1720 cm⁻¹. The peak obtained at 1458.96 cm⁻¹ is assigned to the asymmetric bending of CH₂ group, while the next recorded band around 1378.26 cm⁻¹ is related to CH₃ group in accordance to the previous studies of Sabarinathan et al (2018) and Narayanan et al (2021). Finally, the additional peaks centered between 1000 cm⁻¹ and 1300 cm⁻¹ is related to the stretching of the C–O ester bond (Narayanan et al 2021). In conclusion, the current FTIR pattern (figure 5) is matched with previous reported FTIR spectrum of PHB (Ramezani et al 2015).



3.4. HPLC analysis

Figure 6 shows HPLC diagrams of standard PHB (Sigma) and the PHB produced by *Haloarcula* sp strain NRS20. The produced PHB was detected at 7.5 min with intensity exceed the standard PHB at 8.0 min the present results revealed that strain NRS20 as promising efficiency for the production of PHB as compared with related species motioned in previous work (Soni *et al*, 2012). Moreover, Karray *et al* (2021) concluded that genera *Haloarcula* and *Halorubrum* were considered as promising candidates for PHB-production.

4. Conclusions

The promising archaeal isolate, *Haloferax* sp strain NRS20 (MZ520352) was obtained in pure culture from the hypersaline environment located at the southern coast of Jeddah city, Saudi Arabia. *Haloferax* sp strain NRS20 was able to use starch, CMC, sucrose, glucose and glycerol as the sole carbon sources for PHB-production. The highest yields of PHB-synthesis were 23.83%, 14% by using starch and CMC respectively at 37° C, pH 7, and 25% NaCl (w/v). FTIR pattern revealed significant groups which are typical characteristics feature of PHB. Future research will focus on optimization employing other low-cost feedstocks to improve both quality and PHB productivity.

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Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

Conflicts of interest

The authors declare that there are no conflicts of interest.

ORCID iDs

Nashwa Hagagy  <https://orcid.org/0000-0002-8725-5588>

Hend M Tag  <https://orcid.org/0000-0001-9417-1844>

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