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Potential Biotechnological Applications of Products from the Haloarchaeon *Haloterrigena turkmenica*

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ABSTRACT

The extreme halophilic archaeon Haloterrigena turkmenica showed capacity of producing several classes of molecules of high interest in biotechnological fields. It was able to produce pigments and polyhydroxyalkanoates (PHA) when grown in selected growth conditions. In the present work, the microorganism was cultivated in halobacteria growth medium added with 1% (w v^{-1}) glucose (HMG1), and in the same medium devoid of some components (nutrient deficient medium-NDMG1). Cells of H. turkmenica exhibited an orange colour when grown in HMG1, and after extraction with methanol, the membrane extract showed an absorption spectrum with the characteristic bell shape of yellow pigments. HPLC analysis allowed to identify astaxanthin among the pigments produced. Synthesis of PHA was followed in NDMG1 by optical microscopy with Nile Blue A, a dye which specifically bounds to poly-3-hydroxybutyrate (PHB). The biopolymer was proved to be produced during the stationary growth phase. These preliminary data show the high versatility of H. turkmenica in producing different valuable compounds when cultivated in defined growth medium conditions.

Keywords: Astaxanthin, *Haloterrigena turkmenica*, Polyhydroxyalkanoates, Pigments

ABBREVIATIONS

HMG1: Halobacteria Medium with 1% Glucose; NDMG1: Nutrient Deficient Medium with 1% Glucose; PHA: Polyhydroxyalkanoates; PHB: Poly-3-hydroxybutyrate; PHBV: Poly(3-hydroxybutyrate-co-3-hydroxyvalerate); UV/Vis: Ultraviolet/Visible

INTRODUCTION

Among the vastness of compounds produced by plants, animals and microorganisms, pigments have received significant attention due to their industrial applications and their potential beneficial effects on human health. They are powerful natural antioxidants and many of them are also used as colorants in the food industry to enhance the colour in farmed salmons, such as astaxanthin and canthaxanthin [1]. Among halophiles, *Dunaliella salina* represents the better known carotenoid-producing microorganism [2], although haloarchaea are widely recognized as highly active pigment producers [3]. These microorganisms have colours varying from yellow-orange to pink-red as consequence of the kind of pigments they synthesize. Characteristic pigments produced by haloarchaea are bacterioruberin, a red C_{50} carotenoid, and its anhydro derivatives. *Halorubrum* sp. SH1, isolated by de la Vega et al. [4] in the Southwest of Spain, produced bacterioruberin, bisanhydrobacterioruberin and trisanhydrobacterioruberin, whereas *Haloferax mediterranei* was proved to synthesize bacterioruberin, monoanhydrobacterioruberin and bisanhydrobacterioruberin [5]. Other carotenoids such as phytoene, β-carotene, canthanxanthin and astaxanthin are also produced by several of these species [6].

Interest in pigments of microbial origin is rising because of their many biotechnological applications and of the great attention by consumers toward natural health-protective compounds [3].

Polyhydroxyalkanoates (PHA) are very attractive macromolecules from a biotechnological point of view. They are renewable biopolymers synthesized by several species of Bacteria as well as halophilic Archaea and are accumulated inside the cells under peculiar growth conditions, namely when an excess of the carbon source occurs and when an essential nutrient such as nitrogen or phosphorous is depleted, thus providing an internal reserve of carbon and energy [7]. PHA possess features similar to those of synthetic plastics, such as thermoplastic and elastomeric properties, but contrarily to conventional plastics, they are biodegradable [8]. In the last decades, PHA has received great attention as potential material for medical uses, for instance surgical or medical scaffolding material, due to their biocompatibility [9]. Due to their significant properties, studies about PHA biosynthesis and applications are continuously increasing, and the research for microorganisms able to produce PHA is active. Recently, Salgaonkar and Braganca [10] reported on the capacity of the extreme halophilic archaeon *Halogeometricum borinquense* strain E3, to synthesize the co-polymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV). This halophile accumulated a large quantity of the biopolymer inside the cells (73.51% of cell dry weight). In addition, as the high costs of production limit PHA diffusion, liquid and solid residues from industries are exploited in order to substitute the carbon source with cheaper ones. Molasses, cheese whey, glycerol and olive mill wastewater (OMW) are only few examples of by-products used for PHA production [11,12]. As example, the haloarchaeon *Haloferax mediterranei* exhibited growth capacity in the presence of OMW with 43% PHBV cell dry weight^{-1}, and accumulated PHBV at 70% of cell dry weight when cultivated with 25% pretreated vinasse [13,14].

Haloterrigena turkmenica is a haloarchaeon belonging to the euryarchaeal family of *Halobacteriaceae*, isolated from a sulphate saline soil in Turkmenistan, and requiring at least 2 M NaCl for growth. As the microorganism showed capacity to synthesize exopolysaccharides when grown in halobacteria medium added with different sugars [15], we investigated the ability of producing additional valuable compounds. This short report deals with preliminary results concerning production of pigments and PHA when *H. turkmenica* was grown in different medium conditions, thus attesting the great versatility of the microorganism and the possibility to direct the production of specific classes of compounds in relation to the growth medium used.

MATERIALS AND METHODS

Chemicals

Chemicals for the preparation of the growth media, Nile Blue A, astaxanthin (98%), and beta-carotene (>95%) were purchased from Sigma-Aldrich Co. Methanol (99.8%) and acetone were from Carlo Erba Reagents. Acetonitrile (99.99%) was obtained from Fisher Chemical. Canthaxanthin (99%) was from Roche and lutein (10%) was from FloraGLO, Kemin. All other reagents were of the highest grade commercially available.

Media and growth conditions

Haloterrigena turkmenica (DSM-5511), obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), was revitalized following the manufacturer's instructions, and then grown aerobically in a rotary water-bath shaker (Aquatron, Infors AG, Switzerland) at 37°C and 180 rpm. For pigment production, halobacteria medium M372 (DSMZ catalogue) was added with 1% (w v^{-1}) glucose (HMG1), whereas for PHA biosynthesis a modified medium (nutrient deficient medium-NDMG1) was prepared (Table 1). Archaeal growths were followed by optical density measurement at 600 nm (Varian spectrophotometer DMS-200, Varian Analytical Instruments, Leini, Torino, Italy).

Pigment detection

For pigment detection, cells from 500 mL HMG1 growth culture were harvested after 120 h from the beginning of the growth by centrifugation at 17,000 rpm for 30 min at 4°C. For the extraction of pigments, the frozen cells (1 g) were thawed and treated with 1.2 mL methanol for 60 min under magnetic stirring. The yellow supernatant was separated by centrifugation at 13,200 rpm for 30 min at 4°C and the cells were re-suspended in 1.2 mL methanol, stirred, and centrifuged again. The whole extract was analyzed by UV/Vis scanning and the spectrum was recorded between 300 and 600 nm (Cary 100, Varian Analytical Instruments). The amount of pigments in the extract was expressed as µg per gram of dry cells. All steps were performed in the dark to avoid isomerization phenomena.

Pigment analysis

Pigment separation was performed by using HPLC (Agilent 1200 Series system, Agilent Technologies, Tokyo)

Table 1: *Haloterrigena turkmenica* growth media

HMG1		NDMG1	
Nutrient	**Quantity**	**Nutrient**	**Quantity**
Yeast extract	5.0 g L^{-1}	Yeast extract	1.0 g L^{-1}
Casamino acids	5.0 g L^{-1}	Casamino acids	-
Sodium glutamate	1.0 g L^{-1}	Sodium glutamate	1.0 g L^{-1}
Potassium chloride	2.0 g L^{-1}	Potassium chloride	2.0 g L^{-1}
Sodium citrate	3.0 g L^{-1}	Sodium citrate	-
Magnesium sulphate	20.0 g L^{-1}	Magnesium sulphate	20.0 g L^{-1}
Manganese chloride tetrahydrate	0.36 mg L^{-1}	Manganese chloride tetrahydrate	0.36 mg L^{-1}
Iron (II) chloride tetrahydrate	36.0 mg L^{-1}	Iron (II) chloride tetrahydrate	36.0 mg L^{-1}
Sodium chloride	200.0 g L^{-1}	Sodium chloride	200.0 g L^{-1}
Glucose	10.0 g L^{-1}	Glucose	10.0 g L^{-1}
pH	7.2	pH	7.2

equipped with a µ-Bondapack C18 (250 × 4.6 mm) column and a detector UV/Vis (Agilent Technologies, Tokyo). The elution was carried out for 90 min at a flow rate of 0.5 ml min^{-1} using a mobile phase composed of 99.8% methanol/acetonitrile (3:1 v v^{-1}). Peak identification was performed at 477 nm by comparing the retention times with those obtained for individual standards (astaxanthin, canthaxanthin, lutein and beta-carotene), using the LC3D ChemStation software (Rev.A.10.02 [1757], 1990-2003, Agilent Technologies, USA).

PHA detection

In order to follow the production of PHA, aliquots from 500 mL NDMG1 growth culture were withdrawn at different stages of *H. turkmenica* cultivation. Nile Blue A was used as dye to observe PHA granules inside the cells by microscope. Briefly, a drop was placed onto the object slide, and stained with 1% (w v^{-1}) Nile Blue A at 55°C for 15 min. Then, the slide was washed with water and submerged in 8% acetic acid for 1 min. After covering with the cover slide, the growth was observed in dark field under fluorescent light (Leica inverted microscope DMI 6000; objective PL APO HCS 100x).

RESULTS AND DISCUSSION

Preliminary studies on *H. turkmenica* cultivation in HMG1 were carried out with the purpose to establish the most appropriate time of cell harvesting for pigment extraction. Figure 1 shows the growth curve of the microorganism over 11 days. Exponential growth phase started at 24 h and after 144 h the growth stopped ($OD_{600\ nm}$ 18.6). From that point, the decline phase began, with a slow cell density decrease up to 216 h (OD_{600nm} 16.2). Optical density continued to decrease until 264 h reaching the value of 13.4. Based on these observations, we decided to stop *H. turkmenica* growth after 120 h (OD_{600nm} 17.9), to avoid loss of pigments due to possible cell lysis phenomena. The collected orange biomass was treated with organic solvents in order to extract the pigments. Methanol was a solvent better than acetone for pigments removal as the biomass resulted discolored after the extraction steps. The total amount of pigments produced by *H. turkmenica* was 32.6 µg g dry $cell^{-1}$. Although this quantity may seem quite low, the yield among extreme halophilic microorganisms is very variable. In fact, the pigment yield of *Haloarcula japonica* was 335.0 µg g dry $cell^{-1}$, whereas *Halobacterium salinarum* produced 45 µg g dry $cell^{-1}$ [16,17].

The yellow extract was analyzed by UV/Vis scanning and the resulting absorption spectrum showed the characteristic shape of the pigments astaxanthin and canthaxanthin (Figure 2). HPLC analysis revealed several peaks at 477 nm (Figure 3); however, it was possible to confirm only the presence of astaxanthin in the extract sample after comparison of the retention times (rt) with those of four different available standards: astaxanthin (rt: 10 min), lutein (rt: 11.5 min), canthaxanthin (rt: 17.5 min) and beta-carotene (rt: 87 min). Astaxanthin identification was in agreement with what already described in literature for other halophilic Archaea. In fact, although the main pigments of halophilic Archaea are the C_{50} carotenoids bacterioruberin and its derivatives, the presence of astaxanthin has been reported [18,19].

Production of PHA was observed in NDMG1 medium. Usually the biosynthesis of PHA is connected to growth stressed conditions [20], and in the case of *H. turkmenica*, the biopolymer was produced under limitation of nitrogen source (Table 1). The changed cultivation conditions with respect to HMG1 medium lowered the biomass yield and slowed down the whole time of growth, indicating a condition of stress (Figure 4). In fact, the time required for reaching the stationary phase was more than doubled in comparison to the HMG1 growth. Samples, withdrawn at different stages of growth, were subjected to Nile Blue A staining for PHA detection. Usually, the PHA produced by haloarcahea

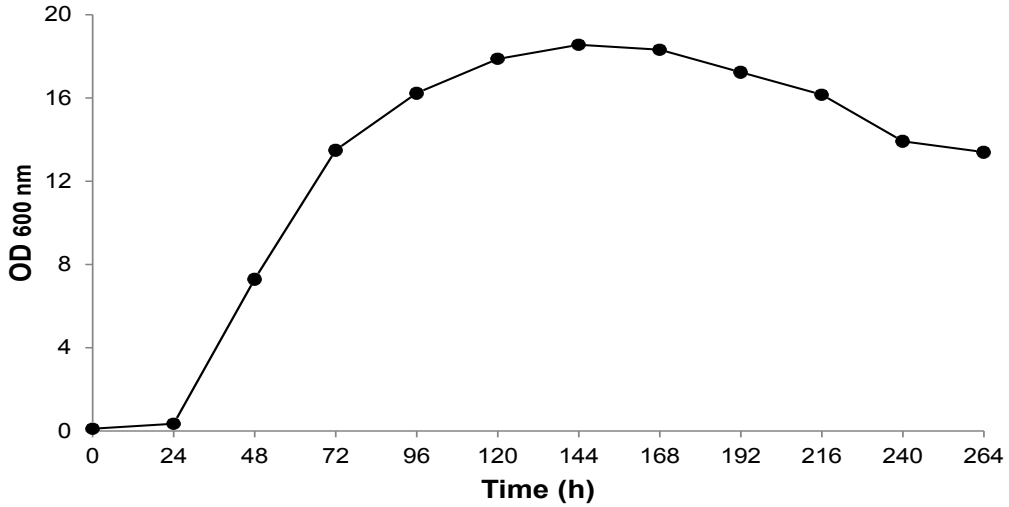

Figure 1: Growth curve of *Haloterrigena turkmenica* in HMG1 medium

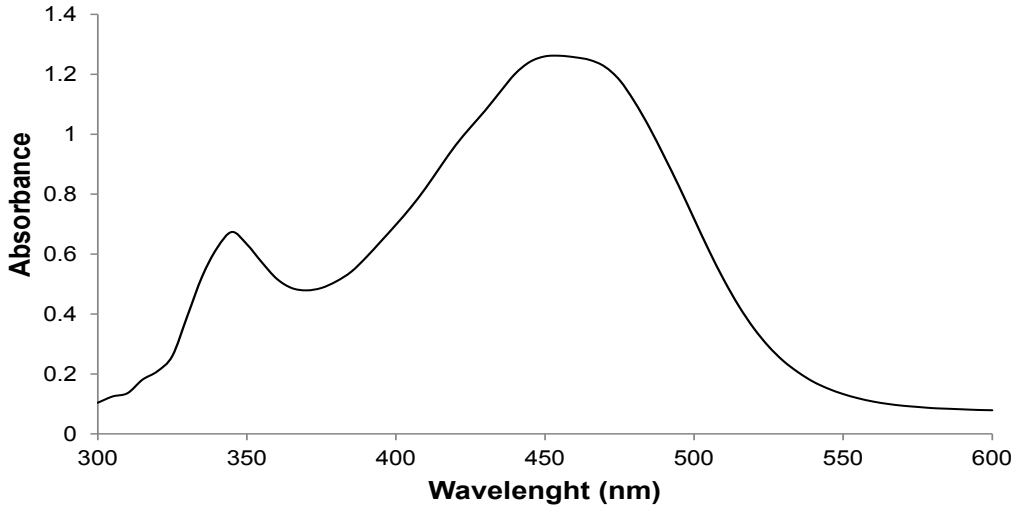

Figure 2: UV/Vis spectrum of *Haloterrigena turkmenica* extract in methanol

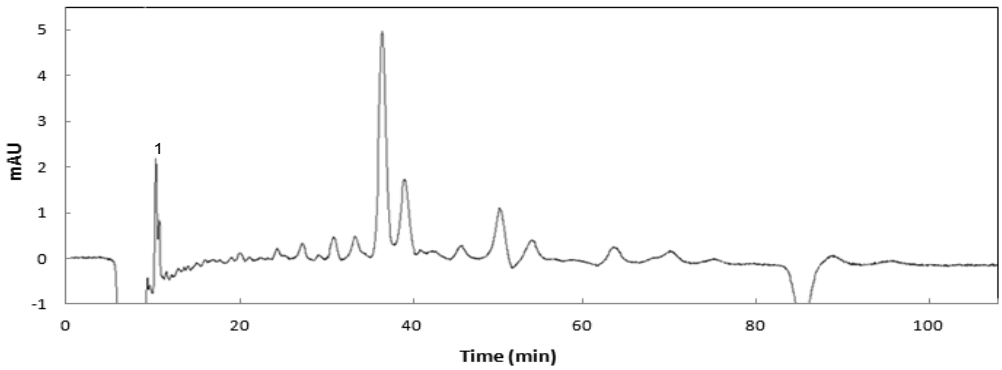

Figure 3: HPLC chromatogram of the extract from *Haloterrigena turkmenica* recorded at 477 nm. 1: astaxanthin

is the poly-3-hydroxybutyrate (PHB), but it is not known if this capacity is a general rule that can be applied to all members. Based on that, we chose the fluorescent dye Nile Blue A for the PHA detection as Ostle and Holt [21] reported that it has a high specificity for PHB. Microscopic analysis evidenced, in the sample from the stationary phase, the synthesized biopolymer which appeared as orange bright roundish granules when observed in dark field under fluorescent light (Figure 5a). The overlap with the same image observed under bright field (Figures 5b and 5c) allowed demonstrating that the biopolymer was accumulated in the cells characterized by a coccoid morphology,

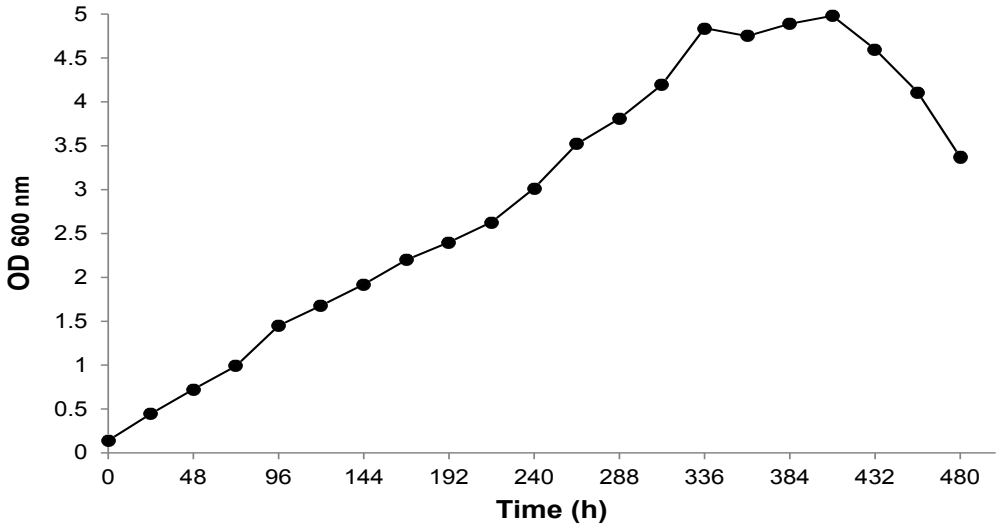

Figure 4: Growth curve of *Haloterrigena turkmenica* in NDMG1 medium

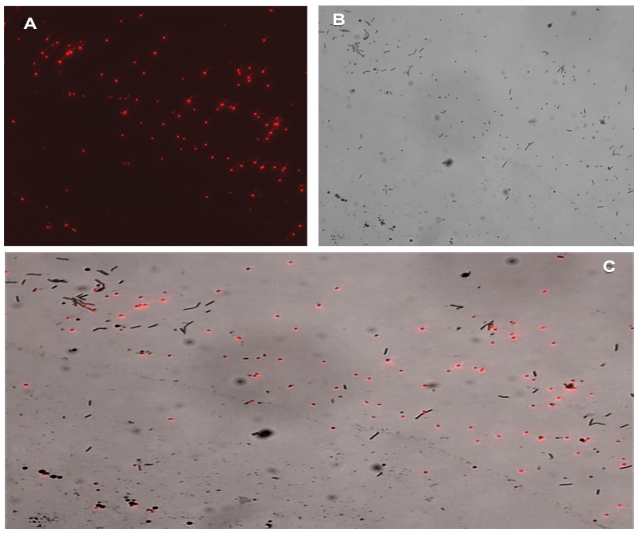

Figure 5: PHA detection following staining with Nile Blue A. Dark field under fluorescent light (a). It shows PHA as bright roundish granules. Bright field (b). Overlap of (a) and (b) images (c). Image (c) allowed to establish that the polymer was produced and accumulated inside cells with coccoid morphology which are associated to stressed growth conditions

typical of stressed conditions and mainly associated to the stationary phase. Moreover, the specific staining allowed us to establish that *H. turkmenica* was capable of producing PHB.

CONCLUSION

H. turkmenica is a halophilic Archaeon with high potentiality in the biotechnological field because it is able to produce several valuable compounds that can find utilization in diverse sectors. In this short report, authors has proved that the microorganisms can synthesize pigments of high value for the food industry and PHB, that can be used as biodegradable plastic, when cultivated in diverse selected conditions. The high adaptability of *H. turkmenica* to different growth conditions allowed to direct the production of the different compounds in a specific manner. However, though properties of the described compounds make *H. turkmenica* an interesting candidate to be used in different areas, their yield is still insufficient for a wide application. Studies aiming to obtain higher productions are currently under investigation.

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COMPETING INTEREST

The authors declare that they have no competing interests.

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