International Journal of Biological Macromolecules 118 (2018) 1942–1947

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: http://www.elsevier.com/locate/ijbiomac

Purification and biochemical characterization of photo-active membrane protein bacteriorhodopsin from *Haloarcula marismortui*, an extreme halophile from the Dead Sea

Diya Alsafadi [a,*], Fawwaz I. Khalili [b], Hassan Juwhari [c], Bashar Lahlouh [c]

[a] Royal Scientific Society, Amman 11941, Jordan
[b] Department of Chemistry, The University of Jordan, Amman 11942, Jordan
[c] Department of Physics, The University of Jordan, Amman 11942, Jordan

ARTICLE INFO

Article history:
Received 7 June 2018
Received in revised form 11 July 2018
Accepted 11 July 2018
Available online 12 July 2018

Keywords:
Bacteriorhodopsin
Retinal proteins
Haloarcula marismortui
Halophilic archaea

ABSTRACT

Bacteriorhodopsin (BR) is an exciting photo-active retinal protein with many potential industrial applications. In this study, BR from the extremely halophilic archaeon *Haloarcula marismortui* (*Hm*BR) was purified successfully using aqueous two phase extraction method. Absorption spectroscopy analysis showed maximum absorption peak of *Hm*BR retinal protein (λ_{max}) at 415 nm. The purified *Hm*BR was visualized by SDS-PAGE, with a subunit molecular mass of 27 kDa, and its identity was confirmed by resonance Raman spectroscopy, Fourier transform infrared spectroscopy and atomic force microscopy. The effect of pH and salt concentration on the absorption spectrum of *Hm*BR was evaluated. Red-shifted in λ_{max} of *Hm*BR was recorded at acidic condition (pH 5) and *Hm*BR showed remarkable optical activity under high salinity condition. The photoelectric activity of *Hm*BR was evaluated by measuring the DC-voltage generated from *Hm*BR coated on indium tin oxide (ITO) glass when light illumination was applied.

1. Introduction

The extremely halophilic archaeal organisms thrive in extreme environments of ~4.5 M salt such as solar salterns, the Great Salt Lake and the Dead Sea [1]. These organisms have evolved strategies to tolerate the osmotic stress exerted by highly saline environments. The "salt in" strategy involves the accumulation of high concentrations of salt, predominantly KCl, in the cell cytoplasm, thereby balancing the external osmotic pressure [2]. A special feature of the halophiles is the presence of purple hue due to a specialized region in their cell membrane that contains bacteriorhodopsin (BR). BR is a protein chromophore (retinal) complex which serves as a light-driven proton pump. BR has attracted much biotechnological and photoelectrical interest for their high degree of cyclicity, thermal stability and quantum efficiency [3]. As a result, BR finds applications in the artificial retinas, photochromic data storage, holo-graphic cameras and information technology and processing [4]. However, the industrial applications of BR are restricted by insufficient production yield, expensive production cost [5] and the use of time-consuming purification processes such as the sucrose density gradient ultracentrifugation process [4].

For their biotechnological application to be realized, methods for efficient production and purification of BR are essential. While *Escherichia coli* (*E. coli*) is an industrially attractive host, which has been successfully employed for the overexpression of BR protein [6, 7], a frequent consequence of overexpression of such protein in *E. coli* is the formation of insoluble inclusion bodies that require solubilization and reactivation [7]. Halophilic archaea, as a result of their ability to thrive in seemingly harsh environments, are exciting sources of BR with industrial applications. Additionally, most of halophilic archaea have simple growth requirements, with relatively rapid doubling time [5]. So far, the isolation and biochemical characterization of BRs from halophilic Archaea are limited to *Halobacterium salinarum* [8], *Haloarcula* sp. IRU1 [9] and *Haloquadratum walsbyi* [3]. Photo-activated membrane protein BR from *Haloarcula marismortui* (*Hm*BR) is relatively less explored. *Haloarcula marismortui* (*H. marismortui*), an extremely halophilic archaeon, isolated from the Dead Sea in the 1930s [10]. The genome sequence of *H. marismortui* proposed the existence of novel dual-BR systems [11], and this was confirmed by cloning and heterologous overexpression of *Hm*BR in *E. coli* [7, 12]. Studies in the literature have reported on the identification and the biochemical characterization of active proteins from *H. marismortui* such as dissimilatory nitrate reductase [13], alcohol dehydrogenase [14] and catalase-peroxidase [15].

Recently, the complicated purification process of the BR containing protein membrane from halophilic microorganisms has been successfully replaced by simple, non-toxic, environmentally friendly and easily scalable extraction process with two aqueous phase system [4]. The new purification process opens up the possibility for isolation of BR

* Corresponding author.
E-mail address: Diya.safadi@rss.jo (D. Alsafadi).

https://doi.org/10.1016/j.ijbiomac.2018.07.045
0141-8130/

from protein membrane of native halophilic organism such as *H. marismortui*. In order to take advantage of this technique, this study reports the first isolation and biochemical characterization of BR from native *H. marismortui*. The effect of pH and salt concentration on the light-driven proton pump of BR is also discussed. Finally, the photo activity of the purified *Hm*BR was measured to ensure the production of active purified BR.

2. Materials and methods

2.1. Chemical and reagent

All chemical reagents, unless stated otherwise, were purchased as analytical grade. DNase I was obtained from Promega. [(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS) and the dialysis bag (cutoff 12,000–14,000) were obtained from Sigma. Poly ethylene glycol 600 was purchased from JHD. A broad range protein marker, P7712S, (11–245 kDa) was purchased from New England Biolabs, USA.

2.2. Cell cultivation

Haloarcula marismortui DSM 3752 was obtained from German Collection of Microorganisms and Cell cultures (DSMZ). *Haloarcula marismortui* (*H. marismortui*) was cultivated in 300 mL of nutrient-rich AS-168 medium containing (per liter) 200 g NaCl, 20 g $MgSO_4 \cdot 7H_2O$, 2 g KCl, 3 g trisodium citrate, 1 g sodium glutamate, 50 mg $FeSO_4 \cdot 4H_2O$, 0.36 mg $MnCl_2.4H_2O$, 5 g casaminoacids, 5 g yeast extract, pH 7.2 [16]. The culture was grown under illumination of light in a shaker incubator with constant shaking (170 rpm) at 37 °C. The cell growth was analyzed spectrophotometrically by measuring the optical density at 600 nm (OD_{600}nm), using a Biochrom Libra S50 UV–visible spectrophotometer. The cells were cultured for 3 days to OD_{600} nm values of approximately 2.0.

2.3. Bacteriorhodopsin purification

Bacteriorhodopsin from *H. marismortui* (*Hm* BR) was purified using aqueous two phase extraction method developed by Shiu et al. [4, 5] with addition of some modifications on the method. Briefly, *H. marismortui* cells were harvested by centrifugation at 1960 ×*g* for 15 min using MPW-352 centrifuge. The produced pellets were suspended in 100 mM Tris–HCl buffer, pH 7.5, containing NaCl (2 M) and disodium EDTA (2 mM) (1 mL buffer was used to resuspend 50 mg cells). Cells were disrupted by sonication at 6 W, 4 °C at intervals of 30 s until the lysate appeared transparent. The lysate was filled in a dialysis bag and 100 μl DNas I was added to digest the genomic DNA. Dialysis bag was left in water under stirring for 24 h at 4 °C. The cell lysate was removed from the dialysis bag and stored at −20 °C. 5 mL of cell lysate was taken and mixed with 10 mL 50% Poly ethylene glycol and 10 mL of 24% potassium phosphate buffer containing 22 g monobasic potassium phosphate, 2 g dibasic potassium phosphate, pH 8. The lipids that are associated with the BR out layer can be stripped off by using the ionic detergent CHAPS. Therefore, 100 μl 60 mM CHAPS was added and the mixture left for 1 h with shaking (170 rpm) at 25 °C. The two phases were separated by centrifugation at 1960 ×*g* for 15 min. The red band formed at the interface of the two phases was collected in Eppendorf tube and centrifuged at 13,835 ×*g* for 15 min. After centrifugation, red *Hm*BR was obtained and the colorless supernatant was decanted.

2.4. Analytical methods and bacteriorhodopsin characterization

BR-containing samples were analyzed by SDS-PAGE using 12% polyacrylamide gels, stained with Coomassie brilliant blue R250. A broad range protein marker, P7712S, (17–100 kDa) (New England Biolabs, USA) was used for determination of relative molecular weight. The amount of BR recovered by the purification processes was calculated based on *H. marismortui* culture volume using the hydroxylamine method [4]. UV–visible absorption spectra of the BR-containing samples were monitored on Biochrom Libra S50 UV–visible spectrophotometer. BR samples were previously incubated for 1 h in the dark under various conditions and the final BR concentration was 100 μg/mL. The absorbance spectrum, scanning from 250 to 750 nm, was measured for each sample. The fluorescence spectrum of BR sample was monitored on Resonance Raman Spectrometer RaPort® Handheld analyzer with liner CCD array detector. The parameters for spectra are 532 nm excitation wave length, 200 millisecond exposure time, 12 number of frame and 50 mW power. Fourier transform infrared (FT-IR) spectra for *Hm*BR was obtained in the range 400 cm^{-1} to 4000 cm^{-1} using IR-Prestige-21 Shimadzu FT-IR spectrophotometer. For atomic force microscopy (AFM), sample of 50 μl BR was layered over silica gel and dried at room temperature. The image was taken using contact mode Bruker Innova AFM [17].

2.5. Effect of pH and salt concentration on the retinal chromophore absorption band of BR

Samples of *Hm*BR were incubated at pH 6.0, 8.0 and 10.0 for 2 h at room temperature in the dark. The absorbance spectrum, scanning from 250 to 750 nm, was measured for each sample. The following buffers were used: 50 mM KCl-HCl buffer pH 2, 50 mM citric acid–K_2HPO_4 buffer pH 5.0, 50 mM Tris–HCl buffer pH 7.0 and 50 mM glycine–KOH buffer 9.0. The effect of salt concentration was evaluated by incubation *Hm*BR samples in 50 mM Tris–HCl buffer pH 7.0, containing various concentration of salt (1 M NaCl, 2 M NaCl, 3 M NaCl and 4 M NaCl) for 24 h at room temperature in the dark. The absorbance spectrum, scanning from 250 to 750 nm, was measured for each sample.

2.6. Photoelectric activity measurement

Indium tin oxide coated glass is one of the standard substrates used to construct Bio solar cells. ITO is a low resistance conductive oxide that can be used to carry the generated charge carries in a constructed solar cell. The ITO used in this work is a 6 Ω coating on a 20 mm × 20 mm × 1.1 mm glass substrate. The ITO coating was cleaned with phosphate-free detergent and left with DI water in a sonication bath for 15 min. Isopropyl alcohol and methanol were used sequentially to wash the surface of ITO. The cleaned ITO glass was further dried with N_2 gas. A Laurell Technologies spin coater (WS-400B) was used to spin a uniform layer of purified *Hm*BR onto a pre-cleaned ITO-coated glass slide. The spun layer was left to naturally dry under room temperature. The photocell was constructed by placing a U-shaped glass separator, of 1 mm thickness, on top of the dried *Hm*BR coating and a counter ITO-coated glass electrode was placed on the opposite side of the U-shaped separator to complete the photocell. The U-shaped separator was filled out with 10 mM K_2HPO_4–KH_2PO_4 buffer, pH 8.5, containing 100 mM KCl. The optical response of the photocell, with and without the buffer solution, was evaluated using a UV–visible spectrophotometer. The photoelectric activity of the cell was further tested by using a commercial voltmeter, just as a proof of concept, were the generated DC-voltage under room lighting conditions was compared to that generated under direct sun-light.

3. Results and discussion

3.1. Purification and characterization of bacteriorhodopsin

Photo-active membrane protein bacteriorhodopsin (BR) from *H. marismortui* (*Hm*BR) was purified using aqueous two phase extraction system as described in the Materials and methods part. Very good phase separation was achieved and red band containing the purified *Hm*BR was formed at interface of two phases (Fig. 1A). The formation

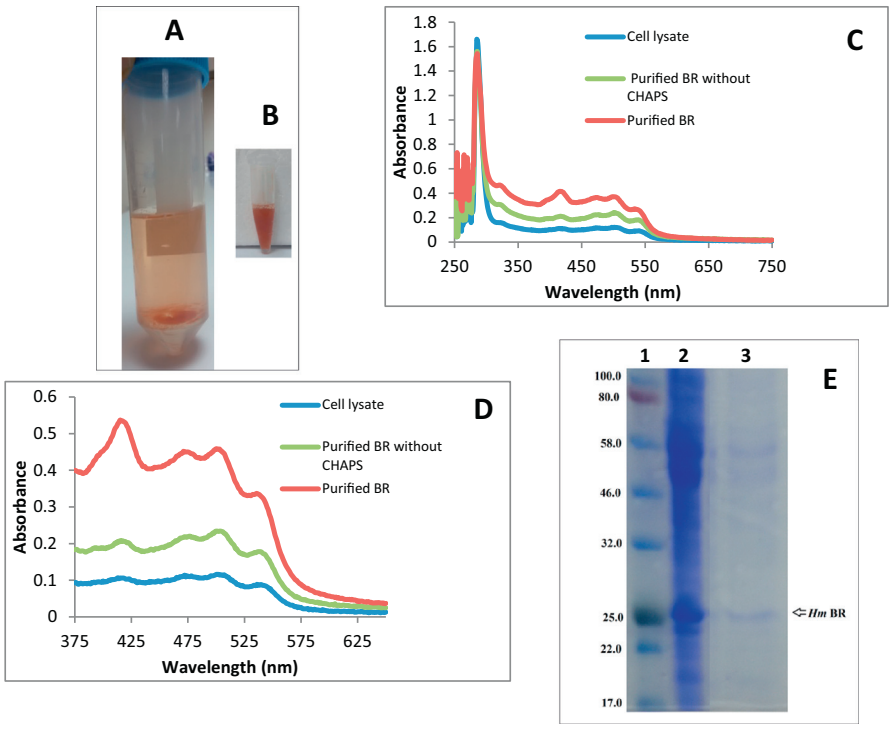

Fig. 1. Purification and characterization of bacteriorhodopsin (A) Image for purification of bacteriorhodopsin from *H. marismortui* (*Hm*BR) using aqueous two phase extraction system (B) Purified *Hm*BR (C) Absorption spectra in the range 250–750 nm of *Hm*BR from different preparation processes (D) Absorption spectra in the range 375–625 nm of *Hm*BR from different preparation processes (E) SDS–PAGE analysis of *Hm*BR from different preparation processes; Lane 1 (left) broad range protein marker P7712S, (17–100 kDa), Lane 2 crude protein extract, Lane 3 purified *Hm*BR.

of colored band was also observed in a previous works for the purification of BR from *Halobacterium salinarum* [4, 5]. The purified *Hm*BR was collected and used for further analysis as shown in Fig. 1B. The UV–visible absorption spectrum of *Hm*BR from different preparation processes were detected in 50 mM Tris–HCl buffer pH 7.0 at 25 °Cas shown in Fig. 1C. The cell lysate showed very low chromophore absorbance in the range 375–550 nm and strong absorbance at 280 nm. The purified *Hm*BR sample showed three maximum absorption peaks at 471 nm and 501 nm and 537 nm (Fig. 1D), these peaks could be attributed to the presence of red pigment bacterioruberin, the abundant pigment in many extreme halophiles [18]. The major function of the bacterioruberin in halophiles cell is to protect DNA damage against UV light [19]. The maximum absorption peak of the retinal protein *Hm*BR (λ_{max}) was observed at 415 nm, this peak showed strong red-shift if compared to the bacteriorhodopsin from *Halobacterium salinarum* (550 nm) [20] and the recombinant *Hm*BR heterologously expressed in *E.coli* (552 nm) [7]. The red shift in maximum absorbance was also observed in bacteriorhodopsin isolated from *Halobacterium halobium* [21].

It was reported that the ionic detergent CHAPS was included in the purification process to remove the lipids from the out layer of the BR [4]. To estimate the direct effect of CHAPS, purified *Hm*BR was prepared without the addition of CHAPS during the purification process as previously descried in the method. The UV–visible absorption spectrum of

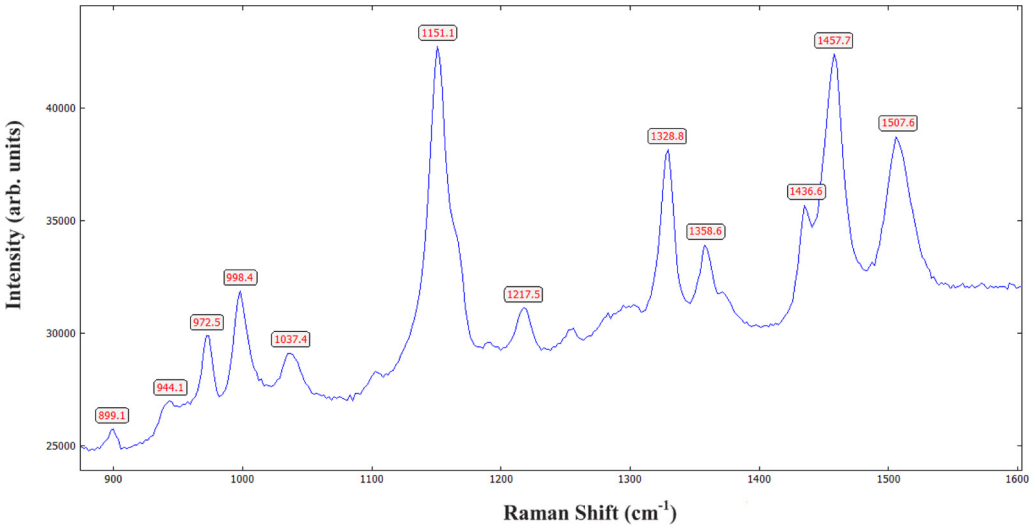

Fig. 2. *Hm*BR resonance Raman spectrum with 532 nm excitation wavelength, 200 millisecond exposure time, 12 number of frame and 50 mW power.

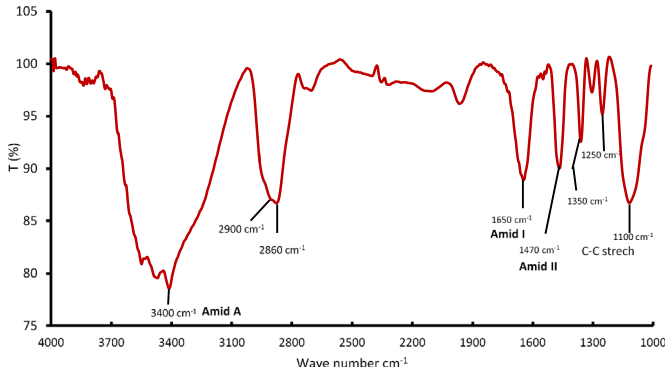

Fig. 3. FTIR spectra of *Hm*BR in 50 mM Tris–HCl buffer pH 7.0 at 25 °C.

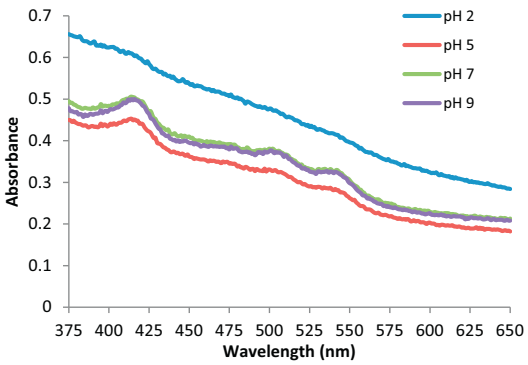

Fig. 5. Absorption spectra of *Hm*BR at different pHs.

the preparation showed low chromophore absorbance in the range 375–625 nm compared to the *Hm*BR preparation in the presence of CHAPS (Fig. 1C and D). This indicates that the lipids associated with BR have been stripped off after treatment with CHAPS.

In order to assess the level of purity for the purified BR, an aliquot of the crude and pure BR preparations were visualized by SDS-PAGE (Fig. 1E). The SDS-PAGE shows a main band approximately 27 kDa corresponding to the subunit molecular mass of native *Hm*BR. The observed molar mass was overestimated than the molar mass of BR produced from heterologous overexpression of *Hm*BR in *E. coli* (20 kDa) [7]. This can be attributed to the presence of excess negative charge over the surface of halophilic proteins, which affects the migration of halophilic proteins through the gel matrix [22]. As expected, the molecular mass of native *Hm*BR in this study is very close to the reported BR molecular mass from halophilic organisms such as the archaeon *Halobacterium salinarum* [5] and *Haloquadratum walsbyi* [3]. In previous studies, BR has been heterologously overexpressed using different expression system and microbial strains with varying degree of success. For example, Nekrasova et al. [6] reported expression level of 120 mg/L culture for BR from *Halobacterium salinarum* in *E. coli.* Fu et al. [12] showed a low level of wild type BR from *H. marismortui* expressed in *E. coli* (3–10 mg/L culture). Further improvements in BR yield were obtained using a mutated BR from *H. marismortui* (*Hm*BRI/D94N) as a fusion partner giving expression of 70 mg/L [7]. Here, the BR was successfully obtained from *H. marismortui* in good yield 77 mg/L culture using simple and cost-efficient method. The main advantages for production and purification of BR from *H. marismortui* over heterologous expression of BR are the following (i) production and characterization BR in native form (ii) avoiding production inclusion bodies that required solubilization and reactivation following the heterologous overexpression [8] (iii) prevent BR inhibition by imidazole which has been used as eluting agent in the purification of His tag BR [23].

The structure of *Hm*BR was investigated by resonance Raman spectroscopy as shown in Fig. 2. In general the fingerprint regions for native, *Hm*BR can be divided into four groups the C═C stretches (1400–1600 cm^{-1}), the C—C—H in-plane rocks (1200–1400 cm^{-1}), the C—C stretches (1100–1200 cm^{-1}), and the hydrogen out-of-plane wags (900–1000 cm^{-1}). The bands at 1507 cm^{-1} and 1151 cm^{-1} can be assigned to in-phase C═C frequencies (ν_1) and C—C stretching frequencies (ν_2) vibrations from the polyene chain of carotenoids [24]. Resonance Raman spectroscopy reveals the major features of BR structure, confirming the purity of BR isolated from *H. marismortui*.

FT-IR has also been employed for the assessments of *Hm*BR structure (Fig. 3). The major protein vibrational modes were observed for amide I (C═O) at 1650 cm^{-1}, amide II (C═N and N—H) at 1470 cm^{-1} and amide A at 3400 cm^{-1}. These bands indicated that the recombinant *Hm*BR has a native well-folded protein secondary structure [20]. The bands for C—C stretches and C—C—H bends were observed in the range of 1100–1350 cm^{-1} (fingerprint region). The FT-IR spectrum confirms the BR nature of the sample.

AFM imaging is a high-resolution structural method that allows for the visualization of single protein molecules under physiological conditions (non-destructive method). The 3D topography image of dried *Hm*BR patches is shown in Fig. 4. The image reveals narrow and sharp aggregates of *Hm*BR. The sharp morphology of *Hm*BR aggregates could be related to the removal of the lipids that surround the BR and resulting

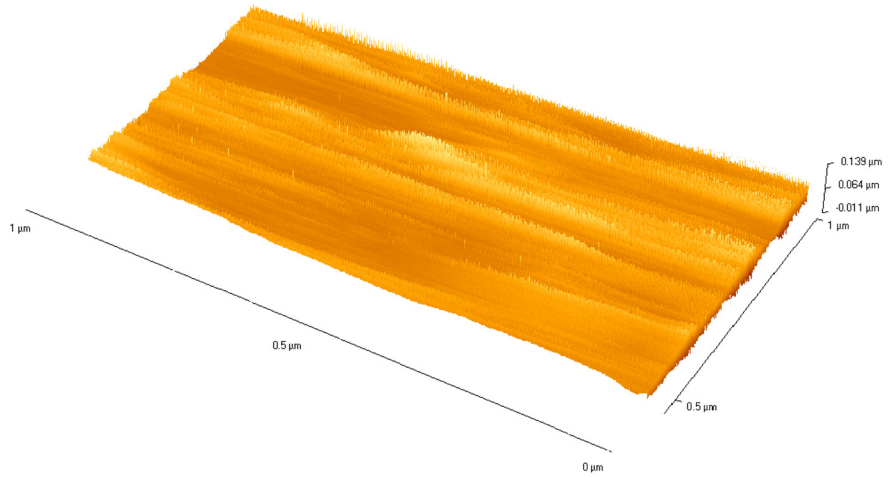

Fig. 4. AFM image of *Hm*BR immobilized on silica gel surface.

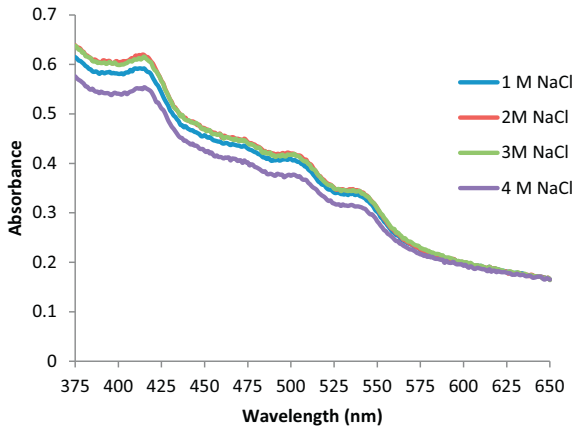

Fig. 6. Absorption spectra of *Hm*BR at different salt concentrations.

densely packed BR monolayer as reported by Shiu et al. [4] for BR from *Halobacterium salinarum*.

3.2. Effect of pH and salt concentration on retinal chromophore absorption of HmBR

The effect of pH on the retinal chromophore absorption of *Hm*BR was measured at pH range of (2–9) using suitable buffers as described in the Materials and methods. As mentioned before the maximum absorbance band of retinal *Hm*BR (λ_{max}) was observed at 415 nm. Fig. 5 shows that the λ_{max} did not change significantly as the pH varied in the range 7–9. In contrast, λ_{max} showed red-shift with decrease in the absorbance at pH 5. This could be attributed to the protonation of an aspartate residue at the retinal Schiff base (Asp85 in *Hm*BR). The light activity of *Hm*BR at pH 5 might contribute the survival of *H. marismortui* under the acidic environment of the Dead Sea (the average pH is around 5.5). Clearly, no optical properties were recorded when *Hm*BR was incubated at low pH (pH 2). This result is not surprising because the strong acidic environment is not the optimal environment for the proper functioning of most BR proteins [25].

The effect of salt concentration on absorption spectra of *Hm*BR was assessed in 50 mM Tris–HCl buffer, pH 7, containing varying concentrations of NaCl. Fig. 6 shows that salt concentration has no effect on the on λ_{max} of *Hm*BR while a decrease in the absorbance had been observed at 4 M NaCl. The results confirm the optical and functional durability of *Hm*BR under high salinity conditions.

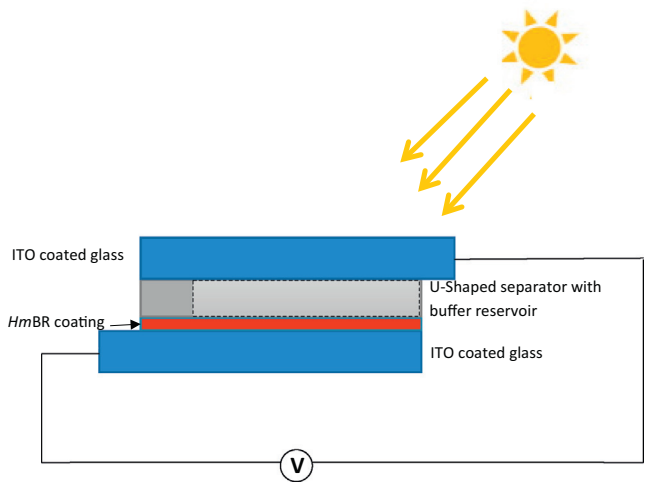

Fig. 7. Schematic structure of home-built photocell, consisting of one ITO with a surface coated with *Hm*BR monolayer as working electrode, U-shaped glass separator with buffer reservoir and another blank ITO as a counter electrode.

3.3. Photoelectric activity of HmBR

The functionality and photoelectric activity of purified *Hm*BR was evaluated by measuring the DC-voltage generated from *Hm*BR coated on ITO glass when sun light illumination was applied. A schematic of the constructed photocell is presented in Fig. 7. The structure of this cell is very simple and it is arranged in such a way to facilitate the subsequent evaluation and test procedures. The central area of the cell kept clear of any obstructions to allow for the optical evaluation of the cell, while the upper and lower edges of the ITO-coated slides were kept clear to facilitate the cell DC-voltage measurements (Fig. 7).

The absorbance spectra of the photocell with and without the buffer solution were measured in the wavelength range 375–625 nm (data not shown). The addition of the buffer solutions has no adverse effects on the absorption characteristics of *Hm*BR. The generated DC-voltage of the cell was measured, as a proof of concept only, using a commercial voltmeter. The measured DC-voltage under room lighting conditions (traditional fluorescent lamps) was around 1.2 mV/cm^2. This value increased to more than 40 times as the cell was moved to direct natural sunlight (51.5 mV/cm^2). The photocell in this study has better photovoltaic performance in comparison to the cell constructed from monolayer of BR functionalized on gold and covered with ITO electrode (9.73 mV/cm^2) [26]. The high photovoltaic generated from the photo cell constructed in this study could be due to the high purity of BR isolated from *H. marismortui*. Also previous studies have shown that the kinetics of the BR photocycle depend on the level of humidity in the BR sample [26]. The photocycle and proton transfer kinetics of dried BR film differ from aqueous, or wet, BR because of dehydration. The U-shaped separator used to construct the test cell in this work was rather thick (~1 mm) and this means that *Hm*BR was covered by thick buffer solution layer. Therefore, the constructed cell was able to perform well in comparison to other reported values of similar bio-solar cells.

4. Conclusion

As a light-driven protein, BR has been found to have various potential photoelectric applications, but its high production cost and low availability limits broader and commercial applications. A cost reduction in BR biosynthesis process can be achieved by searching on new BR producing strains and by developing BR production processes with cheap and simple purification steps. This work presented an extremely halophilic organism, *H. marismortui* as a promising source for the industrial production of BR. The high salinity of *H. marismortui* cultivation media minimises the risk of microbial contamination therefore; cultivation environment has been simplified even without expensive sterilization process. The BR was extracted from microbial biomass by cheap, easy and environmentally friendly aqueous two phase extraction method. Also, resonance Raman spectroscopy, Fourier transform infrared spectroscopy and atomic force microscopy showed that the *Hm*BR has characteristics similar to those of BR-producing Archaea. Further work is still needed to bring BR production step closer to industrial scale production such as cultivation *H. marismortui* under controlled conditions (dissolved oxygen, pH and illumination) and scaling-up the process using continuous feeding of the culture.

Acknowledgments

This research study was financially supported by the European Union, Project 'Support to Research and Technological Development & Innovation initiatives and Strategies in Jordan' (SRTD II) contract No AR 173 and the Scientific Research Support Fund in Jordan project no. Bas/1/01/2014.

References

[1] A. Oren, Molecular ecology of extremely halophilic archaea and bacteria, FEMS Microbiol. Ecol. 39 (2002) 1–7.
[2] M.J. Danson, D.W. Hough, The structural basis of protein halophilicity, Comp. Biochem. Physiol. 117 (1997) 307–312.
[3] S. Lobasso, P. Lopalco, R. Vitale, M. Sublimi Saponetti, G. Capitanio, V. Mangini, F. Milano, M. Trotta, A. Corcelli, The light-activated proton pump Bop I of the archaeon *Haloquadratum walsbyi*, Photochem. Photobiol. 88 (2012) 690–700.
[4] P.J. Shiu, H.M. Chen, C.K. Lee, One-step purification of delipidated bacteriorhodopsin by aqueous-three-phase system from purple membrane of *Halobacterium*, Food Bioprod. Process. 92 (2014) 113–119.
[5] P.J. Shiu, J. Yi-Hsu, H.M. Chen, C.K. Lee, Facile isolation of purple membrane from *Halobacterium salinarum* via aqueous-two-phase system, Protein Expr. Purif. 89 (2013) 219–224.
[6] O.V. Nekrasova, A.N. Wulfson, R.V. Tikhonov, S.A. Yakimov, T.N. Simonova, A.I. Tagvey, D.A. Dolgikh, M.A. Ostrovsky, M.P. Kirpichnikov, A new hybrid protein for production of recombinant bacteriorhodopsin in *Escherichia coli*, J. Biotechnol. 147 (2010) 145–150.
[7] M.F. Hsu, T.F. Yu, C.C. Chou, H.Y. Fu, C.S. Yang, A.H. Wang, Using *Haloarcula marismortui* bacteriorhodopsin as a fusion tag for enhancing and visible expression of integral membrane proteins in *Escherichia coli*, PLoS One 8 (2013) 1–11.
[8] S.V. Kalenov, M.M. Baurina, D.A. Skladnev, A.Y. Kuznetsov, High-effective cultivation of *Halobacterium salinarum* providing with bacteriorhodopsin production under controlled stress, J. Biotechnol. 233 (2016) 211–218.
[9] M. Taran, A. Monazah, N. Asadi, Production of a novel biomacromolecule for nanodevices from glycerol as carbon source in different conditions, Int. J. Biol. Macromol. 49 (2011) 955–957.
[10] M. Oren, B.Z. Ginzburg, L. Ginzburg, B. Hochstein, E. Volcani, *Haloarcula marismortui* (Volcani) sp. nov., nom. rev., an extremely halophilic bacterium from the Dead Sea, Int. J. Syst. Bacteriol. 40 (1990) 209–210.
[11] N.S. Baliga, R. Bonneau, M.T. Facciotti, M. Pan, G. Glusman, E.W. Deutsch, P. Shanno, Y. Chiu, R.S. Weng, R.R. Gan, P. Hung, S.V. Date, E. Marcotte, L. Hood, W.V. Ng, Genome sequence of *Haloarcula marismortui*: a halophilic archaeon from the Dead Sea, Genome Res. 14 (2004) 2221–2234.
[12] H.Y. Fu, Y.C. Lin, Y.N. Chang, H. Tseng, C.C. Huang, K.C. Liu, C.S. Huang, C. Wei Su, R.R. Weng, Y.Y. Lee, W.V. Ng, C.S. Yang, A novel six-rhodopsin system in a single archaeon, J. Bacteriol. 192 (2010) 5866–5873.
[13] K. Yoshimatsua, T. Sakuraib, T. Fujiwaraa, Purification and characterization of dissimilatory nitrate reductase from a denitrifying halophilic archaeon, *Haloarcula marismortui*, FEBS Lett. 470 (2000) 216–220.
[14] L.M. Timpson, D. Alsafadi, C. Mac Donnchadha, S. Liddell, M.A. Sharkey, F. Paradisi, Characterization of alcohol dehydrogenase (ADH12) from *Haloarcula marismortui*, an extreme halophile from the Dead Sea, Extremophiles 16 (2012) 57–66.
[15] F. Cendrin, H.M. Jouve, J. Gaillard, P. Thibault, G. Zaccai, Purification and properties of a halophilic catalase-peroxidase from *Haloarcula marismortui*, Biochim. Biophys. Acta 1209 (1994) 1–9.
[16] J. Han, Q. Lu, L. Zhou, J. Zhou, H. Xiang, Molecular characterization of the phaECHm genes, required for biosynthesis of poly(3-hydroxybutyrate) in the extremely halophilic archaeon *Haloarcula marismortui*, Appl. Environ. Microbiol. 73 (2007) 6058–6065.
[17] A.M. Whited, P.S. Park, Atomic force microscopy: a multifaceted tool to study membrane proteins and their interactions with ligands, Biochim. Biophys. Acta 1838 ((2014) 56–68.
[18] M. de la Vega, A. Sayago, J. Ariza, A.G. Barneto, R. León, Characterization of a bacterioruberin-producing haloarchaea isolated from the marshlands of the Odiel river in the southwest of Spain, Biotechnol. Prog. 32 (2016) 592–600.
[19] H.R. Shahmohammadi, E. Asgarani, H. Terato, T. Saito, Y. Ohyama, K. Gekko, O. Yamamoto, H. Ide, Protective roles of bacterioruberin and intracellular KCl in the resistance of *Halobacterium salinarium* against DNA-damaging agents, J. Radiat. Res. 39 (1998) 262–351.
[20] Z. Cao, X. Ding, B. Peng, Y. Zhao, J. Ding, A. Watts, X. Zhao, Novel expression and characterization of a light driven proton pump archaerhodopsin 4 in a *Halobacterium salinarum* strain, Biochim. Biophys. Acta 1847 (2015) 390–398.
[21] B. Hess, D. Kuschmitz, The photochemical reaction of the 412 nm chromophore of bacteriorhodopsin, FEBS Lett. 74 (1977) 20–24.
[22] D. Madern, C. Ebel, G. Zaccai, Halophilic adaptation of enzymes, Extremophiles 4 (2000) 91–98.
[23] D. Bratanov, T. Balandin, E. Round, V. Shevchenko, I. Gushchin, V. Polovinkin, V. Borshchevskiy, V. Gordeliy, An approach to heterologous expression of membrane proteins. The case of bacteriorhodopsin, PLoS One 10 (2015) 1–16.
[24] C.P. Marshall, S. Leuko, C.M. Coyle, M.R. Walter, B.P. Burns, B.A. Neilan, Carotenoid analysis of halophilic archaea by resonance Raman spectroscopy, Astrobiology 7 (2007) 631–643.
[25] L. Zimanyi, G. Varo, M. Chang, B. Ni, R. Needleman, J.K. Lanyi, Pathways of proton release in the bacteriorhodopsin photocycle, Biochemistry 31 (1992) 8535–8543.
[26] K.M. Al-Aribe, G.K. Knopf, A.S. Bassi, Organic Photovoltaic Cells Based on Photoactive Bacteriorhodopsin Proteins, Proc. SPIE. Microfluidics. BioMEMS. and Medical Microsystems, XI, 2013https://doi.org/10.1117/12.2004018.