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Moderately Halophilic Bacterium *Halomonas* sp. AAD12: A Promising Candidate as a Hydroxyectoine Producer

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Abstract

Properties of osmolytes that help protect biological molecules from stress and preserve their functions impose significant importance to finding new organisms with different osmolyte accumulation strategies. To this end, the moderately halophilic *Halomonas* sp. AAD12 has been characterized for its adaptation to stress conditions with particular emphasis on its osmolyte accumulation strategy. The effect of temperature, salinity, aeration and organic components on the accumulation of osmoprotectants and synthesis of fatty acids were examined in M63 minimal medium. Ectoine, proline and hydroxyectoine were the major osmolytes and palmitic acid (16:0), palmitoleic acid (16:1), and oleic acid (18:1) were the major fatty acids. Overall, ectoine yield was the highest among the three osmolytes at all salt concentrations and temperatures investigated. However, high salinity reduced ectoine yield with a concomitant increase in hydroxyectoine yield. The yield of hydroxyectoine as high as 525 mol/g dry cell mass at 37°C suggested that this microorganism could be a promising candidate as a hydroxyectoine producer.

Keywords: Moderately halophile; Osmolytes; Hydroxyectoine; Ectoine hydroxylase; Fatty acids

Introduction

Extremophiles such as psychrophiles, thermophiles, acidophiles, alkaliphiles, barophiles and halophiles could represent the direct descendants of ancestral forms of life [1]. In this group, moderately halophilic microorganisms have been in the center of industrial interest in the last decades owing to their growth in a wide range of salt concentrations. For survival under high osmotic environments, halophilic microorganisms accumulate compatible solutes (osmolytes) that increase the cytoplasmic osmotic pressure and give a correct turgor pressure to protect the contents of the cytoplasm [1-6].

Compatible solutes are usually small organic molecules such as amino acids or their derivatives, carbohydrates or their derivatives, glycerol and other sugar alcohols [7-12]. Variations among halophilic microorganisms have been reported regarding their osmolyte accumulation strategies. *Chromohalobacter salexigens* accumulates ectoine and hydroxyectoine as osmoprotectants. This behaviour is similar to that reported for *Halomonas salina* and *H. halophila*. *H. pantelleriense* accumulates glycine, betaine, ectoine, hydroxyectoine and glutamate as major osmolytes [6]. Moderately halophilic *H. halophilus* accumulates proline and ectoine during different growth phases [12-14].

The properties of different osmolytes make these molecules desirable for a variety of uses in biotechnology. Among them, ectoine and hydroxyectoine have been widely investigated [5]. The exceptional ability to protect enzymes against a variety of stress factors such as heating, freeze-thawing and freeze-drying places hydroxyectoine, a derivative of ectoine, in the heart of a number of studies [15,16]. Hydroxyectoine is especially suitable as a protector for desiccation of healthy cells during chemotherapy and as a molecular chaperon for Alzheimer`s disease [4]. Recently, Tanne et al. [17] reported hydroxyectoine as a good glass-forming compound and Srinivasan et al. [18] proposed that hydroxyectoine-modified Histidine-Tryptophan-Ketoglutarate (HTK) solutions could be used for preservation of cardiac death donors (DCD) livers. Hydroxyectoine is superior over ectoine in stress protection and preserving various biological functions, hence it is noteworthy to understand the trafficking between ectoine and hydroxyectoine. In this context, Widderich et al. [19] predicted the importance of ectoine and hydroxyectoine and mined the fully sequenced genomes of Bacteria and Archaea for the enzymes of ectoine (EctC) and hydroxyectoine (EctD) synthesis. They reported that among the 6428 microbial genomes searched, out of 440 species producing ectoine, only 272 species could produce hydroxyectoine.

In our former work, we have reported *Halomonas* sp. AAD12 as a moderate halophile that required minimum (w/v) 5% NaCl for growth. Its optimum growth was at 37°C with 10% NaCl in Brown medium, but it tolerated NaCl concentrations up to 20% (w/v) (Ceylan et al., 2011). With trisodium citrate as the carbon source, proline and ectoine were equally important osmolytes at low salinity whereas proline became its major osmolyte at higher salinity [20]. Moreover, at higher salinity hydroxyectoine accumulation was also significant. Model simulations with this microorganism predicted that the carbon source that entered the system as citrate was mainly diverted to hydroxyectoine synthesis at higher salinity.

This work aimed to investigate the osmoadaptation strategy of *Halomonas* sp. AAD12 in M63 minimal medium in response to different environmental stresses with particular emphasis on the synthesis of osmolytes and distribution of fatty acids. The activity of ectoine hydroxylase explained the trafficking between ectoine and hydroxyectoine.

Materials and Methods

Bacterial strain, culture and growth conditions

Halomonas sp. AAD12 was isolated from salt sediments in

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ponds found in Çamaltı Saltern area in İzmir (western Turkey). It was maintained in a medium with (w/v) 0.5% yeast extract, 0.3% sodium citrate, 2% $MgSO_4.7H_2O$, 0.2% KCl, 25 % NaCl and 1% agar adjusted to pH 7.0 [21]. The strain was identified based on its typical cultural, morphological, and biochemical characteristics and on its 16S ribosomal ribonucleic acid (rRNA) gene sequence. The organism is deposited at our research laboratory at Marmara University, Turkey, and the 16S rRNA sequence of the strain AAD12 is been deposited in the NCBI database under the accession no. GU397429 [21].

The effects of temperature, salinity, aeration and organic components on the accumulation of osmoprotectants were examined in M63 minimal medium (KH_2PO_4 13.6 g/L; $(NH_4)_2SO_4$ 2.0 g/L; Fe $SO_4.7H_2O$ 0.5 mg/L). The medium was supplemented with 0.5% glucose as the carbon source. 2% agar (w/v) was added to the medium for growth on plates. Cells for the *inocula* were grown for several generations in M63 media. The effects of salinity and temperature on osmoprotectant accumulation were examined by shifting NaCl concentration up, from 5% (0.85 M) to 15% (2.55 M) and increasing the temperature from 20 to 37°C, keeping rest of the chemical-physical parameters unchanged.

In order to investigate osmolyte uptake during osmoadaptation, in addition to glucose, the medium was supplemented with 1 mM proline, glycine betaine or ectoine. Then glucose was totally replaced with 10-20 mM ectoine, 10-30 mM proline or glycine-betaine. Cell growth was monitored by measuring absorbance at 600 nm. The cells were harvested by centrifugation at the end of exponential phase and used in further steps.

Extraction and analysis of osmolytes

Extraction and determination of intracellular osmolytes were carried out using the method published by Motta et al. [22] and previously described by Ceylan et al. [20]. For the determination of osmolytes, ^{1}H -nuclear magnetic resonance (^{1}H-NMR) spectra were recorded at 25°C on a Varian Unity Inova spectrometer operating at 500 MHz. Chemical shifts were reported in ppm on the scale relative to TMSP (sodium-3-(trimethylsilyl) propionate-2.2.3.3-d_4). Peak for TMSP was at 0 ppm whereas the peaks were at 1.95 - 2.10 ppm for L-proline; at 2.24 - 2.26 ppm for ectoine; and at 2.30–2.32 ppm for hydroxyectoine. These signals for the osmolytes were assigned by comparison with previously published chemical shift values from Motta et al. [22] and confirmed by comparison with ^{1}H -NMR spectra of commercially available pure compounds.

Osmolytes were quantified by integrating the peak areas with respect to the internal standard TMSP. Since the peak area of TMSP is directly proportional to the mass of TMSP analyzed, the mass of L-proline, ectoine, and hydroxyectoine have been calculated using equation 1 [23].

$$m_x = \frac{I_x / N_x}{I / 9} \cdot m \qquad (1)$$

where,

I_x: integral of the metabolite of interest

N_x: equivalent number of protons of the metabolite of interest

m_x: mass of the metabolite of interest

I: integral of TMSP

m: mass of TMSP.

Osmolyte masses, found using equation 1, reflect absolute values obtained from different cell masses, hence normalization was required for comparison. For this reason, total osmolyte masses, calculated using Equation 1, have been divided by dry cell weight.

Determination of ectoine hydroxylase activity

Ectoine hydroxylase activity was determined by the method described by Bursy et al. [24]. Reaction mixture was incubated in the presence of 5 and 15% NaCl at different temperatures. Supernatant was analysed by HPLC (Shimadzu LC Class 10A) using TSKGel HA1000 column and a UV-visible detector set at 210 nm. One unit of enzyme activity was defined as the amount of hydroxyectoine converted to ectoine per minute under the assay conditions.

Identification of fatty acids

Fatty acid methyl esters (FAMEs) were obtained from complex lipids by acid methanolysis given by Romano et al. [6] and analyzed using an Agilent 6890N (Manufacturer address) gas chromatograph fitted with a FID detector. Mixture me-100 (Larodan; Fine Chemicals AB, Malmo, Sweden) was used as a standard.

All experiments were performed at least three times, and the results given were calculated as mean values. Standard errors of mean values were lower than 4%.

Results

Effect of temperature, salinity and oxygen limitation on growth and osmolyte production

Growth profile of *Halomonas* sp. AAD12 in minimal M63 medium at low (5% NaCl, w/v) and high salinity (15% NaCl, w/v) and at two different temperatures, 37 and 20°C, are shown in Figure 1 at 37°C, as NaCl concentration increased from 5 to 15%, specific growth rate increased from 0.18 to 0.25 hr^{-1}. However, final cell density was very low with 15% NaCl when compared to the final cell density with 5% NaCl. Interestingly, at 20°C specific growth rate dropped from 0.22 hr to 0.13 hr^{-1} with increasing salinity. Lag phase at 20°C was significantly long when compared to the lag phase at 37°C. As the temperature dropped from 37 to 20°C, lag phase increased from 7 to 47 hrs in the presence of 5% NaCl and from 16 to 68 hrs in the presence of 15% NaCl. These results indicated that an initial adaptation period is required for cell doubling at lower temperatures with higher salinity.

In M63 media supplemented with glucose, the major osmolytes synthesized by *Halomonas* sp AAD12 were ectoine, proline and hydroxyectoine (Figure 2a and 2b). Yield coefficients ($Y_{osmolyte/X}$, mmol osmolyte/g DCM), amount of osmolyte obtained per biomass, showed that intracellular ectoine and hydroxyectoine contents were directly proportional to salinity and temperature and that ectoine was the major osmolyte at both temperatures and salt concentrations studied, regardless of growth phase (mid exponential or late stationary) (Figure 2a and 2b). In general, at the reduced temperature, osmolyte accumulation was poor.

The change in a given osmolyte synthesized per cell in a given growth phase and temperature was found by calculating the relative change for each osmolyte. Unity was assigned to the case with the lower amount and the increase in that osmolyte was expressed as a fold change relative to unity (Figure 2a and 2b and Table 1). Increasing salt concentration at 20°C resulted in lower amounts of osmolyte accumulation in cells in mid-exponential phase whereas in late-stationary phase, osmolyte accumulation increased with salinity. The changes recorded for the

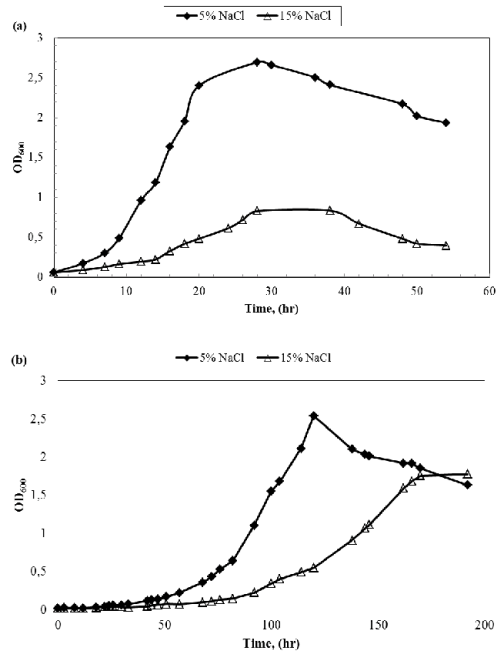

Figure 1: Growth curve for *Halomonas* sp. AAD12 in the presence of 5 and 15 % NaCl at (a) 37°C and (b) 20°C. Experiments were carried out in minimal media supplemented with 0.5% glucose. Initial pH was adjusted to 7.00.

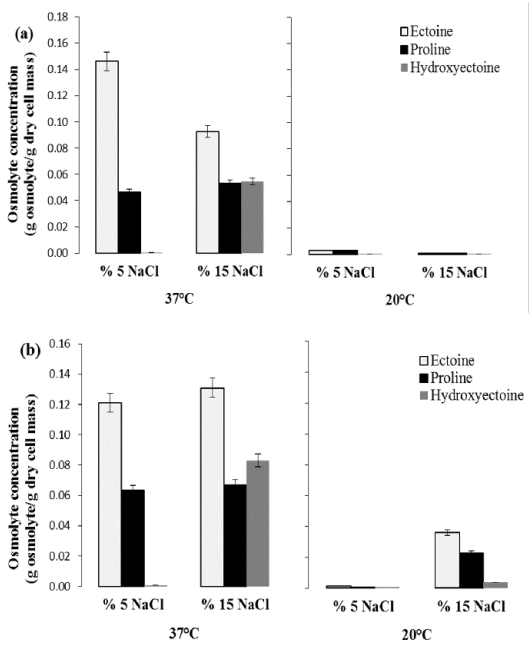

Figure 2: Distribution of osmolytes during different growth phases, (a) Mid-exponential phase, (b) Late stationary phase. Experiments were carried out with 5 and 15 % NaCl in minimal media supplemented with 0.5% glucose. Initial pH was adjusted to 7.00. Growth temperatures were 20 and 37°C.

three osmolytes in both growth phases were very similar, e.g., with the transition to higher salinity all osmolytes increased approximately 30-fold (Table 1). Interestingly, with increasing salinity, proline and ectoine accumulation remained almost constant in either growth phase at 37°C. Moreover, increasing salinity at this temperature resulted in a dramatic increase in hydroxyectoine amount, about 100-fold in both growth phases (Table 1).

The conversion reaction of ectoine to 5-hydroxyectoine, catalyzed by ectoine hydroxylase, does not go to completion. Thus the effects of salinity and temperature stress on ectoine hydroxylase activity have also been investigated in an attempt to increase hydroxyectoine yield. Enzymatic analysis of ectoine hydroxylase has shown that at the three different temperatures investigated, 20, 37 and 40°C, salinity always enhanced ectoine hydroxylase activity, with the highest value at 37°C (Figure 3). With 5% NaCl, increase in temperature resulted in a slight enhancement in ectoine hydroxylase activity. However, with 15% NaCl, maximum ectoine hydroxylase activity was at 37°C. At 20 and 40°C, the increase in ectoine hydroxylase activity was about 35% when salt concentration increased from 5 to 15%, whereas the increase in activity was 140% at 37 °C. The activity of ectoine hydroxylase remained almost unchanged at 20°C; consequently hydroxyectoine amount was almost constant.

Investigation of growth and osmolyte production under oxygen limited conditions has shown that specific growth rate dropped to one tenth of its original value at 37°C with 5% NaCl. At the same time, the concentrations of all the osmolytes dropped sharply with oxygen limitation. Ectoine concentration dropped sharply from 0.0494 (0.347) to 0.0011 (0.0077) g/g dry cell weight (mmol/ g dry cell mass), while proline concentration dropped from 0.0353 (0.306) to 0.0054 (0.0469) g/g dry cell weight (mmol/g dry cell mass). Moreover, under the oxygen-limited conditions, there was no hydroxyectoine synthesis, at all.

Growth and osmolyte synthesis in the presence of proline, ectoine and glycine-betaine

At 37°C with 5% NaCl, in addition to 0.5% glucose, when the

Growth phase	Relative increase in osmolyte concentration*			
	20°C		37°C	
	5% NaCl	15% NaCl	5% NaCl	15% NaCl
	Proline			
Mid exponential	**2.39**	1.00	1.00	1.15
Late stationary	1.00	**29.20**	1.00	1.06
	Ectoine			
Mid exponential	**2.59**	1.00	1.57	1.00
Late stationary	1.00	**28.64**	1.00	1.08
	Hydroxyectoine			
Mid exponential	**2.18**	1.00	1.00	**95.63**
Late stationary	1.00	**31.47**	1.00	**102.27**
Growth phase	**Relative increase in osmolyte concentration***			
	20°C		**37°C**	
	5% NaCl	**15% NaCl**	**5% NaCl**	**15% NaCl**
	Proline			
Mid exponential	**2.39**	1.00	1.00	1.15
Late stationary	1.00	**29.20**	1.00	1.06
	Ectoine			
Mid exponential	**2.59**	1.00	1.57	1.00
Late stationary	1.00	**28.64**	1.00	1.08
	Hydroxyectoine			
Mid exponential	**2.18**	1.00	1.00	**95.63**
Late stationary	1.00	**31.47**	1.00	**102.27**

*(Relative increase of osmolyte concentration = (High osmolyte concentration/ low osmolyte concentration) x 100; low amount of osmolyte was taken as "1")

Table 1: Relative increase of osmolyte concentration at different temperatures and NaCl concentrations.

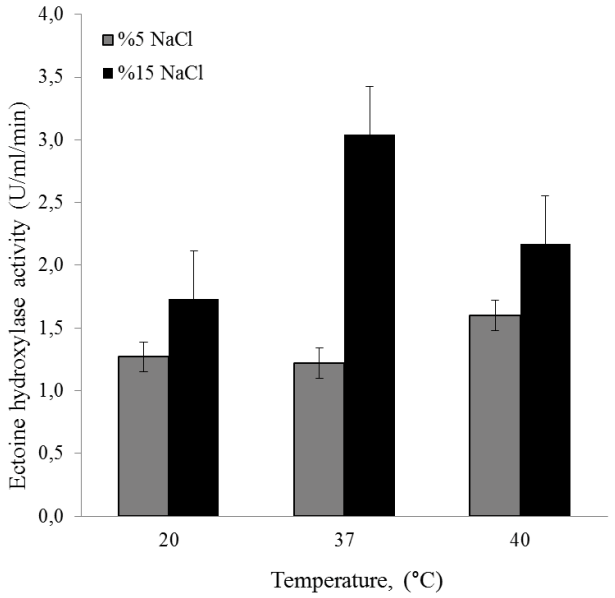

Figure 3: Ectoine hydroxylase activity. Activity measurements were carried out at three different temperatures as 20, 37 and 40°C. Salt concentration in the assay was kept at 5 or 15 % NaCl.

medium was supplemented with 1 mM proline, glycine-betaine or ectoine, specific growth rate increased significantly only with glycine-betaine (Table 2). Under the same growth conditions, glucose was replaced with proline, glycine-betaine, ectoine or hydroxyectoine to investigate whether AAD12 could utilize osmolytes as carbon sources. Although AAD12 can grow in M63 medium with 15% NaCl, growth at this salinity was slower than the growth at 5% NaCl. Hence, utilization of osmolytes as carbon sources has been investigated in the presence of 5% NaCl. Control cells were grown with glucose as the carbon source. As seen in Figure 4, all the selected osmolytes could be catabolized by *Halomonas* sp. AAD12 and as a general rule, an increase in the concentration of an osmolyte enhanced growth rate. When the growths with different osmolytes were compared with the growth with glucose, exponential growth started much earlier with glucose. The lag phase with glycine-betaine was significantly short when compared to the lag phases in the presence of the other osmolytes. An initial adaptation period was probably required for cell doubling with proline, ectoine and hydroxyectoine.

As for the production of osmolytes during utilization of proline, glycine-betaine, or ectoine as the carbon source, total intracellular osmolyte amount was the highest in the presence of ectoine (Figure 5). Among the osmolytes tested as carbon sources, hydroxyectoine yield was highest with 10 mM ectoine. Intracellular osmolyte accumulation was lowest with glycine-betaine as the carbon source but increasing its initial amount enhanced proline synthesis, while ectoine and hydroxyectoine amounts remained almost constant.

Effect of temperature and salinity on fatty acid methyl ester synthesis

Since stress conditions affect fatty acid compositions of halophilic microorganisms, the percentage of fatty acids were determined under stress conditions. Fatty acid analyses of *Halomonas* sp. AAD12 grown

Carbon source	Specific growth rate
0.5% Glucose + 1 mM Proline	0.10 hr^{-1}
0.5% Glucose + 1 mM Betaine	**0.39 hr^{-1}**
0.5% Glucose + 1 mM Ectoine	0.11 hr^{-1}
0.5% Glucose + 1 mM 5-Hydroxyectoine	0.12 hr^{-1}

Table 2: Specific growth rate of *Halomonas* sp. AAD12 with glucose and osmolytes together as carbon source (Experiments were carried out at 5% NaCl, pH: 7.00 and 37°C).

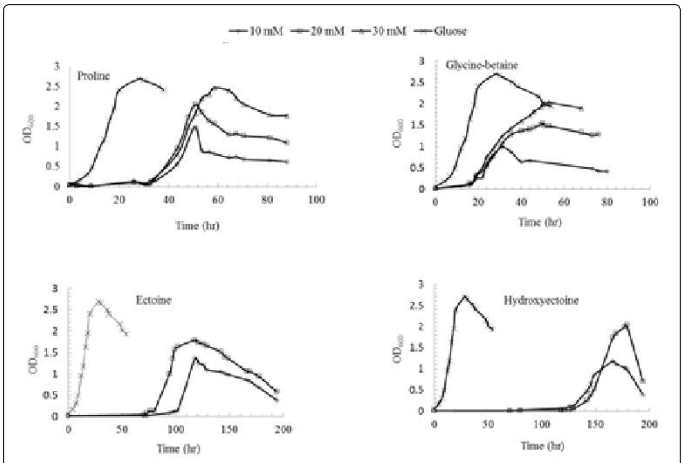

Figure 4: Utilization of compatible solutes as a carbon source by *Halomonas* sp. AAD12.

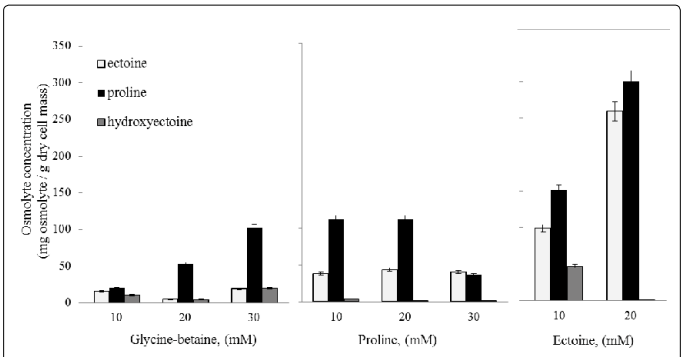

Figure 5: Distribution of osmolytes during growth at 37°C in the presence of ectoine, proline and hydroxyectoine as the carbon source. Salt concentration was adjusted to 5% NaCl and the initial pH was adjusted to 7.00.

under different temperatures with variations in salinity are presented in Table 3. As with many different moderate halophiles [25-28], the major fatty acids found in *Halomonas* sp. AAD12 were palmitic acid (C16:0), palmitoleic acid (C16:1) and oleic acid (C18:1). At 37°C, the saturated, C16:0 and the monounsaturated, C18:1 dominated. There were substantial amounts of longer fatty acids C20:1, C21, and C23. At this temperature with an increase in salt concentration, there could be a tendency to decrease membrane fluidity. The relative abundance of C16:0 increased significantly, with only a slight increase in C18:1. There was no substantial change in the abundances of longer fatty acids. When the temperature was lowered to 20°C, fatty acid unsaturation increased tremendously with the percentage of C16:1 and C18:1 together accounting for at least 65% of total fatty acids. This result should be an outcome of the general tendency of microorganism to increase membrane fluidity at lower temperatures. The increase has

Fatty acid methyl ester	Temperature, (°C)			
	37°C		20°C	
	NaCl Concentration			
	5 %	15 %	5 %	15 %
C4	6.83	2.51	8.5	1.2
C10	2.46	1.80	ND	ND
C11	2.74	2.02	ND	ND
C12	2.8	2.15	0.2	0.4
C14	3.05	2.43	0.5	0.7
C14:1	ND	ND	0.4	0.7
C15	3.77	2.78	ND	ND
C16	**15.41**	**20.34**	**17.8**	**24.2**
C16:1	3.59	3.69	**18.5**	**19.1**
C16:3	ND	ND	ND	0.4
C17	4.73	3.41	ND	ND
C18	0.69	1.26	0.3	1.5
C18:1	**25.97**	**27.16**	**47.7**	**44.7**
C18:3	0.71	1.70	0.5	2.8
C20:1	5.89	4.09	0.4	0.3
C21	6.28	4.22	ND	ND
C22:1	ND	ND	1.0	ND
C22:2	ND	ND	0.2	0.7
C22:6	1.95	1.87	ND	0.6
C23	5.51	4.45	ND	ND

Table 3: Effect of temperature and salinity on fatty acid methyl ester composition (%).

been achieved at the expense of decreased amounts of all minor fatty acids. As with the analysis at 37°C, at 20°C with increasing salinity fluidity decreased. The abundance of C16:0 increased significantly whereas the abundances of C16:1 and C18:1 dropped slightly.

Discussion

Growth of *Halomonas* sp. AAD12 in minimal M63 medium supplemented with glucose was sensitive to salinity and temperature however there was no regular pattern in the changes. In general osmolyte addition enhanced growth, with glycine-betaine being the best. Vargas et al. [11] reported that overall, growth with osmolytes was slower when compared to growth in glucose-supplemented medium. When *C. salexigens* was grown at 37°C with 1.5 M NaCl in media supplemented with a mixture of 10 mM glucose and one of these osmolytes, the presence of ectoine and hydroxyectoine enhanced growth [11]. Hanelt and Mullet [14] reported that both glutamate and proline supported the growth of the halophilic bacterium *Halobacillus halophilus* but this microorganism could hardly use ectoine as a nutrient since it does not possess the genes for its utilization. Although *Halomonas* sp. AAD12 and *C. salexigens* were very similar, while *Halomonas* sp. AAD12 could grow in M63 media with 5% (≈0.85M) NaCl and 20 mM of an osmolyte as the carbon source (proline, betaine and ectoine), *C. salexigens* could not grow at all in the same media with 0.75 M NaCl and any of the osmolytes [11].

The major osmolytes synthesized by *Halomonas* sp. AAD12 are ectoine, proline and hydroxyectoine. In our previous work, we have reported that at 37°C with trisodium citrate as the carbon source, proline and ectoine were equally important osmolytes at low salinity whereas proline was the major osmolyte at higher salinity [20]. In contrast, ectoine was the dominating osmolyte in M63 media supplemented with glucose. An interesting finding regarding osmolytes synthesis in this microorganism was that, at 37°C, salinity triggered hydroxyectoine synthesis which suggested that in addition to the increase in the flux from oxaloacetate to ectoine, flux from ectoine to hydroxyectoine increased tremendously. At the same temperature and salinity, although the same osmolytes were accumulated, the priority for each osmolyte changed. Hence medium composition is a significant factor that strictly affects distribution of intracellular osmolytes.

Regarding the osmoadaptation strategy in *Halomonas* sp. AAD12, the relative importance of the three osmolytes remains similar in different growth phases at 20°C. However at 37°C, the importance of hydroxyectoine stands out at high salinity. In contrast to our findings, Saum and Müller [12] reported that the moderately halophilic *Halobacillus halophilus* changed its osmolyte accumulation strategy through exchanging proline by ectoine in the transition from exponential to stationary phase. Another moderate halophile, *C. salexigens*, accumulated maximum ectoine at 37°C with 17.4% NaCl and maximum hydroxyectoine at 45°C with 14.5% NaCl [29]. Maximum hydroxyectoine accumulation was achieved with 15% NaCl by both *C. salexigens* and *Halomonas* sp. AAD12. Hydroxyectoine yield in the engineered *C. salexigens* was as high as 883 mol/g bacterial dry matter with 4.35% NaCl at 37°C [29].

In the absence of any genetic engineering for increased yield, *Halomonas* sp. AAD12 produced 525 mol hydroxyectoine/g dry cell mass. This amount is pretty much comparable to the hydroxyectoine yield by the engineered *C. salexigens* [29]. Hydroxyectoine yield can further be enhanced by engineering the strain [29,30]. Therefore, *Halomonas* sp. AAD12 could be evaluated as a potential 5-hydroxyectoine producer. Vargas et al. [11] reported that glycine-betaine as the sole carbon source, completely prevented ectoine synthesis in *C. salexigens*. The negligible intracellular ectoine and hydroxyectoine in *Halomonas* sp. AAD12 under this condition suggested that a fraction of the taken-up betaine could have been used as an osmoprotectant under the salt-stress conditions.

Intracellular conversion of ectoine to hydroxyectoine is catalysed by the enzyme ectoine hydroxylase (EctD) and the extent of hydroxyectoine conversion is highly sensitive to reaction conditions [19]. In order to correlate changes in hydroxyectoine yield with changes in temperature and salinity in *Halomonas* sp. AAD12, ectoine hydroxylase activity was measured at different temperatures and salinities. As for the *in-vitro* activities of the biosynthetic enzymes from *H. elongata*, [10] the ectoine hydroxylase in *Halomonas* sp. AAD12 was sensitive to salinity. Moreover, the optimum temperature of the enzyme was between 32 and 40°C, as reported by Widderich et al. [19]. Synthesis of hydroxyectoine via ectoine is a reversible reaction [31]. However, under physiologically relevant conditions, ectoine hydroxylase catalyzes this reaction only in one direction. [19] We propose that the increase in intracellular hydroxyectoine during growth in 15% NaCl and at 37°C should be a result of the increase in ectoine hydroxylase activity since this enzyme is responsible for hydroxyectoine synthesis [31].

The conversion reaction of ectoine to 5-hydroxyectoine, catalysed by ectoine hydroxylase, is an oxygen-dependent reaction [13,19,32]. Consequently, as expected, a re-distribution in osmolytes under oxygen-limited conditions has been found with limited oxygen. Moreover, since ectoine hydroxylase is dependent on the availability of oxygen for its activity, the absence of hydroxyectoine synthesis under this condition was not unexpected.

In the fight with the lethal effects of high salt concentrations, while osmolyte accumulation protects the cell interior, cellular lipids are modified to maintain the integrity of the microorganism. An important factor that dictates the physical properties of membrane lipid matrix is the fatty acid composition of the phospholipids. Specifically, fatty acid

length, unsaturation and, branching are involved in the maintenance of proper membrane fluidity under stress conditions [33-35]. To this end, we tackled the effect of salinity and temperature on the abundances of fatty acids of *Halomonas* sp. AAD12. In general, both salinity and temperature increased the abundance of saturated fatty acids in *Halomonas* sp. AAD12. Our results are in agreement with the study carried out by Valderrama et al. [27]. In a closely related microorganism *H. salina*, as a consequence of increasing salinity the relative proportion of saturated fatty acids increased with a concomitant decrease in the monounsaturated fatty acids, which suggests decreased membrane fluidity [27]. Reports concerning the fatty acids of the moderate halophiles *H. halophila* and *Pseudomonas halossaccharolytica* showed similar distributions [25,26].

Conclusions

Halomonas sp. AAD12 is a moderate halophile that changes its osmolyte accumulation strategy and membrane fluidity in protection from stress. As with many moderate halophiles, it accumulated changing concentrations of ectoine, proline and hydroxyectoine to protect its cytoplasm when exposed to stress, such as salinity, temperature, and oxygen limitation. Its major fatty acids were palmitic acid (16:0), palmitoleic acid (16:1), and oleic acid (18:1). Fatty acid distributions suggested that as a response to salt and temperature stress membrane fluidity decreased. Interestingly, hydroxyectoine accumulation reached a value as high as 525 µmol/g dry cell mass at 37°C with 15% NaCl. Increased intracellular ectoine hydroxylase activity explained the increase in hydroxyectoine yield. On the other hand, oxygen limitation totally abolished the synthesis of hydroxyectoine. The wide range of adaptive capabilities of *Halomonas* sp. AAD12 with high yield hydroxyectoine of suggests that the potential of this microorganism as a producer of hydroxyectoine should be exploited.

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