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Screening of Moderately Halophilic Bacteria Producing Ectoine Resulting in Selection of *Virgibacillus salarius* BHTA19

Running title: Screening of Ectoine-Producing Bacteria

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SUMMARY

Research background. Ectoine is a desirable molecule with high application potential, particularly in the cosmetics and pharmaceutical industries. The current production method involves microorganisms that require high salinity. Therefore, product purification is expensive, complex, and requires proper equipment. To overcome these obstacles, we were searching for new moderately halophilic, ectoine-producing bacteria.

Experimental approach. The bacteria were isolated from high salinity environments: the vicinity of Tyrawa Spring, Złockie vicinity of Na Mokradłach Spring, and Rajcza all in Poland. Their capacity for biosynthesizing ectoine and additionally hydroxyectoine in a 10% premixed seawater environment was assessed semi-quantitatively using mass spectrometry (MS). The growth of bacteria was also compared under these conditions. Then the most promising strains were identified based on 16S rDNA sequence, and their morphological, biochemical, and physiological features were described. The biosynthesis of ectoine was conducted based on the collected data and the preferences of individual strains. Final product concentrations were estimated by HPLC. Following the screening process, the most suitable strain was pointed out.

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Results and conclusions. Fifty-six bacterial strains were isolated. Most produced insignificant amounts of ectoine or hydroxyectoine in the presence of 10 % salt. However, ten, all isolated from Tyrawa Spring, showed promising properties and were used in further studies. Based on the 16S rDNA sequence four were as assigned as *Halobacillus* sp., two *Virgibacillus* sp. and one from *Bacillus* sp., *Pseudalkalibacillus* sp., *Salimicrobium* sp., and *Thalassobacillus* sp. The basic biochemical and physiological features as well as the ability to grow in the presence of NaCl, KCl, $(NH_4)_2SO_4$ and $MgSO_4$ were described. The biosynthesis of ectoine was conducted following the best parameters estimated for each strain. Based on the results *Virgibacillus salarius* BHTA19 was singled out as a new potential producer of ectoine.

Novelty and scientific contribution. We isolated promising ectoine producer, moderately halophilic bacteria - *Virgibacillus salarius* BHTA19. The BHTA19 is a wild-type strain that produces significant quantities of ectoine in environments with moderate salt concentrations. It shows great potential and possibility of industrial application.

Keywords: halophilic bacteria; ectoine; hydroxyectoine; biosynthesis; screening

INTRODUCTION

Living organisms that have colonised planet Earth for billions of years have created mechanisms and strategies that let them inhabit environments which, from an anthropocentric point of view, are potentially harmful or lethal. One of the organisms' groups that had the longest evolution time to adapt to rough habitats is bacteria. These, inhabit environments the most lethal and unfriendly for humans and are in general named as extremophiles. Depending on the factor limiting the growth of microbes they are categorised *i.e.* as thermophiles (elevated temperature), psychrophiles (low temperature), acidophiles (low pH), alkaliphiles (high pH), radiophiles (high radiation resistance), piezophiles and barophiles (high pressure), xerophiles (low water activity) and polyextremophiles (tolerating or preferring more than one extreme factor) (*1-3*). Another interesting group of extremophiles are halophiles. The bacteria grow and proliferate in high-salted environments, for instance, salt mines, ponds and springs or salt deposits on rocks or buildings (*4,5*). Colonization and ability to proliferate in environments exert extreme osmotic pressure is a huge challenge for bacteria. Microorganisms have a low cell volume to surface ratio, which can cause easier loss of intracellular water, and in a consequence making it harder for them to manage the osmotic balance effectively (*6*).

To survive, halophiles have developed specific intracellular mechanisms that enable them to retain intracellular water. In general, they show two main mechanisms of action. The former called the "salt-in strategy" relies on accumulation inside the cell salts whose concentration equilibrates external osmotic pressure and avoids leakage of water. This is common for archaea and certain *Halobacteria* (*7*). The latter one, more often present among procaryotes, is much more complicated, based on synthesised inside the cell small organic molecules called compatible solutes or osmoprotectants. These molecules are produced in cells in high concentration without affection on central metabolism and belong mainly to polyols and carbohydrate derivatives. These compounds are commonly found in bacteria and plant kingdoms, including glycerol, myo-inositol, glucosylglycerol, and trehalose (*8*).

In terms of potential commercial application noteworthy are two hydroxyproline derivatives, osmoprotectants - ectoine and its hydroxylated derivative - hydroxyectoine. Both bind water molecules strongly and are found in bacterial cells. Their concentration depends on the type of bacteria and the environmental conditions. Ectoine levels can rise from a few millimoles per liter to up to 100 mM in response to stress like high salt, drought, or temperature changes (*1*).

Apart from their osmoprotectants feature, they act also as anti-freezing agents, UV filters, soothing and anti-inflammatory molecules (*9*). These properties make both molecules incredibly attractive components for the cosmetic and food industry. In terms of low irritation rate, they are also widely distributed in pharma products including high-purity drugs like eye or nose drops (*10*).

The primary aims of the research were to isolate a wild-type bacterial strain that synthesizes ectoine at low or moderate salt concentration and to conduct preliminary work on finding cost-effective and simple growth media.

MATERIALS AND METHODS

Isolation of halophilic bacteria

Halophilic bacteria were isolated from highly salted environments: the vicinity of Tyrawa Spring, Złockie vicinity of Na Mokradłach Spring, and Rajcza all in Poland. The samples, which included a combination of bottom sediments and water, were kept at 4 °C until needed. For the isolation 10 g of well-mixed sample were added to 100 mL of buffered peptone water (BTL, Lodz, Poland), then the dilutions were shaken for 30 min, at 30 °C and 150 rpm. Screening of halophilic bacteria was performed on nutrient agar (BTL) with the addition of 100 g/L premixed salts Instant

Ocean (Aquarium Systems, Sarrebourg, France). The incubation was conducted at 30 °C, for one to two weeks. Then the single colonies were picked and streaked onto petri dishes with the previously used medium. After 24 h of incubation, at 30 °C the purity of the culture was examined, after Gram staining, under the light microscope at 1000x magnification (BX63, Olympus, Tokyo, Japan). If the culture purity was confirmed the isolated bacteria were kept frozen at -80 °C in glycerol stocks. If the isolated bacteria needed further purification, they were again streaked, incubated, and observed under a microscope.

Recovery of bacteria from the glycerol stocks

The cells collected from the glycerol stock were inoculated onto nutrient agar (BTL) with 100 g/L of premixed salts Instant Ocean (Aquarium Systems) and incubated for 24 h at 30 °C.

Characterisation of bacteria morphology

Microscopic features of bacterial cells were assessed in fresh cultures, obtained directly after the recovery of the bacteria from glycerol stocks. Cell morphology was examined after Gram staining at 1000x magnification using a light microscope (BX63, Olympus).

Determination of oxidase, L-alanine aminopeptidase, and catalase activity

Enzymatic activities were evaluated after recovering the bacteria from the glycerol stocks. The cytochrome oxidase and L-alanine aminopeptidase activity were detected using Bactident® Oxidase and Bactident® Aminopeptidase (both from Merck, Darmstadt, Germany) test strips. The analyses were performed according to the manufacturer's instructions. Catalase activity was determined by placing the bacterial biomass on a microscope slide and applying a drop of 5 % hydrogen peroxide (Chempur). The activity was assessed based on gas formation. The presence of catalase was confirmed by the appearance of gas bubbles.

Bacteria cultivation and ectoine biosynthesis

Cells were recovered from glycerol stock, and one loop of biomass was placed in 50 mL of inoculation medium with nutrient broth (BTL) and 100 g/L seawater (Instant Ocean, Aquarium Systems). Bacteria were incubated overnight. After that 20 mL inoculum was transferred to 200 mL of nutrient broth (BTL) and either 100 g/L or 150 g/L of seawater, where the biosynthesis of ectoine

was performed. The fermentations were conducted at 150 rpm and 30 °C in a shaking incubator (Multithron, Infors HT, Basel, Switzerland).

Impact of various salt concentrations on bacterial growth

Bacteria were recovered from glycerol stocks as previously described. Cells were grown in 50 mL of medium with nutrient broth (BTL) and 100 g/L seawater (Instant Ocean, Aquarium Systems), at 150 rpm and 30 °C in a shaking incubator (Infors HT), then used as an inoculum. After that, tubes with 9 mL of the tested media were inoculated with 1 mL of the culture. In total, 21 types of media were prepared. One contained: 1 g/L beef extract (BTL), 2 g/L yeast extract and 5 g/L peptone (both from VWR, Radnor, Pennsylvania, USA). The other media additionally included one of the following inorganic salts: Instant Ocean (Aquarium Systems), NaCl, MgSO4·7H2O, (NH4)2SO4 (all from Chempur, Piekary Śląskie, Poland), or KCl (VWR). The examination of bacterial growth was carried out using five different concentrations of each salt: 5, 10, 15, 20, and 25 %. The strains were incubated at 30 °C. The study was conducted for 48 h. Turbidity was measured with a densitometer (DEN-1B Densitometer, Biosan, Riga, Latvia) to determine the limiting growth parameters. The results were expressed on the McFarland scale. Then the changes in turbidity were calculated according to Eq.1:

$$\Delta T(\text{seed culture}) = T_{48}(\text{seed culture}) - T_0(\text{seed culture}) \quad /1/$$

where ΔT is the change in turbidity, T_0 is the initial turbidity and T_{48} is the turbidity after 48 h of incubation. Based on the results, the cardinal salt concentrations were estimated.

Evaluation of bacteria growth at various temperatures and pH

The test was performed analogously to the evaluation of the impact of various salt concentrations. Each tube contained 9 mL of nutrient broth (BTL) supplemented with 100 g/L of pre-mixed salts from Instant Ocean (Aquarium Systems). This time, the pH of the medium in each tube was different and was adjusted to 3, 4, 5, 6, 7, 8 or 9. The cultures were incubated at 30 °C for 48 h. Additionally, for pH 7, the changes of turbidity were measured also after incubation at 10, 15, 20, 25, 37 and 44 °C. To define limits of growth, and optimum temperature and pH, the increase in turbidity was estimated according to Eq. 1.

Molecular identification of selected bacteria

Genomic DNA was extracted using NucleoSpin® Microbial DNA kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the manufacturer's recommendations. The concentration and

purity of the isolated DNA were assessed by spectrophotometry method (Nanovue Plus, Biochrom, Cambridge, UK). Universal bacteria primer sets 27F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R: 5'-TACGGTACCTTGTTACGACTT-3' were used for the amplification of the 16S rRNA genes. The PCR master mix mixture contained 4 µL genomic DNA, 1 µL of each primer (concentration 10 µM), 25 µL PrimeSTAR® Max DNA Polymerase (Takara, Kyoto, Japan), and water so that the final volume was 50 µL. The PCR was carried out in Agilent SureCycler 8800 (Agilent Technologies, Santa Clara, California, United States) after 30 cycles under the following conditions: 10 s of initial denaturation at 98 °C, 30 cycles of denaturation (10 s at 98 °C), annealing (10 s at 55 °C) and extension (10 s at 72 °C). The PCR products were sequenced by Genomed Inc. (Genomed, Warsaw, Poland) by the Sanger Sequencing method.

Determination of cell growth

The changes in the absorbances were checked at λ=600 nm using Shimadzu UV-1800 UV/Visible Scanning Spectrophotometer (Shimadzu, Kyoto, Japan). To dissolve the salts, present in the medium the samples were firstly diluted ten times with 10 % acetic acid (Chempur) and then in water.

Extraction of ectoine and hydroxyectoine

A volume of 200 mL broth was centrifuged by 3220×*g* (centrifuge 5810 R, Eppendorf, Hamburg, Germany) at 4 °C for 30 min and then the pellets were suspended in 80 % ethanol (Avantor, Gliwice, Poland). Extraction was carried out at 200 rpm, 25 °C on a shaking incubator (Infors HT). After centrifugation by 3220×*g* at 4 °C for 30 min, the supernatant was placed in a vacuum dryer at 60 °C, 30 kPa (VO500 Memmert, Schwabach, Germany), until no further change in mass occurred. The dried extract was applied for further analysis.

MS determination of ectoine and hydroxyectoine

The presence of ectoine and hydroxyectoine was confirmed by comparing the mass spectra of the samples with the standards. The content of ectoine and hydroxyectoine was determined semi-quantitatively based on the number of mass detector counts (cnts) of the searched *m*/*z* values (ion intensity), which is proportional to the analyte concentration. However, the ion intensity may be influenced by the matrix effect, which was not investigated in this case. High-resolution mass spectra were recorded on the mass spectrometer Impact HD UHR-QqTOF (Bruker, Billerica, USA). ESI (+)

ion source was operated at a dry temperature of 200 °C, needle voltage 4.5 kV, with dry gas flow set up at 6 L/min and nebulizer gas pressure of 200 kPa. The mass analyser was working at hexapole rf 60 Vpp, collision rf 500 Vpp, transfer time 60 µs, and pre-pulse storage of 5 µs. The scan range in MS mode was set up from 50 to 1000 m/z and the sample time was 1 s. Collision energy for qualitative analysis was set up at 7 eV. The spectrometer was internally calibrated before each run with a sodium formate clusters calibrant, according to the procedure given by the manufacturer. Ectoine (97.4 % HPLC) and hydroxyectoine (98.1 % HPLC) standards were purchased from Merck (Darmstadt, Germany) and Sigma Aldrich (Saint Louis, USA), respectively. 100 % HPLC grade water was obtained by double distillation and purification using an HYDROLAB HLP20 water purification system (Hydrolab, Straszyn, Poland). To prepare the standard solutions, in 10 mL volumetric flasks 51.4 mg ectoine standard and 21.2 mg hydroxyectoine standard were weighed on an analytical balance, dissolved in water, and filled to a final volume of 10 mL. The solutions were diluted with water to a concentration of 50 µg/mL. To prepare the sample solutions, about 20 mg of each sample was weighed and dissolved in water to a concentration of 20 mg/mL. A small amount of the prepared sample solutions was diluted fourfold to a concentration of 5 mg/mL. All solutions were filtered on 0.2 µm syringe PTFE filters and injected into the mass spectrometer using a syringe pump (180 µL/h).

HPLC determination of ectoine and hydroxyectoine

Concentrations of ectoine and hydroxyectoine were determined in relation to external standards using UltiMate 3000 UHPLC system coupled with UV-VIS detection (Thermo Scientific, Waltham, USA) and fitted with Ultra AQ C18 column 100 mm x 2.1 mm x 3 µm (Restek, Bellefonte, USA). 100 % HPLC grade water was used as a mobile phase. All chromatographic separations were performed at 30 °C using a flow rate of 0.08 mL/min and a run time of 10 min. The detection at 210 nm was selected. The standard solutions prepared according to the procedure above were diluted to a concentration of 10 µL/mL. To prepare the sample solutions, in a 1 mL volumetric flask 10 mg of each sample was weighed on an analytical balance, dissolved in water, filled to a final volume of 1 mL, and diluted 100 times with water. All solutions were filtered on 0.2 µm syringe PTFE filters and transferred to a chromatographic vial.

Data analysis

Calculations, statistical analysis, and graphs were prepared using Microsoft Excel 2010 (*11*). The sequences were analysed with open-source software Chromas and Decipher, then aligned and

compared with sequences deposited in GenBank (*12-14*). The otofControl and Compass Data Analysis software were used to record and analyse mass spectra, appropriately (*15,16*). HPLC data were collected and analysed using Chromeleon software (*17*).

RESULTS AND DISCUSSION

Isolation and characterisation of halophilic bacteria

We isolated 56 bacterial strains able to grow in the presence of a 10 % salt mixture. 15 originated from vicinity of Tyrawa spring (BHTA), 30 from Złockie vicinity of Na Mokradłach Spring (BHMM) and 11 from Rajcza (BHEGO).

Most bacteria strains were Gram-positive and rod-shaped. BHTA20 and BHTA24 were the only strains classified as coccus. Nevertheless, microscopic observations confirmed that we obtained a diversified set of strains. The morphological differences of the isolates are shown in Fig. 1.

The more varied the collection of microorganisms obtained before the screening step, the higher the probability of effective selection. Significant morphological differences between the isolated strains allowed us to expect positive screening results which was isolation of ectoine producer. The isolated bacteria were grown in a nutrient-rich medium containing 10 % seawater. Subsequently, the presence of ectoine and its derivative hydroxyectoine was assessed semi-quantitatively using the LC-MS method. The common logarithm of the number of mass detector counts (cnts) was calculated for easier presentation of the results (Fig. 2). Moderately halophilic bacteria, which were the subject of the search, growth well in a salt concentration range of between 5 and 20 %. Therefore, the absorbance was compared alongside the estimated amount of ectoine (Fig. 2).

Most of the bacteria did not synthesize ectoine and hydroxyectoine or produced them in minimal quantities. Nevertheless, based on the number of mass detector counts we indicated 10 strains able to biosynthesis of significant amounts of desirable products. Strains BHTA2, BHTA18, BHTA19, BHTA21, BHTA22, BHTA24, BHTA25, BHTA30 synthesise mainly ectoine and negligible quantity of hydroxyectoine. In the case of BHTA20 ectoine was the only product detected, in contrast to BHTA8 which synthesises the predominant amount of hydroxyectoine. The majority of the selected microorganisms grew well under aerobic conditions in the medium containing in total 10 % seawater. Only BHTA22 showed a minor biomass concentration. It was considered in further studies. ANOVA analysis was conducted to validate the statistical differences observed between the strains. In all cases, the statistical significance of the results was confirmed, and the p value was less than 0.001.

All strains that produced significant amount of ectoine and its derivative hydroxyectoine were isolated from vicinity of Tyrawa Spring. We performed their identification, based on 16S rDNA sequence and basic biochemical features (Table 1).

The results confirmed the differentiation of the isolated strains. Among the microorganisms, we identified 6 genera of bacteria. Four strains were classified as *Halobacillus* spp., two as *Virgibacillus* spp. and one each as *Bacillus* sp., *Pseudalkalibacillus* sp., *Salimicrobium* sp. and *Thalassobacillus* sp. In addition to the genetic identification, basic biochemical characterisation was also performed (Table 2).

According to current knowledge, *Halobacillus halophilus* is the only ectoine producer that has been previously described in detail (*18*). However, the final amount of ectoine synthesised by the species is low. Therefore, *H. halophilus* has been not industrially applied for ectoine biosynthesis (*18,19*). There is a lack of knowledge about the cultivation conditions and biosynthesis of ectoine by the other strains. Therefore, we decided to analyse the influence of temperature, pH, and various salts concentration on the bacteria (Table 2).

All bacteria showed catalase activity and lacked L-alanine aminopeptidase. Only *P. hwajinpoensis* BHTA18 and *T. devorans* BHTA24 did not present oxidase activity. All strains grew in the whole examined range of temperature. However, the optimal value of this parameter was diversified depending on the strain. Most bacteria preferred temperatures around 30 °C, but *B. velezensis* BHTA8, *P. hwajinpoensis* BHTA18, *Halobacillus* sp. BHTA2 and *H. sediminis* BHTA30 had optimum at 37 °C (Table 2). The pH range was strain dependent. Nevertheless, most of the tested microorganisms preferred slightly alkaline or neutral pH. It is not surprising since most halophiles usually inhabit such environments (*20)*.

The growth of most halophilic microorganisms requires the presence of inorganic salt. High-salt medium is mandatory to stimulate ectoine biosynthesis. On the other hand, cells growth and excretion of product can be limited by high salt concentration (*21*). Moreover, specific ions can affect ectoine metabolism in bacterial cells (*22*). There is no information about the media used for ectoine biosynthesis with the isolated strains. Therefore, to select the inorganic components of the media and to determine their concentration range, the growth of the bacteria was tested in the presence of: NaCl, $MgSO_4$, KCl, and $(NH_4)_2SO_4$ (Table 2).

Halophilic bacteria require the presence of sodium ions for growth and metabolism, some are not able to grow in the absence of salt. Halophiles can be categorized into three primary groups based on their optimal saline preferences: slight (1–3 %), moderate (3–15 %), and extreme (15–30 %) (*23*).

NaCl content, in particular the sodium ions, is known factor responsible for regulation of ectoine biosynthesis. Ectoine production tends to show a positive correlation with increasing NaCl concentration (*24*). NaCl impact is observed also on the molecular scale. It influences cell transport system and expression of genes involved in the biosynthesis of ectoine (*ectABC*) and hydroxyectoine (*ectD*) (*25*). On the other hand, the high value of this factor limits the growth of bacteria.

In the case of KCl, it had been proven that some of the halophilic bacteria *e.g*. *Halanaerobiales* sp. or *Salinibacter* sp. accumulate KCl to provide osmotic balance in cells and stabilise acid proteome. It is known that diaminobutyrate-2-oxoglutarate transaminase (*EctB*) is a pyridoxal phosphate (PLP) dependent enzyme, and the K^+ ions are necessary for its stability and activity (*26*,*27*). Ono *et al.* (*26*) reported that the presence of 0.01–0.5 M KCl increases the activity of *EctB* more than the presence of NaCl. Moreover, K^+ is obligatory for the activity of many enzymes included in extreme halophiles cells, which exhibit a salinity dependent osmoregulation (*28*).

The ammonium ions can increase the expression of the gene cluster *ectABC involved* in the synthesis of ectoine (*29*). It was also assumed that the ammonium sulphate increased the amount of L-aspartate-β-semialdehyde which is the precursor of ectoine, and glutamate which is responsible for the supply of co-substrates for *EctB* (*30*). These actions improve the production of ectoine by increasing the flow of substrate through the ectoine pathway.

Another factor that can influence the metabolism of halophilic bacteria is the presence of Mg^{2+}. It was shown that halophilic bacteria have a higher demand for this ion during their growth than non-halophilic microorganisms (*31*). It was also conjectured that high concentrations of Mg^{2+} protect and stabilise macromolecules during periods of dormancy. Moreover, the ratio between Mg^{2+} and Na^+ varies depending on the physiological state and the presence of nutrients (*32*).

The results presented in Table 2 showed that only *B. velezensis* BHTA8, *P. hwajinpoensis* BHTA18, *V. salarius* BHTA19 and *T. devorans* BHTA24 were able to grow in medium without salt addition. The optimal NaCl concentration range is different for each strain. However, Following the nomenclature proposed by Kushner most of the isolated strains can be classified as moderately halophilic bacteria, because they have an optimum in the range 3–15 % (*23)*. In the case of *Halobacillus sediminis* BHTA 25 and BHTA30 there is some doubt and lower salt concentrations should also be tested.

We observed no growth of *Halobacillus* sp. BHTA2, *H. sediminis* BHTA25 and BHTA30 in the presence of KCl only. Interestingly, the optimal value of this parameter reached 20 % for *S. halophilum* BHTA20. Growth in the presence of KCl varied significantly depending on the strain (Table 2). A

similar influence was observed in the case of $(NH_4)_2SO_4$. *Halobacillus* sp. BHTA2, *H. sediminis* BHTA25 and BHTA30 as well as *V. litoralis* BHTA22 did not grow. In other cases, the optimal concentration of the salt did not exceed 10 %. Most of the tested strains grow well in the presence of $MgSO_4x7H_2O$, and had their optimum in the range of 5-10 % of the salt. The obtained results do not allow for a simple determination of concentration limits. Future studies should take into account synergistic and antagonistic effects between ions. Therefore, artificial seawater (Instant Ocean) was used in further analysis. Artificial seawater is a mixture of various salts that mimics the natural environment, and it is easy to use in grow media. The isolated bacteria grow well when the seawater concentration is up to 15 %. Additionally, *P. hwajinpoensis* BHTA18, *V. salarius* BHTA19, *H. sediminis* BHTA25 and BHTA30 were able to proliferate even in 25 % solution (Table 2).

According to the presented results we carried out biosynthesis of ectoine under optimal temperature and pH selected for each of the strains. If the optimal pH was not strict, we calculated the mean value of this parameter. The fermentation media contained 15 % of Instant Ocean (seawater). During cultivation, the absorbances were measured (Fig. 3). The ectoine and hydroxyectoine were extracted after 48 h of fermentation and their concentration was quantified by HPLC method (Table 3).

All of the isolated strains grow well in described conditions. The highest increase in absorbance was observed in *B. velezensis* BHTA8 and *P. hwajinpoensis* BHTA18 cultures, whereas the lowest was presented by *Halobacillus* sp. BHTA2 and *V. litoralis* BHTA22. Ectoine concentration reached 274, 4.5, 1.5 and 5.3 mg/L, for *V. salarius* BHTA19, *S. halophilum* BHTA20, *V. litoralis* BHTA22 and *H. sediminis* BHTA25, respectively. Hydroxyectoine was not detected. ANOVA analysis confirmed the statistical significance of the results, the p value was less than 0.001.

Ectoine is produced on an industrial scale by *Halomonas elongata* DSM142 through a “bacterial milking” process. Using this microorganism Kunte’s research group obtained 7.4 g/L of ectoine, with productivity 0.22 g/L/h (*8*). The main disadvantage of the process was high salinity, up to 2.57 M, which can cause equipment corrosion, increases the production cost and inhibits the growth of microorganisms. Moreover “bacterial milking” is effective only for Gram-negative bacteria. Therefore, other microorganisms were considered as ectoine producers. At a much lower salt concentration, biosynthesis of ectoine is carried out by *Brevibacterium epidermis* DSM20659. The strain produced 1.42 g/L of ectoine, with a productivity 0.08 g/L/h (*33*). *Halomonas salina* BCRC17875 is another wild type strain used for biosynthesis of ectoine. In this case ectoine production achieved a concentration of 13.96 g/L after 44 h of cultivation, and NaCl concentration of 2 M (*34*). High

concentrations of ectoine can be achieved with the use of genetically modified microorganisms. *Escherichia* coli and *Corynebacterium glutamicum* are commonly used for this purpose. The genetically modified *E. coli* BL21 strain yielded 60.7 g/L with a productivity rate of 1.08 g/L/h, utilizing glucose as the sole carbon source under low salt concentration conditions (*35*). Also metabolically engineered *C. glutamicum* produced significant amounts of ectoine in low salt environment. *C. glutamicum* Ect10 demonstrated the capability to synthesize 115.87 g/L of ectoine, positioning it as one of the most efficient ectoine producers documented thus far (*36*).

The biosynthesis of hydroxyectoine is closely related to the presence of *ectD* gene, which encodes ectoine hydroxylase. Hydroxyectoine complicates the purification of ectoine and decreases its biosynthesis yield, so strains with high ectoine hydroxylase activity need genetic modification to inhibit this function (*37,38*). The presence of *ectD* gen in *V. salarius* has been confirmed (*39*). *V. salarius* BHTA19 synthesized hydroxyectoine, as identified by LC-MS analysis (Fig. 2), but it was not detected through HPLC (Table 3). This suggests that the expression of the *ectD* gene and the activity of ectoine hydrolase depend on culture conditions and require further investigation.

Among the isolated strains, *V. salarius* BHTA19 showed the most promising properties. The strain not only produced the highest amount of ectoine, but also it exhibited broad tolerance to salt type and its concentration as well as temperature and pH. There is a lack of knowledge about the cultivation conditions and biosynthesis of ectoine by *V. salarius*. However, *Virgibacillus* sp. is already known an ectoine producer. According to our knowledge, only a few genera have been described so far. It should be mentioned about *Virgibacillus pantothenticus*, *Virgibacillus halodenitrificans* and *Virgibacillus salarius* 19.PP.SC1.6 (*25*,*39,40*). Unfortunately, their production efficiency is not impressive. Taking into account, that *V. salarius* BHTA19 was cultivated in flask culture, the obtained amount of ectoine seems to be promising. On account of that further studies are required to determine the potential for industrial application of the strain.

CONCLUSIONS

Ectoine is one of the most marketable biotechnological products. There is a consistent increase in the number of its applications, leading to a growing demand for the metabolite. Although ectoine is produced on an industrial scale, the process still needs to be improved. The high salt concentrations used in current technologies require the use of equipment that is suitably resistant. Furthermore, managing production waste that contains significant quantities of salt is a challenge. Ongoing research efforts should prioritize not only the improvement of efficiency and the reduction of

production costs but also the identification of natural ectoine-producing strains that can perform biosynthesis at low or moderate salt concentrations.

As a result of the presented research, a moderate halophile *V. salarius* BHTA19 has been identified as new potential producer of ectoine. The studies showed that after 24 hours of a shake flask culture, the concentration of ectoine reached 274 mg/L. The amount of ectoine is lower than currently reported for other microorganisms, however BHTA19 did not undergo any engineering optimization processes and therefore the results should not be directly compared. Nevertheless the presented research provides a solid foundation for advancing the development of innovative technology.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTIONS

ML, KM, NB and OF conceived and designed research. ML, KM, NB, and OF conducted experiments. KM, TK contributed new reagents or analytical tools. ML, KM, NB and OF analysed data. ML, KM, NB, OF, TK wrote and discussed the manuscript. All authors read and approved the manuscript.

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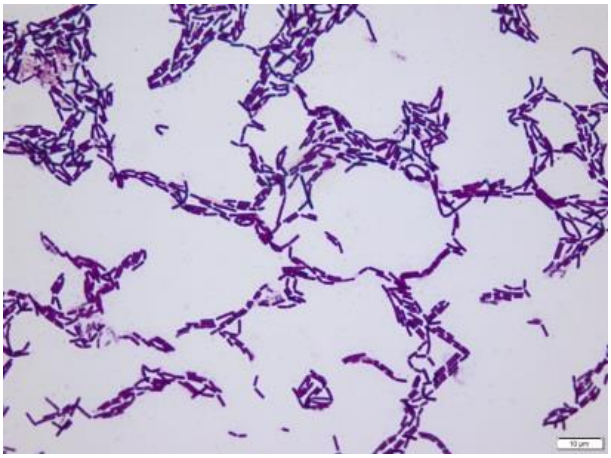

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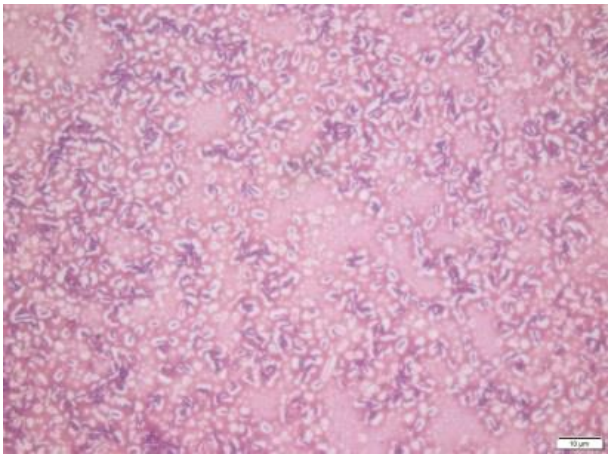

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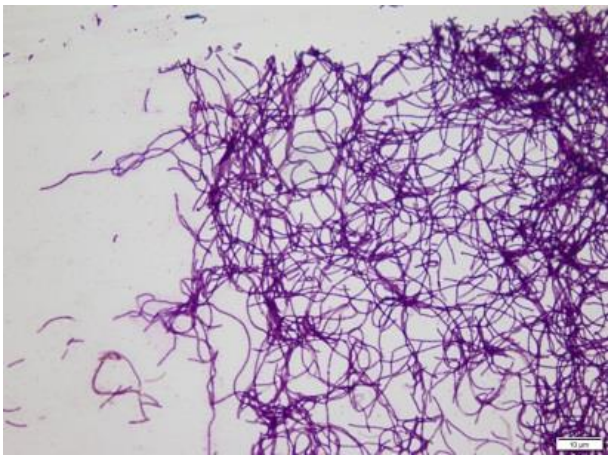

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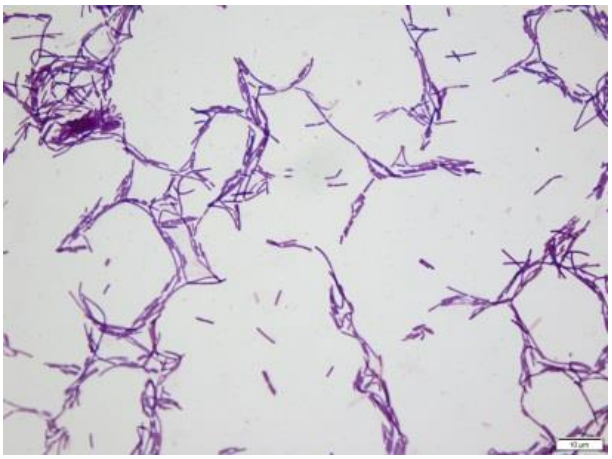

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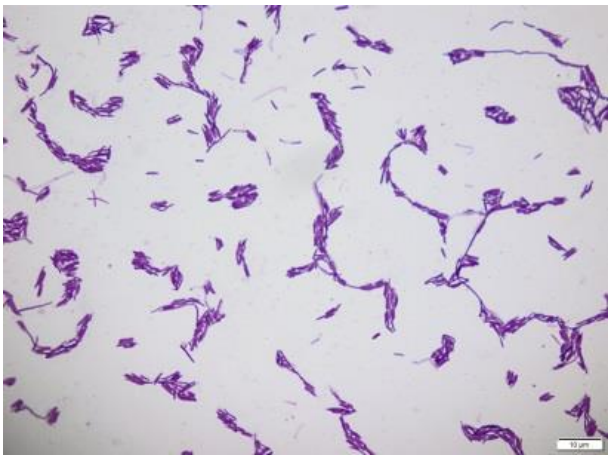

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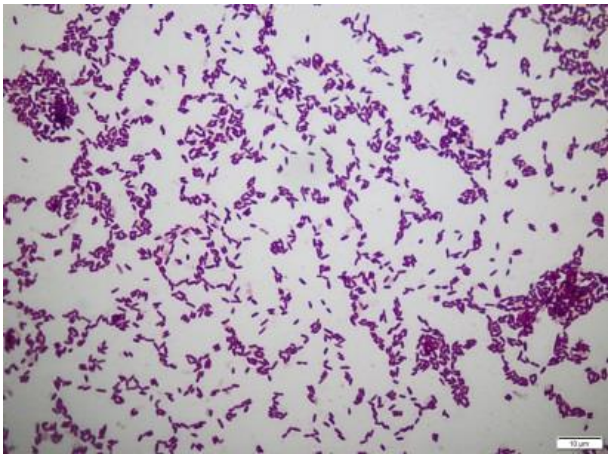
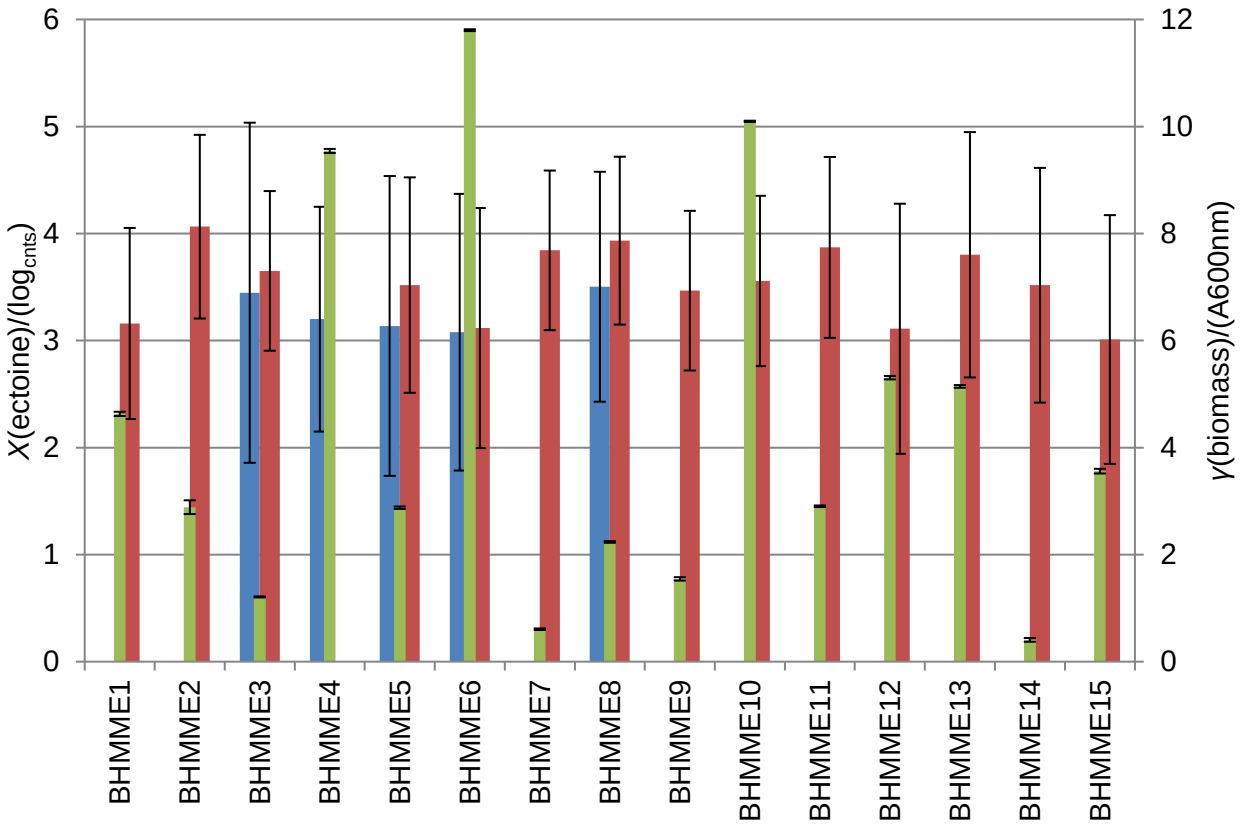


Fig. 1. Examples of microscopic observations of isolated bacteria at 1000× magnification: a) BHMME 1, b) BHMME 8, c) BHTA 12, d) BHTA 19, e) BHEGO 1, f) BHEGO 3

a)



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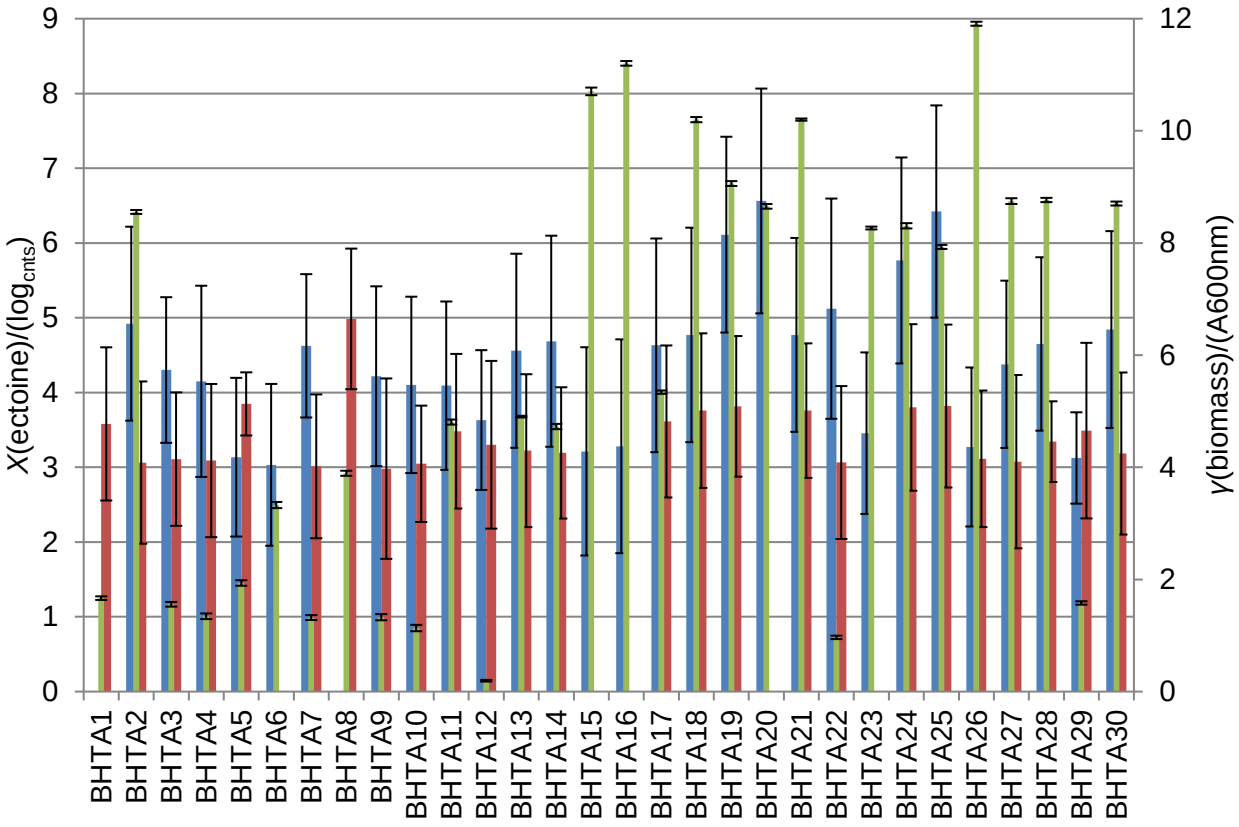

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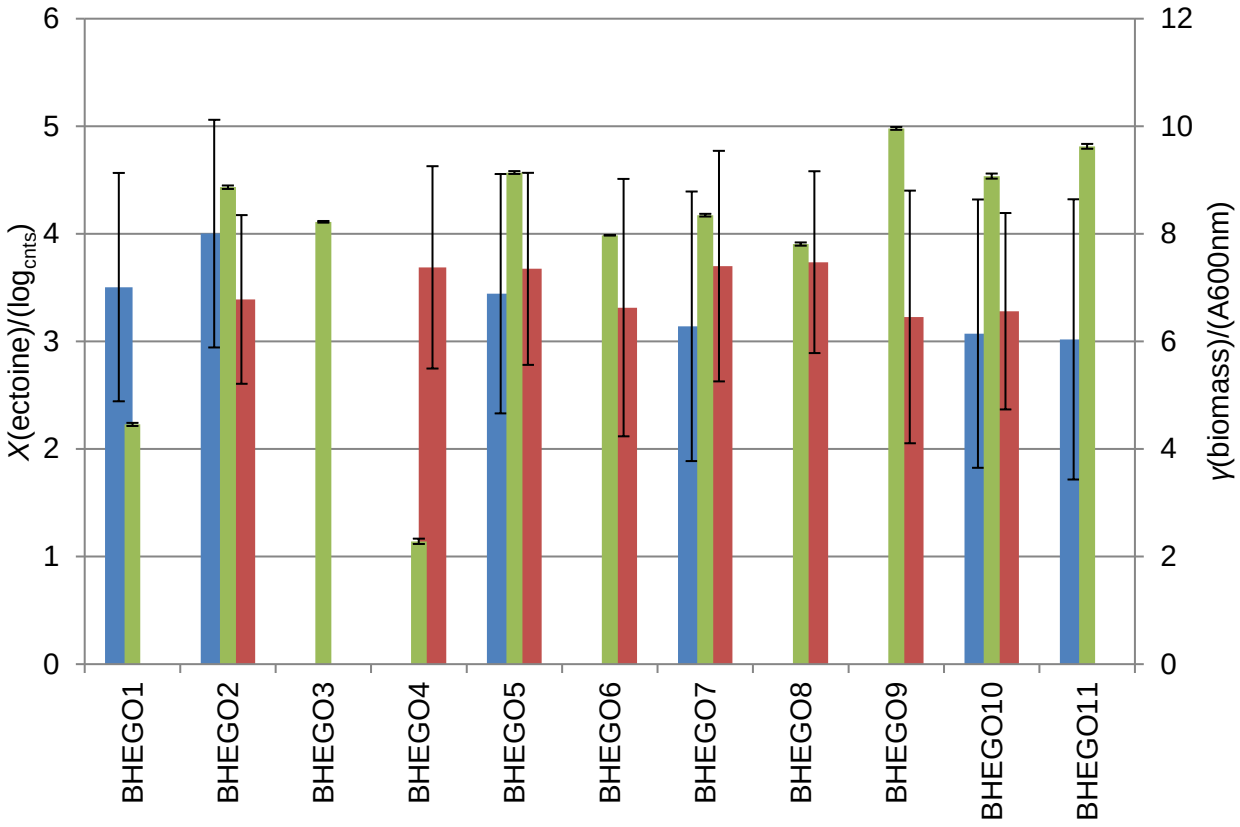

Fig. 2. Comparison of absorbances and the LC-MS estimation of the amount of ectoine and hydroxectoine in the bacterial cultures originating from: a) Złockie vicinity of Na Mokradłach Spring (BHMM), vicinity of Tyrawa spring (BHTA) and Rajcza (BHEGO), Poland. Blue – ectoine number of mass detector counts (cnts), red – hydroxyectoine cnts, green – absorbance

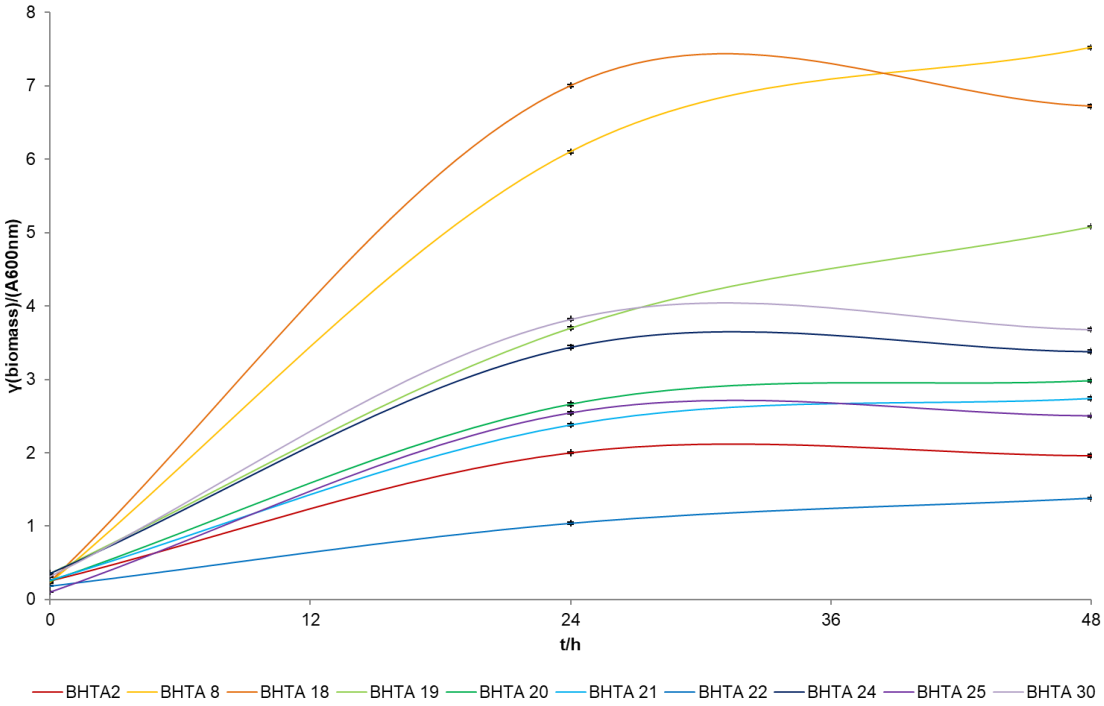

Fig. 3. The growth of selected strains in optimal pH and temperature

Table 1. Identification of selected strains

Strain	Species name	GenBank accession number	Collection number
BHTA2	*Halobacillus sp.*	OR240871.1	KPD 1605*
BHTA8	*Bacillus velezensis*	OR240872.1	KPD 1606*
BHTA18	*Pseudalkalibacillus hwajinpoensis*	OR240874.1	KPD 1607*
BHTA19	*Virgibacillus salarius*	OR240982.1	B/00477**
BHTA20	*Salimicrobium halophilum*	OR240983.1	KPD 1608*
BHTA21	*Halobacillus halophilus*	OR240984.1	KPD 1609*
BHTA22	*Virgibacillus litoralis*	OR241005.1	KPD 1610*
BHTA24	*Thalassobacillus devorans*	OR241017.1	KPD 1611*
BHTA25	*Halobacillus sediminis*	OR241020.1	KPD 1612*
BHTA30	*Halobacillus sediminis*	OR241039.1	KPD 1604*

*Collection of Plasmids and Microorganisms, University of Gdańsk, **Polish Collection of Microorganisms

Table 2. Determination of oxidase, L-alanine aminopeptidase, catalase activity and the influence of temperature, pH, and concentration of various inorganic salt on the bacteria growth

Characteristic	BHTA2	BHTA8	BHTA18	BHTA19	BHTA20	BHTA21	BHTA22	BHTA24	BHTA25	BHTA30
Temperature range (optimum)/°C	10-44 (37)	10-44 (37)	10-44 (37)	10-44 (30)	10-44 (30)	10-44 (30)	10-44 (30)	10-44 (30)	10-44 (30)	10-44 (37)
pH range (optimum)	6-9 (7)	6-9 (8)	5-9 (8)	3-9 (8)	3-9 (9)	6-9 (8)	3-8 (6-7)	6-9 (7-8)	5-9 (7-8)	6-9 (7-8)
Oxidase	+	+	-	+	+	+	+	-	+	+
L-alanine aminopeptidase	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+
Growth in medium without salt addition	-	+	+	+	-	-	-	+	-	-
Instant Ocean (seawater) range (optimum)/%	5-20 (10-15)	5-15 (5)	5-25 (5)	5-25 (10-15)	5-15 (10)	5-20 (10)	5-15 (15)	5-20 (10)	10-25 (10)	5-25 (10-15)
NaCl range (optimum) (%)	5-20 (5-10)	5-20 (5-10)	5-20 (5)	5-20 (5)	5-20 (5)	5-20 (15)	5-15 (10)	5-20 (5)	5 (5)	5 (5)
KCl range (optimum)/%	NG	5-25 (5-10)	5-20 (5)	5-25 (5-10)	5-20 (20)	10 (10)	5 (5)	5-15 (5)	NG	NG

$(NH_4)_2SO_4$ range (optimum)/%	NG	5-20 (5)	5-20 (5)	5-25 (10)	5-10 (10)	5 (5)	NG	5-25 (5)	NG	NG
$MgSO_4x7H_2O$ range (optimum)/%	5-25 (5-10)	5-25 (5-10)	5-15 (5)	5-25 (5-10)	5-25 (5-10)	15-20 (15)	5-15 (5)	5-20 (5-10)	5-15 (15)	5-15 (5)

Assessment of enzymatic activity: - negative reaction, + positive reaction, NG=strain was not grown

Table 3. Concentration of ectoine and hydroxyectoine determined by HPLC method

Strain	Ectoine/(mg/L)	Hydroxyectoine/(mg/L)
BHTA2	ND	ND
BHTA8	ND	ND
BHTA18	ND	ND
BHTA19	274±5.29	ND
BHTA20	4.5±0.30	ND
BHTA21	ND	ND
BHTA22	1.5±0.03	ND
BHTA24	ND	ND
BHTA25	5.3±0.03	ND
BHTA30	ND	ND

ND=not detected