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Regular paper

Halophilic microorganisms in deteriorated historic buildings: insights into their characteristics*

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Historic buildings are constantly being exposed to numerous climatic changes such as damp and rainwater. Water migration into and out of the material's pores can lead to salt precipitation and the so-called efflorescence. The structure of the material may be seriously threatened by salt crystallization. A huge pressure is produced when salt hydrates occupy larger spaces, which leads at the end to cracking, detachment and material loss. Halophilic microorganisms have the ability to adapt to high salinity because of the mechanisms of inorganic salt (KCl or NaCl) accumulation in their cells at concentrations isotonic to the environment, or compatible solutes uptake or synthesis. In this study, we focused our attention on the determination of optimal growth conditions of halophilic microorganisms isolated from historical buildings in terms of salinity, pH and temperature ranges, as well as biochemical properties and antagonistic abilities. Halophilic microorganisms studied in this paper could be categorized as a halotolerant group, as they grow in the absence of NaCl, as well as tolerate higher salt concentrations (*Staphylococcus succinus*, *Virgibacillus halodenitrificans*). Halophilic microorganisms have been also observed (*Halobacillus styriensis*, *H. hunanensis*, *H. naozhouensis*, *H. litoralis*, *Marinococcus halophilus* and yeast *Sterigmatomyces halophilus*). With respect to their physiological characteristics, cultivation at a temperature of 25–30°C, pH 6–7, NaCl concentration for halotolerant and halophilic microorganisms, 0–10% and 15–30%, respectively, provides the most convenient conditions. Halophiles described in this study displayed lipolytic, glycolytic and proteolytic activities. *Staphylococcus succinus* and *Marinococcus halophilus* showed strong antagonistic potential towards bacteria from the *Bacillus* genus, while *Halobacillus litoralis* displayed an inhibiting ability against other halophiles.

Key words: halophilic microorganisms, historic buildings, phenotypic characteristics, biodeterioration

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INTRODUCTION

Excessive moisture, and high salinity are the key factors contributing to the growth of halophilic microorganisms, a unique group of organisms which are able to grow at high salt concentrations. Many classification schemes have been designed according to their response to salt (slightly halophilic: 0.2–0.5 M salt, moderately halophilic: 0.5–2.5 M salt, or extremely halophilic: 2.5–5.2 M salt, and halotolerant strains: not requiring salt for growth but also grow at high salt concentrations). Halophilic microorganisms have developed two mechanisms which determine their tolerance to high salinity. On one hand, they can accumulate inorganic ions (usually K^+ and Cl^-) at isotonic concentrations to the surrounding environment, but on the other hand, they can use the so-called compatible solute strategy in osmo-adaptation, based on the uptake or synthesis of organic molecules (e.g. sugars, polyols, amino acids, ectoine) (Madigan & Oren, 1999; Xiang *et al.*, 2008; Averhoff & Müller, 2010). They have adapted to grow in many different niches (Laiz *et al.*, 2000). Even though saline environment refers to water, considerable research has been carried out on halophiles inhabiting historic buildings (Rölleke *et al.*, 1998; Heyrman *et al.*, 1999; Laiz *et al.*, 2000, 2001; Piñar *et al.*, 2001, 2014; Ripka *et al.*, 2006; Ettenauer *et al.*, 2014).

Many of the historic buildings show signs of excessive moisture and salt efflorescence, which intensifies the corrosion processes, leading to the destruction of the building. Hence, historic buildings provide the environment convenient to the development and proliferation of halophilic microorganisms. Frequently, historic buildings are colonized by bacteria, whose representatives are *Gammaproteobacteria* (e.g. *Idiomarina* sp., *Salinisphaera* sp., *Halomonas* sp.), *Firmicutes* (e.g. *Halobacillus* sp., *Bacillus* sp.) and *Actinobacteria* (e.g. *Rubrobacter* sp.), or archaea (e.g. *Halococcus* sp., *Halobacterium* sp.). Halophilic fungi (e.g. *Wallemia* sp., *Eurotium* sp.) are in the minority (Piñar *et al.*, 2014b; Sterflinger *et al.*, 2014). Within the decayed materials, soluble salts such as carbonates, chlorides, nitrates or sulphates are dispersed. They migrate through the stone with capillary water, and as a result of changing physical parameters, the solution dries out causing the formation of salt deposits on the surface, known as salt efflorescence (Saiz-Jimenez & Laiz, 2000; Ettenauer *et al.*, 2010). Salt crystallization process accompanying the development of halophilic microorganisms causes destruction, crushing and cracking of historic buildings. Lazar (1971) and Bassi & Giacobini (1973) suggested the biological origin of salt efflorescence, apart from a chemical one, and therefore it is important to check conditions that favour growth of halophiles. Halophiles are also responsible for aesthetic changes on surfaces, which is due to their ability of pigment formation, in particular carotenoids, that ultimately leads to the occurrence of characteristic colored biofilms (pink, orange, red) (Lo-

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Abbreviations: DGGE, denaturing gradient gel electrophoresis; PCA, Principal Component Analysis; PCR, polymerase chain reaction; TSB, Tryptic Soy Broth; M, mol/dm³; °McF, McFarland scale; % w/v, weight/volume percentage concentration

banova *et al.*, 2008; Laiz *et al.*, 2009; Jurado *et al.*, 2012; Ettenauer *et al.*, 2014).

The need to improve the condition of historic monuments plays a significant role, hence it is advisable to supplement the knowledge necessary to implement effective restoration and preservation methods with a microbiological aspect, taking into consideration especially halophiles. Unfortunately, using unsuitable media with inappropriate salt concentrations and improper incubation strategy has consequently led to their absence of growth under standard laboratory conditions, and hence resulted in their being overlooked. Although molecular techniques based on PCR reaction, DGGE analysis and clone library construction approach led to the great increase in our knowledge about halophilic microbial communities inhabiting cultural assets (Piñar *et al.*, 2013; Otlewska *et al.*, 2015), in order to obtain the entire information about biodeterioration, it is still important to get an overview on the halophilic organisms' culture conditions and phenotypic features. With this background, the current work was aimed to determine optimal growth conditions of halophilic microorganisms in terms of salinity, pH and temperature ranges, as well as biochemical properties and antagonistic abilities.

MATERIALS AND METHODS

Strains of halophilic microorganisms. Nine strains of microorganisms used in this investigation were isolated from brick, plaster and paint coatings collected from the former Auschwitz II-Birkenau concentration and extermination camp in Oświęcim, and 19th century chateau in Łódź, Poland (Table 1) with visible symptoms of dampness and salt efflorescence. Salinity of the samples was determined by a spectrophotometric method [% sample mass]. Pure cultures were cultivated on TSA medium with both, 10% NaCl (w/v) and 2% $MgSO_4 \times 7H_2O$ (w/v), and incubated aerobically at 30°C for 5 days. The frequency of occurrence of each strain was investigated.

Optimal growth conditions determination. pH profile. Growth at different pH ranges was determined on TSB medium supplemented with 10% NaCl (w/v) and 2% $MgSO_4 \times 7H_2O$ (w/v), wherein the pH was adjusted from 1 to 12 for each strain. The cultures were incubated at 30°C for 5 days. Every 24 hours, a turbidity measurement was performed using a densitometer in order to check the concentration of cells in McFarland scale (°McF).

Salinity profile. Tolerance to NaCl or $MgSO_4$ was studied on TSB medium supplemented with 2% $MgSO_4 \times 7H_2O$ (w/v), where the NaCl concentration ranged from 0–30% (w/v); or 5% NaCl (w/v), where the $MgSO_4 \times 7H_2O$ concentration ranged from 0–30% (w/v), respectively. The cultures were incubated at 30°C for 5 days. A turbidity measurement was performed in the same way as described above.

Temperature profile. Growth temperature was tested in the range of 4, 10, 25, 30, 37 and 44°C on TSA medium supplemented with 10% NaCl (w/v) and 2% $MgSO_4 \times 7H_2O$ (w/v), or 10% $MgSO_4 \times 7H_2O$ (w/v) and 5% NaCl (w/v). Plates were incubated for 5 days.

Biochemical characterization. The utilization of a variety of substrates was tested using commercial API 50CH system (bioMérieux, France) while enzymatic activities were detected with API ZYM test (bioMérieux, France). API tests were performed following the manufacturer's instructions. The approximate number of free nmol hydrolyzed substrate may be estimated according to Nowak & Piotrowska (2012): 0 — no activity; 1 (5 nmol) and 2 (10 nmol) — weak activity; 3 (20 nmol), 4 (30 nmol), and 5 ($\geq$40 nmol) — strong activity.

Antagonistic properties of halophilic microorganisms. For detection of antagonistic activity of halophilic isolates, a diffusion method was applied. Tested strains included other halophilic microorganisms considered in this study, as well as heterotrophic bacteria of the *Bacillus* genus (*B. cereus, B. muralis, B. simplex, B. atrophaeus*), which according to the literature are known to inhabit historic buildings (Laiz *et al.*, 2000; Saiz-Jimenez & Laiz, 2000; Koziróg *et al.*, 2014; Rajkowska *et al.*, 2014). *Bacillus* strains, just as halophilic microorganisms, were isolated from historical sites, and based on 16S rRNA sequencing clustered to the *Bacillus* genus (Piotrowska *et al.*, 2014). Using a 10 mm diameter sterile cork borer, the growing edge of 5-day culture of halophilic microorganisms was aseptically cut and placed at the plate already inoculated with the antagonist being tested. Bacteria of the *Bacillus* genus were plated on TSA medium, while halophilic microorganisms on TSA medium supplemented with 10% NaCl (w/v) and 2% $MgSO_4 \times 7H_2O$ (w/v). Plates were incubated at 30°C and monitored for 5 days. Measurement of inhibition (mm) was taken.

Statistical analysis. The relationship between optimal growth conditions of halophilic microorganisms, as well as their biochemical and enzymatic properties, were performed by Principal Component Analysis (PCA) and phenograms using Statistica v.10.0 (Stat Soft. Inc., USA) software.

Table 1. Halophilic microorganisms considered in the study

Strains	Inhabited area	Salinity of inhabited area (% mass sample)	Relative abundance (%)
Halobacillus styriensis	brick[1]	0.28	8
Halobacillus naozhouensis			50
Halobacillus litoralis			40
Staphylococcus succinus	brick[2]	0.73	3
Halobacillus hunanensis			16
Marinococcus halophilus	brick[3]	1.55	7
Sterigmatomyces halophilus H18			4
Virgibacillus halodenitrificans	plaster with paint coatings[4]	0.82	44
Sterigmatomyces halophilus H11			26

1 — samples collected from historical buildings located in the former Auschwitz II-Birkenau concentration and extermination camp. 2, 3, 4 — samples collected from 19th century chateau in Łódź

RESULTS AND DISCUSSION

Optimal growth conditions determination

Bacteria of the *Staphylococcus succinus*, *Virgibacillus halodenitryficans*, *Marinococcus halophilus* and *Halobacillus litoralis* species showed the widest pH range, up to 9 (Table S1 Supplementary Materials at www.actabp.pl). Regardless of the medium, optimum pH for the studied halophilic microorganisms occurred between 6–7, similarly to what was reported by Spring *et al.* (1996), Ventosa *et al.*, (1998) and Ripka *et al.* (2006). They noted an increase in the counts of halophilic microorganisms of the *Halobacillus* genus in the pH range from 6 to 9.5, while the optimum was between 7.5–8.0. The majority of identified halophilic or halotolerant microorganisms grew best in media with pH from 6.8 to 7.5 (Vreeland *et al.*, 2002; Caton *et al.*, 2004; Schneegurt, 2012). It should be noted, however, that the authors did not observe the growth capacity below 6 and above 9.5. Nevertheless, historic buildings are unusual environment for the growth of halophiles, which typically inhabit saline lakes, marine and inland salterns, saline soils and salted foods (Ettenauer *et al.*, 2010; Ventosa *et al.*, 2012; Piñar *et al.*, 2014a), and as a consequence, it may influence their optimal growth pH values. Building materials are generally characterised by alkaline pH, however, due to biocorrosion it may be lowered (Beech & Gaylarde, 1999; Warscheid & Braams, 2000).

Optimum growth for all analysed strains occurred at 25–30ºC both on medium supplemented with 10% NaCl and 10% $MgSO_4 \times 7H_2O$ (Table S1 Supplementary Materials at www.actabp.pl). Growth in the widest range of temperature (4–37°C) was shown, as previously reported, by *Staphylococcus succinus* and *Virgibacillus halodenitryficans*. According to the literature, halophilic bacteria- *Halobacillus litoralis*, *H. trueperi* and *H. halophilus,* can grow at a wide range of temperatures, from 10 to 43°C. Their optimal growth is estimated at around 37°C (Spring *et al.*, 1996; Ventosa *et al.*, 1998; Ripka *et al.*, 2006), while in contrast, optimum temperature for *Halobacillus naozhouensis* is 25°C (Chen *et al.*, 2009). Usually halophiles are grown at room or slightly elevated temperature (25–30°C) (Oren, 1986; Caton *et al.*, 2004; Schneegurt, 2012).

Taking into consideration the response to high salinity, as reported by Kushner & Kamekura (1988) and Joo & Kim (2005), the investigated microorganisms were divided into halophilic and halotolerant which requires salt for growth or are able to grow in the absence of salt, respectively. The group of halophilic microorganisms included: *Halobacillus styriensis*, *H. hunanensis*, *H. naozhouensis*, *H. litoralis*, *Marinococcus halophilus,* and *Sterigmatomyces halophilus*. Optimum growth occurred in media supplemented with 15–30% (w/v) NaCl (Table S1 Supplementary Materials at www.actabp.pl). No growth was observed without halite supplementation. In contrast, for halotolerant species (*Staphylococcus succinus*, *Virgibacillus halodenitrificans*) optimal salinity range was 0–15% and 0–10% (w/v) NaCl, respectively (Table S1 Supplementary Materials at www.actabp.pl). Bacteria of the genus *Halobacillus* (*H. litoralis*, *H. halophilus* and *H. trueperi*), known as moderately halophilic was observed to grow in a wide range of NaCl concentration (0.5–25%), but the optimum growth was estimated at 10% NaCl (Spring *et al.*, 1996; Ventosa *et al.*, 1998; Ripka *et al.*, 2006). Salt concentration plays crucial role in media composition. Ettenauer *et al.* (2014) for the cultivation of halophilic microorganisms isolated from the Johannes Chapel in Pürgg used media supplemented with 15% NaCl (w/v) whereas Piñar *et al.* (2014a) for the cultivation of halophilic bacteria colonizing the exhibition areas of the Capuchin Catacombs in Palermo applied media with three different NaCl concentrations 3%, 10% and 20% (w/v).

The primary component of efflorescence is not always halite (NaCl), because salinity can also be based on epsomite ($MgSO_4 \times 7H_2O$), gypsum ($CaSO_4 \times 2H_2O$), nitrate ($NaNO_3$) and other salts (Saiz-Jimenez, 2000). Because of the specific composition of salt efflorescence it was found necessary either to check the capacity of analysed strains to grow at various $MgSO_4$ (epsomite) concentration, however, no significant changes were detected (Table S1 Supplementary Materials at www.actabp.pl). Slightly poorer growth was observed when the concentration exceeded 15% (w/v). Laiz *et al.* (2000) detected that 1% to 10% (w/v) epsomite concentration favoured

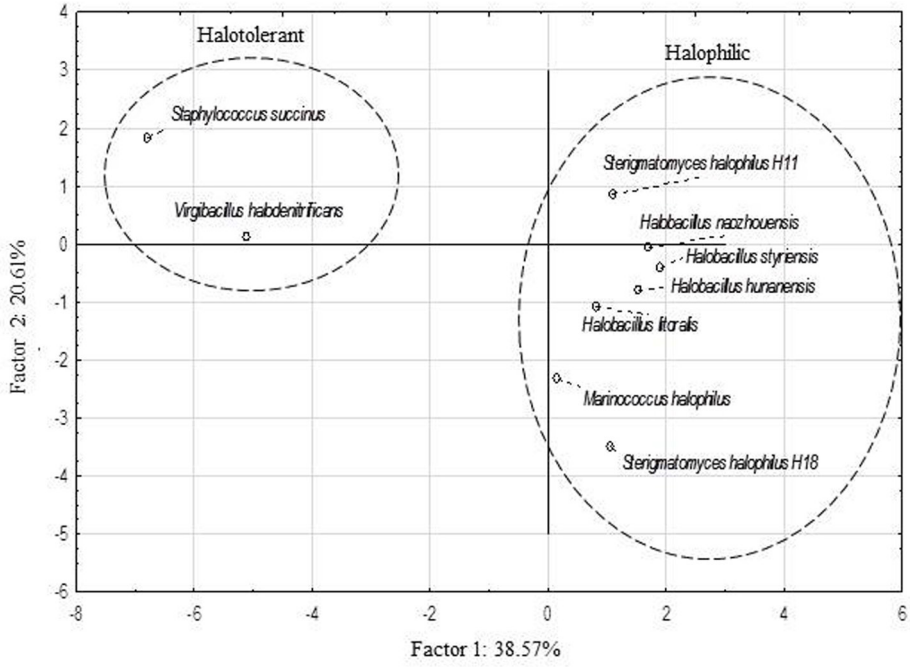

Figure 1. Principal Component Analysis (PCA) concerning pH, temperature, halite and epsomite concentration ranges for all studied halophilic/halotolerant microorganisms

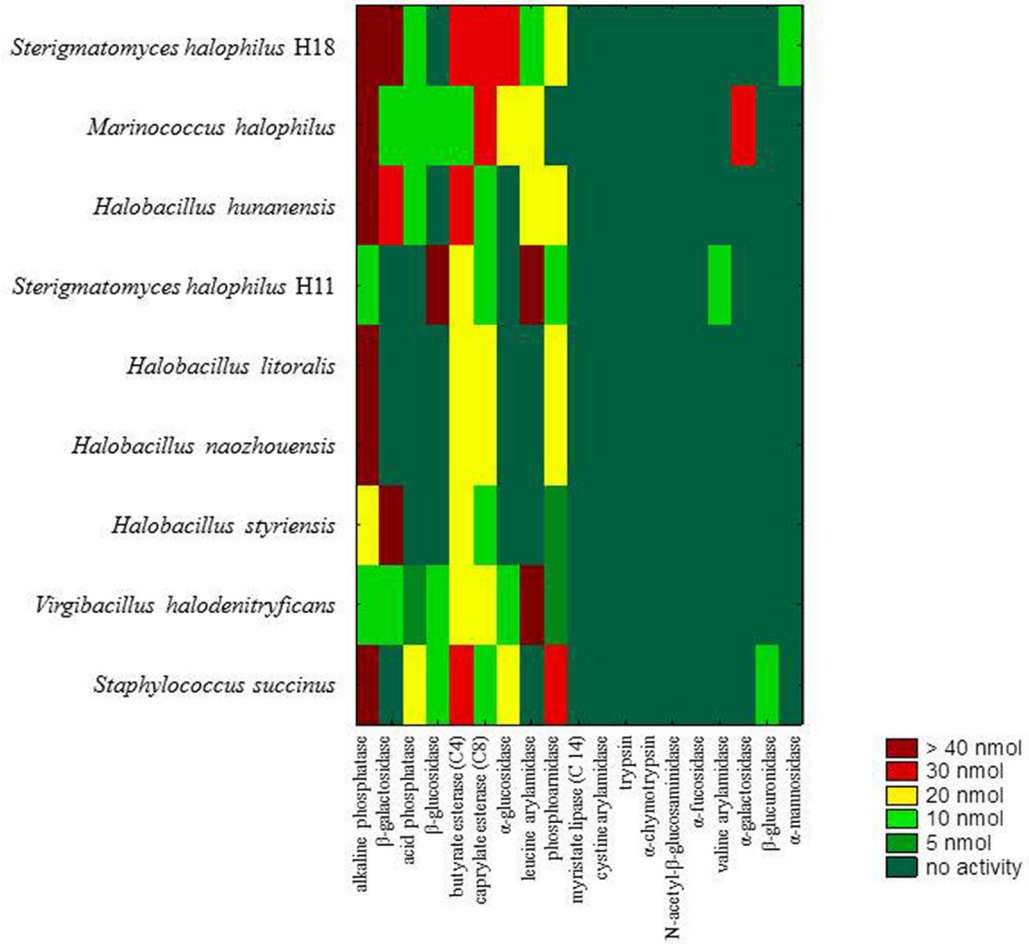

Figure 2. Enzymatic activities of selected strains

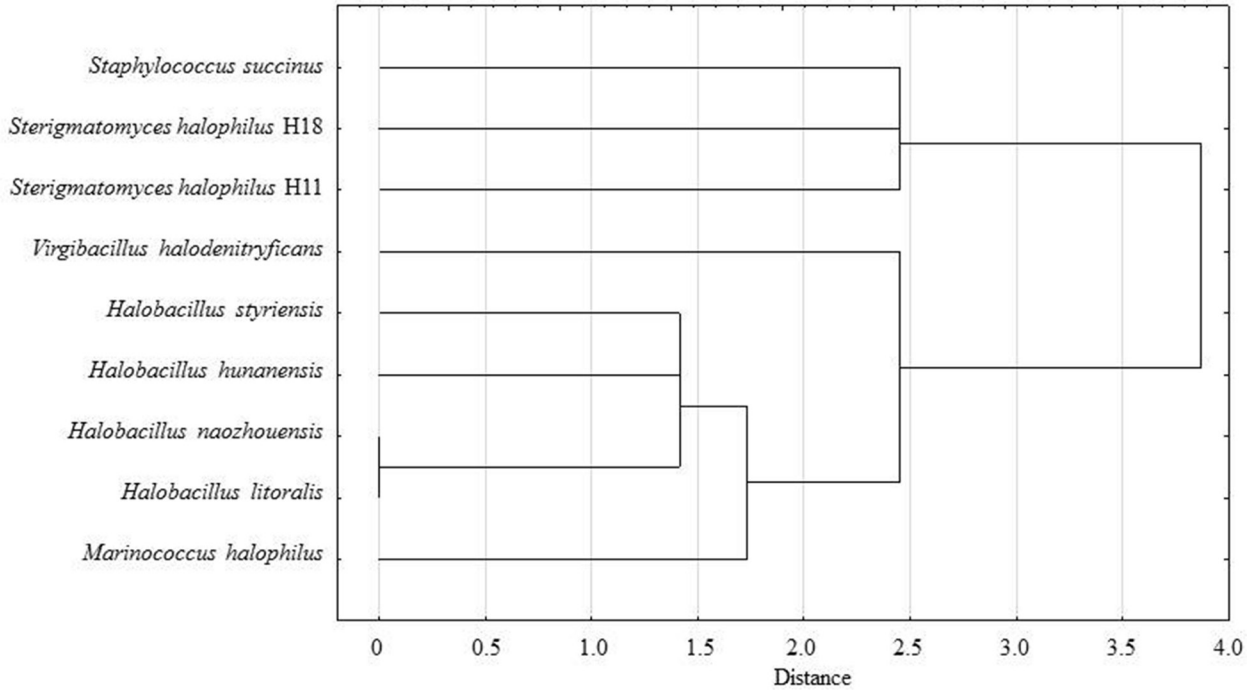

Figure 3. Phenogram showing similarity between studied halophilic/halotolerant microorganisms based upon utilisation of a variety of substrates

Table 2. Antagonistic properties of halophilic microorganisms (X ±S.D. [mm])

Tested strains / Antagonists	*Staphylococcus succinus*	*Virgibacillus halodenitrificans*	*Halobacillus styriensis*	*Halobacillus hunanensis*	*Sterigmatomyces halophilus H11*	*Halobacillus naozhouensis*	*Marinococcus halophilus*	*Sterigmatomyces halophilus H18*	*Halobacillus litoralis*	*Bacillus cereus*	*Bacillus muralis*	*Bacillus simplex*	*Bacillus atrophaeus*
Staphylococcus succinus	×	-	-	-	-	-	-	-	-	4.3±1.5	4.3±1.2	3.0±0	5.0±1.0
Virgibacillus halodenitrificans	-	×	-	-	-	-	-	-	-	4.3±0.6	-	4.3±0.6	1.0±1.0
Halobacillus styriensis	-	-	×	-	-	-	-	-	-	-	-	2.7±0.6	13.0±2.6
Halobacillus hunanensis	-	-	10.7±2.1	×	-	-	-	-	-	2.3±0.6	-	3.0±1.0	12.0±2.0
Sterigmatomyces halophilus H11	-	-	-	-	×	-	6.0±1.0	2.7±1.2	-	0.7±0.6	-	4.6±0.6	7.3±2.0
Halobacillus naozhouensis	-	-	-	-	-	×	-	-	-	-	-	-	-
Marinococcus halophilus	-	-	-	-	-	-	×	-	-	1.0±0	6.7±1.2	5.3±1.5	10.0±1.0
Sterigmatomyces halophilus H18	-	-	-	-	-	-	-	×	-	-	-	1.3±0.6	10.3±2.3
Halobacillus litoralis	13.3±1.2	12.0±2.0	-	7.3±2.5	4.3±2.5	-	-	-	×	-	-	-	-

(-) no inhibition; × not tested; X - mean; S.D., standard deviation

growth of halophiles, which resulted in increasing number of colony forming units (up to 48.3×10^3 cfu/g) with respect to medium without $MgSO_4 \times 7H_2O$.

The next goal of this research was to compress data concerning pH, temperature, halite and epsomite concentration ranges for all studied halophilic microorganisms, and in particular to classify them by highlighting their differences or similarities. For that purpose, Principal Component Analysis (PCA) was performed (Fig. 1). An interesting observation is division of the organisms into two groups: members of the first one are *Halobacillus* sp. (*H. litoralis*, *H. styriensis*, *H. hunanenis*, *H. naozhouensis*), *Sterigmatomyces halophilus*, *Marinococcus halophilus*, whereas the second one consists of *Virgibacillus halodenitryficans* and *Staphylococcus succinus*. This corresponds with an established scheme, according to response to high salinity, proposed by Kushner & Kamekura (1988) and Joo & Kim (2005). The first group involves halophilic microorganisms, the second group halotolerant ones.

Biochemical properties

Enzymatic activities of selected strains are shown in Table S2 (Supplementary Materials at www.actabp.pl) and Fig. 2. All tested strains synthesized alkaline phosphatase; 8 out of 10 microorganisms (except from *Virgibacillus halodenitryficans* and *Sterigmatomyces halophilus* H11) displayed its strong activity. Our findings are similar to the results published by Jurado *et al.* (2012), who observed alkaline phosphatase activity in strains isolated from biodeteriorated monuments, and Chen *et al.* (2009) who detected that *Halobacillus naozhouensis* synthesized this enzyme. Almost all analysed halophilic microorganisms displayed a strong butyrate esterase activity (excluding *Marinococcus halophilus*), but on the other hand weak caprylate esterase activity. López-Miras *et al.* (2013) found that *Virgibacillus* sp., inhabiting oil painting showing signs of biodeterioration, produced esterases cleaving 2-naphtylbutyrate and 2-naphtylcaprylate. None of the strains hydrolyzed ester linkage in 2-naphtylmirystate (myristate lipase activity). Only one out of 5 peptide hydrolases (leucine, valine, and cystine arylamidases, and trypsin and α-chymotrypsin) was detected. *Virgibacillus halodenitryficans* and *Sterigmatomyces halophilus* H11 synthesized leucine arylamidase (strong activity). Proteases synthesised by halophilic microorganisms were applied in industry (Karbalaei-Heidari *et al.*, 2009; Yin *et al.*, 2014). Three enzymes involved in carbohydrate metabolism were detected: β-galactosidase (*Halobacillus styriensis*, *H. hunanensis* and *Sterigmatomyces halophilus* H18 strongly produced this enzyme), α-glucosidase (synthesised mainly by *Staphylococcus succinus*, *Marinococcus halophilus* and *Sterigmatomyces halophilus* H18) and β-glucosidase (*Sterigmatomyces halophilus* H11). It is worth to note that almost all strains (excluding *Marinococcus halophilus*) synthesised a phosphoamidase, which with reference to López-Miras *et*

al. (2013), was found to be excreted by *Bacillus* sp. inhabiting historic paintings. The same enzymatic pattern was obtained only for *Halobacillus naozhouensis* and *Halobacillus litoralis* (Fig. 2).

Among studied halophilic microorganisms, *Staphylococcus succinus* was able to utilise the widest variety of substrates (25 out of 49 tested substrates; Table S3 Supplementary Materials at www.actabp.pl) as a sole source of carbon, nitrogen and energy, and this strain may become a serious threat for historic buildings (Table S3 Supplementary Materials at www.actabp.pl) (Chen *et al.*, 2009; López-Miras *et al.*, 2013). Halophilic yeast *Sterigmatomyces halophilus* was able to utilise 23 substrates, while in contrast *Halobacillus* ssp. and *Marinococcus halophilus* utilized between 1 and 3, depending on the species. Phenogram based on utilisation of a variety of substrates (Fig. 3), shows the relationship between halophilic microorganisms and their biochemical properties. *H. naozhouensis* and *H. litoralis* presented the same biochemical profile, while other strains were more dissimilar.

Antagonistic properties of halophilic microorganisms

Staphylococcus succinus and *Marinococcus halophilus* were the most effective in inhibiting the growth of all tested bacterial strains from the *Bacillus* genus, while the least effective were *H. naozhouensis* and *H. litoralis,* which were not able to inhibit growth of any of the above mentioned strains (Table 2). In contrast, the inhibiting ability of *Halobacillus litoralis* against other halophilic microorganisms (*Staphylococcus succinus*, *Virgibacillus halodenitryficans*, *Halobacillus hunanensis* and *Sterigmatomyces halophilus* H11) considered in this study was observed. The members of the *Halobacillus* genus represent the majority of detected halophilic microorganisms in historic buildings, which may be explained by remarkable osmotic adaptation of bacilli (Piñar *et al.*, 2009).

CONCLUSIONS

Cultivation at temperatures of 25–30°C, pH 6–7, and NaCl concentration for halotolerant/halophilic microorganisms, 0–10% and 15–30% respectively, provides the optimum conditions for their growth. For the successful isolation of halophiles, it is therefore necessary to use specific media with high salt concentration and to apply appropriate incubation strategy. Halophiles described in this study displayed a strong lipolytic activity, as well as weaker glycolytic and proteolytic activities. Two strains, *Staphylococcus succinus* and *Marinococcus halophilus,* showed antagonistic potential towards bacteria from the *Bacillus* genus, while *Halobacillus litoralis* displayed an inhibiting ability against other halophiles.

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