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ORIGINAL PAPER

Relationship between the cell surface hydrophobicity and survival of bacteria *Zymomonas mobilis* after exposures to ethanol, freezing or freeze-drying

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Abstract An inverse linear relationship ($P < 0.01$) was detected between the cell surface hydrophobicity (CSH) and survival of ethanologenic bacteria *Zymomonas mobilis* 113S exposed to elevated (2.55 M) ethanol concentration. In the same way, viable cell counts of relatively hydrophobic *Z. mobilis* were less diminished by growing (0.85–3.40 M) ethanol concentrations as compared to more hydrophobic bacteria. Very similar inverse relationships ($P < 0.01$) were observed between the CSH of intact *Z. mobilis* and survival of cells subjected to subsequent freeze-drying or freezing/thawing cycles thereby affinity substantially lowered ability of hydrophobic bacteria to survive under adverse environments. Observed relationships were supported by significant correlations between independent analytical data of the carbohydrate content within fractions of lipopolysaccharide and surface proteins extracted from cells of varied hydrophobicity. The results suggest that the CSH could be of value to predict the ability of intact bacteria to endure stress conditions and should be monitored towards lower values during cultivation in order to reduce subsequent unwanted structural and physiological disturbances provoked by multiple stress factors.

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Introduction

Ethanologenic α-proteobacteria *Zymomonas mobilis* appear to have a considerable biosynthetic potential in respect to diverse metabolites (ethanol, sorbitol, gluconic acid, levan and fructose oligomers) which makes them promising for technological applications [6, 10, 18]. *Z. mobilis* are of particular interest for industrial production of ethanol due to its faster sugar uptake and conversion rates together with a higher ethanol tolerance in comparison with the yeasts traditionally used for this purpose [21, 23, 24, 28]. However, the production of ethanol using bacteria *Z. mobilis* remains to be commercialized and improved by various approaches with a special emphasis on the metabolic engineering and recombinant DNA technology [24].

Original strain *Z. mobilis* 113S [3] has been reported to possess good productivity characteristics for both fructan and ethanol synthesis [2, 4] under appropriate fermentation conditions.

For all that, any bacterial culture of potent technological usage obviously has to withstand a variety of adverse environmental conditions, and ethanol tolerance of the employed strain is regarded as very important, even critical, in biosynthesis of ethanol [12, 21]. Besides, the effectiveness of common preservation methods (cryoconservation, freeze-drying) for industrial strains to keep their authenticity, vitality, specificity and activity depends on the ability of bacteria to survive under corresponding stress conditions (freezing/thawing or dehydration/rehydration treatments) [17, 25, 33].

Cell surface properties of bacteria are recognized as vital to the organism's survival, since the various surface constituents mediate the contact and interaction of the bacterium with its environment [25]. In turn, the cell surface hydrophobicity (CSH) has been acknowledged as a physical measurable macroscopic characteristic of bacteria, which generally reflects the proportion between hydrophilic and hydrophobic components of the cell envelope [9]. In the microbiological practice, CSH appears to be concerned with diverse processes and interactions of physiological and technological importance [19, 29, 34].

We have recently reported [37] the changes in CSH of *Z. mobilis* 113S in response to varied environmental conditions (temperature and phase of growth, concentration or type of carbon source, etc.), as well as an implication to the impaired barrier function for more hydrophobic cells. This study examines the ability of bacteria *Z. mobilis* 113S to survive during exposure to elevated ethanol concentrations just as under conditions of freezing/thawing and freeze-drying/rehydration of intact cells essentially distinctive by the levels of CSH and characteristics of chemical composition.

Materials and methods

Strain and culture conditions

Zymomonas mobilis strain 113S [3] was maintained and cultivated batch wise without aeration as described previously [37].The independent cultivations were performed at different temperatures (20–40 °C) or at varied concentrations (0.08–0.56 M) of glucose (sucrose) as a sole carbon source in order to modify the cell surface hydrophobicity (CSH) of *Z. mobilis* as reported previously [37].

Preparation of cells, incubation conditions and measurements of cells surface hydrophobicity

The cells from an appropriate growth medium were recovered by centrifugation (7,000×*g*, 10 min, 20 °C), washed twice with phosphate-buffered saline (20 mM phosphate buffer, 0.15 mM NaCl, pH 7.0) or sterile water (pH 7.2) resuspended in the same medium at standardized concentration of cells (OD_{660} = 0.5). Incubation were performed at the same medium and concentration of cells in the absence (control) and the presence of ethanol at the range of concentration 0.85–3.4 M for 0.5 h at 30 °C.

The measurements of cell surface hydrophobicity (CSH) were performed by a modified.

Microbial adherence to hydrocarbon (MATH) method [1] using *o*-xylene (*Sigma*) as described previously [37].

Freezing-thawing procedures and freeze-drying of cells

Five to 10 ml of suspension (8×10^{10} cells ml^{-1}) at the same medium and concentration of cells were subjected to freezing (–20 °C) and subsequent thawing (30 °C, 0.5 h) procedures.

Frozen suspension of cells were lyophilized using a laboratory scale freeze-dryer (LGA 05, VEB MLW, Leipzig, Germany) at a constant temperature of −20 °C and vacuum pressure of 0.4 Pa for 16 h.

Vials of freeze-dried cells were stored in the absence of freezing conditions and external moisture (desiccator over silicagel, 4 °C) for 5–7 days.

Rehydration of freeze-dried cells was performed at 30 °C for 0.5 h using the same amount of sterile water as for initial suspensions.

Determination of viable cell count

Viability of *Z. mobilis* cells after exposures to ethanol, freezing or freeze-drying were examined using a standard serial dilution method and subsequent plating in triplicate onto the medium consisting of (g l^{-1}) KH_2PO_4, 2.5; $(NH_4)_2SO_4$, 1.6; $MgSO_4 \cdot 7H_2O$, 1.0; yeast extract (*Sigma*), 5.0, glucose, 50 and agar, 20 following incubation at 30 °C for 48–72 h. Bacterial counts were carried out as the log number of colony forming units (log CFU ml^{-1}). To measure the effects of stress exposure the log reduction (Δ log CFU) between the control sample and the test sample was compared according to the expression Δ log CFU = log control (CFU ml^{-1}) − log test sample (CFU ml^{-1}).

Analytical measurements

Lipopolysaccharides (LPS) were extracted from the *Z. mobilis* cells using the phenol–water method [36]. The carbohydrate content of the LPS was determined using the phenol–sulphuric acid assay [7, 26] and glucose (20–90 mg ml^{-1}) as the assay standard. The absorbance (A_{485}) of each of the standards and LPS sample after exposure to phenol–sulphuric acid was determined on a spectrophotometer (Shimadzu, UV 260, Japan) and the standard curve constructed of absorbance relative to the concentrations of glucose was used to determine the carbohydrate content of the LPS sample.

Ketodeoxyoctulonic acid (KDO) determinations were performed using the thiobarbituric acid assay [14, 26] after the acid hydrolysis of LPS (0.025 N H_2SO_4 at 100 °C for 1 h) and KDO (*Sigma*) as the assay standard (2–20 μg ml^{-1}). The absorbance (A_{550}) of each of the standards and LPS sample after reaction with thiobarbituric acid was measured and the unknown KDO content of the LPS was calculated from the standard curve.

In order to release proteins from the bacterial envelope a procedure of mild extraction [8] was performed. A protein extract was obtained by stirring the bacterial pellet (1 g) with 5 ml of 50 mM Tris/HCl buffer, pH 9.0 supplemented with 20 mM bezamidinium chloride (*Sigma*) and 0.2 M $MgCl_2$. After stirring for 90 min at 25 °C the bacterial suspension was centrifuged (7,700×*g*, 40 min, 4 °C) and the supernatant defined [8] as the protein extract from the envelopes. Protein content in the extract was assayed by the dye-binding (Coomassie Brilliant Blue G250, *Sigma*) procedure [30].

FT-IR spectroscopy measurements

The Fourier transform infrared (FT-IR) spectra of *Z. mobilis* cells convectively dried under mild (≤50 °C) conditions were obtained with a Bruker Vortex 70 spectrometer (HTS-HT, Hyperion facilities) at the range of 4,000–400 cm^{-1} and resolution of 4 cm^{-1}.

Data processing and analysis

Incubation, freeze/thawing and freeze-drying/rehydration cycles were performed at least in duplicate for cells of each independent cultivation of *Z. mobilis*. All analytical measurements for each sample were performed in triplicate.

The data within individual experiments were subjected to the one-way variance analysis (ANOVA) using the software Statgraphics®Plus (Manugistics, Inc., US) and SPSS 11.0 for Windows (SPSS Inc. Ill., US) to assess the significance of effects attained by varied growth conditions on the integral characteristics (hydrophobicity, survival, composition) of cells under study.

The combined data sets were processed by correlation (parametric and non-parametric) and linear regression analysis using the same software control. At least 200 CFU (±7% from the mean) for each viable cell count was employed to compute the log reduction (Δ log CFU).

Results

The cell surface hydrophobicity (CSH) of bacteria *Zymomonas mobilis* 113S substantially varied in response to changes of cultivation conditions displaying significant ($P < 0.01$) effects (Figs. 1, 2) of the same kind as reported previously [37]. In fact, increased CSH indices were attained for *Z. mobilis* grown at elevated temperatures or concentration of the carbon source (glucose, sucrose) in the medium and the use of sucrose instead of glucose promoted the formation of more hydrophobic cells [37]. At the same time, biochemical characteristics for cells of varied CSH displayed commensurable changes and, as a result, significant correlative relationships were ascertained between the data (Figs. 1, 2). Thus, a gradual reduction of the carbohydrate content was detected in the lipopolysaccharide (LPS) fraction of more hydrophobic *Z. mobilis* cells (Fig. 1) concomitantly with a proportional decrease of ketodeoxygulonic acid (KDO) concentration in corresponding LPS

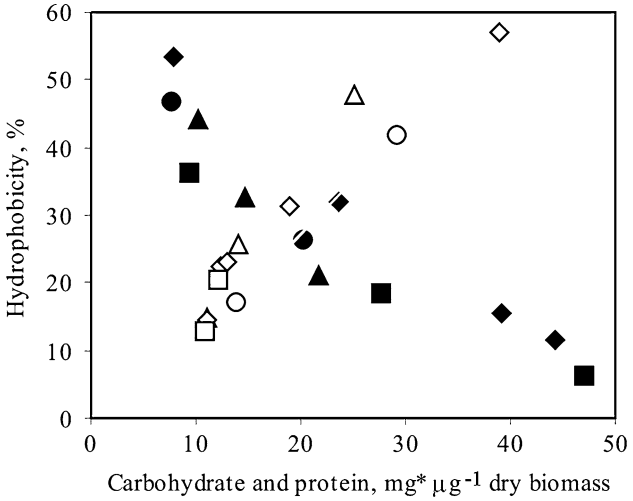

Fig. 1 The relationship between the cell surface hydrophobicity and the carbohydrate level (*filled symbols*, $r = -0.957$, $P < 0.001$) of lipopolysaccharides, and surface protein content (*open symbols*, $r = 0.971$, $P < 0.001$) of *Z. mobilis* 113S cells. Different symbols indicate the data of 4 independent experiments under varied growth conditions: *filled diamond* and *open diamond* sucrose 0.08, 0.16, 0.28 and 0.56 M, 30 °C; *filled triangle* and *open triangle* glucose 0.28 M, 20, 25 and 37 °C; *filled circle* and *open circle* glucose 0.28 M, 30 and 37 °C; *filed square* and *open square* glucose 0.08, 0.16 and 0.56 M, 30 °C

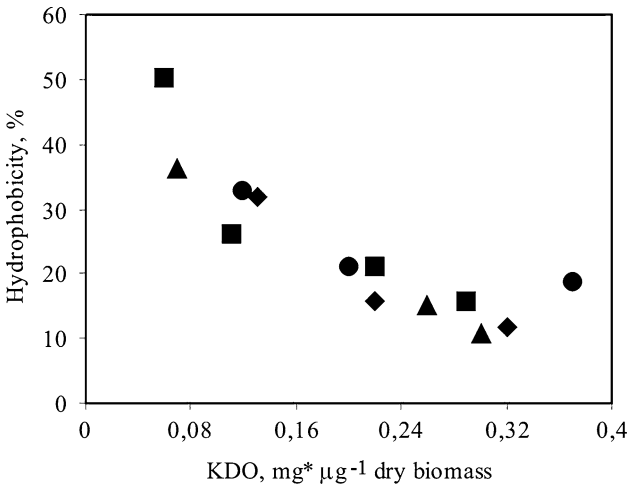

Fig. 2 The relationship between the cell surface hydrophobicity and the ketodeoxyoctulonic acid (KDO) content of lipopolysaccharides of *Z. mobilis* 113S cells ($r = -0.929$, $P < 0.001$). Different symbols indicate the data of 4 independent experiments under varied growth conditions: *filled diamond* glucose 0.28 M, 20, 25 and 37 °C; *filled triangle* glucose 0.28 M, 20, 25 and 37 °C; *filled square* sucrose 0.08, 0.16, 0.28 and 0.56 M, 30 °C; *filled circle* glucose 0.16, 0.28 and 0.56 M, 30 °C

samples (Fig. 2). On the contrary, the content of extractable cell surface proteins graduated in direct proportion to the rise of *Z. mobilis* CSH level (Fig. 1). Observed significant ($P < 0.01$) relationships, therefore, indicate that proportions of both hydrophilic (LPS) and hydrophobic (surface proteins) constituents of the cell envelope could be responsible for the overall CSH of *Z. mobilis* 113S.

An examination of FT-IR spectra attained for *cells* of varied CSH revealed several deviations of indices in the range between 2,800 and 3,000 cm^{-1}, which is dominated by the absorption modes of lipids aliphatic chains [19]. A significant direct relationship was detected (Fig. 3) between CSH of *Z. mobilis* 113S and the ratio of absorbance (A) intensities assigned to asymmetric and symmetric stretching vibrations of methyl groups [ν_{as} (CH_3) and ν_{sym} (CH_3), respectively], mainly on account of decreasing ($r = -0.533$, $P < 0.1$) ν_{sym} (CH_3) absorbance. In addition, the band position (frequencies) of ν_{as} (CH_3) were found to vary within a range of 1.62 cm^{-1} (2,959.48–2,961.10 cm^{-1}, coefficient of variation 35.8%) in direct proportion to increasing CSH values.

Other representative indices [19, 35] such as the asymmetric or symmetric stretches of methylene groups [ν_{as} (CH_2) and ν_{sym} (CH_2), respectively] varied at the comparable range of frequencies (coefficients of variation 35.8 and 42.5%, respectively), however, did not correlate with the corresponding CSH values as well as the proportions (CH_3/CH_2) between absorbance values assigned to vibrations of methyl- and methylene groups (data not shown).

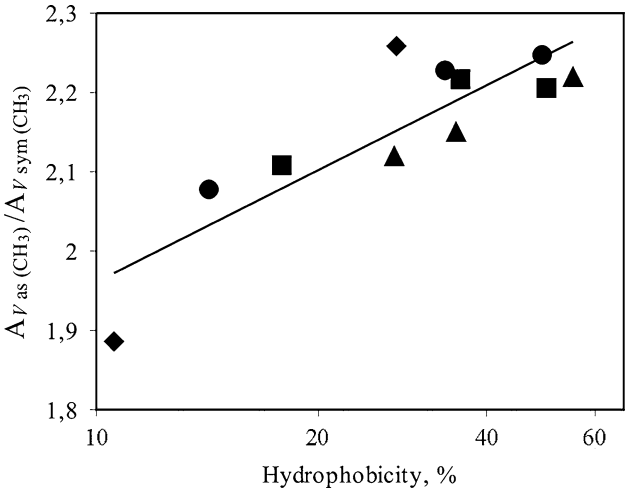

Fig. 3 The relationship ($r = 861$, $P < 0.001$) between the cell surface hydrophobicity and the ratio of absorbance (A) intensities assigned by FT-IR measurements to asymmetric and symmetric stretching vibrations of aliphatic methyl groups [ν_{as} (CH_3) and ν_{sym} (CH_3), respectively] of *Z. mobilis* 113S plotted on the semilogarithmic scale. Different symbols indicate the data of 4 independent experiments under varied growth conditions: *filled diamond* sucrose 0.08 and 0.28 M, 30 °C; *filled triangle* glucose 0.28 M, 30, 37 and 40 °C; *filled circle* glucose 0.28 M, 20, 37 and 40 °C; *filled square* glucose 0.08, 0.44 and 0.56 M 30 °C

The ability of *Z. mobilis* 113S to survive under adverse conditions of freezing/thawing, freeze-drying/rehydration cycle and exposure to elevated ethanol concentration was found to decrease with an increase of CSH values for intact bacterial cells. Observed almost linear relationships of the same kind (Figs. 4, 5), nevertheless, reflected a distinctive tolerance of intact bacteria *Z. mobilis* 113S in regard to various subsequent treatments. Thus, the smallest reduction of survival (0.135-1.917 log units) was observed during exposure of intact cells to the fixed (Fig. 5) or growing (Fig. 4) correlation of ethanol, particularly at the range of low (24-56%) CSH values. The subjection of intact bacteria to the freezing/thawing cycle at invariant conditions resulted in a more pronounced reduction of survival rates over the whole range of CSH values (Fig. 5), however staying above the highest losses (1.6-4.0 log units) in survival caused by freeze-drying and rehydration procedures of even relatively hydrophobic cells (CSH values 15%-53%) of *Z. mobilis* 113S.

Discussion

Observed relationships between the cell surface hydrophobicity (CSH) of *Z. mobilis*, the content of carbohydrates and ketodeoxygulonic acid (KDO) in the corresponding preparations of lipopolysaccharides (LPS), as well as the amount of cell surface proteins (Figs. 1, 2) appear in agreement with a common view on the hydrophobic character of constituents in the polymorphic composition of cell envelopes from proteobacteria [11, 22, 26]. In fact, the range of carbohydrate concentration (7.82-47.12 μg mg^{-1} dry mass) corresponds to an apparent content of LPS about 1.1–7.3% of the cell mass [11] being very comparable to that from various proteobacteria [22, 26] including *Z. mobilis* ATCC 10988 [32].

On the other hand, KDO, the well-known constituent of LPS composition in proteobacteria [11, 16, 22, 26] was not detected in *Z. mobilis* ATCC 10988 [32]. However, mentioned apparent absence of KDO might be due to the elevated cultivation temperature (37 °C) employed in this study [32], which could substantially reduce the amounts of LPS and, hence, KDO level in more hydrophobic [37] cells, possibly, below the limits of analytical determination. Besides, a varied KDO content could be considered as a strain-specific feature of particular proteobacteria [16].

LPS is generally thought to be a determinant in the structure of cell envelope rendering proteobacteria resistant to a variety of adverse environments [37]. The leaflet of LPS consists of closely packed oligosaccharide core and highly ordered hydrocarbon chain region which provide the hydrophilic and hydrophobic protective barriers, respectively [20]. In turn, pronounced lateral interaction between the

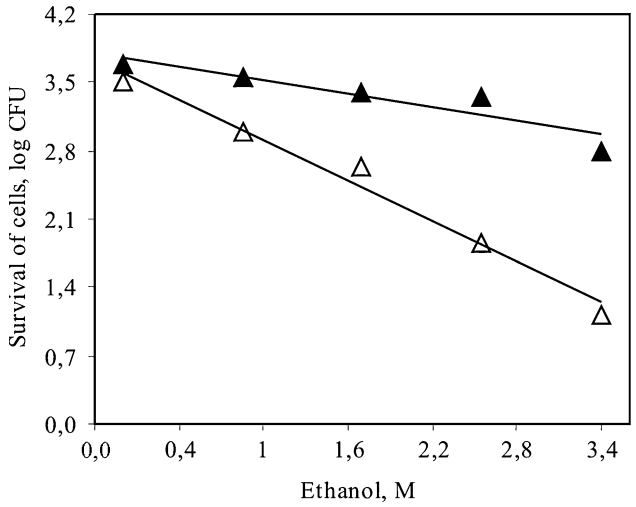

Fig. 4 The viable cell count of *Z. mobilis* 113S with different cell surface hydrophobicity (CSH) affected by elevated ethanol concentrations in the incubation medium [CSH of 24% ($r = -0.918$, $P < 0.02$) and 56% ($r = -0.989$, $P < 0.01$), *filled triangle* and *closed triangle*, respectively]. Growth conditions: *filled triangle* glucose 0.28 M, 30 °C and *closed triangle* glucose 0.28 M, 40 °C

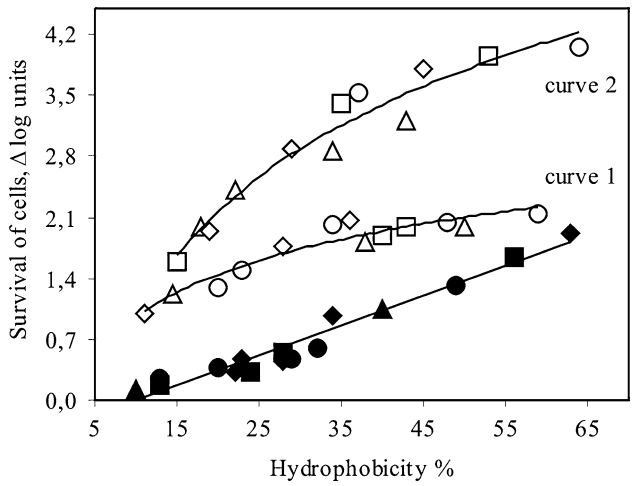

Fig. 5 The relationship between the cell surface hydrophobicity of intact *Z. mobilis* 113S and viability of cells subjected to subsequent freezing/thawing, freeze-drying/rehydration procedure [*open symbols*, *curve 1* ($r = 0.973$, $P < 0.001$) and *curve 2* ($r = 0.958$, $P < 0.001$), respectively] or affected by 2.55 M ethanol concentration (closed symbols, $r = 0.976$, $P < 0.001$). *Different symbols* indicate the data of 4 independent experiments under varied growth conditions: *filled diamond* sucrose 0.08, 0.16, 0.28, 0.44 and 0.56 M, 30 °C; *filled triangle* glucose 0.28 M, 20 and 37 °C; *filled circle* glucose 0.28 M, 20, 25, 30, 37 and 40 °C; *filled square* glucose 0.08, 0.16, 0.28 and 0.56 M, 30 °C; *open diamond* glucose 0.28 M, 20, 30 and 37 °C; *open triangle* glucose 0.08, 0.16, 0.44 and 0.56 M, 30 °C; *open circle* sucrose 0.08, 0.16, 0.28, 0.44 and 0.56 M, 30 °C; *open square* glucose 0.28 M, 20, 37 and 40 °C

hydrocarbon chain determine a gel-like state of strongly diminished fluidity and, hence, a very low permeability of intact proteobacteria [20]. For all that, an essential increase of cells permeability is generally acknowledged as a common decisive factor being responsible for the loss of their viability under diverse stress conditions including freeze-drying and freezing/thawing processes, exposure to ethanol and other chaotropic agents [13, 17, 20, 25, 27]. On the other hand, an impaired permeability barrier of cells should drive to the reduced fermentation power of bacteria, in particular to the diminished ethanologenic capacity of *Z. mobilis* [23].

A variety of structural rearrangements in bacterial envelopes caused by changes of the H-bonding, charge/dipole, hydrophobic and van der Waals interactions, etc., have been reported to impair the permeability barrier and therefore, the vital function of cells [5, 20]. From this point of view, observed changes of FT-IR spectral indices assigned to ν_{as} (CH_3) and ν_{sym} (CH_3) vibrations could indicate alternations in the orientation of methyl groups and alterations in the chain packing mode of aliphatic residues [31] with the growth of *Z. mobilis* 113S CSH values (Fig. 3) since a sensitive orientation information can be gleaned from observation of FT-IR methyl vibrations [15]. In turn, the absence of correlation between the CSH values and noticeable changes in the vibrational frequencies of methylene groups, ν_{as} (CH_2) and ν_{sym} (CH_2), suggests that concomitant formation of *gauche* conformers [19] could not make appreciable differences in hydrophobicity of *Z. mobilis* 113S cells.

It should be noted that an increase of *Z. mobilis* 113S permeability in direct proportion to growing CSH as a leakage of intracellular proteins into the medium has been observed in previous study [37], which could be responsible for generally acknowledged inhibition of ethanol formation [23] by damaged *Z. mobilis* cells.

Notable distinctions of tolerance upon exposures of intact *Z. mobilis* 113S to ethanol, freeze/thawing and freeze-drying/rehydration in principle could be expected taking into account marked differences in the amount of stress conditions presumably involved into above treatments [13, 17, 25, 27].

The results of the present study strongly suggest that the index of hydrophobicity as a general characteristic of cell surface properties could be of value to predict the ability of intact *Z. mobilis* to endure extreme environments and should be monitored towards lower values during cultivation in order to reduce subsequent unwanted structural and physiological disturbances provoked by multiple stress factors.

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