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Operating parameter optimization of cell surface hydrophobicity test for ureolytic bacteria

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Abstract: As one of the main non-covalent relations in microbiological-based systems, cell surface hydrophobicity (CSH) can be observed as a relevant parameter for biodegradation capability and suggested bacterial behaviour and biofilm formation during a bioremediation process. On the other hand, the role of ureolytic bacteria in bioremediation has subsequently led to the examination of this bacterial type in different engineering fields. In order to optimize the operating parameters of microbial adhesion to hydrocarbons test (MATH) for ureolytic bacteria, Box–Behnken experimental design was conducted for five ureolytic bacteria isolated from soils, as well as for the reference strain *Sporosarcina pasteurii* DSM 33. The optimization was completed with and without the essential substrate for the targeted metabolic reaction, with the aim to compare differences in bacterial hydrophobicity. A vortex time of 2 min, a hydrocarbon volume of 0.5 mL, and a phase separation time of 15 min are recommended as MATH operating parameters for all tested ureolytic bacteria. Although all bacteria are hydrophobic, lower CSH values in the presence of urea were observed for the same bacterium, which could be explained by the interaction of urea with the organic phase of the separation system, as well as a rapid ureolysis process that also occurs during the application of ureolytic bacteria in biotechnology systems.

Keywords: MATH test; adhesion potential; phase separation time; hydrocarbon volume; vortex time; *Sporosarcina pasteurii*

INTRODUCTION

The evidence of the contribution of ureolytic bacteria in bioremediation processes has subsequently led to the examination of these bacteria in a variety of engineering fields. Applications of ureolytic bacteria are found in traditional techniques of soil bioremediation and removal of metals ions and colours from

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industrial effluents.[1–3] In addition to these conventional strategies that are dependent on the biodegradation of organic pollutants, the use of ureolytic bacteria has been suggested for civil engineering bio-based processes, such as biocalcification, biomineralization or bioconsolidation of different materials (from repairing of cultural heritage substrates to development of a new generation of cement-based materials).[4–7] In the past decades, isolation of ureolytic bacteria from nature has supported the investigation of metabolic activity as well as the potential role of viable ureolytic cells in targeted engineering processes. Besides the widespread application possibility of ureolytic bacteria, the role of concentration and bacterial cell in a bioremediation system were always the main questions for engineers.

The hydrophobicity of the surface of ureolytic bacterial cell could contribute to adhesion and biofilm formation on different abiotic and biotic surfaces, as well as it could improve beneficial processes, such as degradation of organic contaminants in nature or removal of heavy metal ions in industrial waste effluents.[8] Furthermore, direct hydrophobic interaction with the substrate allows ureolytic bacteria to modify the surface contact and affect a modification in bacterial behaviour. One of the necessities for a bioremediation process is using non-pathogenic microorganisms that have defined behaviour in terms of biofilm potential,[9] and determination of cell surface hydrophobicity could be one of the initial parameters of the appearance of biofilm at the treated site during bioremediation.

Cell surface hydrophobicity has a significant role in the adhesion potential of bacteria on a surface. Understanding this phenomenon is essential for the application of a specific type of bacteria in different engineered processes, such as food processing, environmental engineering, biological-based systems, *etc.*[10,11] Bacteria exist predominantly as a population of microbial biofilms in the environment, due to their high adhesion capacity and possibility to aggregate on a different surface.[12] Since microbial adhesion is considered as the initial stage of biofilm formation, hydrophobic bacteria have been involved in the interaction with surfaces, accumulation of different pollutants around cells, and their decomposition.[8] A high level of cell surface hydrophobicity strongly affects biofilm formation, influencing the sustainability of diverse and complex social cooperation and coordination inside a biofilm.[13]

As a laboratory assay, the microbial adhesion to the hydrocarbons test (MATH) is often used for defining the cell surface hydrophobicity (CSH) of different bacteria, such as *Pseudomonas*, *Bacillus*, *Escherichia*, *Staphylococcus*, *etc.*[11,14–16] This test is based on the partitioning of bacterial cells between the hydrocarbon and aqueous phase of the separation system. The results of spectrophotometric measurement of the aqueous phase before and after vortex mixing of a bacterial suspension could be used as a quick assessment of hydrophobicity and therefore has been used in different fields, such as bioremediation in a waste-

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water system, biofiltration, biomedical science, food and dairy industry, *etc.*[17–19] As a result of the MATH test, cell surface hydrophobicity is presented as the percentage of bacterial cells separated into a hydrocarbon phase.[18]

Although the MATH test is frequently used in laboratory conditions, this method is liable to be influenced by variable operating conditions whereby the obtained values could vary by more than ten times and there is no unique or standard protocol in any scientific paper. For example, for vortex time as one of main MATH operating parameters is recommended from 10 s[20] through 60 s[21] and 120[22] to 300 s.[23] A similar situation exits for the employed phase separation time which is often 5, 15, 30 or 45 min,[14,24–26] as well as from 0.04 to 4 mL for the hydrocarbon volume.[11] Additionally, Saini[11] reported that the selection of hydrocarbon, wavelength, and suspension media could be used as variables in the MATH test, but the mentioned parameters do not exert a high influence on the results compared to the vortex time, hydrocarbon volume, and phase separation time as the main MATH operating parameters.

The optimization of laboratory assay, such as the MATH test, allows a comparative analysis of the influence of operating parameters and their interaction through quantification of the effect of operating parameters on the final result. A limited number of scientific studies have described a comparative analysis of operating parameters for the MATH test. It should be noticed that no specific experimental design has been established for this laboratory assay setting. Furthermore, no growth media with and without the addition of essential substrate for optimization of MATH operating parameters have yet been established for specific bacteria.

Therefore, this study aims to compare the effects of three MATH operating parameters (vortex duration, phase separation time, and hydrocarbon volume) on cell surface hydrophobicity by a Box–Behnken experimental design for six ureolytic bacteria. Considering that urea is always required as a nitrogen source when it comes to the utilization of ureolytic bacteria in engineering processes, the additional aim was observing differences of cell surface hydrophobicity between incubation of bacterial cultures in nutrient media with and without the addition of urea.

EXPERIMENTAL

The bacterial culture of five ureolytic bacteria from soil[27] and the well-known ureolytic strain *Sporosarcina pasteurii* DSM33 (DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were used for this experiment. The selected bacterial isolates (named as II_8, II_{10}, III_{11}, III_{15} and IV_5) previously showed high ureolytic activity compared to the mentioned reference strain.[27] The nutrient media for bacterial growth were TSB (Tryptone Soya Broth, HiMedia, Mumbai, India) with and without the addition of 20 % urea. The bacterial cultures were aerobically incubated overnight at 30 °C and centrifuged at 13000 rpm for 15 min in a Hettich Rotina 380 R centrifuge (Tuttlingen, Germany). The cell pellets were washed and resuspended in the same growth media.

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The MATH setup was performed at room temperature. Controls for the MATH test experiments ($A_{control}$) were the absorbance of prepared microbial suspensions in previous steps. An aliquot of 4 mL bacterial suspension (approx. 10^6 CFU mL^{-1}) was added in the selected hydrocarbon volume in a glass tube, vortexed, and hydrocarbon–aqueous phase separation was allowed. Hexadecane (Sigma–Aldrich) was used as the hydrocarbon. The aqueous phase was used for measuring the absorbance (A_{MATH}, wavelength = 600 nm) using a UV--1800 UV/Vis spectrophotometer (Shimadzu, Kyoto, Japan). All measurements were performed in triplicate. The non-inoculated nutrient media were used as the blank for the absorbance measurements and their absorbance was compared to the control cultures. The change in aqueous phase absorbance due to hydrocarbon addition represented as a measure of bacterial cells that separated into the hydrocarbon phase. Cell surface hydrophobicity (*CSH*) was calculated using Eq. (1):

$$CSH = 100\frac{A_{control} - A_{MATH}}{A_{control}} \quad (1)$$

Three operating parameters (X_1 – vortex time, X_2 – hydrocarbon volume, and X_3 – phase separation time) were independent factors in the selected Box–Behnken experimental design. The cell surface hydrophobicity was chosen as the dependent factor (Y_k). The experimental design is given as % in Table I with three levels for each independent factor, coded as –1, 0, and +1, which corresponded to the lower, middle, and higher levels, respectively. The response surface method was used to evaluate the influence of the MATH operating parameters on cell surface hydrophobicity for each bacterial strain. The relations between the independent factors and the responses were calculated by a second-order polynomial, Eq. (2):

$$Y_k = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{i=1, j=i+1}^{3} b_{ij} X_i X_j \text{ , k=10} \quad (2)$$

where Y_k is the defined response, b_0 is the intercept, b_i, $b_{ii,}$ and b_{ij} are the linear, quadratic, and interaction regression coefficients, respectively, while X_i and X_j are the varied factors.

Standard scores were calculated for each assay and were used for complex comparison of observed samples, regarding the obtained results for cell adhesion hydrophobicity. The ranking procedure between different samples was performed, based upon the ratio of raw data and extreme values for each applied assay,[28] according to Eq. (3) in case of "the higher, the better" criteria (used for cell adhesion hydrophobicity parameters) or Eq. (4) in case of "the lower, the better" criteria (used for factor scores), where x_i represents the raw data:

$$\overline{x_i} = \frac{x_i - \min\limits_i x_i}{\max\limits_i x_i - \min\limits_i x_i}, \quad \forall i \quad (3)$$

$$\overline{x_i} = 1 - \frac{x_i - \min\limits_i x_i}{\max\limits_i x_i - \min\limits_i x_i}, \quad \forall i \quad (4)$$

The higher cell adhesion hydrophobicity was considered as a positive score, while the lower factors scores were considered as positive for final product properties. The separate SS values for nutrient media without urea and nutrient media with the addition of 20 % urea were calculated. An optimization procedure was performed using Microsoft Excel 2007 to determine the workable optimum conditions.

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TABLE I. Box–Behnken experimental design for the MATH assay

Experiment	Coded factor level			Varied factor value		
	X_1	X_2	X_3	Vortex time min	Hexadecane volume mL	Phase separation time min
1	–1	–1	0	0.5	0.5	30
2	1	–1	0	4	0.5	30
3	–1	1	0	0.5	4	30
4	1	1	0	4	4	30
5	–1	0	–1	0.5	2	15
6	1	0	–1	4	2	15
7	–1	0	1	0.5	2	45
8	1	0	1	4	2	45
9	0	–1	–1	2	0.5	15
10	0	1	–1	2	4	15
11	0	–1	1	2	0.5	45
12	0	1	1	2	4	45
13	0	0	0	2	2	30
14	0	0	0	2	2	30
15	0	0	0	2	2	30

Statistical analyses were done using Statistica software, v. 13.2 (Dell, Round Rock, TX, USA). The influence of examined factors, as well as their interaction, was studied by comparing the sum of squares values for each of the coefficients in the second-order polynomial (SOP) model. The response surface plots were drawn using the same software for a constant value of hydrocarbon volume (2.25 mL) and varied values of the other two factors (vortex time and phase separation time).

RESULTS AND DISCUSSION

To the best of our knowledge, no MATH operating parameters have to date been established for the determination of cell adhesion hydrophobicity (CSH) of ureolytic bacterial strains isolated from nature,. Additionally, using an essential substrate during the experimental setup allows a comparative analysis of cell surface hydrophobicity and defines potential bacterial behaviour of selected bacteria during the bioremediation process. This step could be very important because ureolytic bacteria always require urea as a nitrogen source to realize the stated targeted metabolic process– ureolysis.[3]

In this study, the MATH operating parameters and tested range for CSH examination of the selected ureolytic bacteria (reference and wild strains) were chosen from relevant scientific papers.[18–24] The vortex time, hydrocarbon volume, and phase separation time are the most variable operating parameters that strongly affected the results of the MATH test.[11] In order to determine the MATH operating parameters for the targeted bacterial type, experiments were performed according to Box Behnken design. The obtained results for all six bacteria in both tested nutrient media (with and without the addition of 20 % urea) are given in Table II.

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TABLE II. The obtained results for cell adhesion hydrophobicity (%) of ureolytic bacteria by the Box–Behnken experimental design; –U: without urea addition, +U: with the addition of 20 % urea

Design	Reference strain		Wild ureolytic strain									
	S. pasteurii DSM33		II_8		II_{10}		III_{11}		III_{15}		IV_5	
	–U	+U	–U	+U	–U	+U	–U	+U	–U	+U	–U	+U
1	8.2	3.7	9.1	9.2	2.4	1.6	11.1	8.0	1.9	4.5	21.0	18.3
2	23.1	15.2	27.1	26.7	11.2	10.5	21.4	18.4	5.6	21.1	18.3	15.9
3	7.6	5.1	8.3	7.7	1.9	1.3	12.3	9.4	0.7	14.2	22.2	19.4
4	25.0	17.1	31.2	30.7	7.0	–0.2	19.4	19.5	2.6	–0.5	19.3	16.0
5	3.4	1.9	10.2	11.4	1.2	6.9	5.6	5.7	–0.3	10.9	17.6	15.4
6	17.1	13.7	19.4	18.8	10.1	2.2	17.0	17.8	1.2	8.8	18.7	15.9
7	9.2	4.9	11.1	11.7	3.3	8.7	15.2	11.1	4.7	15.3	24.7	21.6
8	20.2	13.3	26.7	25.0	12.6	2.8	14.7	10.1	2.8	5.6	21.8	19.1
9	10.1	7.1	17.7	16.6	4.0	5.2	15.2	13.3	1.5	10.7	22.4	19.6
10	14.1	7.7	17.2	16.9	7.5	5.6	19.2	14.4	3.0	11.7	19.2	16.6
11	14.9	5.9	22.1	19.0	8.2	6.4	22.4	16.9	3.3	13.8	23.1	16.7
12	16.2	9.0	23.0	24.5	9.5	6.5	21.4	17.0	4.1	14.1	25.0	20.2
13	16.5	9.85	23.9	24.7	9.1	6.0	22.0	17.4	4.4	13.8	20.0	16.8
14	16.35	9.71	24.0	24.5	8.9	5.9	23.1	16.8	3.9	13.3	18.5	17.8
15	16.2	9.9	23.9	23.8	8.8	6.0	22.7	17.2	4.05	13.4	19.6	16.2

Principal component analysis (PCA) of the presented data explained that the first two components (PC1 and PC2) accounted for 75.09 % of the total variance (PC1: 53.69 % + PC2: 21.40 %) in the twelve variables (cell surface hydrophobicity of the different bacterial strains). Considering the map of the PCA performed on the data (Fig. 1), the cell surface hydrophobicity of the reference strain incubated in nutrient media with and without urea (which contributed 11.7 and 14.0 % of the total variance, based on correlations, respectively), bacterial strains II_8 (14.0 and 14.1 % for nutrient media with and without urea, respectively), III_{11} (12.4 and 11.2 % for nutrient media with and without urea, respectively) as well as II_{10} and III_{15} incubated in the absence of urea (12.2 and 7.1 %, respectively) exhibited positive scores according to the first principal component.

The positive contribution to the second principal component calculation was observed in the following cases: bacterial strains II_{10} incubated with urea addition (24.6 % of the total variance, based on correlations), III_{15} incubated without urea addition (15.3 %), III_{15} incubated with urea addition (26.6 %), and IV_5 incubated with and without urea addition (10.6 and 12.1 %, respectively).

ANOVA calculation was conducted for the obtained second-order polynomial (SOP) models for incubation of selected ureolytic bacteria in nutrient medium TSB without the addition of urea, and the response variables were tested against the impact of factor variables (Table III).

According to the obtained results, ANOVA analysis revealed that the linear term of vortex time (X_1) considerably influenced the SOP model predictions of the

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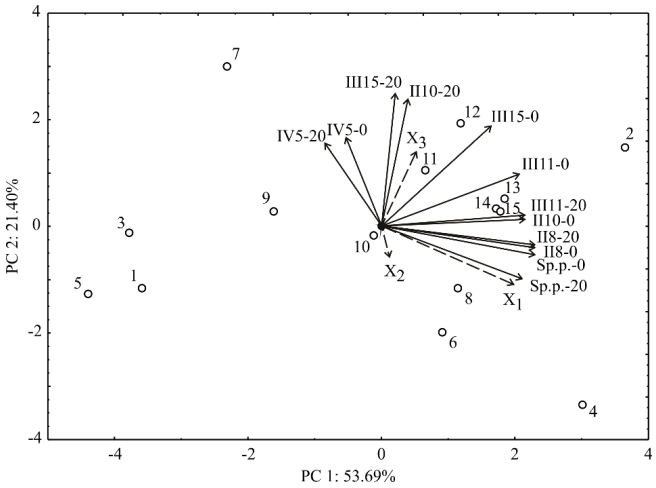

Fig. 1. PCA ordination of the variables based on component correlations.

TABLE III. ANOVA calculation for experiments with ureolytic bacteria nutrient media without urea (the sum of squares is presented); $^{+}$: statistically significant at $p<0.01$ level; *: $p<0.05$ level; **: $p<0.10$ level

Variable	Degrees of freedom	Reference strain	Wild ureolytic strain				
		S. pasteurii DSM33	II_8	II_{10}	III_{11}	III_{15}	IV_5
X_1	1	407.568^{+}	548.560^{+}	122.367^{+}	96.193^{+}	3.024	6.834**
X_1^2	1	12.933*	100.911^{+}	22.450*	178.869^{+}	5.612	0.360
X_2	1	6.040	2.592	0.038	0.392	0.576	0.093
X_2^2	1	0.500	3.623	7.230	0.038	0.205	5.836
X_3	1	27.668*	46.649*	13.616**	25.000*	9.734**	34.810^{+}
X_3^2	1	33.352^{+}	32.974**	0.214	33.117*	2.829	11.679*
$X_1 \times X_2$	1	1.862	9.592	5.379	2.216	0.826	0.012
$X_1 \times X_3$	1	1.655	9.738	0.017	36.230^{+}	2.449	3.364
$X_2 \times X_3$	1	2.037	0.638	1.074	6.118	0.246	5.698
Error	5	9.455	30.772	12.159	8.203	11.942	7.603
r^2		0.981	0.957	0.933	0.978	0.684	0.901

reference strain *Sporosarcina pasteurii* DSM33, as well as the bacterial strains II_8 and II_{10} calculation, and they were statistically significant at the $p<0.01$ level. The quadratic term of vortex time was also influential in the SOP models for these response calculations, statistically significant at $p<0.01$ or $p<0.05$ level. The linear term of phase separation time (X_3) was influential only for the reference strain and bacteria II_8, statistically significant at the $p<0.05$ level, while the quadratic term was only influential for the reference strain calculations, statistically significant at the $p<0.01$ level. The linear and the quadratic terms of vortex time were statistically significant at $p<0.01$ level in the SOP model, while the linear and the quadratic terms of phase separation time were statistically significant at the $p<0.05$ level in the SOP model for strain III_{11} calculation. The inter-

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change term of vortex time and phase separation time ($X_1 \times X_3$) was also influential for bacterial strain III_{11} calculation, statistically significant at $p<0.01$ level. As shown in Table III, for bacterial strain IV_5, the linear and the quadratic terms of phase separation time were statistically significant at $p<0.01$ and $p<0.05$ level in the SOP model.

The coefficients of determination (r^2) for the SOP models were rather good (0.684–0.981, Table III). According to the results presented in Table III, the higher r^2 values were attributed to the SOP models in which the nonlinear terms had less effect and the linear terms were more pronounced. The relatively inaccurate results of the SOP models indicate that some other models would improve the validity of the model predictions.

ANOVA analysis for the experiment in which selected ureolytic bacteria were incubated in nutrient media with urea (Table IV) revealed that the linear term of vortex time considerably influenced the SOP model predictions of the reference strain, as well as strains II_8 and III_{11} calculations, and they were statistically significant at the $p<0.01$ level. The quadratic term of vortex time was also influential in the SOP models for bacterial strains II_8 and III_{11} calculations, statistically significant at the $p<0.01$ or $p<0.05$ level. The quadratic term of phase separation phase was influential for the reference strain calculation, statistically significant at the $p<0.01$ level. The coefficients of determination for the SOP models for nutrient media with urea were relatively good (0.500 – 0.990, Table IV).

TABLE IV. ANOVA calculations for experiments with ureolytic bacteria nutrient media with the addition of 20 % urea (sum of squares is presented); $^+$: statistically significant at $p<0.01$ level; *: $p<0.05$ level; **: $p<0.10$ level

Variable	Degrees of freedom	Sum of squares					
		Reference strain	Wild ureolytic strain				
		S. pasteurii DSM33	II_8	II_{10}	III_{11}	III_{15}	IV_5
X_1	1	**237.420**$^+$	**477.817**$^+$	2.609	**124.093**$^+$	25.227	7.863
X_1^2	1	0.006	**100.226***	10.124	**68.611**$^+$	30.197	0.202
X_2	1	**6.237***	10.555	17.499	1.754	27.761	0.289
X_2^2	1	0.615	11.394	2.104	0.563	0.694	0.424
X_3	1	0.810	40.069**	2.250	0.436	3.725	13.764**
X_3^2	1	**16.409**$^+$	43.943**	2.855	17.715**	0.528	3.458
$X_1 \times X_2$	1	0.123	10.928	24.186	0.053	**239.477**$^+$	0.366
$X_1 \times X_3$	1	2.531**	8.056	0.346	46.529	15.490	1.331
$X_2 \times X_3$	1	1.297	7.506	0.028	0.070	0.055	8.483
Error	5	2.710	49.035	59.914	17.030	52.173	15.448
r^2		0.990	0.929	0.500	0.935	0.861	0.692

In order to present the interactions of the MATH operating parameters and define the recommendation of their value in the MATH protocol for ureolytic bacteria, three-dimensional response (RSM) surface plots were created. All RSM

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plots show the interaction of two tested parameters on the cell surface hydrophobicity, while the third parameter was sustained at the central value from the Box–Behnken experimental design. The effect of vortex time and phase separation time on the CSH at a constant volume of hydrocarbon (2.25 mL) is presented in Fig. 2a–f for all tested bacteria incubated in nutrient media without urea, as well as in Fig. 3a–f for all bacteria incubated in nutrient media with urea addition.

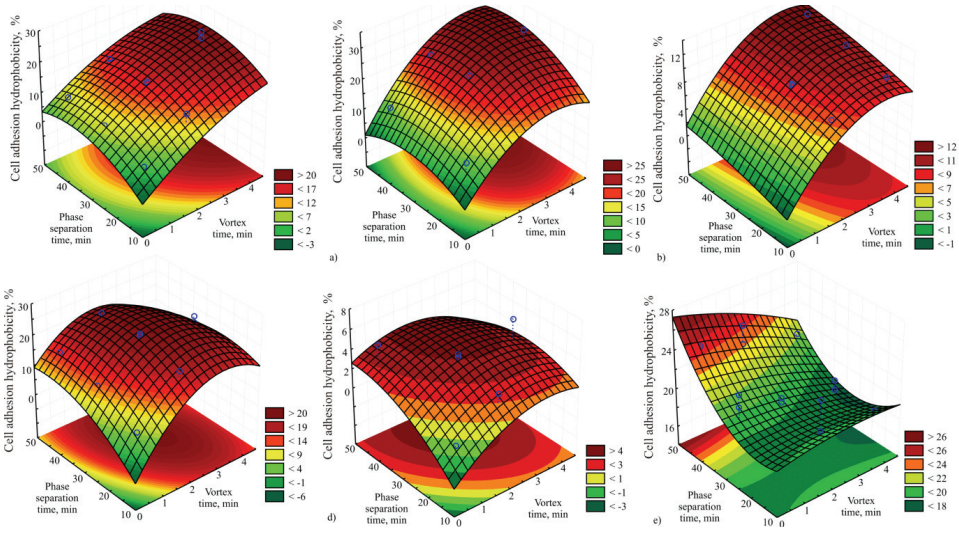

Fig. 2. The effects of the MATH operating parameters on cell surface hydrophobicity using nutrient media without urea: a) *S. pasteurii* DSM33; b) II_8; c) II_{10}; d) III_{11}; e) III_{15}; f) IV_5.

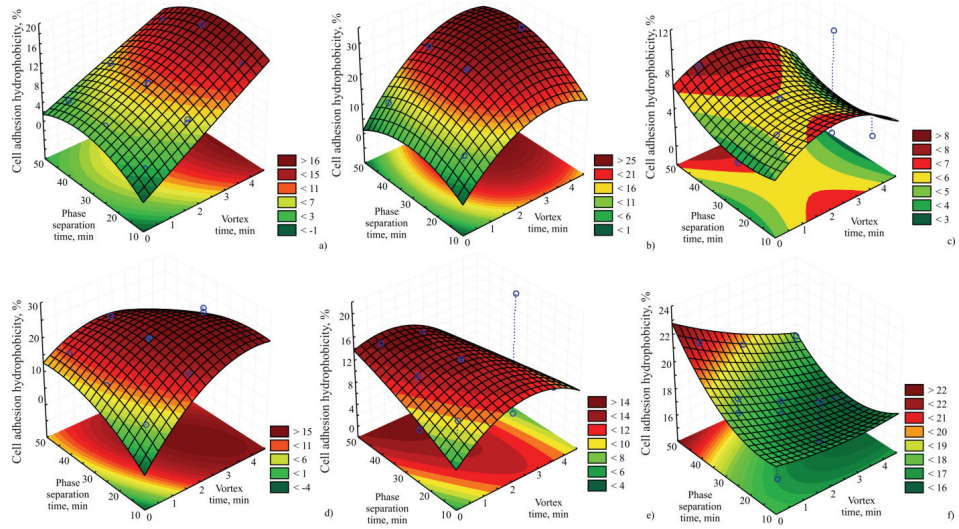

Fig. 3. The effects of the MATH operating parameters on cell surface hydrophobicity using nutrient media with 20 % urea: a) *S. pasteurii* DSM33; b) II_8; c) II_{10}; d) III_{11}; e) III_{15}; f) IV_5.

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Based on the obtained results for the referent ureolytic strain (Figs. 2a and 3a), it is evident that the maximum value of CSH (about 20 %) was achieved at the maximum vortex time and at any value of the separation time. A similar effect of vortex and separation time was observed for bacterial strain II_8 (Fig. 2b and 3b). In the case of bacterial strain II_{10} incubated in nutrient media without urea (Fig. 2c), the maximum CSH (about 12 %) was detected at the highest values of both presented parameters. On the other hand, the maximum value of CSH (about 8 %) for the same bacterial strain incubated in the presence of urea, a vortex time of 2 min, and a separation time of 45 min were the most suitable values (Fig. 3c).

According to the results presented in Fig. 2d and 3d, it could be concluded that the maximum CSH of bacterial strain III_{11} is equally achieved at medium values of vortex and separation time (2 and 30 min, respectively) and the maximum values of mentioned parameters (4 and 45 min, respectively).

It could be noticed that the presence of urea in the nutrient media makes differences in the obtained results for all the tested bacteria. Namely, urea has a strong impact on hydrophobic interaction by the transfer of hydrophobes from aqueous solutions to a pure liquid hydrocarbon phase,[29] as well as inducing an immediate metabolic hydrolysis reaction. Due to these details and the fact that urea is an essential nitrogen source for ureolytic bacteria, the presence of urea is required to obtain more representative values of cell surface hydrophobicity.

Standard score analysis (Fig. 4) revealed that the optimal MATH operating parameters for the determination of the CSH of ureolytic bacteria are: a vortex time of 2 min, a hydrocarbon volume of 0.5 mL, and a phase separation time of 15 min for both the tested experiment settings. This treatment was recommended for all tested ureolytic bacteria, with an *SS* value of 0.634 for the nutrient media without urea, and an *SS* value 0.677 for nutrient media with the addition of 20 % urea. The highest value of standard score has bacterial strain IV_5 with values of 0.677 and 0.649 for incubation with and without the addition of urea, respectively. As shown in Table I, this bacterial strain is the most hydrophobic, while II_{10} has the lowest value of CSH.

Comparing the ureolytic bacterial strains from nature with the reference strain *S. pasteurii* DSM 33, three of them (II_8, III_{11} and IV_5) are more hydrophobic, while the cell surface hydrophobicity of the other strains (II_{10} and III_{15}) could be compared that of the referent.

It could be noticed that urea addition decreased the value of the hydrophobicity in almost all experiments, but did not affect change in the MATH operation parameters. Due to this fact, the gained optimized MATH operation parameters (vortex time, hydrocarbon volume, and phase separation time) could be recommended for all ureolytic bacteria. Additionally, using urea in the nutrient media as a nitrogen source, which is required for biotechnological use of ureo-

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lytic bacteria, could provide more realistic values of CSH in a real-time process catalyzed by ureolytic bacteria.

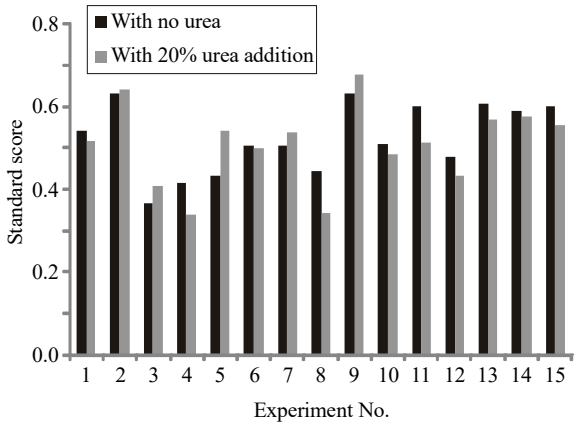

Fig. 4. Standard score analysis for cell adhesion hydrophobicity.

CONCLUSIONS

According to the results obtained in this study, the tested ureolytic bacteria showed hydrophobicity of cell surface in the range to 31.2 % as well as similar behaviour in the case of urea addition in the system. After the optimization process of the MATH test for six different ureolytic bacteria by the Box–Behnken experimental design, three main operating parameters (vortex time, hydrocarbon volume, and phase separation time) were adapted for the universal MATH protocol for ureolytic bacteria. The recommended values of these parameters are 2 min for the vortex time, 0.5 mL of hexadecane as an appropriate hydrocarbon, and 15 min for phase separation time. Despite the lower values of cell surface hydrophobicity of all tested ureolytic strains when urea was present in the system, incubation in nutrient media with urea addition is suggested for use in the MATH test, because this substrate is an essential nitrogen source for application of ureolytic bacteria in bioremediation systems.

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И З В О Д

ОПТИМИЗАЦИЈА ПРОЦЕСНИХ ПАРАМЕТАРА ТЕСТА ЋЕЛИЈСКЕ ХИДРОФОБНОСТИ ПОВРШИНА ЗА УРЕОЛИТИЧКЕ БАКТЕРИЈЕ

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Хидрофобне интеракције су једне од главних нековалентних веза у биолошким системима, те хидрофобност ћелијских површина представља један од кључних параметара за дефинисање способности биодеградације и понашања бактерија током процеса биоремедијације. С друге стране, улога уреолитичких бактерија у биоремедијацији је довела до испитивања карактеристика ћелијских површина као битног фактора адхезије ове бактеријске групе на абиотичке и биотичке површине и потенцијала стварања биофилма. У циљу оптимизације теста микробиолошке адхезије хидрокарбонатима (microbial adhesion to hydrocarbons test, MATH) за уреолитичку групу бактерија, урађен је Бокс–Бенкенов (Box–Behnken) експериментални дизајн за пет уреолитичких изолата из земљишта и референтни сој *Sporosarcina pasteurii* DSM 33. Оптимизација процесних параметара је урађена за хранљиву подлогу са и без додатка есенцијалног супстрата за циљану метаболичку активност (уреа), са циљем да се упореде разлике у хидрофобности истих уреолитичких сојева. Као оперативни параметри MATH теста, без обзира на присуство урее у систему се препоручују време мешања од 2 min, запремину хидрокарбоната од 0,5 mL и време раздвајања фаза од 15 min. Иако је за све тестиране бактерије доказана хидрофобност ћелијске површине, ниже вредности ове карактеристике у присуству урее за исти бактеријски сој. Овакав резултат може бити објашњен интеракцијом урее са органском фазом у систему за раздвајање и скоро тренутном индукцијом метаболичке реакције хидролизе што се неминовно дешава и у току употребе уреолитичких бактерија у биотехнолошким системима.

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REFERENCES

1. C. H. Kang, S. J. Oh, Y. Shin, S. H. Han, I. H. Nam, *Ecol. Eng.* **74** (2016) 402 (https://doi.org/10.1016/j.ecoleng.2014.10.009)
2. D. Arias, L. A. Cisternas, C. Miranda, M. Rivas, *Front. Bioeng. Biotechnol.* **6** (2018) 209 (https://doi.org/10.3389/fbioe.2018.00209)
3. S. Bibi, M. Oualha, M. Y. Ashfa, M. T. Suleiman, N. Zouari, *RSC Adv.* **8** (2018) 5854 (https://doi.org/10.1039/C7RA12758H)
4. R. Siddique, R. N. K. Chahal, *Constr. Build. Mater.* 25 (2011) 3791 (https://doi.org/10.1016/j.conbuildmat.2011.04.010)
5. H. Min Son, H. Y. Kim, S. M. Park, H. K. Lee, *Mater.* **11** (2018) 782 (https://doi.org/10.3390/ma11050782)
6. H. Pang, G. Wang, H. Yang, H. Wu, X. Wang, Y. Chen, *IOP Conf. Ser.: Earth Environ. Sci.* **252** (2019) 052131 (https://doi.org/10.1088/1755-1315/252/5/052131)
7. J. M. van der Bergh, B. Miljevic, O. Šovljanski, S. Vučetić, S. Markov, J. Ranogajec, A. Bras, *Constr. Buil. Mater.* 248 (2020) 118557 (https://doi.org/10.1016/j.conbuildmat.2020.118557)
8. A. Krasowska, K. Sigler, *Front Cell Infect. Microbiol.* **4** (2014) 112 (https://doi.org/10.3389/fcimb.2014.00112)

(CC) 2021 SCS.

9. A. Vidaković, *Denitrifier PhD Thesis*, University of Novi Sad, Novi Sad, 2019 (in Serbian)
10. C. O. Obuekwe, Z. K. Al-Jadi, E. Al-Saleh, *FEMS Microbiol. Lett.* **270** (2009) 214 (https://doi.org/10.1111/j.1574-6968.2007.00685.x)
11. G. Saini, *PhD Thesis*, Oregon State University, Corvallis, OR, 2010
12. L. Hall-Stoodley, J. W. Costerton, P. Stoodle, *Nat. Rev. Microbiol.* **2** (2004) 95 (https://doi.org/10.1038/nrmicro821)
13. Z. A. Mirani, A. Fatima, S. Urooj, M. Aziz, M. N. Khan, T. Abbas, *Iran. J. Basic. Med. Sci.* **21**(2018) 760 (https://doi.org/10.22038/IJBMS.2018.28525.6917)
14. C. H. Bolster, S. L. Walker, K. L. Cook, *J. Environ. Qual.* **35** (2006) 1018 (https://doi.org/10.2134/jeq2005.0224)
15. P. Tribedi, A. K. Sil, *J. App. Microbiol.* **116** (2013) 295 (https://doi.org/10.1111/jam.12375)
16. P. Lather, A. K. Mohanty, P. Jha, A. K. Garsa, *Biochem. Res. Intern.* **60** (2016) 1091290 https://doi.org/10.1155/2016/1091290
17. Y. N. Sardessai, S. Bhosle, *Biotechnol. Prog.* **20** (2008) 655 (https://doi.org/10.1021/bp0200595)
18. K. Hori, H. Watanabe, S. I. Ishii, Y. Tanji, H. Unno, *Appl. Environ. Microbiol.* **74** (2008) 2511 (https://doi.org/10.1128/AEM.02229-07)
19. S. Torres, A. Pandey, G. R. Castro, *Biotech. Adv.* **29** (2011) 442 (https://doi.org/10.1016/j.biotechadv.2011.04.002)
20. M. H. Ly, M. Aguedo, S. Goudot, M. L. Le, P. Cayot, J. A. Teixeira, T. M. Le, J. M. Belin, Y. Wache, *Food Hydrocolloids* **22** (2008) 742 (https://doi.org/10.1016/j.foodhyd.2007.03.001)
21. F. Gaboriaud, E. Dague, S. Bailet, F. Jorand, J. Duval, F. Thomas, *Colloids Surf., B* **52** (2006) 108 (https://doi.org/10.1016/j.colsurfb.2006.04.017)
22. C. O. Obuekwe, Z. K. Al-Jadi, E. S. Al-Saleh, *Can. J. Microbiol.* **53** (2007) 252 (https://doi.org/10.1111/j.1365-2672.2008.03887.x)
23. C.R. Bunt, D.S. Jones, I.G. Tucker, I.G., *Int. J. Pharm.* **113** (1995) 257 (https://doi.org/10.1016/0378-5173(94)00205-J)
24. B. M. Hsu, C. Huang, *Colloids Surf.* **201** (2002) 201 (https://doi.org/10.1016/S0927-7757(01)01009-3)
25. S. L. Walker, J. E. Hill, J. A. Redman, M. Elimelech, *Appl. Environ. Microbiol.* **71** (2005) 3093 (https://doi.org/10.1128/AEM.71.6.3093-3099.2005)
26. K. Myszka, K. Czaczyk, *Pol. J. Food. Nutr. Sci.* **61** (2007) 173 (http://dx.doi.org/10.2478/v10222-011-0018-4)
27. O. Šovljanski, A. Tomić, L. Pezo, S. Markov, *J. Sci. Food Agric.* **100** (2020) 1155 (https://doi.org/10.1002/jsfa.10124)
28. R. L. Prior, X. Wu, K. Shaish, *J. Agric. Food Chem.* **53** (2005) 4290 (https://doi.org/10.1021/jf0502698)
29. T. A. Shpiruk, M. Khajehpour, *Phys. Chem. Phys.* **15** (2013) 213 (https://doi.org/10.1039/C2CP42759A).

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