applied microbiology

Supplementary material

Toward Effects of Hydrophobicity on Biosurfactant Production by *Bacillus subtilis* Isolates from Crude-Oil-Exposed Environments

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Supplementary data S1

Biosurfactants screening methods

The culture medium and SDS (1%, v/v) were considered for negative and positive control, respectively.

Oil displacement area

A crude oil layer was formed on distilled water using a Petri dish by adding 20 µL of the crude oil to 50 mL of water. A volume of 10 µL of the culture broth or culture supernatant was put into the centre point of the crude oil layer. The diameter of the clear zone on the surface of the oil is related to the activity of the surfactant called oil spreading activity. For pure biosurfactants, there is a linear relationship between the amount of surfactant and the diameter of the clear zone [35].

Drop collapse assay

The assay was performed on a 96-well microplate. Each well was filled with 2 µL of crude oil and left at room temperature for a few hours. Subsequently, 5 µL of the culture supernatant was gently placed on the oil layer. The state of the drop was observed after one minute. In case, the droplet was spread or collapsed, the result was reported as positive, while a stable droplet was described as a negative result [79, 80].

Hemolysis of red blood cells

The respective culture was spread on a plate with blood agar (5% sheep blood) and was grown for 48 hours at 30 °C. Afterwards, lysis of erythrocytes was analyzed by screening for transparent area around the colonies [35].

Forming insoluble ion pairs (blue agar plate)

A mineral salt medium containing 1% glucose 1.5% and agar was used with 0.2 g/L cetrimonium bromide. The medium was gradually tinted light blue with 0.005 g/L methylene blue. The assay was investigated in two ways: (I) 30 μL of the culture supernatant was poured into the wells embedded in the plates and (II) different isolates were cultured on the plates. All plates were incubated for 48 hours at 37 °C. Observing a dark blue halo around the wells and colonies indicated the production of anionic biosurfactants [81].

Emulsification index (E24%)

A volume of 2 mL supernatant culture was poured into a glass tube with 1 mL n-hexane. The tubes were vigorously vortexed for 2 minutes and kept at room temperature for 24 hours. The emulsification index was obtained with the following equation [35]:

$$E24\% = he/(ht \cdot 100)$$

(he – height of the emulsified layer; ht – total height of the solution)

Supplementary data S2

Table S1. Results of screening tests for crude oil-degrading and biosurfactant-producing isolates.

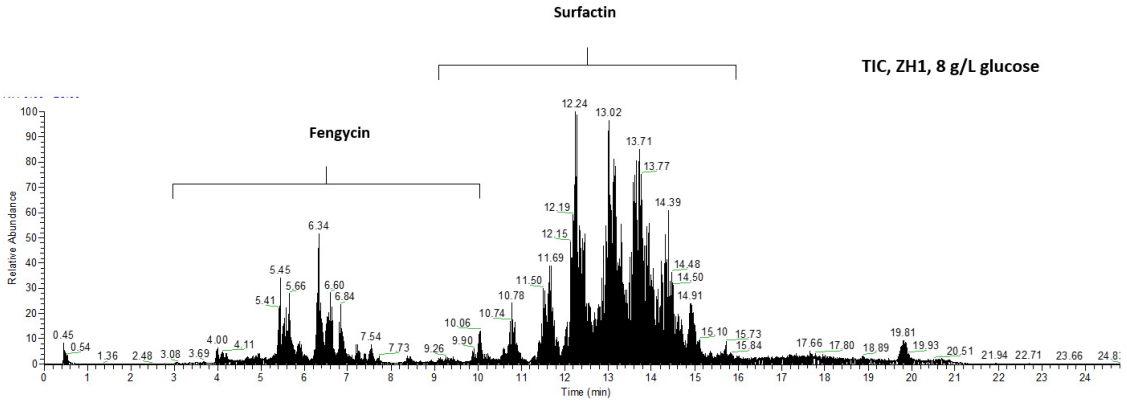
	Medium (negative control)	1 % (v/v) SDS (positive control)	Strain ZH1	Strain P7
Oil spreading assay (mm)	3	70	10-45	15-40
Drop collapse assay	-	+++	++	++
Hemolytic assay	-	++	+ (beta)	+ (beta)
Formation of insoluble ion pairs (blue agar plate)	-	+++	-	-
Emulsification index (E24%)	0	100	56	62
Crude oil degradation	-	*	+	+

Table S2. Characteristics of the isolates

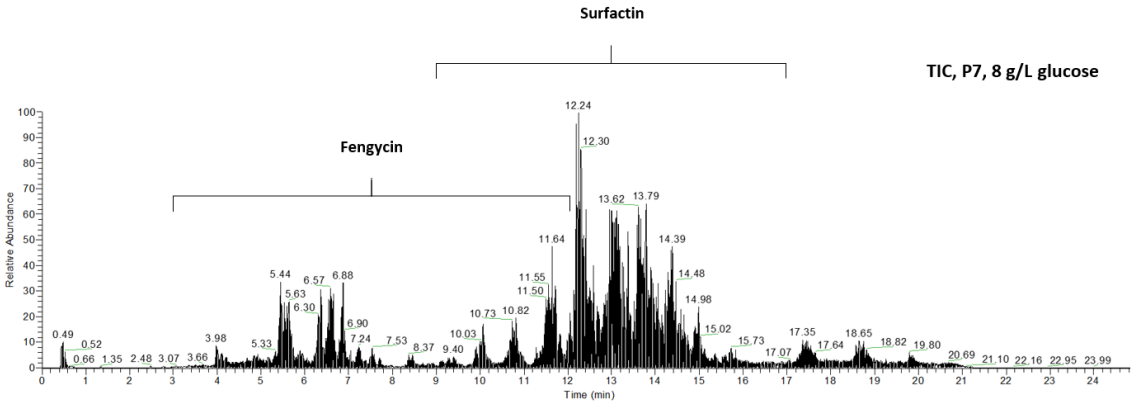
Biochemical tests and identification information	P7	ZH1
Gram staining	+	+
Spore formation	+	+
Catalase	+	+
Anaerobic growth	+	+
Nitrate reduction	+	+
Carbohydrates fermentation (glucose, melibiose, sucrose, mannitol)	+ + + +	+ * + +
Citrate, starch, and casein hydrolysis	-,+,+	-,+,+

MR/VP test	-/+	-/+
16S rDNA identity	*Bacillus subtilis*	*Bacillus subtilis*
Gene bank accession number	ON652357	ON678054
DSMZ ID	11744	11741

Supplementary data S3



(A)



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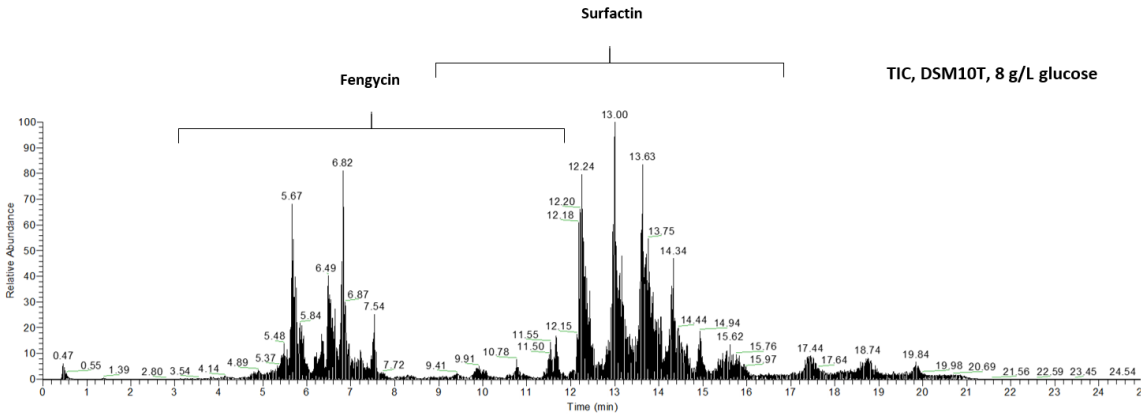


(C)

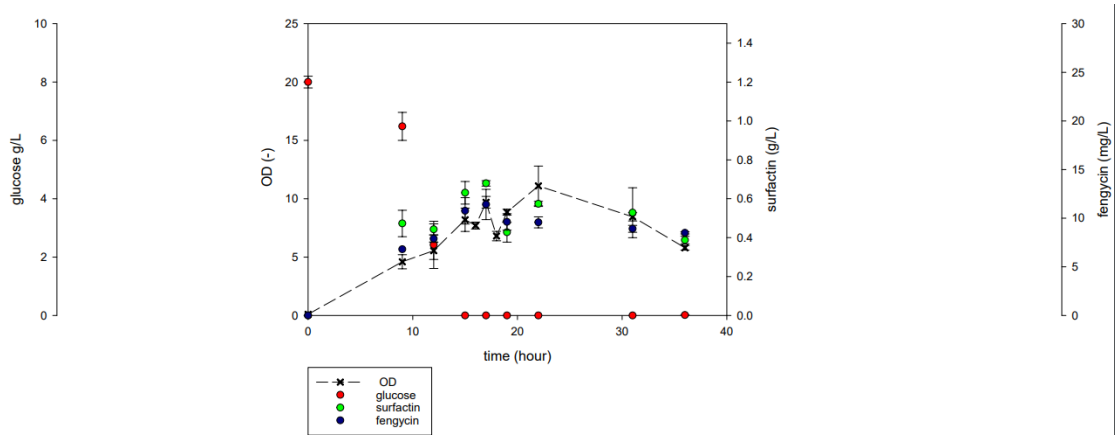
Figure S1. LC-ESI-MS analyses with corresponding total ion chromatograms (TIC) of the lipopeptides produced by *Bacillus subtilis* strains ZH1 (A), P7 (B), and DSM10T (C).

Table S3. Distribution of surfactin and fengycin variants produced by *B. subtilis* isolates P7, ZH1 and wild-type control strain DSM10T.

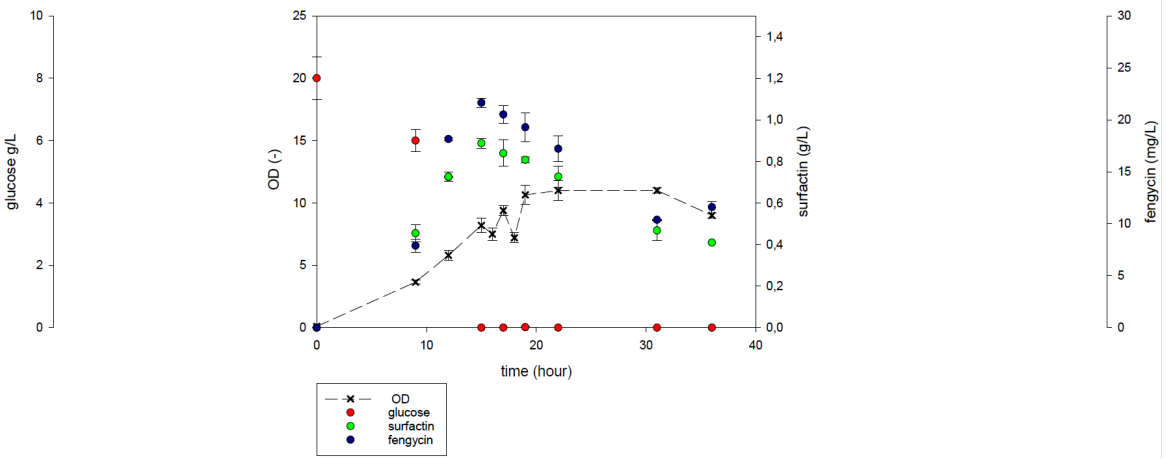
Name of variants	Ratio (%)		
	DSM10T	P7	ZH1
Surfactin C15	33.03	20.39	31.01
Surfactin C14	23.28	22.27	22.72
Surfactin C13	18.70	18.96	14.56
Surfactin C16	9.20	10.48	8.46
Surfactin C12	2.71	8.78	5.26
Surfactin C17	1.99	2.04	2.29
Surfactin [Val7] C13	1.92	3.52	2.28
[AME5] Surfactin C16	1.86	1.35	1.67
Surfactin [Val2] C14	1.70	1.70	3.05
Surfactin [Val7] C16	1.44	1.22	1.18
Surfactin [Val2] C14, Surfactin [Ala4] C15	1.32	2.97	2.67
Surfactin C14/Surfactin [Val2] C15	1.22	1.52	1.35
[AME5] Surfactin C15	0.61	1.10	0.88
[AME5] Surfactin C14	0.40	1.24	1.09
Surfactin [Val2] C13, Surfactin [Ala4] C14	0.29	1.04	0.95
[AME5] Surfactin C17	0.11	0.16	0.20
Surfactin C18	0.06	0.10	0.10
Surfactin [Val7] C14	0.06	1.06	0.20
Fengycin B1 C17 sFA	36.70	32.09	29.21

Fengycin A1 C17 sFA	20.60	21.75	21.88
Fengycin B1 C16 sFA	13.18	10.94	12.03
Fengycin B1 C16 usFA	12.25	14.19	17.86
Fengycin A1 C16 sFA	7.69	7.57	5.99
Fengycin B1 C15 usFA / Fengycin A1 C17 usFA	3.78	5.46	5.52
Fengycin A1 C15 usFA	2.43	5.62	4.74
Fengycin B1 C15 sFA	1.94	1.20	1.51
Fengycin A1 C14 usFA	0.51	0.51	0.54
Fengycin B1 C18 sFA	0.40	0.46	0.49
Fengycin B1 C14 usFA	0.30	0.11	0.10
Fengycin A1 C14 sFA	0.16	0.05	0.08

Supplementary data S4



(A)



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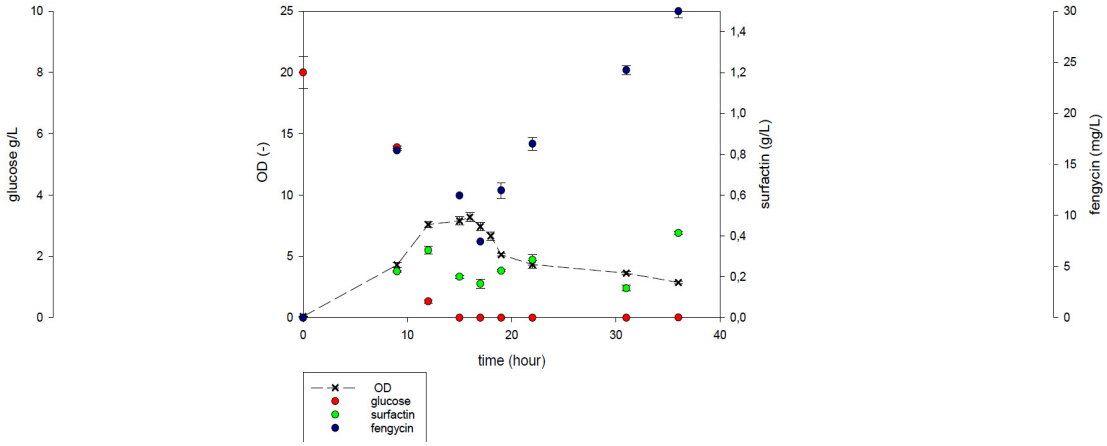

(C)

Figure S2. Effect of 1% (v/v) hexadecane supplementation on lipopeptide production and growth behavior. Using chemically defined mineral salt medium with 8 g/L glucose, effects of additional supplementation of hexadecane as a defined hydrocarbon substrate was analyzed using the *B. subtilis* strains P7 (A), ZH1 (B), and the wild-type control strain DSM10T (C).

Supplementary data S5

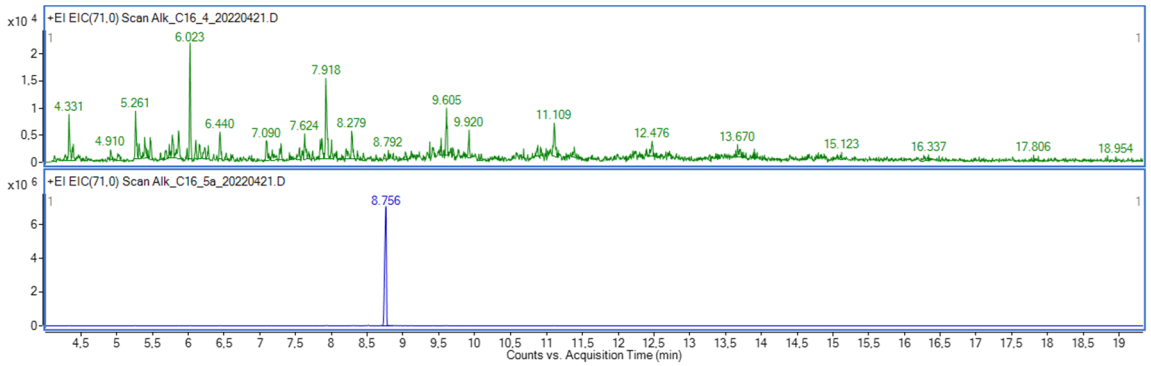

Figure S3. The chromatogram of the abiotic control (blue) compared to one of the strains (ZH1, P7, green) shows the decomposition of hexadecane in the cultivation broth sample. There is only one peak at 8.75 min (hexadecane, blue) and several peaks at 4-19 min (other hydrocarbons, green) which are decomposition products.