Biodegradation of naphthalene mediated by the plant growth promoting rhizobacteria

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Abstract: We compared the ability of two bacterial strains, *Paenibacillus polymyxa A*26 and *P. polymyxa* A26Sfp, for biodegradation of naphthalene (NAP). The studies were performed under simulated laboratory conditions, in liquid medium and soil with different carbon sources, pH and salt contents. Changes in the luminescence inhibition of *Aliivibrio fischeri*, as an indicator of the baseline toxicity, were observed in degradation mixtures during 7 days of incubation. While both strains expressed the best growth and NAP degradation ability in the minimal salt medium containing sucrose and 5% NaCl at pH 8, the mutant strain remained effective even under extreme conditions.

A26Sfp was found to be an efficient and potentially industrially important polycyclic aromatic hydrocarbon degradation strain. Its extracellular polysaccharide production is 30% and glucan production twice that of the wild type A 26. The surface tension reduction ability was ascertained as 25–30% increased emulsification ability.

Introduction

Crude oils contain many major and minor constituents. The properties of the constituents influence how the spilled oil behaves and determines the fate and effects of the spill in the environment [1]. Among the components of crude oil, polycyclic aromatic hydrocarbons (PAHs) are a class of organic contaminants that contain two or more fused benzene rings; they are

considered very important after oil spill accidents because they are toxic, mutagenic, carcinogenic, and relatively persistent in the environment [2 3]. Of the various forms of PAHs in crude oil, 16 PAHs including naphthalene are considered as priority pollutants by the environmental protection agencies [4,5 6 7].

Many studies have focused on the isolation of bacteria that produce biomolecules that promote biodegradation of the PAH and other pollutants, and remove pollutants from the environment [8,9 10,11 12-16]. Our wild barley (*Hordeum spontaneum*) rhizosphere isolate *Paenibacillus polymyxa A* 26 originates from a habitat exposed to various stress factors at the Evolution Canyon (EC) South Facing Slope (SFS) [17,18] (Table 1 and Fig S1). The *P. polymyxa* A26 Sfp-type 4-phosphopantetheinyl transferase deletion mutant strain (A26Sfp) enhanced plant drought stress tolerance[19]. The mutant, compared to its wild type A26, is 30% enhanced in its biofilm exopolysaccharide (EPS) production [20-22]. This correlates with the improved drought stress tolerance conferred by the strain and the enhanced biocontrol ability [19-23]. *P. polymyxa* is a bacterium widely used in agriculture, industry, and environmental remediation because it has multiple functions [24-38]. The species has very high metabolic diversity which results in great differences in the bacterial potential for bio-technological applications [25]. *P. polymyxa* strains from the harsh South Facing Slope (SFS) in comparison to the moderate North Facing Slope (NFS) at 'Evolution Canyon', Israel, show huge differences in their metabolism, drought tolerance enhancement and biocontrol ability [29,26,39]. Our *P. polymyxa* strain A26 is isolated from the stressful SFS and has been shown to be capable of moderate drought stress tolerance enhancement [25,29]. An important pool of the bioactive compounds of great interest for biotechnology are non-ribosomal peptides/polyketides. The spectrum of application of both classes of compounds is large. Non-ribosomal peptides produced by non-ribosomal peptide synthetases (NRPS) and polyketides are produced by polyketide synthetases (PKS). Both are very diverse families of natural products with an extremely broad range of biological activities [40-48]. These molecules exhibit a broad range of structural diversity and display biological activities that include adaptation to unfavorable environments, and communication or competition with other microorganism in their natural habitat [30].

The *A. fishery* bioluminescence inhibition test constitutes a simple and economic technique, frequently applied for ecotoxicological screening [49]. The test is sensitive, easy to apply and reproducible, thereby facilitating testing for the ecotoxicity screening of different compounds. Several commercially available types of this assay have been developed to determine the acute

and chronic toxicities of diverse chemicals.

In the present investigation, we compared the intrinsic ability of two bacterial strains, A26 and its mutant A26Sfp, for degradation of NAP in a defined mineral medium and axenic soil. While the slightly alkaline environment (pH 8), sucrose and 5% NaCl in the medium were most favorable for both strains, the mutant showed 20–35% higher biodegradation efficiency and could function under extreme conditions. We explored a possible link, involving improved emulsification, between the strain's biodegradation ability and the bacterial EPS production.

Methods

Chemicals

The polycyclic aromatic hydrocarbon naphthalene, kerosene, hexadecane, was purchased from Sigma Aldrich.

Biodegradation experiments

The experiments were performed in 250 ml Erlenmeyer flask bottles containing 100 ml DF salt minimal medium (MM) medium as described earlier [50].

Five different carbon sources, including glucose, galactose, fructose, maltose and sucrose, were added individually into MM medium at a concentration of 1.5%.

The bacterial strains were grown on TSB, washed, and resuspended in MM medium (A600, 0.6). 10 ml of the resuspension was inoculated into Erlenmeyer flasks and incubated in a 200 rpm shaker for 24 h at 28±2°C. Furthermore, the NAP (100 mg/L) were added individually to the culture flasks and incubated in the dark at 200 rpm for 7 days at 28±2°C. Bacterial growth was detected by measuring the optical density at 600 nm at predetermined time intervals during the 7 days. All experiments were performed in triplicate. Growth in media without NAP were used as controls.

Analysis of NAP biodegradation efficiency by A*liivibrio fischeri* luminescence inhibition biotest

Changes in the luminescence inhibition of *Aliivibrio fischeri*, as an indicator of the baseline toxicity, were observed in photodegradation mixtures. The BioTox™ Kit was used for the determination of toxicity of samples. The inhibitory effect of the sample on the light emission of luminescent bacteria, *A. fischeri* (formerly *Vibrio fischeri*), was measured with a luminometer.

We reconstituted lyophilized aliquots of *A. fischeri* containing NaCl 3% w/v by adding 1 mL of distilled water and resuspended them in 10–30 mL of the nutrient broth. Two hundred microliters of the bacteria suspension and 100 µL of each sample were added to the microplate wells. The controls consisted of 200 µL of the bacteria plus 100 µL of a 3% NaCl solution in tap water. The emitted light was recorded by a Victor Light 1420 microplate luminometer (Perkin-Elmer, Norwalk, Conn. USA) at fixed intervals between 0 and 48 h. Five replicates were prepared for each sample, and the light emission values expressed as relative luminescence units (RLU). Biodegradation efficiency was calculated by measuring the luminescence of the bacteria after 30 min of contact with the contaminant. To determine the toxic effect, we compared the emitted light from the samples at the various dilutions with the control solution. The less light emitted, the greater the toxicity of the sample. Therefore, the relative biodegradation efficiency of the bacterial strain is expressed as the percentage inhibition of the sample (I%sample) divided from the control sample with the NAP where the bacteria were not used (I%control).The biodegradation efficiency percentage (BE%) was used to express the toxicity of the tested samples and calculated according to:

$$BE\% = I\%control - I\%sample$$

Effect of different carbon sources on PAHs biodegradation

Five different carbon sources, including glucose, galactose, fructose, maltose, and sucrose were added individually into MM medium at a concentration of 1.5%. The isolate grown as described above was resuspended in MM medium (A_{600}, 0.6) and the resuspension was used as an inoculum for this experiment. The inoculated flasks were incubated in a shaking incubator (as described above). Samples were withdrawn at pre-determined time intervals and NAP degradation efficiency of the culture was determined by luminescence inhibition (using the BioTox kit as described above).

Effect of pH on NAP biodegradation

Batch experiments were performed to study the effect of pH on NAP biodegradation. Briefly, 100 mg/L NAP was mixed with 100 mL of MM containing 3% inoculums. The pH of the culture medium was adjusted from 5 to 10. The flasks were incubated in a shaking incubator as described above (200 rpm) at 27 ± 2°C for 7 days. Samples were withdrawn at predetermined time intervals and luminescence inhibition was determined.

Effect of NaCl on NAP biodegradation

The effect of NaCl on biodegradation of NAP was performed in batch experiments. Briefly, NaCl (1–15%) was added individually to MM bottles containing NAP and the bottles were incubated in a shaking incubator as described above for 7 days. Samples were withdrawn at fixed time intervals and luminescence inhibition was determined.

EPS extraction. EPS extraction was performed as described earlier with small modifications [51]. Briefly, bacterial cultures were diluted 1:5 with distilled water and centrifuged for 30 min at 17,600 g at 20 °C to separate cells. Then, EPS were precipitated by slowly pouring the supernatant into two volumes of isopropanol while stirring at 200 rpm. The filtered polysaccharide was suspended in a digestion solution consisting of 0.1M $MgCl_2$, 0.1 mg/ml DNase, and 0.1 mg/ml RNase solution, and incubated for 4 h at 37°C. Samples were extracted twice with phenol-chloroform, and lyophilised using a Virtis SP Scientifc 2.0 freeze dryer. For the emulsification, drop collapse and oil spreading assay the dialysed EPS were taken into initial volume in double distilled sterile water.

Glucan production assay

The bacterial strains were incubated for 5 days as described above. The glucan production assay was performed as described earlier [52]. Briefly, after incubation, glucan was recovered by adding an equal volume of 0.6N NaOH with 30 min stirring to the production medium. The bacterial cells were removed from the production medium by centrifugation at 10,000 rpm for 10 min. The supernatant was neutralized to pH 7 by adding 4N acetic acid. Then the material was washed with water until the pH was neutralized, causing the precipitation of the curdlan, which was thereafter lyophilized.

Emulsification assay

The emulsification assay used was previously described by Cooper and Goldenberg [53] . Cell-free culture broth/bacterial EPS or glucan (200 µl), obtained as described above, was used to determine the emulsification of NAP in an Eppendorf tube containing 600 µl of distilled water. 1.2 ml of NAP was mixed with each sample in triplicate. For two min the mixture was vortexed, and the emulsion was allowed to stand for 24 h. Water and NAP served as negative controls. The height of the emulsion layer was then measured. The emulsification index was calculated based on the ratio of the height of the emulsion layer and the total height of the liquid [EI % = (emulsion/ total h) x 100]. To determine the stability of the emulsification ability of the biosurfactant, the emulsification index was also

determined after 3 and 7 days.

Drop-collapse test

Both bacterial strains' culture filtrates and A26Sfp EPS and glucan extracts were submitted to a drop-collapse test, using the procedure described by Jain et al. [54]. For a period of 7 days the strains were grown in MM medium and 5 µl of the cell-free culture broth (supernatant centrifuged 13,000 x g, 15 min) was dropped on a glass slide covered with crude oil. The result was considered positive for biosurfactant production when the drop diameter was at least 1 mm larger than that produced by distilled water (negative control).

Oil spreading assay

10 µl of crude oil was placed on the surface of a Petri dish that contained 40 ml of distilled water. A thin layer of oil was formed, as described earlier [10]. Culture supernatants, EPS and glucan extracts (10 µl, obtained as above) were then placed in the center of the oil layer. If the oil is displaced by an oil-free clearing zone, then biosurfactant is present in the supernatant. A negative control was performed with distilled water (without biosurfactant), and no oil displacement or clear zone was observed.

NAP degradation in soil

Sterile uncontaminated peat soil was artificially contaminated by adding the defined NAP mixture, prepared in DCM, to a sterile jar, allowing the solvent to evaporate, and then adding soil to the jar. After thorough mixing, the homogeneity of NAP distribution was confirmed by testing using the luminescence inhibition assay in five random samples of the soil. The soil was subdivided into 200 g (dry weight) lots of 1.5-liter jars. The jars were then inoculated to provide a bacterial population of 106 cells per g of soil. Controls, which contained NAPs but lacked inoculum, were set up similarly. All soil cultures were supplemented with sterile MM solution with 5% NaCl, 1.5% sucrose, pH 8 (see above) to approximately 65% of the soil's water-holding capacity and were incubated at 25°C in the dark.

Three samples of 1 g of soil from each jar were collected periodically for analysis of NAP biodegradation and measurement of the microbial population as described by us earlier [55]. Briefly, aliquots of 10 mM of primers 1492R (5-GGTTACCTTGTTACGACTT-39') and 27F (5'-AGAGTTTGATCCTGGCTCAG-3´) and 1 ml of template were used. The reaction was performed in 10 ml. The reaction conditions were 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 20 s, primer extension at 72°C for 1 min,

191 followed by the final extension at 72°C for 5 min. For sequencing, the PCR products were
192 purified with a QIAquickTM Gel Extraction kit (QIAGEN, Hilden, Germany).

193 Data confirmation and validation.

194 To ensure reproducibility, three biological replicates of every treatment, were performed.
195 Replicated data were studied for normal distribution and analysed by MiniTab17 ANOVA. The
196 treatment effects were considered statistically significant, $p \leq 0.01$ (indicted by different letters)
197 or nonsignifcant . Univariate analysis of selected samples involving distribution and variability
198 of distribution was performed using the Unscrambler X10.4.1 descriptive statistics.

199

200 **Results**

201 Biodegradation in the presence of NAP

202 The growth of A26 and A26Sfp in the presence and absence of NAPs at an initial
203 concentration of 100 mg/L was determined as optical density at 600 nm (Fig 1). The time
204 course indicates that both strains are capable of utilizing NAP. It is often reported that
205 biodegradation is dependent on survival, characterized by ODs. However, this measure would
206 also indicate adaptation. Hence biodegradation was analyzed using the bioluminescence
207 inhibition test (Fig 2). Generally, microorganisms require suitable growth conditions (as
208 regards e.g. carbon sources, nutrients, pH) since these strongly affect their growth. Higher
209 degradation efficiency was observed in flasks containing sucrose as a carbon source (Fig 3).
210 Minimum degradation was observed in fructose media (Fig 3).

211 Our results show that maximum degradation efficiency was reached at 5th day. A26Sfp and
212 A26 efficiently degraded NAP by 69 and 49%, respectively, in 5 days of incubation in liquid
213 medium and soil (Fig 2).. Somewhat reduced numbers in the degradation efficiency in the case
214 of degradation in soil (55 and 44%) were observed (Fig 2). Our results show that the two strains
215 used in the study perform best in the medium supplemented with sucrose (Fig 3) and 5% NaCl
216 (Fig 4). at pH 8 (Fig 5). While the A26 biodegradation efficiency is significantly reduced at
217 the pH 5, 6, and at 15% NaCl, the mutant remains effective even at extreme conditions
218 of pH and salinity (Fig 4 and 5).

219 Emulsifying activity

220 Emulsifying activity was tested in both bacterial strain culture filtrates as well as the A26Sfp

EPS and A26Sfp glucan extracts. The standard assay for emulsifying activity was based on a modification of the method of Cooper and Goldenberg [53]. The tested compounds included kerosene and hexadecane. The results with A26 and A26Sfp culture filtrates, A26Sfp EPS and A26Sfp glucan extracts show 63%, 45%, 44%, 20%, degradation ability and with hexadecane and 59%, 45%, 45%, 21% degradation ability with kerosene, respectively. (Table 2). All compounds that showed emulsification activity were also positive in the drop and oil spreading assays (Table 2).

Glucan production

The polysaccharide glucan production was estimated after weighing the lyophilized material produced by the A26 and A26Sfp strains. While the A26 produced glucans at 0.62 mg/L the mutant ability was two times higher at 1.2 mg per L. (Table 2).

Discussion

Our study reveals that A26Sfp and A26 efficiently degraded NAP by 69% and 49%, respectively, after 5 days of incubation in liquid medium and soil. Somewhat reduced numbers for the degradation efficiency in the case of degradation in soil reflect that additional factors such as soil physical properties and texture porosity may influence the degradation process. Generally, microorganisms require suitable growth conditions (e.g. as regards carbon sources, nutrients, pH) which strongly affect their growth. Carbon sources in the growth medium are considered to be key factors for PAH degrading bacteria. For use in bioremediation, PAH degraders should ideally mineralize and grow on PAHs as sole carbon and energy source. This would minimize the production of toxic, water-soluble degradation by-products. In our experiments the strains that used sucrose and 5 % NaCl were most efficient at pH 8. The pH is one of the most important factors for PAH degradation in the culture medium, as it affects bacterial enzymatic activity as well as nutrient solubility [9,56] [4]. The mutant strain can remain effective even at extreme conditions of pH, and salinity.

Most often, chemical parameters are employed to evaluate and analyze PAHs in the environment. The chemical methods are accurate and sensitive for specific molecules, but do not give information regarding biological influence within the ecosystem. Chemical methods do not consider synergistic effects from compound mixtures and there is therefore a risk that they underestimate the toxic potential of a particular sample. Therefore, evaluation of biological influence using rapid simple and economic methods can provide information about all compounds and incorporate these important toxicity parameters. The bioluminescence

inhibition method has been shown to correlate well with the total level of PAHs and has been used for NAP ecotoxicity studies [57].

Several microbes have a great potential for the production of bioactive secondary metabolites associated with PAH biodegradation and various pathways have been revealed [8,9 10,11 12-16].. However, owing to the hydrophobic nature of the PAHs the first step must be surface tension reduction [4] . Therefore, the biodegradation of PAH is dependent on biosurfactants, i.e. compounds that lower interfacial tension between PAH and the soil water face.

Different classes of surfactants have been discovered, and the surfactin group encoded by the *sfp* gene belongs to the group of non-ribosomal compounds that has been considered as one of the most powerful biosurfactant groups [15]. The A26 surfactin production was inactivated and non-ribosomal proteins including surfactin are not produced by the mutant strain[19]. Polysaccharides (glucans) with the ability to emulsify and reduce surface tension of cells were very surprisingly overproduced by the mutant strain (Table 2). The surfactin ability can be partly linked to the increased glucan production. The A26Sfp strains can emulsify kerosene and hexadecane 60% and 59%, respectively, and A26 45 % and 50%, respectively. The property is comparable to a 0.5 solution of the nonionic surfactant Tween 80 [56] and can be linked with, but not limited to, the A26Sfp production of relatively high amounts of EPS and glucans (Table 2). Polysaccharide surfactants are an emerging class of biodegradable nontoxic and sustainable alternatives to conventional surfactant systems [3,5,58,59] .

Here we show that besides non-ribosmally produced lipopeptides of *P. polymyxa* (represented by the *Sfp* gene products), there are other most efficient EPS surfactants produced by A26Sfp. Polysaccharide-based emulsifiers of microbial origin have attracted attention, as they offer several advantages over synthetic emulsifiers, including lower toxicity, higher degradability, and better compatibility with the environment. In addition, they can remain effective even at extreme conditions of pH and salinity. These properties increase their scope for application in a diverse range of biotechnological areas. It has been repeatedly shown that the bacteria from extreme environments offer good candidates for efficient PAH biodegradation and biosurfactant producers [10]. To the best of our knowledge, it has not been reported earlier that Sfp-type 4-phosphopantetheinyl transferase deletion, making the strain incapable of NRP and PKS

production, can cause a significant change in polysaccharide production and composition.

Conclusion: It may be concluded from this investigation that both the bacterial strains A26 and A26Sfp showed significant ability to degrade NAP. The degradation ability of the mutant is high and may be of industrial importance. A26Sfp is easy and safe to use, as the mutant is non-sporulating, which abolishes the possibility for secondary contamination. These compounds produced by the mutant strain are involved in surface tension reduction but may not be the only ones of great importance. The rich composition of the *Paenibacillus polymyxa* A26 Sfp exopolysaccharides deserves attention.

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Figure Legends

Figure 1. Growth (optical density at 600 nm) of *Paenibacillus polymyxa* A26 *and P. polymyxa* A26Sfp in naphthalene (100 mg/L) containing medium. Different letters indicate statistically significant differences See Material and Methods

Figure 2. Degradation potential of *Paenibacillus polymyxa* A26 and *P. polymyxa* A26Sfp exposed to 100 mg/L of naphthalene in mineral medium (a) and in soil (b). Different letters indicate statistically significant differences See Material and Methods

Figure 3. Effect of carbon sources on biodegradation of naphthalene *by Paenibacillus polymyxa* A26 and *P. polymyxa* A26Sfp. Different letters indicate statistically significant differences See Material and Methods

Figure 4 Effect of pH on biodegradation of naphthalene *by Paenibacillus polymyxa* A26 and *P. polymyxa* A26Sfp. Different letters indicate statistically significant differences See Material and Methods

Figure 5. Effect of salinity on biodegradation of naphthalene *by Paenibacillus polymyxa* A26 and *P. polymyxa* A26Sfp. Different letters indicate statistically significant differences See Material and Methods

Figure S1 The Evolution Canyon (EC) model

Schematic diagram of the Evolution Canyon at Lower Nahal Oren, Mount Carmel (source [60] Nevo, 2012 Evolution Canyon," a potential microscale monitor of global warming across life, PNAS 109; 8) (Photo by S. Timmusk).

Table 1. Strains used in the study

name	origin	publications
Paenibacillus polymyxa A26	**Wild barley rhizosphere, the** Evolution Canyon, Haifa, Israel	Timmusk *et al.*, 2011
Paenibacillus polymyxa A26Sfp	Wild barley **rhizosphere**, the Evolution Canyon, Haifa, Israel	Kim and Timmusk 2013 Timmusk *et a*l. 2013 Timmusk *et al*. 2015

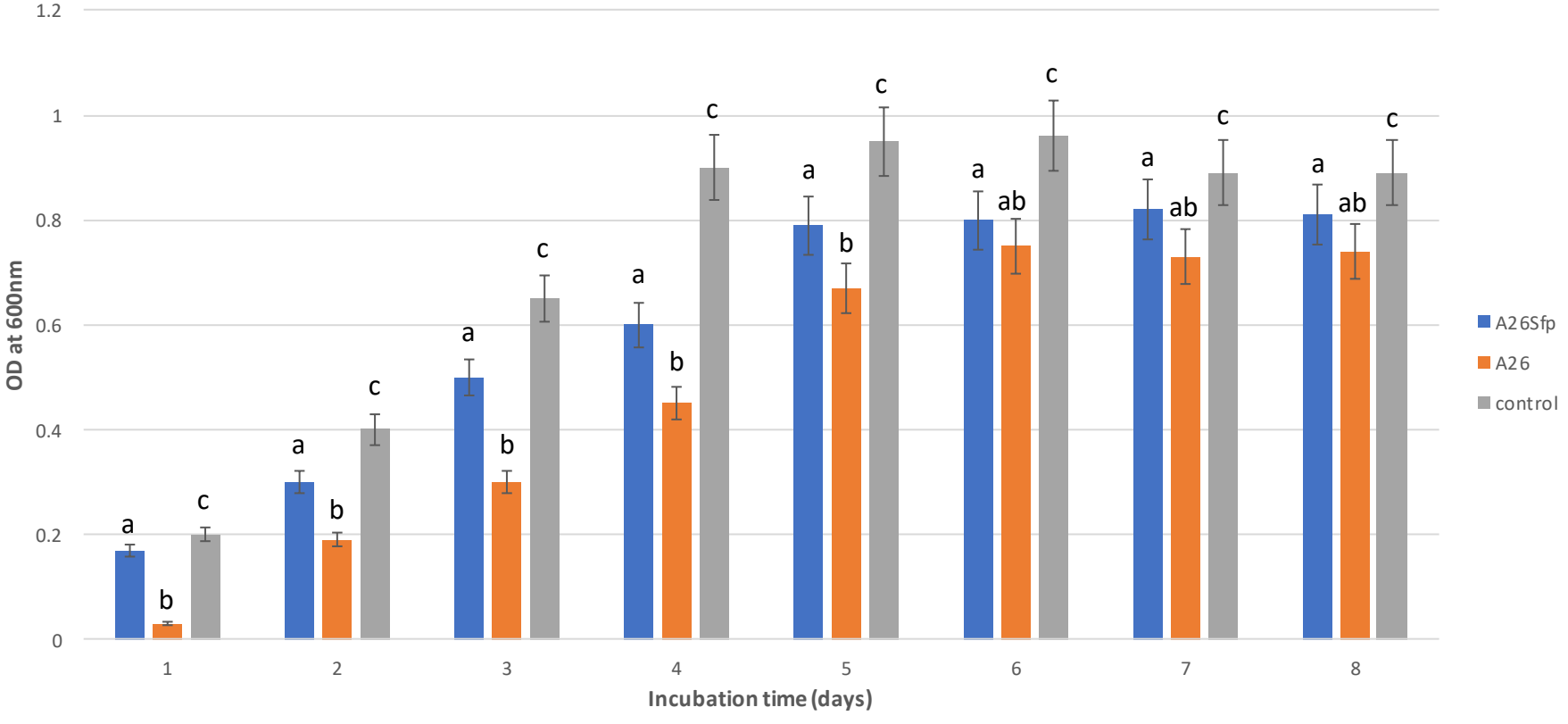
Table 2 Emulsion indices (%) of the strains using kerosene and hexadecane, glucan titer and other characteristics

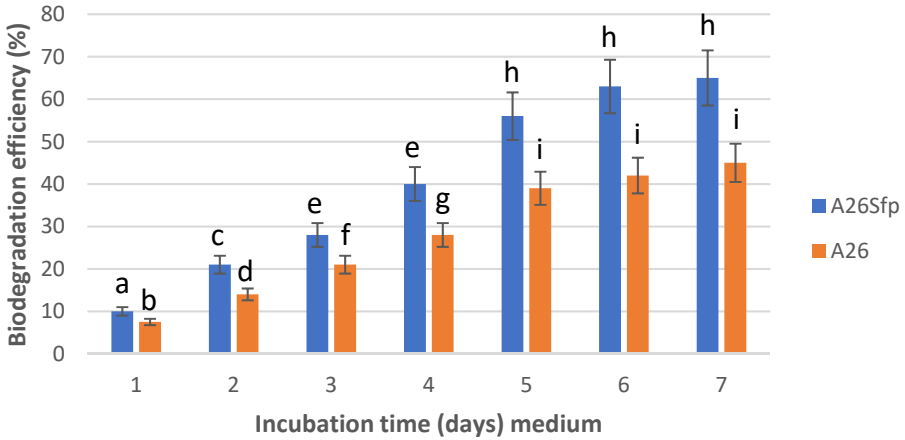
Strains	Emulsion index % hexadecane (day)			Emulsion index % kerosene (day)			*DC*	*OS*	Glucan titre (mg/L)	EPS titre (mg/L)
	1	3	7	1	3	7				
A26Sfp	60±4.2	63±5.2	60±5.2	63±5.2	59±5.2	60±4.1	+	+	1.2±0.1	15±0.2
A26	42±5.2	44±5.2	45±3.2	43±5.2	45±2.2	43±3.2	+	+	0.62±0.1	10±0.2

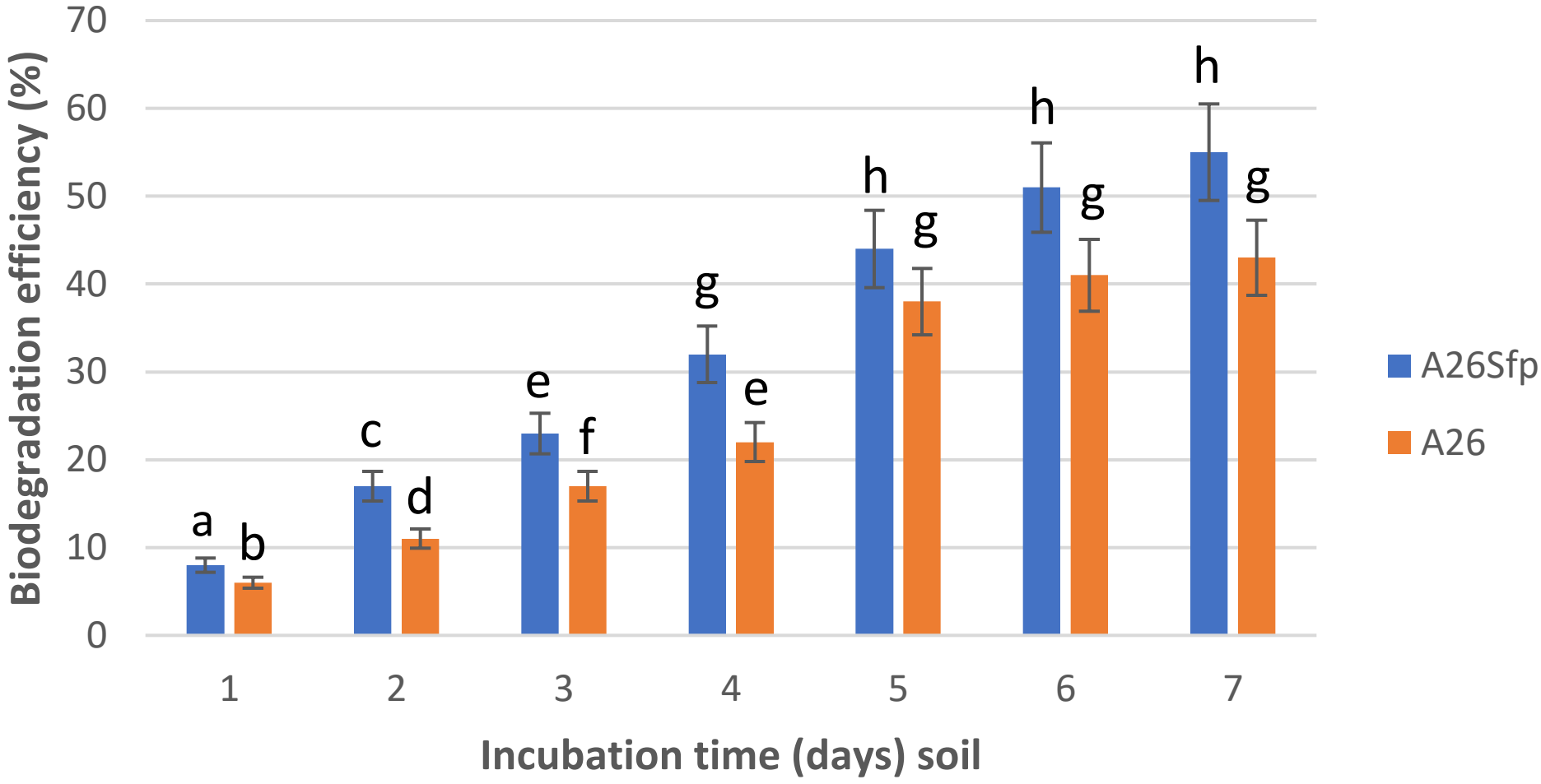
EPS A26Sfp	41±2.2	45±2.2	44±2.2	43±2.2	44±3.2	45±2.2	+	+	ND	ND
Glucan A26Sfp	20±2.0	20±2.2	21±2.2	21±2.2	21±2.1	23±2.2	+	+	ND	ND

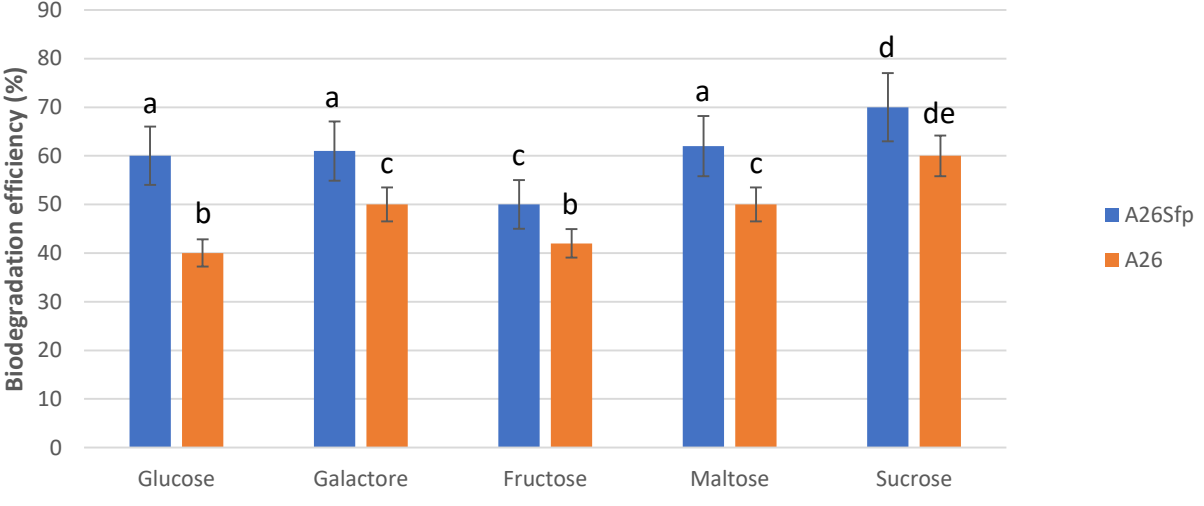
501

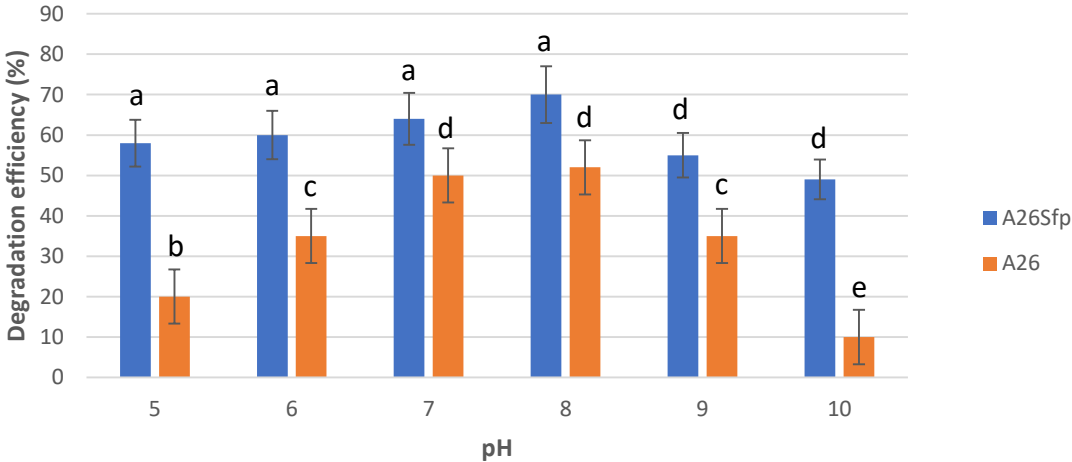
502

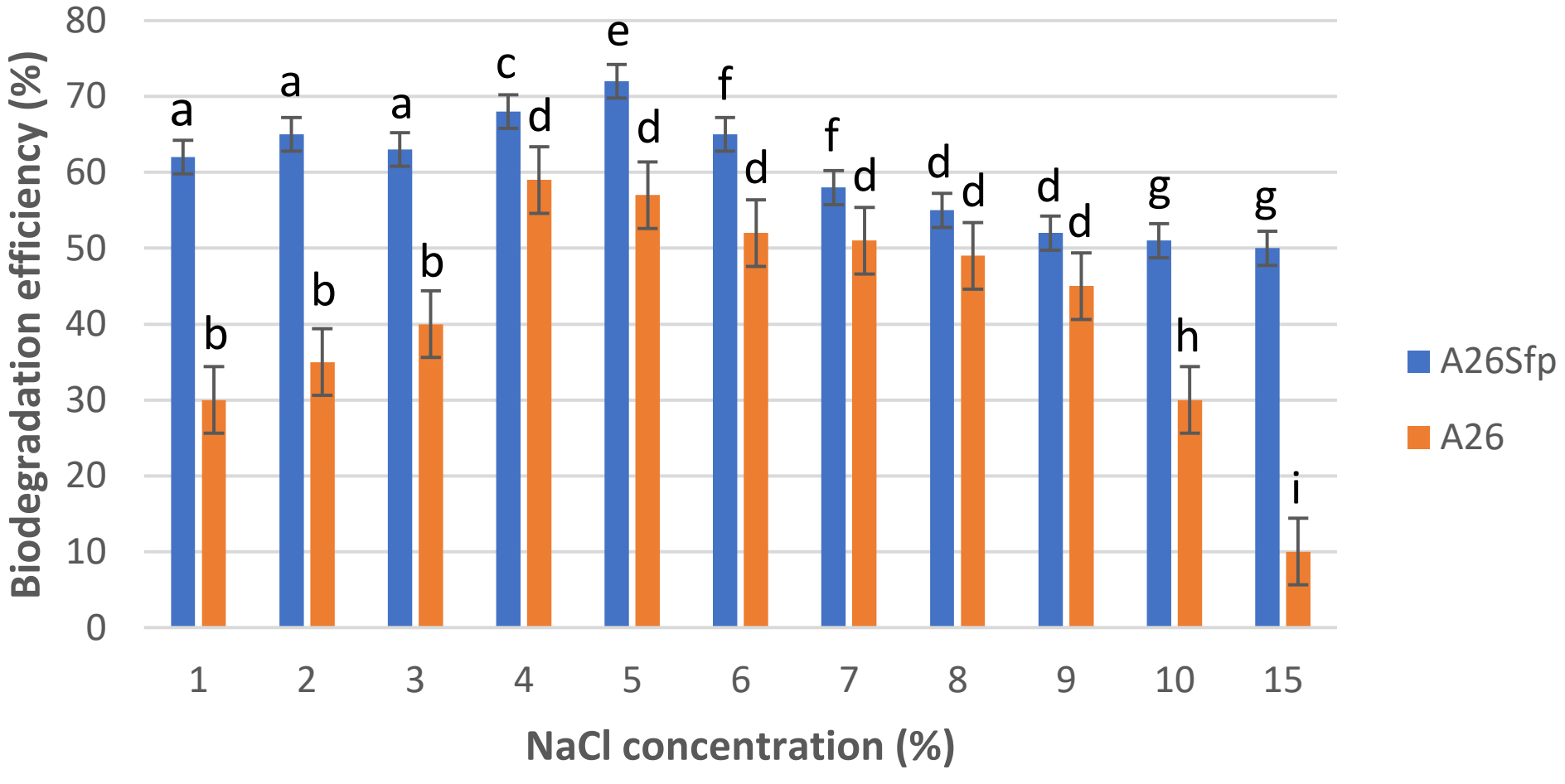

1.2
1
0.8
0.6
0.4
0.2
0
OD at 600nm
1
2
3
4
5
6
7
8
Incubation time (days)
a
b
c
ab
A26Sfp
A26
control




70
60
50
40
30
20
10
0
Biodegradation efficiency (%)
a
b
c
d
e
f
g
e
h
g
h
g
h
g
1
2
3
4
5
6
7
Incubation time (days) soil
A26Sfp
A26


Biodegradation efficiency (%)
90
80
70
60
50
40
30
20
10
0
a
b
a
c
c
b
a
c
d
de
Glucose
Galactore
Fructose
Maltose
Sucrose
A26Sfp
A26


90
80
70
60
50
40
30
20
10
0
Degradation efficiency (%)
a
b
a
c
a
d
a
d
d
c
d
e
5
6
7
8
9
10
pH
A26Sfp
A26


Biodegradation efficiency (%)
80
70
60
50
40
30
20
10
0
a
b
a
b
a
b
c
d
e
d
f
d
f
d
d
d
d
d
g
h
g
i
1
2
3
4
5
6
7
8
9
10
15
NaCl concentration (%)
A26Sfp
A26