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Emulsifying activity in thermophilic and extremely thermophilic microorganisms

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Thermophilic and extremely thermophilic enrichments from several different environments produced cell-associated emulsifiers as did several pure cultures of *Archaea*. The bioemulsifiers were effective over a wide range of pH, at NaCl concentrations up to 200 g L^{-1}, and at temperatures up to 80° C. The emulsifying activity in cell-free extracts of *Methanobacterium thermoautotrophicum* was a cell-associated protein with a molecular weight greater than 5000 Da. This emulsifier formed viscous emulsions, but did not reduce the surface tension of water or the interfacial tension between water and hexadecane. The emulsifier had the greatest activity with alkanes with carbon numbers greater than 10. The characteristics of the bioemulsifier from *M. thermoautotrophicum* makes it suitable for use in saline or thermophilic oil reservoirs as a mobility control agent, or in well-bore clean up processes.

Keywords: emulsifiers; thermophiles; oil recovery; archaebacteria

Introduction

There are several mechanisms by which microorganisms and/or their products can improve oil recovery [19,25]. In microbial well-bore cleanout and stimulation technologies [26,28,32], the *in situ* production of acids, gases, solvents, emulsifiers and/or biosurfactants removes paraffin and scale deposits in or near the well-bore which improves drainage of oil into the well [11]. In microbially enhanced waterflooding, the production of biosurfactants and/or solvents in the reservoir releases oil entrapped in the reservoir [8,24]. The growth of microorganisms in high permeability regions of the reservoir blocks water channels and improves the sweep efficiency of a waterflood [20]. In addition to *in situ* processes, microbial polysaccharides such as xanthan gum are used as mobility control agents to improve the performance of waterfloods [15].

There have been many studies on mesophilic microorganisms that produce bioemulsifiers [9,10,13,30,31,40]. However, it is not known whether thermophilic microorganisms have the ability to produce useful products such as emulsifiers and surfactants [39]. Worldwide, there are many thermophilic reservoirs that could be potential targets for microbial treatment. The objective of this work is to determine if thermophilic microorganisms produce bioemulsifiers and/or biosurfactants, and whether these compounds are effective at the high salinities and temperatures found in many oil reservoirs. The emulsifying activity detected in cell-free extracts of *Methanobacterium thermoautotrophicum* was characterized.

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Materials and methods

Sources of inocula and organisms

Samples were collected aseptically from an aerobic trickling filter, a primary anaerobic digestor, and a secondary anaerobic digestor at the municipal sewage treatment plant in Norman, OK, USA. Subsurface material from a well drilled in Gravberg, Sweden, at a depth of 5500 m, was obtained from Dr J Suflita, University of Oklahoma. This material was originally obtained from Dr T Gold, Cornell University and no special precautions with regard to sterility or anaerobiosis were taken in the maintenance of the sample. A sample from a shallow aquifer underlying the Norman landfill was also obtained from Dr Suflita [3]. A halophilic, methanogenic enrichment inoculated by suspending a soil sample from an oil and brine spill at the Southeast Vassar-Vertz Sand Unit, Payne County, OK, in basal medium [34] with 10% NaCl, and an 80% H_2 : 20% CO_2 head space was also used as an inoculum. The enrichment was incubated at 37° C for one month prior to subsampling for this work.

Pure cultures of *Methanobacterium thermoautotrophicum* Marburg (DSM 2133), *Halobacterium halobium* (ATCC 43214), and *Sulfolobus solfataricus* (ATCC 35091), were obtained from Dr D Nagle, University of Oklahoma. *Bacillus stearothermophilus* (ATCC 12980) was isolated from a steam sterilization control ampule.

Media compositions and conditions of cultivation

Anaerobic procedures for the preparation and use of media and solutions were those of Bryant [6] as modified by Balch and Wolfe [2].

A mineral salts basal medium [34], prepared under an 80% N_2 : 20% CO_2 gas phase, was used for the enrichment and growth of thermophilic, anaerobic bacteria. Anoxic, sterile glucose was added to the cooled, sterile medium to give a final concentration of 30 mM. Light crude oil, from the Southeast Vassar-Vertz Sand Unit, Payne County, OK, was added to the basal medium prior to autoclaving the medium for 20 min at 121° C. Oil was added to determine

visually those cultures that produced emulsifiers. Duplicate bottles containing 50 ml of medium and 5 ml of crude oil were each inoculated with 5 ml of the sample. Autoclaved controls, and unamended controls without glucose were prepared for each sample. All enrichments were incubated at 80° C without shaking; only enrichments inoculated with the three sewage sludges were incubated at 60° C with shaking (200 rpm).

The basal medium with 10 ml L^{-1} of rumen fluid [7], 30 mM glucose, and 30 g L^{-1} of purified agar was used for the isolation of fermentative bacteria. Methanogenic bacteria were isolated using the above medium without glucose and with an 80% H_2 : 20% CO_2 gas phase. Pure cultures were grown in liquid medium and maintained on slants using medium with the same composition as that used for their isolation. Cultures of methanogens were repressurized with H_2/CO_2 every day. One hundred grams of frozen cells (–20° C) of *M. thermoautotrophicum* were obtained from W Lorowitz and R S Tanner, University of Oklahoma. The organism was grown in the basal medium without rumen fluid in a 100-L fermentor sparged with an 80% H_2 : 20% CO_2 gas phase. *B. stearothermophilus* was grown at 65° C in the liquid basal medium with 10% (vol/vol) rumen fluid, 30 mM glucose, and an 80% N_2 : 20% CO_2 gas phase. *H. halobium* was grown aerobically at 40° C with shaking (200 rpm) in the following medium (pH of 7.2) (g L^{-1}): NaCl (200), $MgSO_4 \cdot 7H_2O$ (20), sodium citrate·$3H_2O$ (3), yeast extract (10), Casamino acids (7.5). *Sulfolobus solfataricus* was grown at 75° C with magnetic stirring in the following medium (pH of 4.0) (g L^{-1}): yeast extract (1), Casamino acids (1), KH_2PO_4 (3.1), $(NH_4)_2SO_4$ (2.5), $CaCl_2 \cdot 2H_2O$ (0.25), $MgSO_4 \cdot 7H_2O$ (0.2), $MnCl_2 \cdot 4H_2O$ (1.8×10^{-3}), $Na_2B_4O_7 \cdot 10H_2O$ (4.5×10^{-3}), $ZnSO_4 \cdot 7H_2O$ (0.22×10^{-3}), $CuCl_2 \cdot 2H_2O$ (0.05×10^{-3}), $NaMoO_4 \cdot 2H_2O$ (0.03×10^{-3}), $CaSO_4 \cdot 7H_2O$ (1×10^{-5}).

Isolation of the strains

Strains from the aerobic trickling filter enrichment at 60° C were isolated using the agar roll tube method [16]. The roll tubes were incubated at 60° C for two weeks or until colonies appeared. Organisms with different colony morphologies were picked and transferred to liquid medium and incubated until growth was evident. The roll tube method was repeated until pure cultures were obtained. Each strain was tested for its ability to produce an emulsifier, and the isolates that had the best emulsifying activity were characterized further.

Preparation of the cell suspensions and cell-free extracts

The cell-free spent medium fraction was prepared by removing the cells by centrifugation at $10000 \times g$ for 15 min. Cell suspensions were prepared by harvesting the cells by centrifugation as described above, and washing the cells twice by resuspending the pellet in 10 mM phosphate buffer (pH 7) and recentrifuging them. The final pellet was resuspended in 10 ml of the same buffer. Cell-free extracts were obtained by resuspending washed cells in 2 ml of the above buffer, breaking the cells twice (three times for the *B. stearothermophilus*) using a French pressure cell at 20000 psi, and centrifuging the lysate as above to remove unbroken cells and cellular debris.

Emulsifying activity

Enrichments were visually screened for emulsifying activity by observing whether the oil was dispersed in the aqueous phase. For enrichments grown at 80° C, the bottles were shaken by hand for 1 min prior to observation. For enrichments grown at 60° C, the bottles were held at room temperature for 10 min without shaking prior to observation.

Emulsifying activity was quantified using a modification of the method used by Cooper and Goldenberg [10]. The sample was serially diluted in 10 mM phosphate buffer (pH 7.0) and 2 ml of each dilution was dispensed into duplicate glass tubes (120 × 12 mm), each of which contained 2 ml of dodecane. Each tube was mixed for 30 s using a vortex set on high speed. The tubes were left undisturbed overnight before the height of each phase was measured with a ruler to the nearest millimeter. The percent emulsification was calculated by dividing the height of the emulsion by the total liquid height. Serially diluted uninoculated medium in phosphate buffer (pH 7.0) was the control. Fractions obtained from the different enrichments and pure cultures were serially diluted until the amount of cellular material added was linearly proportional to the percent emulsification (< 50%). One unit of emulsifying activity was defined as the amount of emulsifier that gave a percent emulsification of 20.

Characteristics of the emulsifiers

The effect of pH on the emulsifying activity of Tween 80 and the bioemulsifiers in cell-free extracts of the aerobic sludge enrichment incubated at 60° C and *M. thermoautotrophicum* was determined after 24 h at room temperature using a triple buffer system that contained 10 mM each of sodium succinate (pK 4.00), HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pK 7.01), and CAPS (3-(cyclohexylamino)-1-propanesulfonic acid) (pK 10.08). The pH of the triple buffer system was adjusted using 1 N solutions of HCl or NaOH. The effect of NaCl concentrations up to 200 g L^{-1} and temperatures up to 80° C was determined using the triple buffer system at the optimum pH determined for each emulsifier. The effectiveness of the bioemulsifier in cell-free extracts of *M. thermoautotrophicum* with different hydrocarbons was determined at the optimal pH as described above.

The viscosities of the emulsions formed by Tween 80 and cell-free extracts of *M. thermoautotrophicum* were measured using a digital viscometer. Each emulsion was prepared at room temperature with dodecane and the triple buffer system (pH of 5.0) without salt added. The effect of cell-free extracts of *M. thermoautotrophicum* on the surface and interfacial tensions of water were determined as described previously [17]. For the interfacial tension measurement, hexadecane was used as the hydrophobic phase. The diameters of 20 randomly chosen drops were measured microscopically at 400× using a calibrated ocular micrometer to determine the average droplet diameter [40].

Determination of the biochemical nature of the methanogen emulsifier

Forty milliliters of cell-free extracts of *M. thermoautotrophicum* were extracted using a modification of the method of Bligh and Dyer [5] to determine whether the emulsifying activity was hydrophobic. The effect of protease activity (Pronase E) on emulsifying activity was determined by incubating 10 ml of *M. thermoautotrophicum* cell-free extract with 10 mg of Pronase E (4 U mg^{-1}) at 37° C for 3 h.

The emulsifying activity in cell-free extracts of *M. thermoautotrophicum* was subjected to size exclusion using Sephadex G-25 (Pharmacia Inc, Piscataway, NJ, USA) equilibrated with 10 mM sodium succinate buffer (pH 5.0). After washing the column with the buffer, 10 ml of the cell-free extract was applied to the column and eluted with the same buffer. Ten-milliliter fractions were collected and assayed for emulsifying activity. The void volume was determined using Blue Dextran (200000 MW). A silicic acid column was prepared as described previously [14]. The active fraction obtained after size exclusion chromatography was applied to the silicic acid column and eluted with 10 mM sodium succinate buffer until no absorbance at 280 nm was detected in the effluent. The column was then treated with 20 ml of each of the following solvents: methanol, acetone, and chloroform. The emulsifying activity of each fraction was determined, and corrected for the amount of emulsification in controls containing only the solvents used for elution.

Analytical methods

Growth of the enrichments was followed by determining total cell count per field every week using a phase contrast microscope. Growth of the pure cultures was followed spectrophotometrically at 600 nm. The production of acid by the enrichments was determined with a glass electrode after equilibrating a 5-ml sample with air for 30 min at room temperature. Methane and carbon dioxide were measured by gas chromatography [18]. The amount of protein was determined by the method of Peterson [29].

Results

Characteristics of the enrichments

All enrichments, except that inoculated with Norman aquifer material and incubated at 80° C, grew and produced acids (pH <6.5) and CO_2 (>20%) (data not shown). Large quantities of CH_4 (>10%) were detected only in enrichments inoculated with the aerobic and primary sludges incubated at 60° C. None of the autoclaved controls had any increase in cell number. The pH of unamended or autoclaved controls varied between 6.5 and 7.5. Gas phase concentrations of CO_2 above 20%, or the presence of CH_4 were not observed in any of the controls.

None of the enrichments incubated at 80° C showed any visual signs of emulsification of the oil layer, although emulsifying activity was detected in some of these enrichments with the quantitative assay (G Trebbau de Acevedo, MS Thesis, University of Oklahoma, 1983). Partial emulsification of the oil layer was observed in the primary and secondary sludge enrichments incubated at 60° C. The aerobic sludge enrichment incubated at 60° C completely emulsified the oil layer and had the highest percent emulsification (53%) of any of the enrichments tested. None of the controls had a percent emulsification greater than 10. The surface tensions of the cultures were similar to that of uninoculated medium (>50 mN m^{-1}), indicating that biosurfactants were not produced.

The emulsifying activity of the aerobic sludge enrichment incubated at 60° C was cell-associated since washed cells had 1.4 U ml^{-1} of emulsifying activity and no emulsifying activity was found in cell-free spent medium. Breakage of the cells increased the emulsifying activity by about 4-fold (5 U ml^{-1}).

Isolation of emulsifying strains

Six morphologically different fermentative strains were isolated from the aerobic sludge enrichment. Only one strain, G-1, had emulsifying activity (about 10 U ml^{-1}). Strain G-1 was a Gram-negative, short, curved rod that produced acid from glucose (final pH of 6.1). Seven different methanogens were isolated and strain M-6 had the best emulsifying activity (30 U ml^{-1}). The other methanogenic isolates had emulsifying activities between 5 and 10 U ml^{-1}. M-6 was a Gram-positive rod that produced methane (10% of the head space). The emulsions obtained with cell-free extracts of M-6 were viscous, had a milky appearance, and were stable at room temperature for more than a week (data not shown). The emulsions obtained with cell-free extracts of strain G-1 were not as viscous as those of M-6, and were not stable after 3 days.

Emulsifying activity of other microorganisms

Other *Archaea* and a thermophilic eubacterium were tested for emulsifying activity (Table 1). Cell suspensions of *Methanobacterium thermoautotrophicum*, *Sulfolobus solfataricus*, and isolate M-6 had high specific emulsifying activities (>24 U mg^{-1}), which increased when cell-free extracts were used. Cell suspensions of *Bacillus stearothermophilus* had some activity which did not increase after cell breakage. Neither cell suspensions nor cell-free extracts of *Halobacterium halobium* had emulsifying activity.

The emulsions prepared with cell-free extracts of *S. solfataricus* had the smallest average drop size of any of the organisms tested (Table 1). None of the biological emulsifiers produced emulsions with droplet sizes as small as that produced by Tween 80.

Biochemical nature of the emulsifier produced by M. thermoautotrophicum

The biochemical nature of the emulsifier present in cell-free extracts of *M. thermoautotrophicum* was determined because large amounts of cellular material were readily available (a gift from W Lorowitz and R S Tanner). Most of the emulsifying activity present in cell-free extracts of *M. thermoautotrophicum* was detected in the aqueous phase after lipid extraction (Table 2). The emulsifying activity was susceptible to the action of a protease. The total units of emulsifying activity after treatment with the protease were 400 while those in the untreated control were 32000. Emulsifying activity in cell-free extracts of *M. thermoautotrophicum* were detected only in the void volume after size

Table 1 Emulsifying activity and average drop size of emulsions produced by pure cultures of *Archaea* and a thermophilic bacterium

Organism	Whole cells (U mg^{-1})[a]	Cell-free extracts (U mg^{-1})[a]	Drop size[b] (μm)
Sulfolobus solfataricus	24.6	123.1	12 ± 1
Methanobacterium thermoautotrophicum	62.5	85.1	41 ± 5
Strain M-6	42.5	58.3	54 ± 5
Halobacterium halobium	0	0	NA
Bacillus stearothermophilus	5.0	5.0	80 ± 8
Tween 80[c]	NA[d]	60.3	3 ± 0.5

[a] Specific activity per mg dry weight
[b] Average diameter of drops in the emulsions
[c] Tween 80 (0.20 mg) in 10 mM phosphate buffer (pH 7.0)
[d] NA, not applicable

Table 2 Localization of the emulsifying activity in cell-free extracts of *M. thermoautotrophicum* after solvent extraction

Fraction	Total units	Total protein (mg)	Specific activity (U mg^{-1})	Yield (%)
Cell-free extract	32000	880	36	100
Chloroform phase	800	266	3	2
Aqueous phase	6200	190	32	20

exclusion chromatography, and it eluted from the silicic acid column with polar solvents (buffer and methanol) rather than with less polar solvents such as acetone and chloroform.

Characterization of the emulsifier

The effects of pH, NaCl concentration, and temperature on the emulsifying activities of Tween 80 and of cell-free extracts of the aerobic sludge enrichment incubated at 60° C and *M. thermoautotrophicum* are shown in Figure 1. Tween 80 formed stable emulsions at acidic pH values, but not at alkaline pH values (Figure 1a). Both bioemulsifiers had an optimal pH of 5; the methanogenic bioemulsifier was effective at alkaline pH, retaining about 40% of its activity at pH 10. Increasing salt concentrations greatly affected the emulsifying activity of Tween 80 (Figure 1b). However, both bioemulsifiers were effective at salt concentrations up to 200 g L^{-1}. The bioemulsifier from the methanogen did not lose activity until the salt concentration exceeded 150 g L^{-1}. All three materials were active at temperatures from 4 to 80° C (Figure 1c). The bioemulsifiers lost about 30% of their activities when the temperature was 60° C or higher. The emulsifying activity in cell-free extracts of *M. thermoautotrophicum* was most effective with alkanes with more than 10 carbons (Figure 2). Lower activities were seen when alkanes with carbon numbers less than 10 or aromatic compounds were used.

The viscosity of the emulsion formed by cell-free extracts of *M. thermoautotrophicum* was 44.4 ± 0.1 centipoise, while that of Tween 80 was 8.3 ± 0.1 centipoise. The methanogenic bioemulsifier reduced the surface tension of water by about 20% (from 65 to 39.5 mN m^{-2}), and the interfacial tension of water only by 10% (from 23 to 20 mN m^{-2}).

Discussion

The bioemulsifiers from *Methanobacterium thermoautotrophicum* and the aerobic sludge enrichment have many properties that make them suitable for use in saline and thermophilic oil reservoirs. The bioemulsifiers are active over a wide range of pH (from 5 to 10), at very high salt concentrations (up to 200 g L^{-1}), and at temperatures up to 80° C. The fact that cells enriched from many different environments have emulsifying activity suggests that the ability to produce emulsifiers is widespread, and a process based on the stimulation of indigenous microbial populations is feasible. The fact that a methanogen was the most effective emulsifying organism isolated from our enrichments makes this supposition more credible, since methanogens are present in many oil reservoirs [1,4,12,27].

Sulfolobus solfataricus which contains the highest percentage of tetraethers of all the *Archaea* tested [21–23,36] had the best emulsifying activity and formed emulsions with the smallest average drop size (Table 1). *Halobacterium halobium* which contains only diethers did not have emulsifying ability. These data suggest that the tetraethers might act as emulsifiers. However, characterization of the emulsifying activity from *M. thermoautotrophicum* showed it to be a cell-associated protein. Almost all of the emulsifying activity was lost after treatment with a proteolytic enzyme and most of the activity was in the aqueous (hydrophilic) phase rather than the organic (hydrophobic) phase after lipid extraction (Table 2). The emulsifying activity eluted from the silicic acid column with polar solvents (water and methanol), and had a molecular weight greater than 5000 Da.

In enhanced oil recovery, emulsifiers and the emulsions that they form are used to reduce the capillary forces that entrapped oil within the pores of the rock, and as a mobility control agent to improve the sweep efficiency of a waterflood, respectively [33,35]. The emulsifier in cell-free extracts of *M. thermoautotrophicum* did not reduce the surface tension of water, or the interfacial tension between hexadecane and water. Thus, it is not a surfactant-type of emulsifier and would not be useful for applications that require a reduction in capillary forces. The emulsions formed by the bioemulsifier from *M. thermoautotrophicum* are viscous, more viscous than those formed by Tween 80, suggesting that it could be used as a mobility control agent.

A more likely application for microbial bioemulsifiers is

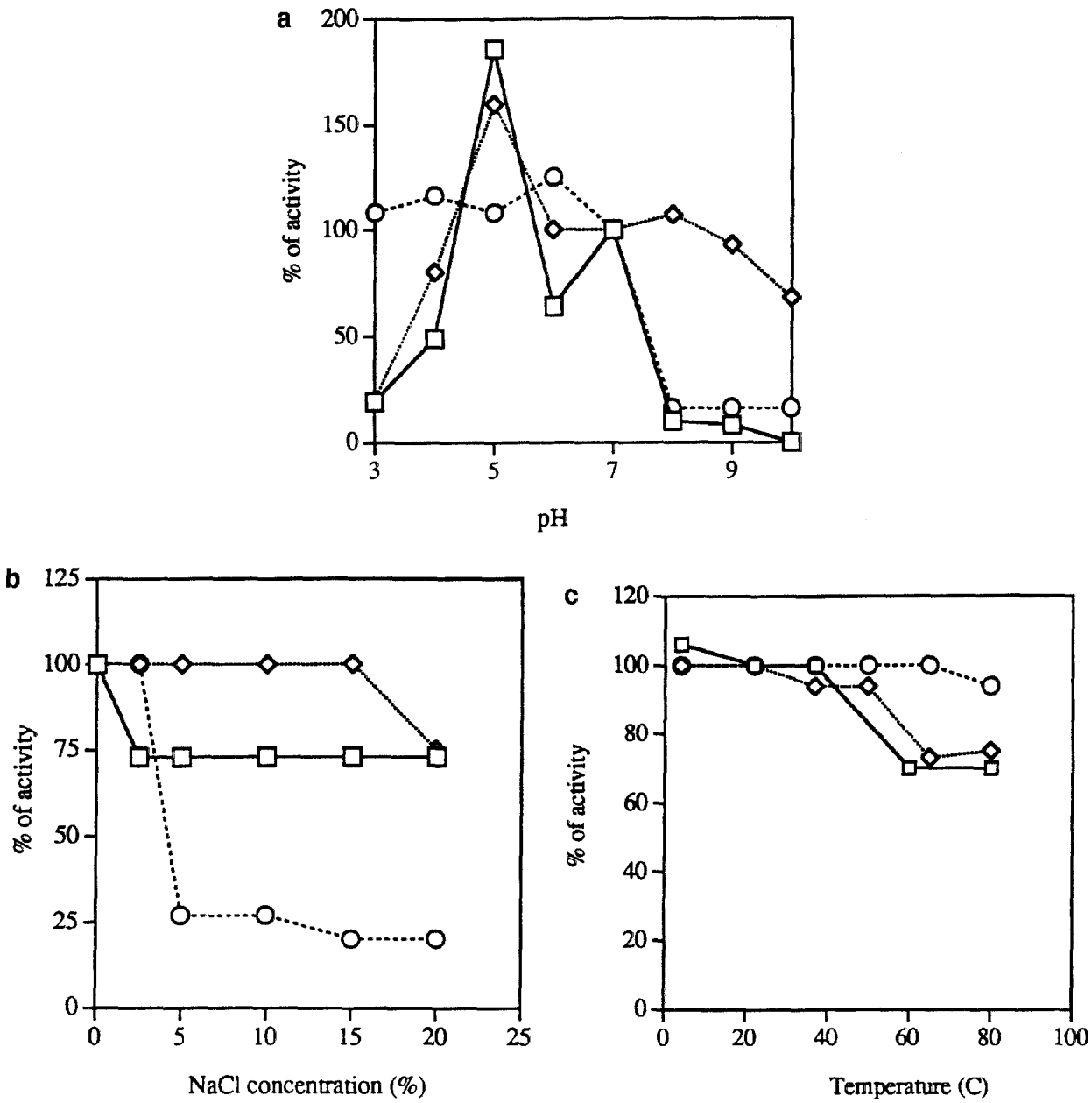

Figure 1 Effect of pH (a), NaCl concentration (b), and temperature (c) on the emulsifying activity of Tween 80 and of cell-free extracts of *Methanobacterium thermoautotrophicum* and the aerobic sludge enrichment incubated at 60° C. Assays were performed with 0.1 mg ml^{-1} of Tween 80, 0.2 mg (dry weight) per ml of the aerobic sludge enrichment, and 0.2 mg (protein) per ml of the methanogen extract. The 100% values correspond to the following specific activities (U mg^{-1}) for Tween 80, the aerobic sludge enrichment and the methanogen, respectively: 20, 22.7, and 5.9 for the effect of pH; 25, 36.4, and 11 for the effect of NaCl; and 26.7, 22.7, and 9.7 for the effect of temperature. ○, Tween 80; □, sludge enrichment extract; ◇, methanogen extract

the stimulation of oil production in marginal wells which have approached their economic limit of operation. A large proportion of US domestic oil production is from marginal fields [37,38], which cannot support the expense of many enhanced oil recovery technologies. Recent studies [26,28,32] report increases in oil production and more profitable operations of marginal wells after treatment with cells and nutrients. Donaldson [11] postulated that removal of particulates and deposits of paraffins and asphaltenes in or near the well-bore by microbial activity increases the pore entrance size distribution of the rock, which shifts the capillary pressure curve, and improves drainage of oil into the well. Our work shows that thermophilic microorganisms produce cell-associated emulsifiers that are active with alkanes with carbon numbers greater than 10 and are released with cell lysis. These observations may explain why the stimulation of microbial growth in oil wells improves oil production.

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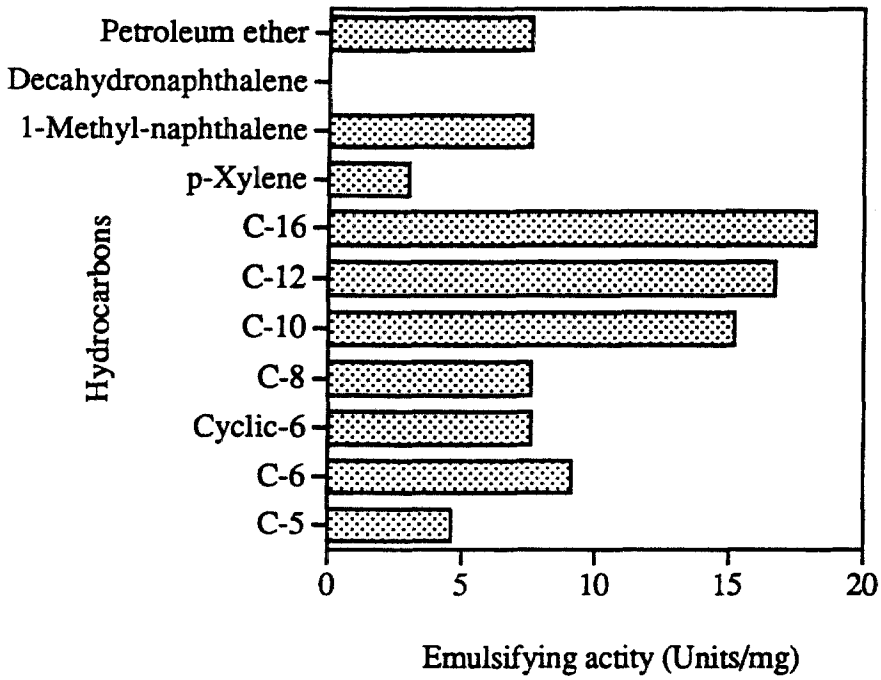

Figure 2 The effectiveness of the emulsifying activity in cell-free extracts of *M. thermoautotrophicum* with different hydrocarbons

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