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Screening of Soil Bacteria for Production of Biocleaner

Haruko Takeyama, Masumi Wada, and Tadashi Matsunaga*

Department of Biotechnology,
Tokyo University of Agriculture and Technology,
2-24-16 Naka-cho, Koganei, Tokyo 184-8588, Japan,
E-mail: tmatsuna@cc.tuat.ac.jp

Abstract

Soil bacteria were studied for the production of biodegradable cleaning agents. Among 86 bacterial strains resistant to liquid paraffin, 58 showed hemolytic activity. These strains were cultured, and the supernatant of culture broths was evaluated for cleaning activity against a dirty porcelain tile. Potent activity was exhibited in 18 strains. The lowest value of surface tension was obtained from *Bacillus* sp. NKB03 suggesting the presence of a biosurfactant. *Aeromonas* sp. NKB26c and *Bacillus cereus* NKB46b exhibited enzymatic cleaning activity. A cleaning efficiency of 82% was achieved when using a mixture of supernatants from culture broths of *Bacillus* sp. NKB03 and *Aeromonas* sp. NKB26c in synthetic minimal media. The cleaning efficiency using this mixture was higher than that of sodium dodecyl sulfate. These results suggest that a mixture of supernatants from culture broths of *Bacillus* sp. NKB03 and *Aeromonas* sp. NKB26c has potential for commercial use as a biocleaner.

Index Entries: Biocleaner; NIH Image analysis; soil bacteria; biosurfactant; hemolytic activity.

Introduction

Cleaning agents have been used for an ever-increasing number of purposes. The main components are surfactant and enzyme. Hydrophobic and hydrophilic groups of surfactants permit the solubilization of oils and proteins into aqueous solution. Enzymes such as lipase and protease degrade insoluble oils and proteins into water-soluble products. Modern

*Author to whom all correspondence and reprint requests should be addressed.

cleaning agents in common use are very powerful and economical. However, most of them are synthesized from crude oil or natural gas and are not easily biodegraded. Therefore, synthetic surfactants may accumulate in the environment and damage the ecosystem *(1)*. Natural products, by contrast, are more easily biodegraded and less likely to accumulate in the environment.

Many kinds of surfactants produced by microorganisms, plants, and animals have been reported. Bacterial biosurfactants include rhamnolipids from *Pseudomonas aeruginosa (2)*, surfactin from *Bacillus subtilis (3)*, and peptide lipids from *B. licheniformis (4)*. They are produced in large amounts by simple bulk culture *(5)* and are expected to increase in usage for a wide variety of fields *(6)*. Certain biosurfactants are now routinely used in cosmetic and medical industries and as food additives *(7–10)*. Compared with synthetic surfactants, biosurfactants exhibit substrate specificity *(11)*, are required in small quantities, and are effective for a broad range of oils *(6,12)*. However, the manufacturing cost for biosurfactants is 3 to 10 times higher than that of synthetic surfactants *(13)*. Purification accounts for up to 60% of the total production cost of biosurfactants. Because of economic considerations in the oil industry, most biosurfactants would require either whole-cell culture broths or crude preparations.

The surfaces of buildings in areas of heavy traffic rapidly become soiled with soot particles (carbon) bonded by *n*-paraffins (C_{19}–C_{22}) and polyaromatic hydrocarbons (PAHs) from exhaust emissions. This dirt cannot be removed by rain or water. Large amounts of powerful synthetic cleaner are therefore used to clean such buildings and are discharged into the environment. In the present study, we isolated soil bacteria that produce biodegradable cleaning agents and evaluated their cleaning efficiency using artificial building dirt prepared with crude oil and liquid paraffin.

Materials and Methods

Bacterial Screening and Cultivation

Soil samples collected from rivers, plants, and coal mines were diluted with sterilized water. The diluted samples were inoculated on agar plates of twofold diluted Luria-Bertani (LB) medium (10.0 g of tryptone, 5.0 g of yeast extract, 5.0 g of NaCl in 1 L at pH 7.2) layered with 200 µL of liquid paraffin and incubated at 37°C. Hemolytic activity of isolated bacteria was tested using blood agar plates. Three synthetic minimal media were used with the following compositions (all components per liter of medium): Ps medium (30 g of glycerol, 5g of $NaNO_3$, 4.5 g of Na_2HPO_4; 0.7 g of KH_2PO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of yeast extract) *(14)*, M9 medium (ATCC culture medium 1281 without casamino acid, 20 g of sucrose, 2.5 g of NH_4Cl, 15 g of Na_2HPO_4, 7.5 g of KH_2PO_4, 1.2 g of $MgSO_4 \cdot 7H_2O$, 1.5 g of $CaCl_2 \cdot 2H_2O$, 1.3 g of NaCl, 0.7 g of yeast extract), and Spizizen medium (5 g of glucose; 2 g of $(NH_4)_2SO_4$, 14 g of K_2HPO_4, 6 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$,

1 g of sodium citrate dihydrate) *(15)*. Bacterial strains were inoculated onto Ps, M9, and Spizizen agar plates. Colonies were subcultured into respective liquid media at 30°C for 24 h, then cultured again with fresh media for another 20–40 h.

Assay for Cleaning Activity of Bacterial Culture

Dirt from car exhaust emissions consists of soot particles (carbon) bonded by *n*-paraffins(C_{19}–C_{22}) and PAHs. We used a mixture of crude oil and liquid paraffin to imitate this dirt. Porcelain tiles (circular diameter = 1.5 cm, INAX Co.), commonly used to ornament the surfaces of buildings, were submerged in a mixture of crude oil and liquid paraffin (1:1 [v/v]), then air-dried at room temperature for 7 d. The resulting dirty tiles were used to assay cleaning activity.

Bacteria exhibiting hemolytic activity were cultured for 7 h in half-strength LB (1/2 LB) medium. The culture was centrifuged (15 min, 13,500*g*, room temperature), and the supernatant was assayed for cleaning activity. The biosurfactant-producing bacteria *P. aeruginosa* DSM2659 *(16)* and *B. subtilis* IAM1213 *(3)* were also used as controls to assay cleaning activity. Sodium dodecyl sulfate (SDS) was used as a representative synthetic surfactant. The concentration (16 m*M*) of SDS used was twice the critical micelle concentration. A single dirty tile was placed into a well of a 24-well microtiter plate, immersed with 2 mL of spent bacterial culture medium, and then incubated for 30 min. Cleaning activity was evaluated by measuring the cleaned tile area using NIH Image analysis, a public domain image processing and analysis program for Macintosh, developed by the Research Services Branch of the National Institutes of Health. The cleaning activity was expressed as the percentage cleaning efficiency:

$$\text{Percentage of cleaning efficiency} = \left(1 - \frac{\text{uncleaned area on the surface of tile}}{\text{surface area of tile}}\right) \times 100$$

Measurement of Surface Tension

Bacteria were cultured for 7 h in 1/2 LB medium, then centrifuged (15 min, 13,500*g*, room temperature). The surface tension of the supernatant was measured using an automatic surface tensiometer (CBVP-Z; Kyowa).

Identification of Bacterial Isolates

Bacterial strains exhibiting strong cleaning activity were examined for Gram reaction using a kit (Color Gram 2; bioMerieux) and cell morphology when grown on 1/2 LB agar. Cell sporulation was observed using a microscope (BH2; Olympus). Either an API 20NE or API 50CHB (API system S.A.) was used to identify isolates according to the manual. Isolates grown for 24 h were used for identification.

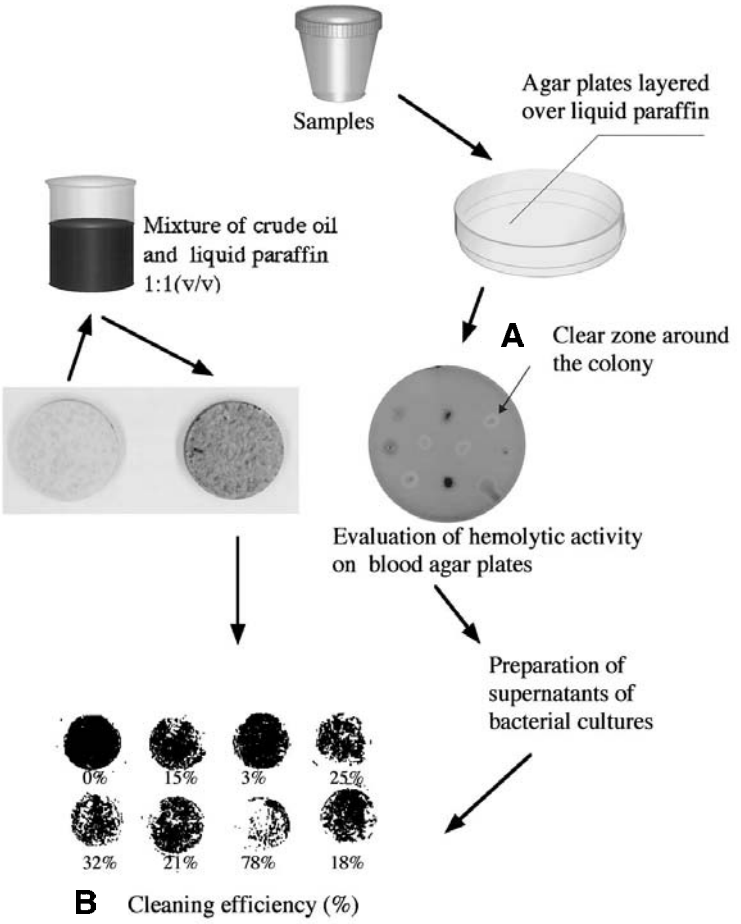

Fig. 1. Experimental procedure for screening of **(A)** biosurfactant-producing soil bacteria and **(B)** cleaning ability assay of bacterial cultures. The production of surfactant was confirmed by the formation of a clear zone around colonies on blood agar plate. The surface of tiles treated with the supernatant of culture broths was imaged using NIH Image. The numerical values (percentage) indicate cleaning efficiency.

Results and Discussion

Screening of Soil Bacteria for Hemolytic Activity and Cleaning Efficiency

Figure 1 shows the experimental procedure (for screening of soil bateria for hemolytic activity and cleaning efficiency). A total of 86 paraffin-resistant bacterial strains were isolated, of which 58 strains formed clear zones around the colony on blood agar plates (Fig. 1A). Hemolytic activity is indicative of the production of surfactants. Those 58 strains were assayed for cleaning activities. The surface of the tiles after treatment with the supernatant from culture broths was imaged (Fig. 1B) and cleaning ability was calculated. Table 1 summerizes the results from the 58 strains tested.

Table 1
Cleaning Efficiency and Surface Tension of Supernatant of Bacterial Cultures[a]

Strain	Cleaning efficiency (%)	Surface tension (mN/m)
S-3	51	33
S-9a	15	67
S-9b	41	68
S-9c	62	69
S-10a	39	69
S-12a	75	58
S-13d	64	62
S-14b	3	68
S-19a	70	70
S-19b	10	67
S-23a	72	59
S-24a	34	70
S-24b	64	71
S-26c	78	69
S-32	57	70
S-39c	7	70
S-44	14	67
S-46b	76	67
Water	0	72
1/2 LB	0	62
Other 40 samples	0	—[b]

[a]Bacteria were cultured for 7 h in 1/2 LB medium.
[b]Not examined.

Eighteen of the strains exhibited cleaning ability that varied in strength. Cleaning efficiency >50% was observed with the supernatants from 10 strains. The supernatant from culture broth prepared from strain S-26c possessed the highest cleaning ability (78%).

Surface Tension of Supernatants in Bacterial Culture Broth

Surface tension was measured in the supernatants from culture broths (Table 1). Microbial surfactants in aqueous solution yielded surface tensions of 27–38 mN/m *(6)*. The supernatants from three strains (S-3, S-12a, and S-23a) exhibited lower surface tensions than 1/2 LB medium (surface tension of 62 mN/m), suggesting the presence of a biosurfactant. However, only the supernatant of strain S-3 reduced the surface tension significantly (33 mN/m). The supernatants from three strains (S-23a, S-26c, and S-46b) exhibited higher cleaning efficiency, but no significant decrease in surface tension. Cleaning activities in the supernatants of strains S-26c and S-46b were inactivated by heating at 90°C for 20 min (data not shown), suggesting the presence of enzymes. By contrast, cleaning activities of strain S-3 did not decrease.

Species Identification of Soil Bacteria

Both strains S-3 and S-46b were Gram positive while strain S-26c was Gram negative. Strains S-3 and S-46b were identified as members of the genus *Bacillus* based on endospore formation and physiologic characterization using the API50CHB kit, and they were renamed as *Bacillus* sp. NKB03 and *B. cereus* NKB46b, respectively. Strain S-26c was characterized as *Aeromonas* sp. using API20NE kit and renamed *Aeromonas* sp. NKB26c.

Cleaning Efficiency of Supernatants in Bacterial Culture Broths Cultivated in Synthetic Media

The pungent odor from bacterial cultures cultivated in LB makes it unsuitable for direct use as an on-site cleaner. It has been reported that production of biosurfactant by *B. subtilis* grown in mineral salts medium with an appropriate carbon source is relatively higher than that when grown in nutrient broth *(17)*. Cultivation in synthetic minimal media was therefore studied for higher yield of cleaning activity. *Bacillus* sp. NKB03, *Aeromonas* sp. NKB26c, *B. cereus* NKB46b and the biosurfactant-producing bacteria *P. aeruginosa* DSM2659 and *B. subtilis* IAM1213 were cultured in three different synthetic media, and the cleaning efficiency of the supernatants from culture broths was measured. The optimal media yielding the highest cleaning efficiency were different for each strain (Table 2). *P. aeruginosa* DSM2659 and *Bacillus* sp. NKB03 yielded the highest cleaning efficiency in Ps medium, *B. subtilis* IAM1213 and *B. cereus* NKB46b in M9 medium, and *Aeromonas* sp. NKB26c in Spizizen medium. Although cleaning efficiencies of supernatants from culture broths of all strains were not increased compared with those when using LB media (cleaning efficiency of the supernatants in culture broths of *P. aeruginosa* DSM2659 and *B. subtilis* IAM1213 grown in 1/2 LB medium was 62 and 30%, respectively), the pungent odor was noticeably reduced.

Cleaning Efficiency of Mixed Supernatants of Bacterial Culture Broths

Soil bacteria that utilize hydrophobic organic compounds produce not only enzymes for degradation but also biosurfactants to desorb hydrocarbons from soil and organic matter *(18,19)*. Surfactants enhance the chemical removal and emulsification of hydrophobic organic compounds from solid surfaces, resulting in stimulation of biodegradation of these compounds. It was envisaged that a combination of biosurfactants produced by *Bacillus* sp. NKB03, *P. aeruginosa* DSM2659 or *B. subtilis* IAM1213, and the enzyme produced by *Aeromonas* sp. NKB26c or *B. cereus* NKB46b might increase the cleaning activity. Six combinations of 1:1 (v/v) mixtures of the supernatants from these various culture broths were prepared and the cleaning activities measured (Table 3). High cleaning efficiencies were observed in a supernatant mixture of *Aeromonas* sp. NKB26c and *Bacillus* sp. NKB03 culture broths (82%) and *Aeromonas* sp. NKB26c and *B. subtilis*

Table 2
Cleaning Efficiency in Supernatant of
Bacterial Cultures Cultivated in Synthetic Media

Strain	Cleaning efficiency (%)		
	M9 medium	Ps medium	Spizizen medium
P. aeruginosa DSM2659	26	58	25
B. subtilis IAM1213	29	—[a]	0
Bacillus sp. NKB03	0	25	0
Aeromonas sp. NKB26c	17	23	71
B. cereus NKB46b	61	7	—[a]

[a]Not examined.

Table 3
Cleaning Activity of Mixed Supernatant of Two Bacterial Cultures

Strain	Cleaning efficiency (%)
P. aeruginosa DSM2659	51
B. subtilis IAM1213	32
Bacillus sp. NKB03	21
Aeromonas sp. NKB26c	71
B. cereus NKB46b	66
Aeromonas sp. NKB26c + *P. aeruginosa* DSM2659	3
Aeromonas sp. NKB26c + *B. subtilis* IAM1213	78
Aeromonas sp. NKB26c + *Bacillus* sp. NKB03	82
B. cereus NKB46b + *P. aeruginosa* DSM2659	25
B. cereus NKB46b + *B. subtilis* IAM1213	18
B. cereus NKB46b + *Bacillus* sp. NKB03	50
Synthetic cleaner	74
SDS[a]	53
Water	0

[a]The concentration of SDS was 2× critical micelle concentration. *P. aeruginosa* DSM2659 and *Bacillus* sp. NKB03 were cultured with Ps medium. *B. subtilis* IAM1213 and *B. cereus* NKB46b were cultured with M9 medium. *Aeromonas* sp. NKB26c was cultured with Spizizen medium.

IAM1213 culture broths (78%). These values were higher than those of spent media of *Aeromonas* sp. NKB26c, *Bacillus* sp. NKB03, and *B. subtilis* IAM1213 cultured alone. These cleaning efficiencies were also higher than that of SDS. The supernatant mixture of culture broths from *B. cereus* NKB46b and *Bacillus* sp. NKB03B yielded a cleaning efficiency of 50%. By contrast, the remaining three mixtures yielded significantly decreased cleaning activities (3–25%), all of which were lower than those of the component media.

The stability of cleaning activity in the supernatant mixture (NKBG26c/NKB03) was evaluated by storage at both 4 and 25°C for 1 wk. At 4°C, no effect on cleaning efficiency was observed. However, at 25°C, approx 20%

of the cleaning activity was lost. These results suggest that a 1:1 (v/v) supernatant mixture from culture broths of *Bacillus* sp. NKB03 and *Aeromonas* sp. NKB26c has potential for commercial use as a biocleaner.

We have developed a potential biocleaner that is safer for the environment. The optimization of culture conditions for effective production of cleaning agents might contribute to their practical application toward on-site cleaning with lower cost.

Conclusion

We have described the screening of soil bacteria for production of cleaning agents using a simple and practical method. The supernatants from culture broth of three bacteria, *Bacillus* sp. NKB03, *Aeromonas* sp. NKB26c, and *B. cereus* NKB46b, which have been isolated from natural soil samples, possess efficient cleaning abilities against porcelain tiles dirtied with a mixture of crude oil and liquid paraffin. Although the bioactive component in each case has not been determined, we suspect it to be either a surfactant or an enzyme. Furthermore, we have demonstrated that a supernatant mixture of culture broth resulted in higher cleaning activity than those of the component media. These biocleaners are good candidates for removal of hydrophobic organic compounds from the surfaces of buildings.

References

1. Fiechter, A. (1992), *Trends Biotechnol.* **10,** 208–217.
2. Mulligan, C. N. and Gibbs, B. F. (1989), *Appl. Environ. Microbiol.* **55,** 3016–3019.
3. Arima, K., Kakinuma, A., and Tamura, G. (1968), *Biochem. Biophys. Res. Commun.* **31,** 488–494.
4. Horowitz, S., Gilbert, J. N., and Griffin, W. M. (1990), *J. Ind. Microbiol.* **6,** 243–248.
5. Cameotra, S. S. and Makkar, R. S. (1998), *Appl. Microbiol. Biotechnol.* **50,** 520–529.
6. Desai, J. D. and Banat, I. M. (1997), *Microbiol. Mol. Biol. Rev.* **61,** 47–64.
7. Desai, A. J., Petel, R. M., and Desai, J. D. (1994), *J. Sci. Ind. Res.* **53,** 619–629.
8. Klekner, V. and Kosaric, N. (1993), in *Biosurfactants: Production, Properties, Applications*, Kosaric, N., ed., Marcel Dekker, NY, pp. 229–272.
9. Shepherd, R., Rockey, J., Shutherand, I. W., and Roller, S. (1995), *J. Biotechnol.* **40,** 207–217.
10. Velikonja, J. and Kosaric, N. (1993), in *Biosurfactants: Production, Properties, Applications*, Kosaric, N., ed., Marcel Dekker, NY, pp. 419–446.
11. Kosaric, K. (1992), *Pure Appl. Chem.* **64,** 1731–1737.
12. Jenny, K., Kappeli, O., and Fiechter, A. (1991), *Appl. Microbiol. Biotechnol.* **36,** 5–13.
13. Mulligan, C. N. and Gibbs, B. F. (1993) in *Biosurfactants: Production, Properties, Applications*, Kosaric, N., ed., Marcel Dekker, NY, pp. 329–371.
14. Arino, S., Marchal, R., and Vandecasteele, J.-P. (1996), *Appl. Microbiol. Biotechnol.* **45,** 162–168.
15. Anagnostopoulos, C. and Spizizen, J. (1961), *J. Bacteriol.* **81,** 741–746.
16. Koch, A. K., Käppeli, O., Fiechter, A., and Reiser, J. (1991), *J. Bacteriol.* **173,** 4212–4219.
17. Cooper, D. G., MacDonald, C. R., Duff, S. J. B., and Kosaric, N. (1981), *Appl. Environ. Microbiol.* **42,** 408–412.
18. Zhang, Y. and Miller, R. M. (1995), *Appl. Environ. Microbiol.* **61,** 2247–2251.
19. Déziel, E., Paquette, G., Villemur, R., Lepine, F., and Bisaillon, J.-G. (1996), *Appl. Environ. Microbiol.* **62,** 1908–1912.