APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1984, p. 747–750
0099-2240/84/100747-04$02.00/0

Vol. 48, No. 4

Isolation of a Bioemulsifier from *Candida lipolytica*†

MICHAEL C. CIRIGLIANO AND GEORGE M. CARMAN*

Department of Food Science, Cook College, New Jersey Agricultural Experiment Station, Rutgers, The State University, New Brunswick, New Jersey 08903

Received 14 May 1984/Accepted 10 July 1984

The yeast *Candida lipolytica* produced an inducible extracellular emulsification activity when it was grown with a number of water-immiscible carbon substrates. Negligible emulsification activity was produced by this yeast when it was grown with glucose as the carbon substrate. In hexadecane-supplemented cultures, emulsification activity was first detected after 36 h of growth, with maximum production after 130 h. A water-soluble emulsification activity was partially purified by repeated solvent extractions of the culture filtrate. This emulsifier, which we named liposan, was primarily composed of carbohydrate. Maximum emulsification activity was obtained when the ratio of hexadecane to liposan was 50:1. Maximum emulsification activity was obtained from pH 2 to 5. Liposan was heat stable to temperatures up to 70°C, with a 60% loss in activity after heating for 1 h at 100°C. Liposan effected stable oil-in-water emulsions with a variety of hydrocarbons.

A number of microorganisms that are capable of assimilating hydrocarbons are also capable of emulsifying these hydrocarbons during the substrate degradation process (6, 8, 13, 27). There have been several reports describing the isolation of extracellular emulsifying agents produced by hydrocarbon-utilizing bacteria (10, 16, 20, 23–26, 28). Some yeasts also utilize hydrocarbon substrates (2) and produce extracellular emulsifying agents (5, 7, 11, 12, 14, 17, 18).

Candida lipolytica has previously been shown to grow on agar plates containing a variety of water-immiscible carbon substrates (4). When grown in the presence of hexadecane, *C. lipolytica* produces emulsifying agents which reduce the interfacial tension in the medium (18, 21). During the early stages of fermentation, the reduction in interfacial tension is mainly attributed to the release of fatty acids by *C. lipolytica* (18, 21), with changes in the interfacial tension in the latter stages of fermentation attributed to other metabolites produced by the organism (18).

We were interested in isolating a bioemulsifier from *C. lipolytica* that could potentially be used in food systems. In this paper we show that cultures of *C. lipolytica*, when grown in the presence of water-immiscible carbon substrates, produce an emulsification activity capable of stabilizing oil-in-water emulsions. A water-soluble emulsifying agent, which we call liposan, was partially purified from culture filtrates, and some of its properties were studied.

MATERIALS AND METHODS

Growth conditions. *C. lipolytica* ATCC 8662 was obtained from the American Type Culture Collection. Stock cultures of the organism were maintained on yeast mold agar slants and transferred once a month. A 300-ml batch of YNB medium (0.6% yeast nitrogen base, pH 5.0) supplemented with 1% hexadecane was inoculated with 10^2 CFU of a 72-h culture of *C. lipolytica* grown in yeast mold broth per ml. The culture was incubated for 130 h at 27°C on a rotary shaker at 220 rpm. Growth of the culture was monitored by plate counts on yeast mold agar.

Isolation of liposan. The 130-h culture was refrigerated for 24 h at 4°C to solidify the remaining hexadecane and to effect yeast settling. The culture was filtered by carefully decanting onto a Schleicher & Schuell grade 588 fluted filter. The resulting filtrate was then filtered through a 0.45-μm membrane (Millipore Corp.). The filtration steps were done at 25°C. Approximately 250 ml of the cell-free filtrate was transferred to a 2-foot (61-cm) length of dialysis tubing (diameter, 4 cm; molecular weight cut-off, 3,000) and concentrated to 50 ml by prevaporation at 4°C. The concentrated filtrate (50 ml) was extracted with 500 ml of chloroform-methanol (2:1, vol/vol) in a 1-liter separatory funnel at 25°C, as previously described (19). The aqueous phase was extracted twice more with 500 ml of chloroform-methanol (2:1); a white precipitate formed in the aqueous phase after the third extraction. The precipitate was collected on Whatman no. 42 filter paper and air dried.

Assay of emulsification activity. Samples (3 ml) from shake flask cultures were filter sterilized by using a Millipore 0.2-μm membrane filter. The filtrate (2 ml) was placed in a screw-capped test tube (15 by 125 mm) and diluted with 2 ml of 0.1 M sodium acetate buffer (pH 3.0); 1 ml of hexadecane (750 mg) was added, the tube was capped, and the mixture was shaken for 2 min at 25°C at a rate of 220 strokes per min (stroke length, 3 cm). The resulting uniform emulsion was allowed to sit for 10 min, after which its absorbance was measured at 540 nm with a Bausch & Lomb Spectronic 20 spectrophotometer. The blank used contained 2 ml of sterile YNB medium. The assay mixture for liposan activity contained 15 mg (dry weight) of precipitate dissolved in 4 ml of sodium acetate buffer (pH 3.0) and 1 ml of hexadecane. Liposan was omitted from the blank. One unit of emulsification activity was defined as that amount of emulsifier that effected an emulsion with an absorbance at 540 nm of 1.0.

Analytical methods. Protein was determined by the Coomassie blue dye-binding method of Bradford (3), using bovine gamma globulin as the standard. Carbohydrate was determined by the phenol-sulfuric acid procedure (15), using dextran as the standard. Fatty acids were analyzed by gas chromatography (9).

Materials. All chemicals were reagent grade. Growth media were purchased from Difco Laboratories. Bovine gamma globulin was obtained from Bio-Rad Laboratories. Dextran was from Sigma Chemical Co. Hexadecane was purchased from Eastman Kodak Co. Paraffin oil was obtained from J. T. Baker Chemical Co. Pure vegetable oils

* Corresponding author.
† Publication D-10203-2-84 of the New Jersey Agricultural Experiment Station.

TABLE 1. Emulsification activity produced by *Candida lipolytica* grown with various carbon substrates[a]

Carbon substrate	Activity (U)
Glucose	0.09
Hexadecane	0.88
Paraffin	0.75
Soybean oil	0.98
Olive oil	0.77
Corn oil	0.88
Cottonseed oil	0.50

[a] Cultures of *Candida lipolytica* were grown in 100-ml batches of YNB medium supplemented with 5% carbon substate for 130 h at 27°C. Following growth, 3 ml of each culture was filter sterilized, and a 2-ml sample was analyzed for emulsification activity by using hexadecane, as described in the text.

(with no added preservatives) were obtained from T. J. Lipton Co. Free fatty acids were removed from vegetable oils as previously described (4).

RESULTS

Emulsification activity produced by *C. lipolytica* grown with water-immiscible carbon substrates. *C. lipolytica* has previously been shown to utilize a variety of water-immiscible carbon substrates (4). Culture filtrates of *C. lipolytica* grown on YNB medium supplemented with water-immiscible carbon substrates produced an agent with the ability to emulsify or stabilize hexadecane-in-water emulsions (Table 1). Negligible emulsification activity was produced by the organism when it was grown in a medium with glucose as the primary carbon substrate. The growth of the organism with each of the carbon substrates was comparable. Although greater emulsification activity was produced by *C. lipolytica* when it was grown with soybean oil, further growth studies for emulsification activity production were conducted with hexadecane as the primary carbon substrate, providing a more defined medium. Figure 1 shows the growth and emulsification activity of *C. lipolytica* when hexadecane or glucose was used as the primary carbon substrate. In both media, the growth of the organism was comparable. Low emulsification activity was produced by the culture over 140 h when it was grown in the presence of glucose, whereas emulsification activity occurred in the presence of hexadecane, with maximum production after 80 and 130 h. During growth with hexadecane, *C. lipolytica* appeared to colonize droplets of hexadecane, as previously described by Nakahara et al. (18). The production of two peaks of emulsification activity was observed in three independently run fermentations. The concentration of hexadecane in the medium at the end of the fermentation was not determined.

Cultures of *C. lipolytica* were grown in YNB medium containing varying concentrations of hexadecane. Cell-free filtrates were prepared for each culture, and the emulsification activity for hexadecane-in-water emulsions was determined. Figure 2 shows that an approximately 10-fold increase in emulsification activity was achieved with 1% hexadecane. The addition of concentrations of hexadecane above 1% did not significantly alter the level of emulsification activity produced.

Isolation of liposan. Refrigeration of the culture following fermentation solidified any remaining hexadecane and facilitated hexadecane removal during the filtration step. The 10:1 ratio of solvent (chloroform-methanol, 2:1) to concentrated filtrate was critical in effecting the formation of a precipitate in the aqueous phase during the third extraction step. When lower ratios of solvent to filtrate were used, a fourth or fifth extraction step was required to obtain comparable yields of precipitated material. The average yield of precipitate per 300-ml batch was approximately 600 mg (dry weight). The average yield of emulsification activity from the culture filtrate was 30%. The remaining emulsification activity was associated with the organic solvent fraction and was not investigated in our studies. The precipitate contained about 93 to 98% carbohydrate and 2 to 7% protein. Fatty acids were not detected in the precipitate as analyzed by gas chromatography.

Properties of liposan. The effect of hexadecane on liposan emulsification activity is shown in Fig. 3. Maximum activity was obtained when the ratio of hexadecane to liposan was 50:1 (wt/wt). Ratios greater than 50:1 resulted in less emulsification activity. Maximum emulsification activity was obtained in the pH range from 2 to 5. Liposan was stable to heating for 1 h at temperatures up to 70°C, with about a 60% loss in activity after heating for 1 h at 100°C. At 10 mM concentrations, potassium and sodium ions stimulated activ-

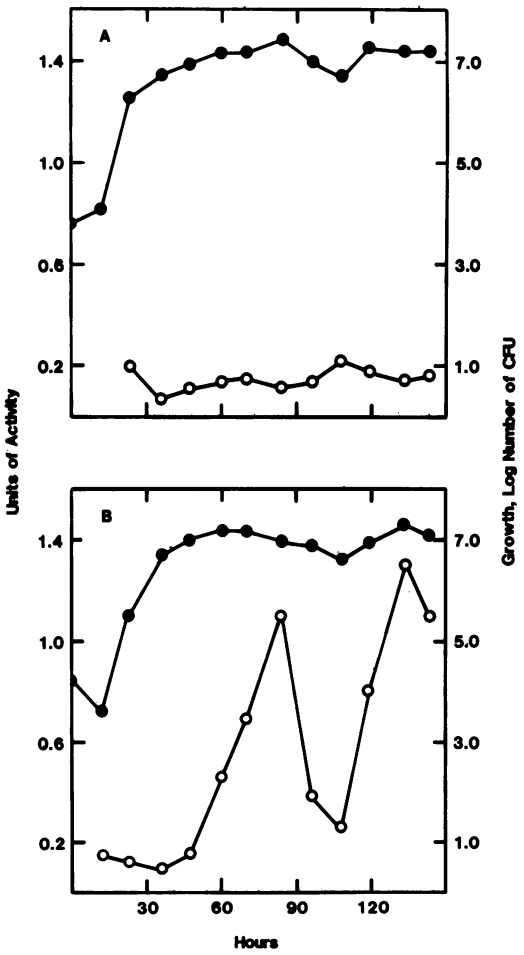

FIG. 1. Emulsification activity production by *Candida lipolytica* grown with hexadecane or glucose as the primary carbon substrate. A 300-ml batch culture of *Candida lipolytica* was grown in YNB medium supplemented with 1% glucose (A) or 1% hexadecane (B), as described in the text. The starting pH of the medium was 5.0, and the final pH was 2.1. Samples were taken at 12-h intervals and analyzed for viable cell counts (●) and emulsification activity (○).

ity 1.2-fold, whereas magnesium, manganese, and calcium ions had little effect on activity. At 1 M ion concentrations, emulsification activity decreased by 1.2- to 2.5-fold. The ability of liposan to stabilize or emulsify a variety of water-immiscible compounds in water is shown in Table 2. When liposan was tested against straight-chain aliphatic hydrocarbons ranging in chain length from 6 to 18, its ability to emulsify appeared to be chain length dependent. No significant activity was observed with chain lengths less than 10. Liposan effected emulsification activity with paraffin and no. 10 oil, as well as with the aromatic hydrocarbons Halowax, toluene, and decahydronaphthalene.

DISCUSSION

The growth of a hydrocarbon-utilizing yeast is limited by the interfacial surface area and the availability of submicron oil droplets in the growth medium (6, 8). A decrease in interfacial tension and an increase in the degree of medium emulsification are critical factors in hydrocarbon fermentation by *C. lipolytica* (18, 21). The decrease in the interfacial tension of hexadecane-containing medium by *C. lipolytica* is presumably due to release of fatty acids during the initial stages of fermentation (18, 21). The production of fatty acids declines by 36 h into the fermentation, but the interfacial tension continues to drop (18). Nakahara et al. (18) suggested that the decrease in the interfacial tension of the medium during the latter stages of fermentation is due to the production of lipoproteins and lipopolysaccharides, as well as to the yeasts themselves. In our studies *C. lipolytica* produced emulsification activity after the organism entered its stationary phase of growth. We did not analyze the growth medium for changes in interfacial tension or for the production of fatty acids, as previously done (18). We did not investigate the nature of the emulsifiers produced in the first peak of emulsification activity during growth on hexadecane (Fig. 1B). There were at least two types of emulsifiers produced in the second peak of activity (Fig. 1B). One type was soluble in organic solvent, and the other type was water soluble. The water-soluble emulsifier, which we have named liposan, was partially purified from the culture filtrate by repeated chloroform-methanal-water phase partitions. This procedure is commonly used to recover microbial gums from bacterial fermentations (19). We have not fully characterized the chemical nature of liposan; however, it appears to be primarily composed of carbohydrate.

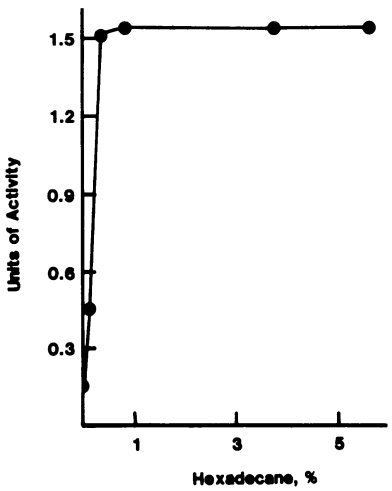

FIG. 2. Induction of emulsification activity by hexadecane. Cultures of *Candida lipolytica* were grown in 100-ml batches of YNB medium supplemented with different concentrations of hexadecane. The cultures were grown for 130 h at 27°C on a rotary shaker at 220 rpm. Emulsification activity was determined in the cell-free filtrate as described in the text.

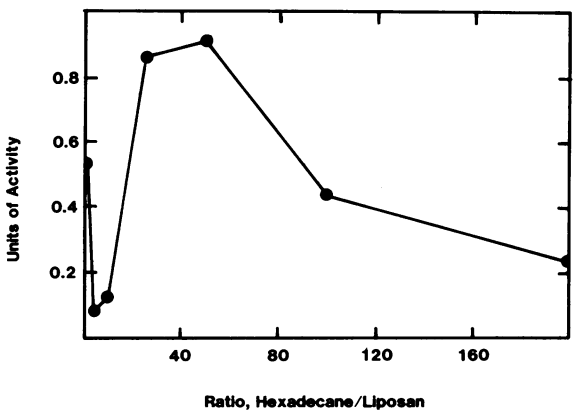

FIG. 3. Effect of hexadecane concentration on liposan emulsification activity. Emulsification activity was measured with 15 mg (dry weight) of liposan and amounts of hexadecane ranging from 15 to 3,000 mg, as described in the text.

Our emulsification assay was modelled after the one described by Rosenberg et al. (23), which is used for emulsan activity. Maximum emulsification activity of liposan was obtained when the ratio of hexadecane to liposan was 50:1. Increasing the hexadecane-to-liposan ratio above 50:1 resulted in a decrease in emulsification activity. Rosenberg et al. (23) reported a similar behavior for emulsan activity and attributed it to a direct interaction between emulsifier and the hydrocarbon, as opposed to an effect on the surface tension of the medium.

The effectiveness of liposan as an emulsifier was limited to the acid to neutral pH range, with maximum activity between pH 2 and 5. Other microbial emulsifiers for which a pH range of activity has been described have also shown the ability to emulsify at low pH values (23, 25).

Liposan was relatively heat stable between 30 and 90°C. Even after boiling for 1 h, liposan retained 40% of its original emulsification activity. The thermal stability of liposan is similar to that of microbial gums (1).

Unlike the bacterial emulsifier emulsan (23), the activity of liposan was not dependent on the presence of a metal ion. The presence of molar concentrations of salts reduced liposan activity, whereas the polymeric emulsifier produced by *Corynebacterium hydrocarboclastus* is quite tolerant to salt (25).

The ability of liposan to emulsify hydrocarbon, unlike emulsan (22), was not dependent on the presence of both aliphatic and cyclic components. When liposan was tested with straight-chain aliphatic hydrocarbons (C_6 to C_{18}), its effectiveness as an emulsifier was dependent on chain length.

In summary, we have shown that *C. lipolytica* can be induced to produce emulsification activity when it is grown with a variety of water-immiscible carbon substrates. This organism produced a water-soluble emulsifier and a chloroform-soluble emulsifier. A water-soluble emulsifier, named liposan, was partially purified and was capable of stabilizing a variety of oil-in-water emulsions. Work is in progress to

TABLE 2. Emulsification of water-immiscible compounds by liposan[a]

Compound	Activity (U)
Paraffin	0.70
Kerosene	0.30
Gast S1 No. 10 oil	0.80
Tributyrin	0.30
Hexane	0.01
Heptane	0.02
Octane	0.04
Nonane	0.05
Decane	0.15
Dodecane	0.98
Tetradecane	1.01
Hexadecane	1.20
Octadecane[b]	0.60
m-Xylene	0.30
Toluene	0.63
Halowax no. 1000	1.25
Decahydronaphthalene	0.75

[a] Emulsification activity was measured with 750 mg water-immiscible compound and 15 mg (dry weight) of liposan, as described in the text.
[b] Solid at 25°C; assay done at 45°C.

purify liposan to homogeneity so its chemical and physical properties may be studied.

ACKNOWLEDGMENTS

This work was supported by New Jersey state funds, by the U. S. Hatch Act, and by T. J. Lipton, Inc.
We thank Rose Ann Ullrich for typing the manuscript.

LITERATURE CITED

1. **Baird, J. K., P. A. Sanford, and I. W. Cottrell.** 1983. Industrial applications of some new microbial polysaccharides. Bio/Technology **1**:778–783.
2. **Bos, P., and W. E. DeBoer.** 1968. Some aspects of the utilization of hydrocarbons by yeast. Antonie van Leeuwenhoek. J. Microbiol. Serol. **34**:241–243.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
4. **Cirigliano, M. C., and G. M. Carman.** 1983. A plating technique for the selective isolation of yeast utilizing water immiscible carbon. J. Food Sci. **48**:1554–1555.
5. **Cooper, D. G., and D. A. Paddock.** 1984. Production of a biosurfactant from *Torulopsis bombicola*. Appl. Environ. Microbiol. **47**:173–176.
6. **Erickson, L. E., and T. Nakahara.** 1975. Growth in cultures with two liquid phases: hydrocarbon uptake and transport. Process Biochem. **10**:9–13.
7. **Guire, P. E., J. D. Friede, and R. K. Gholson.** 1973. The microbial degradation of oil pollutants. *In* D. G. Ahearn and S. P. Meyers (ed.), Publication LSU-SG73-01. Centre for Wetland Resources, Baton Rouge, La.
8. **Gutnick, D. L., and E. Rosenberg.** 1977. Oil tankers and pollution: a microbiological approach. Annu. Rev. Microbiol. **31**:379–396.
9. **Hanson, R. S., and J. A. Phillips.** 1981. Chemical composition, p. 328–364. *In* P. Gerhardt (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
10. **Hisatsuka, K., T. Nakahara, N. Sano, and K. Yamada.** 1971. Formation of rhamnolipid by *Pseudomonas aeruginosa* and its function in hydrocarbon fermentation. Agric. Biol. Chem. **35**:686–692.
11. **Iguchi, T., I. Takeda, and H. Ohsawa.** 1969. Emulsifying factor of hydrocarbon produced by a hydrocarbon-assimilating yeast. Agric. Biol. Chem. **33**:1657–1658.
12. **Ito, S., and S. Inoue.** 1982. Sophorolipids from *Torulopsis bombicola*: possible relation to alkane uptake. Appl. Environ. Microbiol. **43**:1278–1283.
13. **Jobson, A., F. D. Cook, and D. W. S. Westlake.** 1972. Microbial utilization of crude oil. Appl. Microbiol. **23**:1082–1089.
14. **Jones, D. G.** 1967. Novel macrocyclic glycolipids from *Torulopsis gropengiesseri*. J. Chem. Soc. **C**:479–484.
15. **Keleti, G., and W. H. Lederer.** 1974. Preparation of material for chemical analysis or biological characterization, p. 51. *In* Handbook of micromethods for the biological sciences. Van Nostrand Reinhold Co., New York.
16. **Kretschmer, A., H. Bock, and F. Wagner.** 1982. Chemical and physical characterization of interfacial-active lipids from *Rhodococcus erythropolis* grown on n-alkanes. Appl. Environ. Microbiol. **44**:864–870.
17. **Mimura, A., S. Watanabe, and I. Takeda.** 1971. Biochemical engineering analysis of hydrocarbon fermentation. III. Analysis of emulsification phenomena. J. Ferment. Technol. **49**:255–271.
18. **Nakahara, T., L. E. Erickson, and J. R. Gutierrez.** 1977. Characteristics of hydrocarbon uptake in cultures with two liquid phases. Biotechnol. Bioeng. **19**:9–25.
19. **Nelson, C. J.** 1975. Isolation and purification of lipids from animal tissues, p. 1–22. *In* E. G. Perkins (ed.), Analysis of lipids and lipoproteins. American Oil Chemist Society, Champaign, Ill.
20. **Pines, O., E. A. Bayer, and D. L. Gutnick.** 1983. Localization of emulsan-like polymers associated with the cell surface of *Acinetobacter calcoaceticus*. J. Bacteriol. **154**:893–905.
21. **Prokop, A., M. Ludvik, and L. E. Erickson.** 1972. Growth models of cultures with two liquid phases. VIII. Experimental observations on droplet size and interfacial area. Biotechnol. Bioeng. **14**:587–608.
22. **Rosenberg, E., A. Perry, D. T. Gibson, and D. L. Gutnick.** 1979. Emulsifier of *Arthrobacter* RAG-1: specificity of hydrocarbon substrate. Appl. Environ. Microbiol. **37**:409–413.
23. **Rosenberg, E., A. Zuckerberg, C. Rubinovitz, and D. L. Gutnick.** 1979. Emulsifier of *Arthrobacter* RAG-1: isolation and emulsifying properties. Appl. Environ. Microbiol. **37**:402–408.
24. **Suzuki, T., K. Tanaka, I. Matsubara, and S. Kinoshita.** 1969. Trehalose lipid and α-branched β-hydroxy fatty acid formed by bacteria grown on n-alkanes. Agric. Biol. Chem. **33**:1619–1627.
25. **Zajic, J. E., H. Guignard, and D. F. Gerson.** 1977. Emulsifying and surface active agents from *Corynebacterium hydrocarboclastus*. Biotechnol. Bioeng. **19**:1285–1301.
26. **Zajic, J. E., H. Guignard, and D. F. Gerson.** 1977. Properties and biodegradation of a bioemulsifier from *Corynebacterium hydrocarboclastus*. Biotechnol. Bioeng. **19**:1303–1320.
27. **Zajic, J. E., and B. Supplisson.** 1972. Emulsification and degradation of bunker C fuel oil by microorganisms. Biotechnol. Bioeng. **14**:331–343.
28. **Zuckerberg, A., A. Diver, Z. Peeri, D. L. Gutnick, and E. Rosenberg.** 1979. Emulsifier of *Arthrobacter* RAG-1: chemical and physical properties. Appl. Environ. Microbiol. **37**:414–420.