Selective Removal of Free Fatty Acids in Oils Using a Microorganism

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A microorganism assimilating long chain fatty acids without secreting extracellular lipases was screened from soil and was identified as a *Pseudomonas*. Growth factors, nitrogen sources and trace elements required for growth of the microorganism named *Pseudomonas* strain BG1 were determined. Optimum pH and growth temperature were 6 and 30°C, respectively. BG1 was found to utilize lauric, myristic, palmitic, stearic and oleic acids as carbon sources. BG1 was shown to utilize 0.1% oleic acid almost completely in an emulsion medium within 48 hr. When BG1 was grown in a mixture of triolein and oleic acid, it selectively removed the free fatty acid without loss of triolein and did not produce mono-and diglycerides.

KEY WORDS: Biological refining, crude oil, deacidification, *Pseudomonas*.

Crude fats and oils have traditionally been obtained by pressing, rendering and/or solvent extraction. After extraction, the crude oils are generally subjected to refining processes constituted by degumming, deacidification, bleaching and deodorization to remove objectionable impurities in fats and oils (1).

The main objective of deacidification is to remove free fatty acids, and the most important and generally practical deacidification method by far is alkali refining (2). The alkali refining process is relatively simple and removes free fatty acids and residual phosphatides almost completely. During the alkali refining process, however, there are always considerable losses of neutral oil, sterols, tocopherols and vitamins; furthermore, disposal and utilization of the resulting soapstock may cause environmental pollution problems (3).

Many processes have been suggested to replace alkali refining; including solvent fractionation (4), physical refining (5) and biological refining (6). Biological refining can include lipase systems to esterify free fatty acids into triglycerides (6), or a whole cell microorganism system which selectively can remove and/or assimilate free fatty acids for its own growth.

The objectives of the study are: i) Selection of a microorganism that can assimilate free fatty acids selectively and rapidly without secretion of extracellular lipase; ii) partial optimization of the cultural medium and conditions for growth of the microorganism; and iii) characterization of free fatty acid assimilation patterns and application of the microorganism to the selective removal of free fatty acids in mixtures of triglycerides and free fatty acids.

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MATERIALS AND METHODS

Materials. Oleic acid (95% purity), triolein (65% purity) and lecithin (40% purity) were purchased from Sigma Chemical Co. (St. Louis, MO). Silica gel 60 (70–230 mesh) was obtained from Merck (West Germany). For cultivation of microorganism, triolein was purified with activated silica gel 60 before use. All reagents used were of analytical grade unless otherwise specified.

Screening and identification of microorganism. A 1% agar plate containing a medium consisting of minimal salts (KH_2PO_4, 0.1%; $MgSO_4.7H_2O$, 0.05%; NH_4NO_3, 0.1%; Fe_2SO_4, 0.01%; $MnSO_4.7H_2O$, 0.01%; $CaCl_2$, 0.01%) and 1% oleic acid was used for screening of microorganisms from soil origin. Colonies which were capable of assimilating oleic acid were isolated and then tested for secretion of lipase by the methods of Lillie (7) and Alford (8). Taxonomic tests of selected microorganisms were conducted according to standard procedures (9).

Culture medium and growth conditions. The culture medium was modified to select for more effective cell growth and removal of free fatty acids by modifying the growth factor, the nitrogen source and the trace elements. To select the effective trace elements, the basal medium was composed of 0.1% oleic acid, 0.1% ammonium sulfate, 0.1% casein hydrolyzate and 0.2% lecithin. The autoclaved medium was emulsified aseptically using a tissuemiser (Ultra-turrax t25, Janke and Kunkel) at 9000 rpm for 20 min. The cells were grown in Erlenmeyer flasks at 30°C and pH 6.0 with shaking for 48 hr.

To test for the selective removal of free fatty acids in a mixture with triglycerides, 10 g of purified triolein containing 1% oleic acid was overlaid upon a 100 mL salt solution containing 0.1% casein hydrolyzate, and the culture flasks were kept at 30°C for 48 hr with continuous shaking.

Analytical methods. A sample of the culture broth was taken after every 12 hr cultivation and was centrifuged to measure dry cell weight (DCW). The supernatant fluid obtained after centrifugation was mixed with isopropanol (1:1, v/v) and membrane filtered. A sample of this mixture was directly injected into a high performance liquid chromatograph (HPLC) (Waters Associates, Milford, MA; Model 510) equipped with a UV detector (Model 441) and data module (Model 740). Operation conditions of HPLC were as follows: Column, Novapak C18; elution solvent, acetonitrile/isopropanol/hexane (50:3:2, v/v/v); detector, UV at 214 nm; flow rate, 1 mL/min (10).

RESULTS AND DISCUSSION

Selection and identification of microorganism. Several strains of bacteria that were capable of assimilating free fatty acids without extracellular lipase secretion were isolated from soil. Among them, strain BG1 was

found to utilize free fatty acids most rapidly, and to be minimally growth-inhibited by high concentrations of free fatty acids. The reason for selecting the most rapid utilizing microorganism was to reduce the contact time of lipids with water in order to limit lipid oxidation during the biological deacidification process.

Morphological, cultural and biochemical characteristics revealed that strain BG1 belonged to the genus *Pseudomonas,* but the species was not determined.

Medium optimization and cultivation of Pseudomonas *sp. BG1.* Flask cultures were conducted to select the growth factor, nitrogen source and trace element parameters. The culture medium was composed of the screening medium with substituted testing ingredients. Effects of growth factors at 0.1% level on the fatty acid removal by *Pseudomonas* BG1 are shown in Table 1. Among the growth factors tested, casein hydrolyzate was found to be most effective. After growing with casein hydrolyzate, free fatty acids were completely utilized. Maximum biomass yield, 1.8 g dry cell weight/L, was obtained with casein hydrolyzate. Bacto soytone, beef extract, tryptone, peptone and yeast extract also showed good stimulating effects on free fatty acid removal as compared to the control medium.

Effects of 0.1% nitrogen sources on free fatty acid removal and cell growth are given in Table 2. Among nitrogen sources tested, ammonium sulfate and potassium nitrate were shown to be most effective leaving no residual free fatty acids. When BG1 was grown in a medium containing potassium nitrate, a longer lag time was observed compared to ammonium sulfate medium. Sodium nitrite was shown to inhibit the growth of BG1. Maximum biomass yield, 1.8 g DCW/L, was obtained with ammonium sulfate.

As shown in Table 3, utilization of free fatty acids by BG1 was greatly influenced by trace elements. Magnesium and manganese ions were found to promote the utilization of the free fatty acids. Inhibitory effects on the cell growth shown by ferric, ferrous and cupurous ions were considered due to their high prooxidant activity. It is very well known that oxidized lipids are harmful to most microorganisms (11).

Among cultural media tested, the most effective for cell growth and free fatty acid removal had ingredients as follows: Oleic acid, 0.1%; KH_2PO_4, 0.1%; $(NH_4)_2SO_4$, 0.1%; $MgSO_4.7H_2O$, 1 mM; $MnSO_4.4$-$6H_2O$, 1 mM; casein hydrolyzate, 0.1%; and 0.2% lecithin as an emulsifier.

Pseudomonas BG1 was shown to grow well between pH 3.0 and 8.0. The most effective fatty acid removal was observed at pH 5.0 to 7.0. Strain BG1 grew well over a temperature range between 25 to 40°C. Maximum fatty acid removal was, however, observed at 30°C. When BG1 was cultured in a batch fermentor, BG1 completely utilized free fatty acids. The maximum specific growth rate measured under the given conditions was 0.15 hr^{-1}.

Assimilation of fatty acids. Pseudomonas strain BG1 was found to utilize various fatty acids as shown in Table 4. The short chain fatty acids having carbon numbers below 12 and linoleic acid were not utilized and, furthermore, they sometimes inhibited the growth of BG1. Long-chain saturated fatty acids with carbon numbers of 12 or more and oleic acid were utilized. The rate of removal or utilization of fatty acids was proportional to their solubility in water. When BG1 was grown in an oleic acid medium, oleic acid was most rapidly and fully utilized compared to growth in media containing other fatty acids. This effect may be due to the solubility of oleic acid in water as compared to lauric, myristic, palmitic and stearic acids, which made oleic acid more easily contacted by BG1 (12). However, butyric, valeric, caproic, caprylic and capric acids all have higher solubility in water than oleic acid and were not utilized. This could be explained in part by the toxicity of short chain fatty acids to microorganisms (13). Linoleic acid having a higher solubility in water than oleic acid and two double bonds, was not found to be utilized

TABLE 1

Effects of Growth Factors on Residual Fatty Acid Content and Biomass Production

Growth factor	Residual fatty acid content (%)	Biomass (g DCW/L)
Casein hydrolyzate	0	1.8
Yeast extract	20	1.5
Tryptone	35	1.5
Bacto soytone	40	1.6
Peptone	43	1.5
Beef extract	46	1.5
Malt extract	52	1.4
Control	59	0.5

TABLE 2

Effects of Nitrogen Sources on Residual Fatty Acid Content and Biomass Production

Nitrogen source	Residual fatty acid content (%)	Biomass (g DCW/L)
$(NH_4)_2SO_4$	0	1.6
KNO_3	0	1.5
NH_4Cl	9	1.5
NH_4NO_3	12	1.4
$NaNO_3$	18	1.5
Urea	19	1.7
$NaNO_2$	85	0.4
Control	19	1.5

TABLE 3

Effects of Trace Elements on Residual Fatty Acid Content and Biomass Production

Trace element (1 mM in medium)	Residual fatty acid content (%)	Biomass (g DCW/L)
$MgSO_4.7H_2O$	0	1.2
$MnSO_4.4$-$6H_2O$	0	1.0
$CaCl_2$	18	1.3
$ZnSO_4.7H_2O$	25	0.6
$FeSO_4.7H_2O$	53	0.7
$FeCl_3$	72	0.1
$CuSO_4.7H_2O$	82	0.4
All elements	52	1.2
Control	19	1.3

TABLE 4

Utilizability and Biomass Production Depending on Free Fatty Acids

Free fatty acid	Utilizability	Biomass (g DCW/L)
Butyric acid	N.U[a]	0.1
Valeric acid	N.U	0.1
Caproic acid	N.U	0.1
Caprylic acid	N.U	0.1
Capric acid	N.U	0.1
Lauric acid	U[b]	0.8
Myristic acid	U	0.6
Palmitic acid	U	0.7
Oleic acid	U	1.8
Linoleic acid	N.U	0.1
Stearic acid	U	0.6

[a]N.U, not utilized.
[b]U, utilized.

(Table 4). This could be due to the fact that oxidized lipids were produced from readily oxidizable linoleic acid (14). Maximum biomass, 1.8 g DCW/L, was obtained from oleic acid fermentation.

When *Pseudomonas BG1* was cultured in a medium overlaid with purified triolein containing oleic acid, and the residual oleic acid content remained almost constant for 12 hr and then decreased sharply until it reached about 20%. In other words, only 80% of the oleic acid was removed by BG1 with 48 hr cultivation. This result, that BG1 could not remove oleic acid completely, was considered due to decreased contact area in an overlaid medium compared to an emulsion medium. The reason why the simple overlaid medium was used in this study was to reduce the contact of triolein with water and thereby avoid lipid oxidation.

To check whether mono- and/or diolein were produced during the cultivation of BG1 in triolein and oleic acid, the lipids in the culture broths obtained at the beginning and end of fermentation were analyzed by HPLC. The content of triglycerides remained constant during cultivation whereas free fatty acid content decreased significantly after cultivation. Mono- and diglycerides, with retention times of 2.3 min and 11.5–12 min, respectively, were not detected before and after cultivation. The results implied that mono- and diglycerides were not produced during cultivation through enzymatic or nonenzymatic hydrolysis of triglycerides.

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[Received January 29, 1990; accepted May 3, 1990]