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CHAPTER 8

Microbial Protein from Hydrocarbons

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When an *Achromobacter* sp. was grown in stirred fermenters, initial productivity in continuous culture was lower than desired and only a narrow range of hydrocarbons (C_9 to C_{10}) was utilized. When recycled medium was used, productivity increased and the range of hydrocarbons capable of supporting growth broadened (C_9 to C_{14}). In addition, the power necessary to maintain maximum productivity decreased. Similarly, a species of *Brevibacterium* and a yeast, *Pichia* sp., were studied. Feeding tests to evaluate the protein quality of each microbe were conducted and their results correlated with calculated biological values.

INTRODUCTION

Among the important parameters in production of microbial protein from hydrocarbons are that the organism be capable of assimilating a range of hydrocarbons which are available in quantity, that it grows with high productivity and efficiency of conversion of hydrocarbon to cells at minimal power to the fermenter, and that the protein be nutritionally adequate. We have examined growth in stirred fermenters of a species of *Achromobacter* and, to a lesser extent, species of *Brevibacterium* and *Pichia.*

METHODS

Microbes were isolated by enrichment culture, identified according to conventional taxonomic criteria, and grown in 250-ml shake flasks and in 5-liter and 50-liter New Brunswick Scientific stirred fermenters. The medium had the salts composition listed in Table 1. In the fermenters, temperature was maintained at 35 or 38 C, and pH was controlled by automatic addition of 7 N ammonium hydroxide. Agitation rate was 750 rpm and the air rate was 0.3 vvm for *Brevibacterium,* and 0.1 vvm for *Achromobacter* and *Pichia,* unless otherwise specified. Hydrocarbon was metered in with a Sage pump. An ultrasonic probe was used as a liquid level control. Culture effluent was collected in a refrigerated cart and cells were harvested in a Sharples centrifuge. Feeding tests were conducted using lyophilized cells. Protein was determined by Kjeldahl analysis and amino acids by bioassay. Biological values were calculated according to Oser (1959) and chemical scores, according to Mitchell and Block (1946). Nutritional quality of the proteins was verified in a standard 28-day feeding test (Anon., 1960). Culture dialysis was effected by circulation through six feet of ⅝-inch diameter cellulose tubing immersed in a volume of medium that was ten times the fermenter volume (Gallup and Gerhardt, 1963). Culture turbidity was measured on untreated broth with a Bausch and Lomb Spectronic 20 at 400 mμ, 400 units corresponded to 1 g of cells (dry weight) per liter. Unused hydrocarbon was measured by gas chromatographic techniques.

TABLE 1. *Mineral-salts medium used during fermentation studies*

Salt	G/Liter
$(NH_4)_2SO_4$	2.0
Na_2HPO_4	3.0
KH_2PO_4[a]	2.0
$MgSO_4 \cdot 7H_2O$	0.2
Na_2CO_3	0.1
$CaCl_2$	0.01
$FeSO_4 \cdot 7H_2O$	0.005
Na_2MoO_4	0.002
$CoCl_2$	0.002
$MnSO_4$	0.002

[a] Medium for yeast contained 5.0g KH_2PO_4 only.

RESULTS

Growth of Microbes

1. *Achromobacter.* In batch growth using *n*-decane as the carbon source, the cell doubling time during the logarithmic phase was 5.5 hr. In continuous culture, the maximum productivity was 0.35 g of cells (dry weight) per liter of fermenter volume

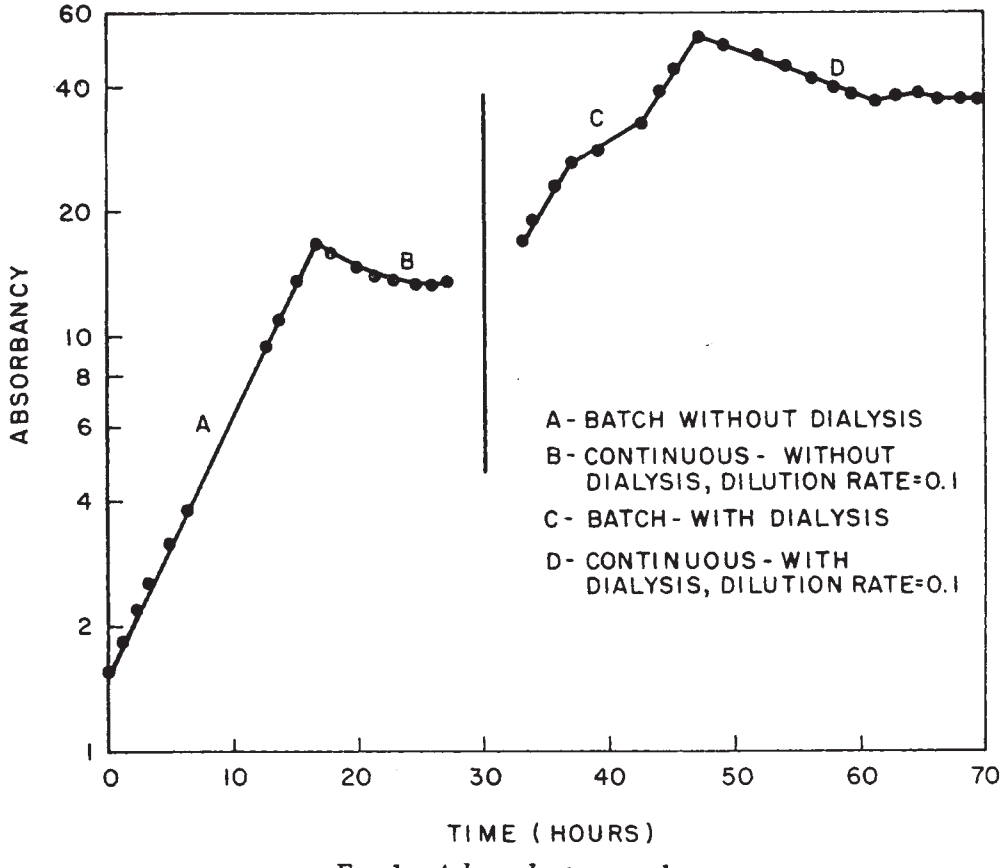

FIG. 1. *Achromobacter* growth.

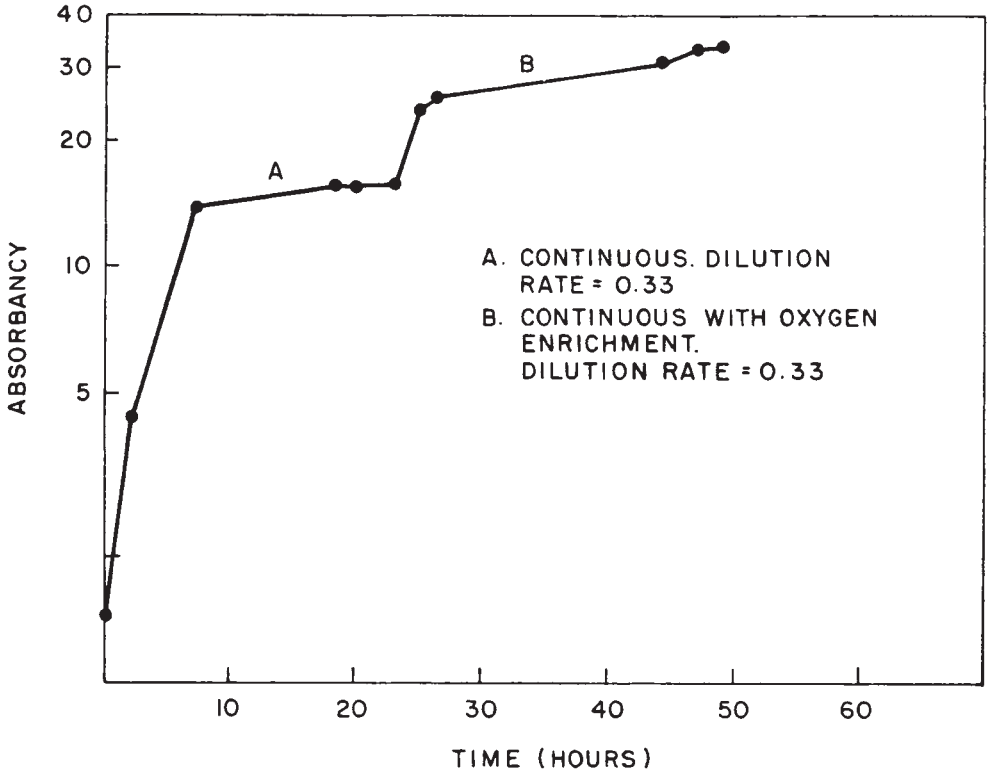

FIG. 2. *Brevibacterium* growth.

per hour at a dilution rate of 0.1 hr^{-1}, corresponding to an average doubling time of 7 hr (Fig. 1). Cell conversion efficiency was found to be 1.12 g of cells per gram of hydrocarbon consumed. The productivity was increased to 0.83 g/hr (Fig. 1) by continuous dialysis of the culture, suggesting that toxic factors were produced which inhibited growth. When the spent medium of the continuous culture system was checked for growth inhibition in shake flask experiments, a marked stimulation of growth was noted (Table 2) and cell doubling time was nearly halved. Moreover, the range of assimilable hydrocarbons was extended downward to C_8 and upward to C_{14} substrates (Table 3). The growth-promoting effect of spent medium was confirmed by recycling cell-free spent medium in a continuous fermenter. Productivity was as high as 1.8 g/hr at a dilution rate of 0.27 hr^{-1}; this corresponded to an average doubling time of 2.6 hr. Supplementation with magnesium ions to the recycled medium was found to be necessary to sustain the observed productivity. Oxygen enrichment studies with this organism were not made.

2. *Brevibacterium.* The limited range of hydrocarbons assimilable by *Achromobacter* prompted investigation of a second organism, *Brevibacterium*, which could assimilate *n*-paraffins in gas-oils. The bacterium, having a doubling time as short as 45 min on hexadecane (Fig. 2), reached a steady state cell productivity of 1.3 g/l/hr. This initially higher productivity may have resulted from faster growth, an earlier formation of emulsifiers in the culture, or other variables. Shake flask experiments again showed

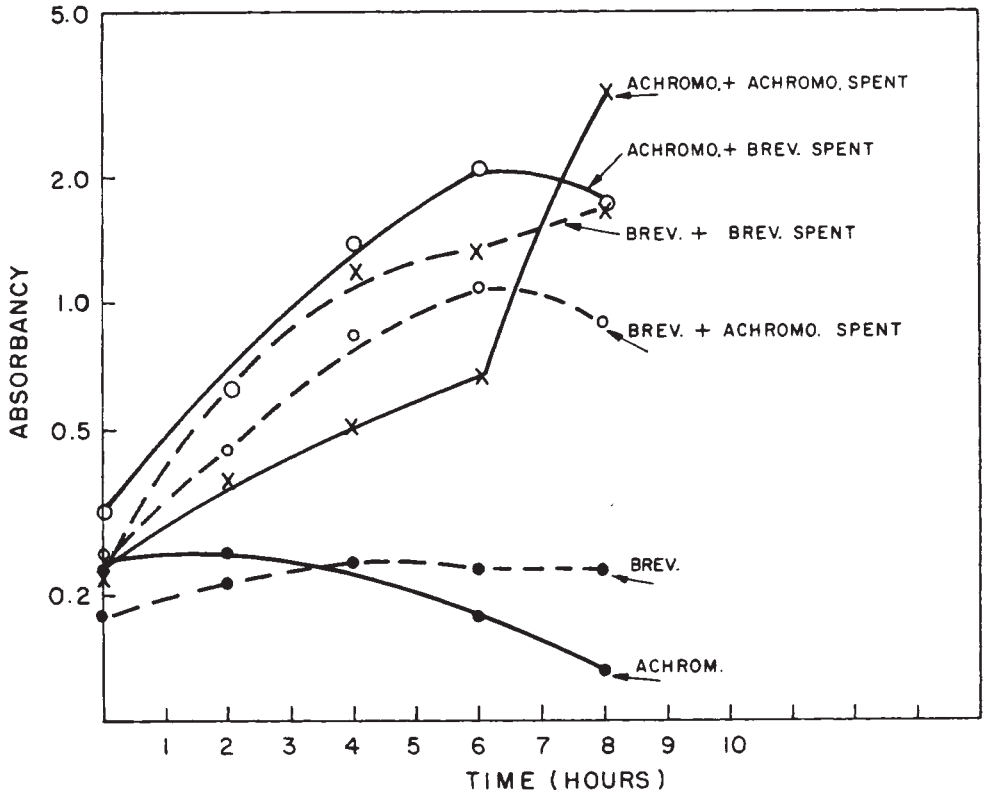

FIG. 3. Growth of *Achromobacter* and *Brevibacterium* in spent media.

TABLE 2. *Stimulation of growth of* Achromobacter *on n-decane in spent media*

Growth Medium	Culture Absorbency (400 mμ)
Spent residue + mineral-salts media.	1.35 units
Spent residue + mineral-salts mediua +0.2 ml *n*-decane.	14.25
Mineral-salts media +0.2 ml *n*-decane.	6.88

TABLE 3. *Specificity of* n-*paraffin utilization by* Achromobacter *with and without spent media*

Carbon Source	Culture Absorbency (400 mμ)	
	Without Spent Media	With Spent Media
n-octane	0.56 units	8.0 units
n-undecane	0.44	12.40
n-dodecane	0.48	21.60
n-tetradecane	0.44	4.35
kerosene	0.32	20.80
naphtha[a]	2.12	24.00

[a] Naphtha—equal parts by volume of *n*-hexane, *n*-heptane, *n*-octane, and *n*-decane.

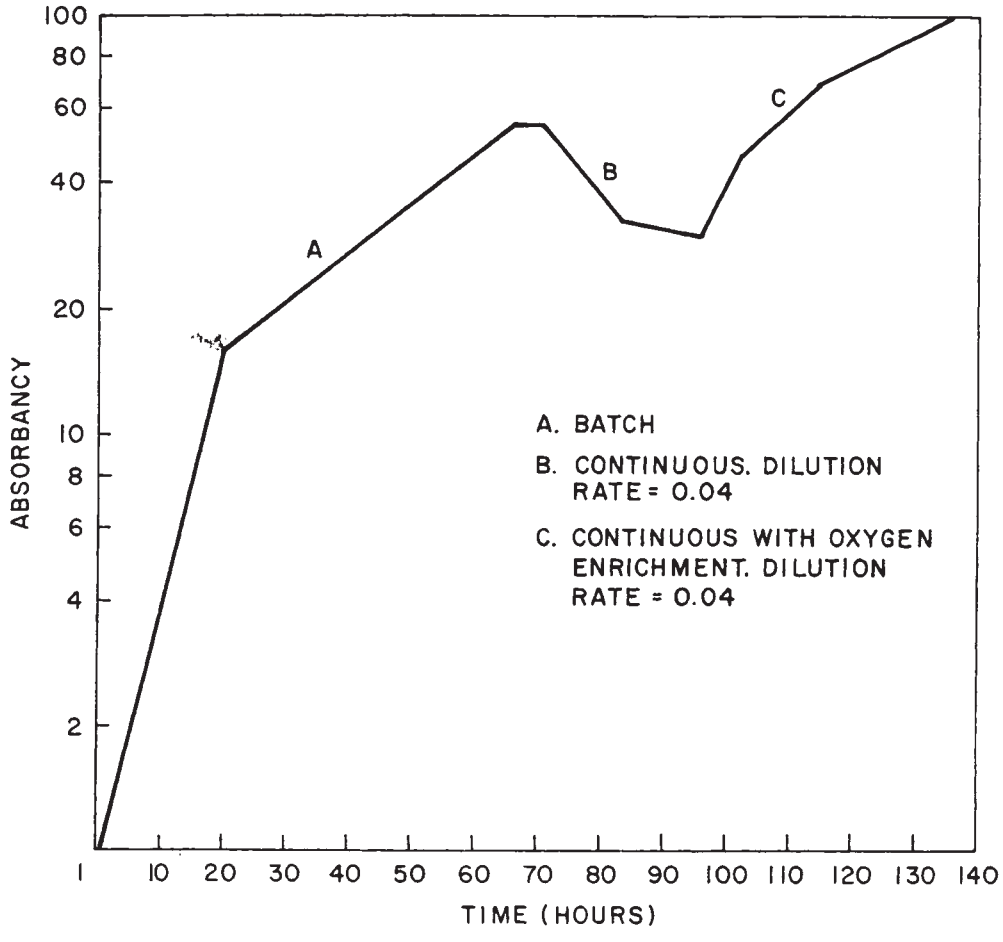

FIG. 4. *Pichia* growth.

that spent medium had a growth stimulation effect. In fact, the spent media from either culture were interchangeable (Fig. 3). However, recycle of cell-free effluent to a continuous fermentation system did not markedly increase cell productivity; this was apparently due to oxygen limitation. When the air was enriched with oxygen to a concentration of 34%, the productivity was increased to 3 g/l/hr in the absence of spent media. There were no adverse effects from medium reuse.

3. *Pichia.* A yeast belonging to the genus *Pichia* was also studied. It could assimilate *n*-paraffins in the boiling range of kerosene and gas-oils with a doubling time of four to five hr. Productivity in continuous culture on C_{16} was 0.4 g/l/hr. Although spent medium similarly enhanced the growth of *Pichia* in shake flasks, there was no rise in cell productivity levels when medium was reutilized in continuous culture. The productivity was again believed to be limited by oxygen supply. This was confirmed by the oxygen enrichment experiment of Figure 4 which shows a productivity increase to about 1

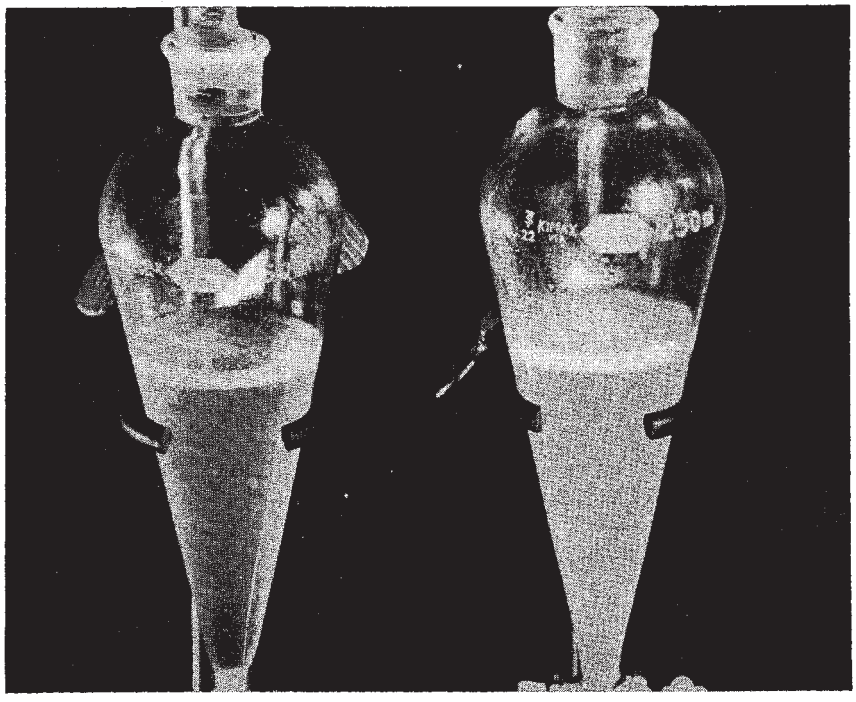

FIG. 5. Hydrocarbon emulsification by spent media after supporting growth of *Achromobacter*.
A. GLUTAMATE CULTURE B. DECANE CULTURE

g/l/hr. The rate of oxygen transport could have been adversely affected by the antifoam agent added to control excessive fermenter foaming and/or diffusion difficulties related to the size of yeast cells (5 μ as compared to 0.1 to 1.0 μ in the case of both bacteria).

4. *Simulation of Spent Media.* A decane-grown culture of *Achromobacter* exhibited a greater capacity to emulsify hydrocarbon than did one grown on glutamate (Fig. 5). This observation led to examination of the effects of several water-soluble, nonionic detergents on hydrocarbon emulsification and growth in shake flask cultures. These detergents, at a concentration of 0.001%, duplicated the activity associated with spent media in stimulating the rate of growth of all three microbes (Leavitt and Heilweil, 1966). For example, growth of *Brevibacterium* (Fig. 6) was comparably stimulated by Renex 688 or spent medium. On increasing the concentration of the detergent Igepal 520, both *Brevibacterium* and yeast assumed longer forms (Fig. 7) and sedimented faster. Growth inhibition was observed above 0.004% surfactant. Spent medium had no influence on cell length.

5. *Effect of Hydrocarbon Concentration.* Increasing the amount of oil to 10% permitted growth at an impeller speed that negated growth at an oil concentration of 0.5% (Fig. 8). An understanding of how excess oil results in a more efficient utilization of power can be seen in Figure 9. At a constant stirring speed, the distribution of oil in water is essentially nil at the lower oil level. Increasing the oil layer from 0.5 to

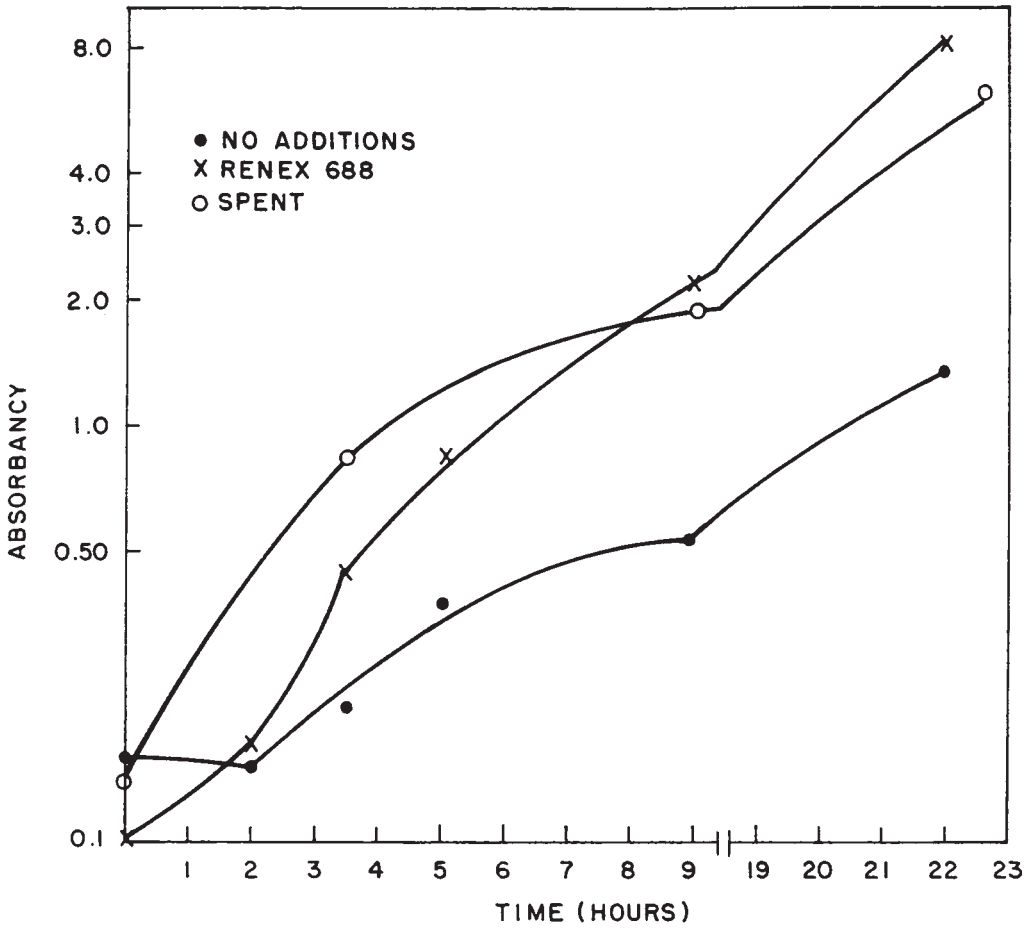

FIG. 6. Growth of *Brevibacterium* in presence of Renex 688 or spent media.

10.0% allows the oil vortex to impinge on the stirrer and the oil becomes distributed. The advantages noted with increases in oil concentration, addition of detergents, and the use of spent media may all be interrelated phenomena which maximize surfaces for growth on a hydrocarbon and thereby reduce power requirements. A beneficial effect on cell recovery was also caused by oil-soluble antifoam agents. If enough hydrocarbon remained in the culture to solubilize the latter, both bacteria and yeast aggregated and settled. If cells metabolized the last traces of oil, clumping ceased with a consequent increase in settling time.

Evaluation of Nutritional Quality

The amount of protein in *Achromobacter*, *Brevibacterium*, and *Pichia* was 55, 60, and 45%, respectively. Thirteen of the amino acids of each sample are listed in Table 4, as are predicted biological values and chemical scores. The latter correlate to the mean weight gain of rats plotted in Figure 10 and to protein efficiency ratios

TABLE 4. *Protein, amino acids, biological value, and chemical score of hydrocarbon-grown cells*

	Achromobacter	*Brevibacterium*	*Pichia*
Protein (% of cell wt.)	55%	60%	45%
Biological value (predicted)	95	78	94
Chemical Score (% of protein)	88	61	73
Amino Acids (% of Protein)			
Arginine	15.0	5.9	10.8
Cystine	0.89	0.2	1.49
Glycine	9.8	7.9	7.4
Histidine	3.8	2.7	5.9
Isoleucine	7.0	4.3	9.5
Leucine	10.7	7.4	11.1
Lysine	8.9	7.2	13.9
Methionine	3.1	2.2	2.3
Phenylalanine	6.0	9.9	9.4
Threonine	6.5	3.4	6.3
Tryptophan	1.5	1.0	2.0
Tyrosine	2.7	18.3	4.7
Valine	9.4	11.1	7.7

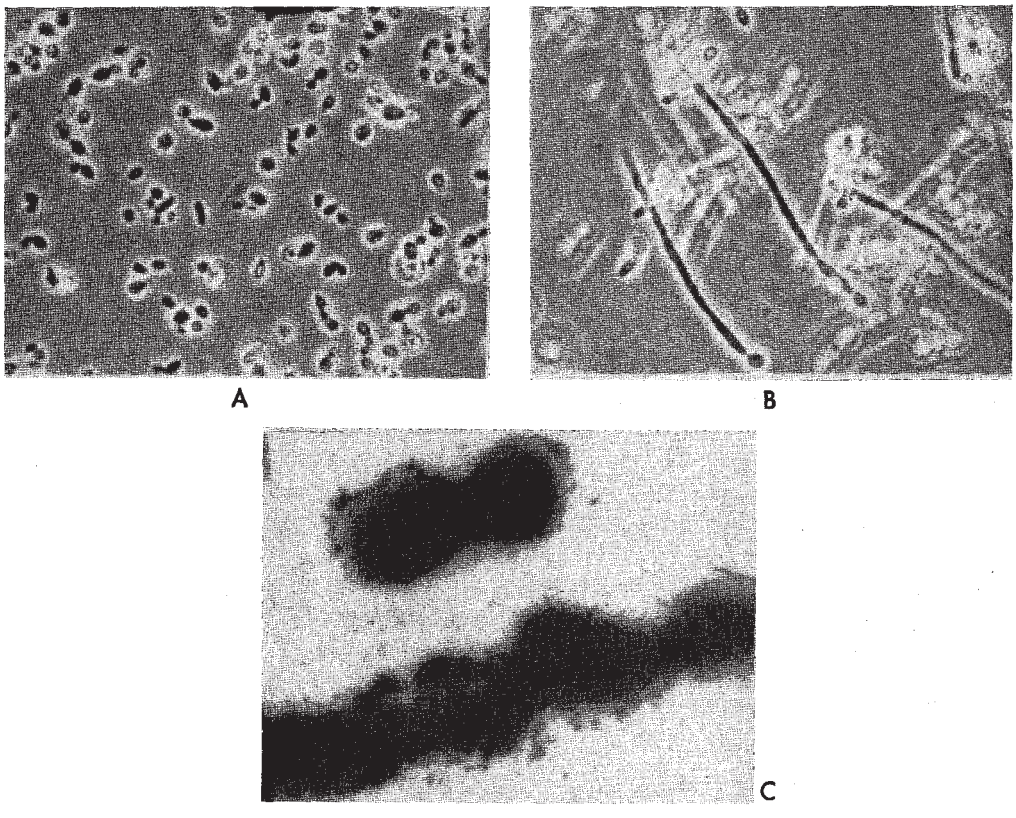

FIG. 7. Effect of detergent on cell morphology.
PICHIA (1000×)
A. No IGEPAL 520
B. IGEPAL 520 (4 ml/liter of a 1% preparation)
BREVIBACTERIUM (7000×)
C. IGEPAL 520 (2 ml/liter of a 1% preparation)

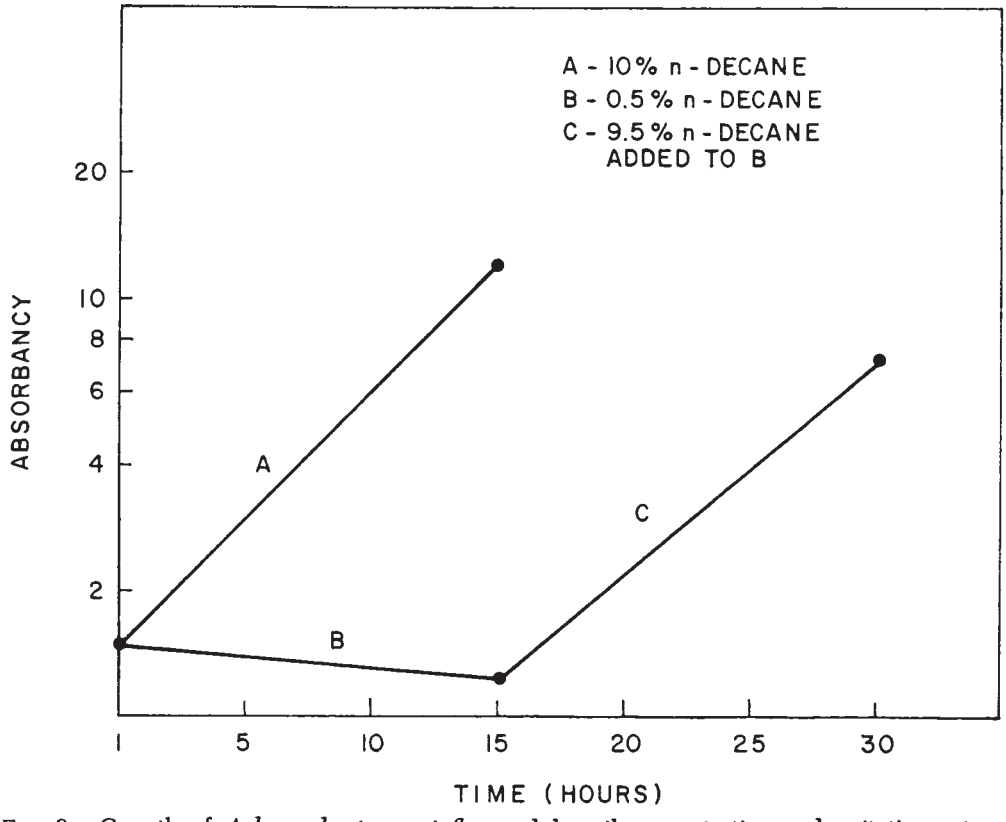

FIG. 8. Growth of *Achromobacter* as influenced by oil concentration and agitation rate of 400 rpm.

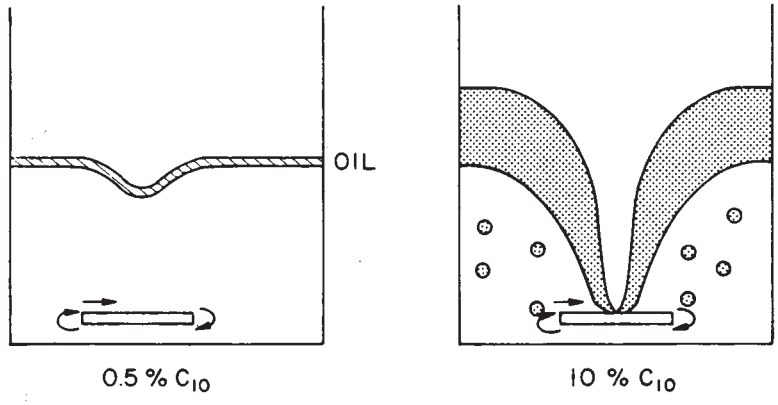

FIG. 9. Effect of hydrocarbon concentration on ease of emulsification.

of 3.2, 3.1, 1.15, and 1.8, respectively, for casein, *Achromobacter*, *Brevibacterium*, and *Pichia*. Tryptophan may have limited growth on *Brevibacterium* protein. Others have shown that methionine is the limiting amino acid in yeast (McNab and Rey, 1966; Laine et al., 1967). *Achromobacter* protein, therefore, can substitute for casein, the other samples could be used to supplement amino acid deficient materials.

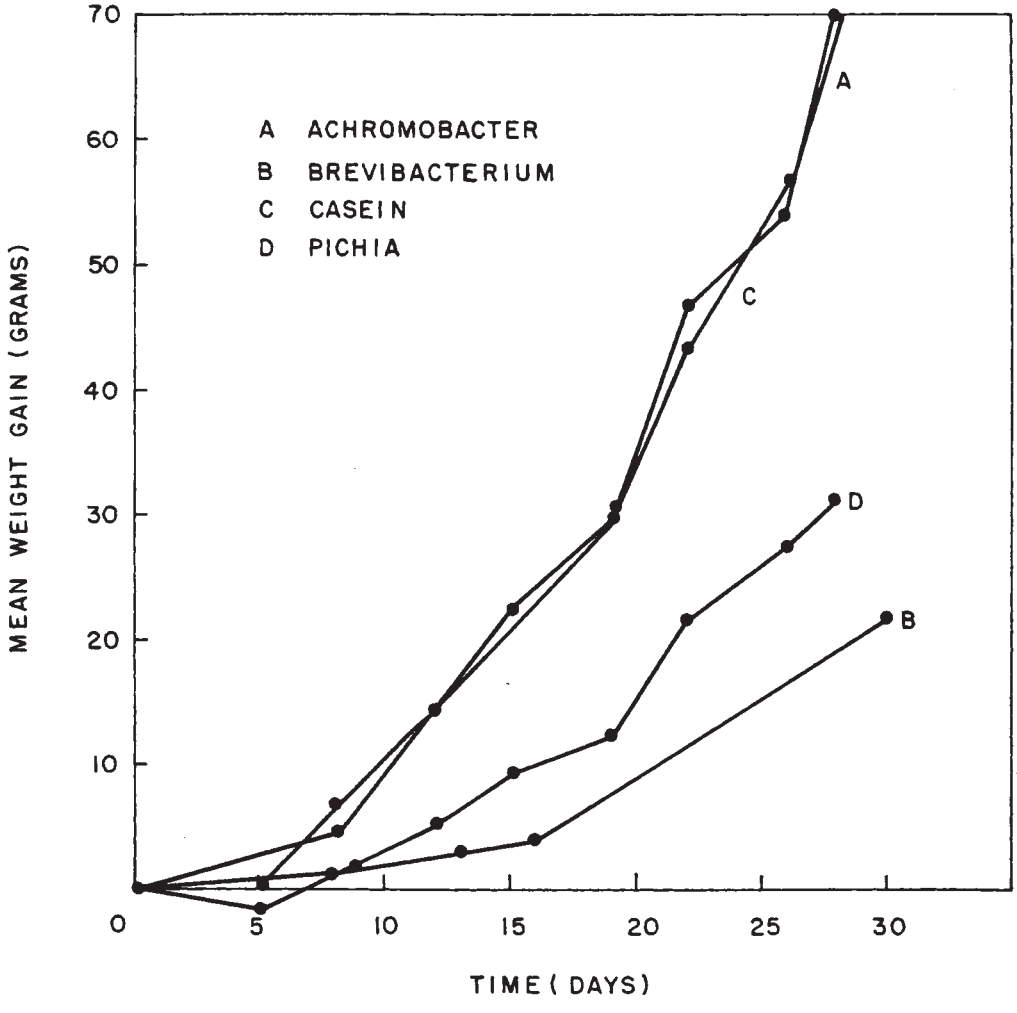

FIG. 10. Mean weight gain (per group) of rats fed either casein, *Achromobacter, Brevibacterium,* or *Pichia* as a protein source.

SUMMARY

Growth of several microbes on *n*-paraffins in the boiling range of kerosenes and gas-oils was followed in stirred fermenters. Growth was optimized by use of spent media, surfactants, increase in partial pressure of oxygen, and dialysis. Power requirements were reduced by some of the foregoing. Several surfactants promoted cell elongation and clumping, thereby enhancing cell recovery. Nutritional quality of the microbial proteins was evaluated from amino acid profiles and feeding tests.

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