MICROBIAL TECHNOLOGY

EDITED BY

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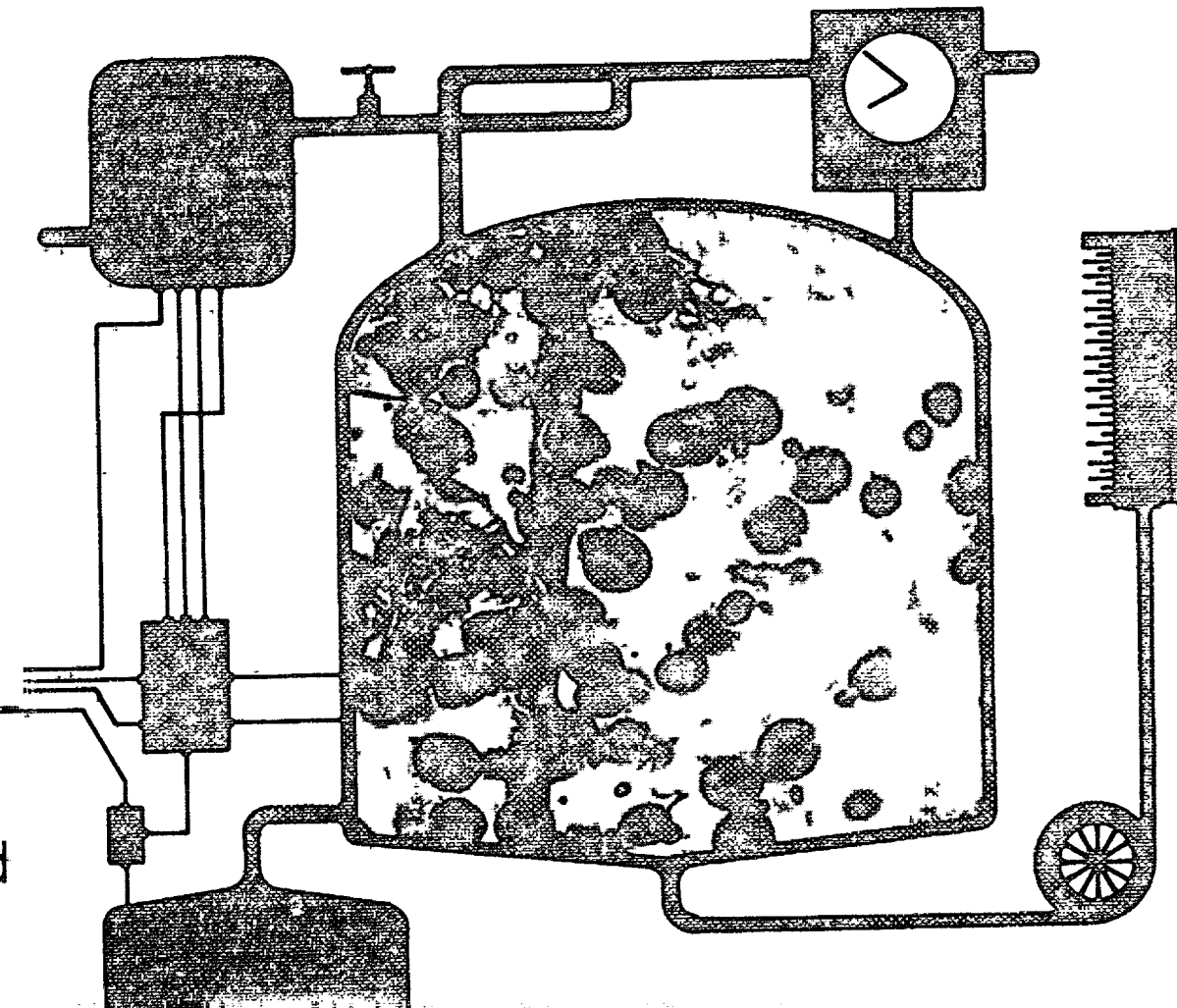

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Figure 5-1

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Contents

CHAPTER 1

Rhizobium Culture and Use

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INTRODUCTION

One of the marvels of Nature is the unique association of certain bacteria with leguminous plants resulting in the combining or fixing of atmospheric nitrogen. In 1886 two German scientists, Hellriegel and Wilfarth, reported their classical discovery that certain bacteria, later called rhizobia, enter the roots of young leguminous seedlings and induce the formation of nodules where they work symbiotically with their host to gather air nitrogen. While the soil is the natural habitat for rhizobia, they are not universally present; moreover, many of those present are often inferior in quality. The need to supply these bacteria artificially led to the development of the legume inoculant industry.

Culture of the nitrogen-gathering nodule bacteria in the laboratory for use in agriculture began shortly after Beijerinck isolated the causal organism from legume nodules in 1888. Early attempts to culture and use rhizobia as soil or seed inoculants for the betterment of legume crop production met with little success; yet many private and public laboratories were soon engaged in producing rhizobial inoculants for distribution to farmers. This period is best described as one of great enthusiasm, ambitious claims, and failure punctuated only by occasional success. Fortunately, vigorous promotion created demand and the legume inoculant industry survived.

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CHAPTER 17

Ethyl Alcohol, Lactic Acid, Acetone-Butyl Alcohol and other Microbial Products

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Among the oldest and most productive of the major industrial fermentations in the United States, ethyl alcohol, acetone-butyl alcohol, and lactic acid have lost the most ground to the nonfermentative, chemosynthetic routes. In the past decade particularly, the fermentation tonnage of industrial alcohol and solvents has declined to the point where each is only a minor factor in the total output. Thus, nonbeverage fermentation alcohol constitutes less than 10% of the total industrial alcohol production of nearly 2 billion pounds.[46,72] Similarly, the acetone capability of the lone domestic fermentation plant amounts to less than 5% of the current market of 1.2 billion pounds.

The lactic acid industry, an exclusive microbial production for many years, has also changed. Five years ago there were four major producers. Today there is only one,[33,45] and its dominant position in the 6 million pound domestic market is being challenged by a new, direct chemical process[43] capable of meeting the total demand.

Despite the decline of some of the older fermentations, mainly because of the high cost of raw materials, renewed efforts in research and development continue to brighten the outlook for microbial processing. Many lines

of research are taking another look at past projects and vigorously exploring the biosynthetic capabilities of a wide variety of microorganisms, cheap energy-rich substrates, and various technological designs and operations. While much of this activity is in the experimental stage, it merits brief mention here.

ETHYL ALCOHOL

Alcoholic fermentation is more generally known and more widely practiced than any other microbial technology. It flourished for centuries before the role of living yeast was established by Pasteur, a century ago, and pure culture principles were applied to improve the quality of the various end products. Today, despite the rising cost of raw materials, the alcoholic beverage industries—distilled spirits, beer and wines—are thriving (Table 17-1), but fermentation alcohol for nonbeverage uses has steadily lost ground to synthetic routes which now supply more than 75% of domestic needs (Table 17-2).

TABLE 17-1 Production of distilled spirits, beer and wine[71] (millions of gallons)

	1956	1961	1966
Distilled spirits (tax gal)[a]	720.7	801.7	889.3
Denatured alcohol (wine gal)	268.2	289.2	309.6
Beer (barrels × 31)	2811.6	2898.3	3401.8
Wines (wine gal)	159.1	189.1	264.2
Distilling material (wine gal)	344.5	323.8	475.4

[a] "Tax gallon" is equivalent to the "proof gallon" for spirits of 100 proof or over; for spirits less than 100 proof, the "tax gal" is equivalent to the "wine gallon."
"Gallon" or "wine gallon"—U.S. gallon liquid measure equivalent to 231 cu in.
"Proof gallon" is the alcoholic equivalent of a U.S. gallon at 60°F, containing 50% ethyl alcohol by volume.
"Proof" is the ethyl alcohol content of a liquid at 60°F, stated as twice the per cent of ethyl alcohol by volume.
"Barrel," as applied to beer, equals 31 wine gallons.

Of the traditional substrates—grains, fruits, and molasses—only the use of molasses has decreased almost to extinction. Thirty years ago, molasses fermentation accounted for 75% of the ethanol produced.[71] Today, because of high prices and shortages, the largest molasses fermentation plant in the world has been placed on standby.[10,42] Located in Philadelphia, Publicker Industries' plant has a capacity of more than 100 million gallons a year.

TABLE 17-2 Materials used for ethanol production[71]

Raw Material Used	Ethanol Produced: % of Total 1956	% of Total 1966	Millions Proof Gal 1956	Millions Proof Gal 1966
Grain and grain products	1.2	11.54	5.4	80.4
Molasses	25.5	1.59	126.7	11.0
Fruit	<0.01	4.07	—	28.3
Sulfite liquors	1.32	0.90	6.5	6.2
Cellulose pulp; chemical and crude alcohol mixtures	0.52	0.12	2.5	0.8
Whey	0.09	0.06	0.4	0.4
From redistillation	2.12	4.11	10.5	28.6
Ethylene gas	9.80	18.29	48.6	127.6
Ethyl sulfate	59.34	59.32	294.4	413.8
Total	100.00	100.00	496.2	889.3

TABLE 17-3 Manufacturing plants—ethanol production[71]

Plants	1965	1966
Distilled spirits	354	359
Denatured alcohol	46	49
Breweries	197	187
Bonded wine cellars		
Still wines	424	395
Vermouth	121	116
Other special natural wines	69	60
Effervescent wines	151	132

The variety and number of ethanol manufacturing and processing units are summarized in Table 17-3.

Fermentation Methods

Ethyl alcohol—also called ethanol, methylcarbinol, grain alcohol, spirits—can be produced from a variety of sugar-containing materials by fermentation with yeasts. Alcohol-tolerant strains of *Saccharomyces cerevisiae* are usually selected. They convert only hexose sugars to ethanol and carbon dioxide, theoretically yielding 51 and 49% by weight, respectively, as expressed by the Gay-Lussac equation: $C_6H_{12}O_6 \rightarrow 2C_2H_5OH +$

$2CO_2$. Sugars fermented by *S. cerevisiae* include glucose, fructose, mannose, galactose, sucrose, maltose, and raffinose.

MOLASSES ALCOHOL. Molasses, whether the by-product of sugar beet or sugar cane processing, contains about 55% sugars, approximately ⅔ sucrose and ⅓ glucose + fructose, which are easily and economically fermented. In a typical batch process, the molasses is diluted with water (20° C) to a sugar content of about 20% by weight, acidified to pH 4 to 5, and mixed in the fermentor with about 5% by volume of vigorous yeast culture. Acidity increases are adjusted with ammonia, and other nutrients may be added to stimulate yeast action. The rise in temperature during the two-day fermentation is controlled with external water sprays or internal cooling coils. Carbon dioxide evolved is collected for commercial use.

After alcohol accumulates to 8 to 10%, the fermented mash or "beer" is distilled, fractionated and rectified. With normal efficiency, one gallon of 190 proof (95%) alcohol can be obtained from 2.5 gallons of cane molasses (blackstrap). Still residues may be recovered.

In today's market, a gallon of alcohol is worth 52¢ per gal, for a raw material cost of 45¢. Estimated production and delivery expenses of 15¢ bring the total cost to about 60¢ per gal of ethanol.[19,42]

GRAIN ALCOHOL. Corn is the principal cereal grain used for alcoholic fermentation. It accounts for nearly 70% of the grains fermented to distilled spirits and almost 50% of the grains used in brewing.[71]

Since none of the commercial yeasts ferment starch, a grain mash of two or more milled grains is mixed with water, cooked to hydrate and gelatinize the starch, cooled to 62 to 64° C, and converted to fermentable carbohydrates with high amylolytic barley malt (170° Lintner). After cooling, stillage may be added to the mash to adjust acidity and supply yeast nutrients.

Fermentation practices vary widely,[49,69] but usually carefully propagated strains of *S. cerevisiae* (see Chapter 6) are prepared stagewise for inoculation of the main mash. At temperatures controlled near 30° C, and with adequate agitation, a vigorous yeast culture completes fermentation in 40 to 60 hours. The 6 to 8% alcohol in the fermented mash is distilled and purified. Stillage residues not used in cooking and dilution of the mash are recovered and dehydrated for animal feed use.

QTHER RAW MATERIALS. Small but significant amounts of ethanol for industrial use are derived from pulp mill waste and whey (Table 17-2), and a great variety of other materials, including starch milk slurry, enzyme extracts and honey.[71] No unusual techniques are applied, except in the fermentation of whey, which employs a lactose-fermenting yeast *Saccharomyces fragilis* or its imperfect (nonascosporogenous) form *Candida pseudotropicalis*. Alcoholic fermentation of spent sulfite liquor yields about

4 million gallons of ethanol from a single plant, the war-born Bellingham (Washington) fermentors now operated by Georgia-Pacific.[10,37] Detailed reports on procedures for processing and fermenting a wide variety of carbohydrate-containing materials are abundant.[1,37,49,69]

Industrial Alcohol

The production, trade and use of ethanol, both pure and denatured, are regulated by the U.S. Treasury Department's Internal Revenue Service, Alcohol and Tobacco Tax Division. Industrial alcohol is ethanol produced and sold for nonbeverage applications, appearing commercially in the form of pure ethanol and two classes of denatured alcohol: completely denatured alcohol (CDA) and specially denatured alcohol (SDA).

PURE ETHYL ALCOHOL. Pure alcohol meets criteria of U.S.P. XVII[70] and specifications of the American Chemical Society for use as a reagent and solvent in medicines, food products, flavorings, cosmetics, analytical laboratories, and research.

DENATURED ALCOHOL. Specially denatured alcohol is the most important type of industrial ethanol. It comprises over 99% of the 309 million gallons total volume of alcohol denatured in 1966. Of the more than 50 authorized SDA formulas, only four specific compositions account for over 80% of denatured alcohol production: SDA 29, SDA 1, SDA 2-B, SDA 3-A.

SDA 29, the most widely used formula, is denatured with acetaldehyde. Most of it is consumed in the manufacture of acetaldehyde, the raw material for acetic acid, *n*-butyl alcohol, resins, and dyes.

LACTIC ACID

Lactic acid has been made entirely by fermentation since 1881.[21] It became the first industrial fermentation chemical in the United States and preceded European application of Pasteur's discovery and proof, in 1857, that lactic acid fermentation is caused by living microorganisms.[67] Its long-established exclusiveness in fulfilling the domestic requirement of about 8 million pounds (as 100% lactic acid) is now confronted with a competitive chemosynthetic process.[8,43] By coincidence, the initial fermentation operation and the new synthetic process originated with the common objective of serving the baking industry. The former attempted to replace tartrates in baking powder with calcium lactate, and the latter supplies lactic acid to the largest single consumer to make calcium stearyl-2-lactylate, a bread additive.[8]

The combination of static sales prices and increased costs of both fermentable carbohydrates and lactic acid purification methods has no doubt

contributed to the decrease in the number of manufacturers from five to one since 1962.[45,46] Clinton Corn Processing Co. (Clinton, Iowa) is the lone fermenter today.

In the last twenty years, the price of food-grade lactic acid (50% solution) rose 1¢, from 16 to 17¢, while consumption doubled,[57] roughly following the growth of the food business,[41] the principal consumer, for acidulation of foods and beverages.

Fermentation

The usual cheap sources of fermentable carbohydrates—starch hydrolysates, whey, molasses—are suitable substrates when supplemented with nitrogenous nutrients and buffers. The choice of medium greatly influences the quality of crude lactic acid in the mash and the cost of its recovery and purification.[44,45] For the better grades of acid, modern practice prefers refined sugar media, minimal nutrient additions and high-grade calcium carbonate. The process described by Inskeep[33] and Schopmeyer[57a] illustrates the typical procedures involved in making commercial calcium lactate and the principal grades of lactic acid.

MEDIUM AND CULTURE. To 24,000 gallons of mash brought to 50°C is added 1125 gallons of *Lactobacillus delbrueckii* culture built up in successive transfers in mash of the same composition of the trade medium: 15% glucose, 10% calcium carbonate, 0.375% malt sprouts and 0.25% diammonium phosphate. During fermentation, the medium is controlled at 50°C automatically and recirculated constantly to keep the calcium carbonate in suspension. Fermentors and accessory equipment (agitators, coils, pumps, processing tanks) fabricated of type 316 stainless steel best resist the corrosiveness of lactic acid. However, combinations of glass and fluorocarbon resins ("Teflon") are employed in the design of wetted parts for pumps, piping systems, valves, filters, extractors, etc.

ACID RECOVERY. When the residual sugar content approaches 0.1%, in about 4 to 6 days, the batch is heated to 82°C, pumped to settling tanks, and held at 82°C during treatment with hydrated lime at pH 10. Following adequate mixing, the slurry is allowed to settle. The clear calcium lactate solution is decanted and combined with the filtrate of the settling-tank sludge. At this point, the calcium lactate liquor may be spray dried after treatment with sodium sulfide, acidification to pH 6.2 with lactic acid, decolorization, and filtration.

For food-grade acid, the lactate solution is mixed with activated carbon, filtered, concentrated under vacuum at 70°C, and converted to lactic acid by the addition of sulfuric acid. Calcium sulfate is removed by filtration.

The lactic acid filtrate is given a second carbon treatment, filtered, concentrated by evaporation to about 50%, purified with sodium sulfide, and decolorized again with carbon. Following control checks for metal ions, color, odor, and free sulfuric acid, the filtrate is either (1) adjusted to 50% lactic acid and carbon treated a fourth time to yield finished edible acid, or (2) returned to the acid evaporator for further concentration, bleaching, sulfide treatment, and adjustment to 88% lactic acid. The finished acid is stored in glass-lined tanks.

Technical grades of lactic acid (50 and 88%) are produced with the same medium and equipment, but processing involves fewer steps of decolorization and metal ion removal.

Higher grades of acid—for plastics manufacture, special food uses and U.S.P. quality[70]—can be produced by any of several methods:[57] (1) steam distillation under high vacuum, (2) solvent extraction, and (3) esterification with methanol, followed by distillation and hydrolysis.[20,21,57] The marked increase in purification costs is reflected in the relative market prices for four grades of lactic acid: the technical/edible/plastics/U. S. P. price ratio is 1:1.6:1.8:4.0, respectively.

YIELD. Homofermentative, thermoduric lactobacilli used commercially theoretically convert glucose quantitatively to D(−)-lactic acid:[54]

$$C_6H_{12}O_6 \rightarrow 2CH_3 \cdot CHOH \cdot COOH$$

Practically, a 100% yield of crude acid is never achieved, and the finished acid is usually the racemic mixture. Crude acid yields of 93 to 96% of the sugar fermented are normal,[57a] indicating some carbon utilization by lactobacilli and metabolic dissipation by contaminating organisms. The latter may produce racemase, the enzyme responsible for converting optically active lactic acid to the inactive mixture.[49]

Mechanical losses during processing and purification reduce the yield of finished acid to 85 to 90%, depending upon the type of fermentation medium, the yield and quality of crude acid, and the efficiency of the refining methods.

Ordinary commercial lactic acid is also known as DL-lactic acid, lactic acid of fermentation, 2-hydroxypropanoic acid, α-hydroxypropionic acid. The L(+)-isomer, commonly called sarcolactic acid or muscle lactic acid, is produced by some variants of *Lactobacillus casei*[54] and the mold *Rhizopus oryzae*.[49] Both isomers of lactic acid are available in limited quantity for research purposes. Miles Chemical Co. has produced pilot quantities of D(−)-lactic acid by a fermentation route.[8]

ACETONE-BUTYL ALCOHOL

In this year of its fiftieth anniversary as an industrial enterprise, the microbial production of acetone and *n*-butyl alcohol may have already

drifted into commercial obscurity. Recent reports[42] identify only one potential producer, Publicker Industries, Inc., Philadelphia. If the capacity of this plant, rated at 50 million pounds of acetone,[7] was fully utilized, it would account for only 4% of the 1200 million pounds of acetone currently consumed in the United States. Twenty years ago fermentation provided 65% of the butyl alcohol and 10% of the acetone[33a] needed.

Petrochemical routes now supply almost all of the butyl alcohol. Two-thirds of domestic acetone is by-product acetone from the cumene-phenol process; the remainder is derived from isopropanol.[42] Production of acetone and butanol by fermentation of molasses recently also ended in Japan.[12] Other countries abandoned the fermentation route for both solvents earlier.[46]

Fermentation

Many process reviews and patent citations touch upon the extensive research and turbulent industrial development of the acetone-butyl alcohol fermentation.[4a,37a,49] The synoptic technology related to only two principal fermentation processes, employing corn mashes and molasses wort, is outlined below.

CORN MEAL. Degerminated, ground corn, mashed at the rate of 6.5% in 65°C water with about 35% stillage and sterilized by conventional methods, is a complete medium for *Clostridium acetobutylicum*. Seed culture is developed aseptically in 5 to 6.5% corn mash at 37°C through three successive laboratory stages and as many plant stages as are needed to inoculate the trade fermentor (usually 6.5% corn meal) at the rate of about 3% by volume. During the 50 to 60 hour run at 37°C, fermentations are checked for acidity changes and the presence of contaminants. Certain lactic acid bacteria and ubiquitous bacteriophages are of grave concern.

The final beer, containing 17 to 24 grams of solvents per liter, is stripped in a continuous-type still. The crude mixture is separated by fractional distillation.

The yield of total solvents averages 26.5%, based on dry meal. The ratio of *n*-butyl alcohol:acetone:ethyl alcohol is around 6:3:1.

Recoverable by-products include dried still residues for animal feeds, carbon dioxide as dry ice, and hydrogen for fuel.

CANE MOLASSES. Both cane molasses (blackstrap) and high-test molasses (concentrated cane juice) are favorite raw materials when the price is right. Both substrates must be supplemented with ammonium nitrogen and phosphate.

Wort for seed culture and trade fermentations is prepared in a similar manner. Molasses is diluted with water and stillage to a sugar concentration of 5.6%, fortified with ammonium sulfate, calcium carbonate and superphosphate, sterilized at 107°C, and charged to sterile fermentors after cooling to 31°C. Seed cultures of *Clostridium saccharo-acetobutylicum*, or other suitable variants[4a,37a] of *Cl. acetobutylicum*, are built up in molasses wort at 31°C in the same manner described above for corn mash cultures. Following inoculation with 3% seed culture, active fermentation is followed with pH determinations, contamination checks, and gravity (Brix) measurements. Ammonia is added to hold the pH above 5.2 to 5.3 during the 40 to 45 hour fermentation.

Solvent and by-product recovery follows the same procedures used with beer from corn mashes. Yields, based on sucrose, range from 29% for blackstrap to 33% for high-test. Individual solvents in the mixture vary from 68 to 73% for *n*-butyl alcohol, 26 to 32% for acetone, and 1 to 3% for ethyl alcohol.

Former producers of acetone-butanol by fermentation were:

Commercial Solvents Corp., Terre Haute, Ind.
Publicker Industries, Inc., Philadelphia, Pa.
U.S. Industrial Chemicals Co., New York, N.Y.
Western Condensing Co., Adell, Wis.

TABLE 17-4 Range of interest in microbiological research

Cellular Products
(1) Protein:
  (a) Bacteria and yeasts grown on hydrogen, methane, *n*-alkanes, and other hydrocarbons.
  (b) Bacteria and molds propagated on various carbohydrates.
(2) Enzymes:
  (a) Bacterial hydrolases for waste treatment, isomerases.
  (b) Bacterial and fungal rennin.
  (c) Yeast protease for upgrading protein waste.
  (d) L-asparaginase for leukemia treatment.
(3) Deep culture of:
  Protozoa, basidiomycetes, mammalian cells, algae.

End Products
(1) Nucleotides and amino acids: both cellular enrichment for extraction and by direct fermentation.
(2) Polyols: glycerol, arabitol, erythritol, mannitol.
(3) Acids: zymonic acid, gentisic acid, salicylic acid.
(4) Sugars: fructose, sedoheptulose.
(5) Alkaloids.

Processing
(1) Extraction of metals from ores.
(2) Algae and bacterial mutants for sewage and industrial waste modification.
(3) Degreasing formulations.

MISCELLANEOUS PRODUCTS

Pioneering research and development efforts are pursuing many lines of interest: (1) application of large-scale deep culture methods to a wider variety of microorganisms; (2) development of mutants producing end products by direct routes, or strains of increased metabolic activity; (3) exploration of fossil fuels as fermentation substrates. Some of the research areas are listed in Table 17-4.

Cellular Material

To upgrade the quality and augment the quantity of the world's protein supply, a variety of microorganisms have been grown on familiar and unusual energy sources in deep culture systems to assess their potential

TABLE 17-5 Deep culture of microorganisms on large scale

Cellular Material	Energy Source	Reference
Bacteria		
Pseudomonas aeruginosa	*n*-Octadecane	18
Bacillus megaterium	Various sugars	65
Bacillus sp.	Methane	73
Nocardia sp.	*n*-Alkanes	51
Azotobacter vinelandii	Sucrose	36
Hydrogenomonas eutropha	Hydrogen	52, 56
Yeasts		
Saccharomyces platensis	Fish and animal protein	5
Candida intermedia, C. lipolytica	*n*-Alkanes, gas oil, paraffin wax	38
271 strains (amino acid content)	Glucose broth	2, 2a, 15, 39
Fat production	High C:N media	17, 74
Fungi		
Fungi imperfecti	Sugar beets, beet pulp, sweet potatoes, etc.	23, 24
Basidiomycetes	Various carbohydrates	53, 58
Mammalian Cells		
Kidney cells (BHK-21)	Glucose	66
Strains HeLa, L, KD	Glucose	77
Protozoa		
Colpoda steinii	*Escherichia coli*, viable cells	50
Algae		
Chlorella pyrenoidosa	Photosynthesis	61, 74
Spongiococcum excentricum	Glucose	78

value. One extensive survey determined the lysine, methionine and tryptophan content of 86 bacterial cultures[2] and 271 yeast strains.[39] An earlier study of 20 species of yeasts[15] focused on the total sulfur amino acid content and means of increasing it. *Rhodotorula gracilis* produced much more methionine and cystine than commercial yeast. Synthesis of methionine by *Saccharomyces cerevisiae* was not affected by the addition of intermediates—choline, cystine, threonine—to the growth medium.

Unusual microorganisms—autotrophic bacteria, molds, algae—have been suggested as food sources, while the use of natural gas, petroleum fractions, and coal as microbial substrates has exposed new potentials for large-scale development. Table 17–5 is a partial listing of the variety of microorganisms propagated successfully in mass culture for different purposes.

End Products

In the search for new fermentation products and process improvement, much of the major activity has centered on amino acid excretion (Chapter 12) and direct microbial routes for certain purine nucleotides, especially the flavor extenders: inosine-5′-PO_4, guanosine-5′-PO_4 and xanthosine-5′-PO_4. Many bacterial mutants capable of accumulating

TABLE 17-6 Fermentation biosynthesis

End Products	Microorganisms and Substrates
5′-Nucleotides	*Micrococcus glutamicus*; glucose,[16]
	Brevibacterium ammoniagenes,[35, 61]
	Corynebacterium petrophilum; hexadecane[32a]
Salicylic acid	*Pseudomonas sp.*; naphthalene,[75]
	Corynebacterium sp.; naphthalene[30, 31]
Gentisic acid	*Pseudomonas desmolytica*; naphthalene[40]
Zymonic acid	8 genera of yeast; glucose[62, 63]
Glycerol	*Torulopsis magnoliae*; hexoses[27, 28, 48]
Arabitol	*Zygosaccharomyces sp.*; hexoses[48]
Erythritol	*Endomycopsis chodati*; hexoses[28, 29]
Mannitol	*Aspergillus candidus*; glucose[59]
Fructose	*Acetobacter suboxydans*; mannitol[47]
Alkaloids	*Claviceps sp.*; glucose-mannitol[72]
Rennin	*Mucor pusillus*, wheat bran,[4]
	Endothia parasitica,[54a]
	Byssochlamys fulva; whey,[34]
	Bacillus subtilis, B. mesentericus,[68]
	Bacillus cereus; casein digest[19, 60]
L-Asparaginase	*Escherichia coli*[14a]