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Flavor Compounds Produced by Fungi, Yeasts, and Bacteria

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9.1 INTRODUCTION

Microorganisms play an important role in the generation of natural compounds, particularly in the field of food flavors. For a long time, plants were the sole sources of flavor compounds and most of them were isolated from essential oils. However, active compounds are present in low concentrations, which makes their isolation difficult. Another disadvantage of plants as a source of flavors is the dependence on factors that are difficult to control such as the weather and the risk of plant diseases. The production of flavor compounds by biotechnological methods has been an interesting alternative due to consumers' preference for natural ingredients. Microbial processes seem to be the most promising methods for the production of natural flavors.

9.2 MAIN TECHNOLOGIES

Many microorganisms are capable of synthesizing flavor compounds when growing on a culture media. They have the ability to perform conversions that would require multiple chemicals steps. Microorganisms are used to catalyze specific steps. They are also an economical source of enzymes, which can be utilized to enhance or alter the flavors of many food products (Kempler 1983). In this way, biotechnological processes involved in the production of flavor compounds can be divided into two groups: microbiological and enzymatic. Microbiological methods are subdivided into biosynthesis and biotransformation. The first is the production of chemical compounds by cells (fermentation or secondary metabolism). The second refers to the use of microbial cells in the specific modification of chemical structures (Welsh and others 1989).

In fermentation, the production of flavors starts from cheap and simple sources such as sugars and amino acids. The product is generated by the complex metabolism of the microorganism. When microorganisms are used in order to catalyze specific conversions of precursors and intermediates, the process is called biotransformation. Although fermentation requires C and N sources, a specific substrate is necessary for microbial transformation. The enzymatic catalysis precedes a simple and specific transformation of the substrate molecule. The substrate does not have to be "natural"; according to Schreier (1989) "non-natural" substrates can also be biotransformed.

It is important to distinguish research with the purpose of obtaining complex products with natural characteristics from those that try to obtain isolated molecules. The first consists in the experience of nature imitation and in developing a process with one or more microorganisms and enzymes. The second tries to obtain a higher yield of the characteristic components. The choice between them determines the methodology, which will be employed *in vivo* or *in vitro*, through biosynthesis or bioconversions (Delest 1995).

9.3 HOW TO OBTAIN FLAVORS

9.3.1 Flavor from Fermented Foods

The sensory properties of fermented foods are one of the key parameters in distinguishing these products from foods that have undergone undesirable spoilage. The organoleptic properties of fermented foods usually differ from those of the unfermented substrate

and are dependant upon the biochemical activities of the associated microorganisms (Cook 1994).

Fermentation has been practiced for the production of food since ancient times. It has become an effective technology for the production of organic acids, flavor compounds, and other biologically important chemicals. New aroma and flavors includes acids, alcohols, carbonyl compounds, esters, and pyrazines.

The use of microorganisms in the production of food has been practiced for a long time to improve the sensory quality of the food. Products such as beer, wine, distilled beverages, bakery, vinegar, fermented vegetables, milk, soybean, and meat are preserved, modified, and flavored using microorganisms. The flavor compounds of traditionally fermented foods originate from a complex microflora that acts in the chemical precursors of a food matrix (Berger 1995).

Lactic acid and alcoholic fermentations are the two important processes responsible for fermented food flavors. However, in some cases, the flavor is formed by specific fermentations (Joshi and Pandey 1999). The creation of new fermented products can result in the development of novel flavors and textures.

9.3.1.1 Dairy Products. Cheese flavors find application in snacks, sauces, baked goods, and several other products. Yogurt and buttermilk flavors are also useful. The cheese flavor results from the action of microorganisms and enzymes on milk's proteins, fats, and carbohydrates. Numerous breakdown products are formed, among them, short-chain fatty acids, acetic and lactic acids, alcohols, aldehydes, ketones, esters, sulfur and nitrogen compounds (Sharpell 1985). Marilley and Casey (2004) have reported that the use of bacteria strains for cheese ripening with enhanced flavor production is promising. They also mentioned that the catabolism of amino acids is presumably the origin of some major flavor compounds.

The starter cultures used in dairy technology are mainly prokaryotes like *Lactococci*, *Lactobacilli*, *Leuconostocs*, *Bifidobacteria*, *Propionibacteria*, *Streptococci*, and *Brevibacterium linens*.

Yeasts, such as *Kluyveromyces*, *Debaromyces*, *Candida* or *Trichosporon* are present in many manufactured milk products. These microorganisms modify the sensory characteristics of the products by synthesizing or assimilating volatile nitrogen and sulfur compounds.

Several chemical reactions take place in the surface of ripened cheeses such as Camembert and Brie during maturation due to fungi growth. The Fungal mycelium of *Penicillium roqueforti* grows rapidly and the resulting products are used directly for flavoring foods with a blue cheese-type flavor.

9.3.1.2 Alcoholic Beverages. Flavor compounds are produced as byproducts of yeast metabolism during alcoholic fermentation. Many flavor compounds have been identified in alcoholic beverages. The main compounds are listed in Table 9.1.

During alcoholic fermentation, yeasts transform sugars (glucose, fructose, and sucrose) into ethanol and carbon dioxide by the Embden–Meyrhof–Parmas pathway. This is the main bioreaction, but not the only one and, at the same time, several secondary byproducts are formed. Higher alcohols, organic acids, and esters are the main flavor compounds.

Higher alcohols, which contain more than two carbons, are also called fusel alcohols. They constitute the major portion of the secondary products of yeast metabolism. They

TABLE 9.1 Compounds Produced by Yeasts During Alcoholic Fermentation.

Class	Compounds
Esters	Amylacetate, butyl acetate, ethyl acetate, ethyl butyrate, ethyl lactate, ethyl benzoate, ethyl hexanoate, ethyl guaiacol, ethyl-2-methyl butyrate, ethyl-3-methyl butyrate, ethyl octanoate, ethyl octenoate, ethyl decanoate, ethyl dodecanoate, diethyl succinate, 3-methyl propionate
Alcohols	Ethanol, 2-methyl butan-1-ol (amyl alcohol), methyl butan-1-ol (isoamyl alcohol), heptanol, hexan-1-ol, 2-phenyl ethanol, 2-methyl propanol, glycerol, 2,3-butanediol, n-propanol
Carbonyls	Decalactona, decan-2-one, acetaldehyde, butyraldehyde, hexanal, nonanal diacetyl benzaldehyde
Acids	Acetate, butyrate, lactate, malate, succinate, hexanoate, nonanoate, octanoate
Sulfur derivatives	Methionol, ethanetiol, methylthioacetate, dimethyl disulfide, ethyl methyl disulfide, diethyl disulfide, 3-methylthiopropyl acetate, 2-mercaptoethanol, *cis*- and *trans*-2-methylthiophanol
Phenolic compounds	Vinyl phenol, ethyl phenol, ethyl guaiacol, vinyl guaiacol

include n-propanol, isobutyl alcohol, 2-methyl butanol, amyl alcohol, isoamyl alcohol, and 2-phenyl ethanol. Isoamyl alcohol accounts for more than 50% of the total concentration of higher alcohols.

Esters at appropriate concentrations impart flowery and fruity flavors. They are formed by esterification of fatty acyl-CoA or of organic acid by alcohols. Esters are present in very low amounts, near their threshold level. However, ethyl acetate has been found in wine in high concentrations.

Beer. Acetaldehyde, the most important aldehyde in beer, is formed as a metabolic branch point in the pathway from carbohydrate to ethanol. Its level varies during fermentation and ageing of beers, reaching 2–20 mg/L. At concentrations of 20–25 mg/L, acetaldehyde causes "green" or "vegetable" flavor.

Diacetyl and pentane-2,3-dione (vicinal diketones) have a characteristic flavor described as "buttery", "honey" or "toffee-like". They have a very high off-flavor potential, dependent on the fermentation temperature. The threshold for diacetyl in lager-type beers is 0.10–0.14 mg/L. At levels above 1 mg/L, it becomes increasingly "cheese-like" and sharp.

Volatile acids are usually present in beer at concentrations of 20–150 mg/L. Butyric and iso-butyric acids in concentrations of 6 mg/L cause a "butyric" or "rancid" flavor. Valeric and iso-valeric acids cause an "old-hop" and "cheesy" flavor. Fatty acids with 6 to 12 carbon fatty acids give the characteristic flavor of "cheesy", "goaty", or "sweaty" (Smogrovicová and Dömény 1999).

Wine. The chemical composition of wine is determined by many factors, among them grape variety, geographical and viticultural conditions, microbial ecology of the grape, fermentation processes, and winemaking practices. Microorganisms affect the quality of the grape before harvest and during fermentation. They metabolize sugars and other components into ethanol, carbon dioxide, and hundreds of secondary products that contribute to the characteristic flavor of wine (Fleet 2003).

9.3.1.3 Bakery Products. Although *Candida* yeast has occasionally been used for baking and some *Saccharomyces carlsbergensis* strains have been patented for use as baker's yeast, pure strains of *Saccharomyces cerevisae* are almost universally employed.

9.3.1.4 Mushroom Flavors. The commercially important mushrooms belong to the orders *Ascomycetes* and *Basidiomycetes*. Truffles (*Tuber* sp.) and morels (*Morchella* sp.) represent the *Ascomycetes*. *Basidiomycetes* are represented by *Agaricus bisporus*, *A. bitorquis*, *Lentinus edodes* (Shiitake), *Volvariella volvacea*, *Pleurotus* sp., and *Flammulina velutipes*. The main chemical compound responsible for the mushroom flavor is 1-octen-3-ol, although several others, including glutamic acid and 5′-guanylic acid, can modify the flavor, giving each mushroom species its distinctive characteristic. There is interest in growing mushroom mycelium in submerged culture and then utilizing the dried mycelium as a flavor compound (Sharpell 1985).

9.3.2 Biosynthesis of Flavor Compounds

Biochemical reactions as well as several nonenzymatic reactions involving sugars, fatty acids, and amino acids give rise to flavor during fermentation. Several reports and reviews have been published on the production of volatile compounds by microorganisms (Janssens and others 1988, 1992; Berger 1995; Jiang 1995; Christen and others 1997; Bramorski and others 1998; Soares and others 2000; Medeiros and others 2001). Although several bacteria, yeasts, and fungi have been reported to produce flavor compounds, a few species of yeasts and fungi are often preferred. However, only a few of them find application in the food industries due to their GRAS (Generally Recognized As Safe) status.

Flavor compounds derived from microorganisms are often produced in low concentrations. These compounds have low thresholds and can be detected by chromatographic methods in parts per million (ppm, μL/L). The amount and type of compounds secreted by microorganisms depend on the strain, with its enzyme-specific action, chemical composition of the culture medium, pH and temperature control, age of inoculum, and water activity of the substrate.

Flavor compounds produced by *Trichoderma viride, Penicillium roqueforti*, and *Penicillium decumbens* have been detected during the phases of growth or sporulation, depending on the culture medium (Latrasse and others 1985).

Singhal and Kulkarni (1999) presented a schematic representation of the flavor compounds produced by microorganisms (Fig. 9.1). Some examples of flavor compounds produced by microorganisms are also indicated in Table 9.2.

9.3.2.1 Esters. Esters are a very important class of flavor compounds of fresh fruits and fermented foods, which are found in concentrations between 1 and 100 ppm (Janssens and others 1992). The production of the ester ethyl acetate by the yeast *Candida utilis* from glucose is observed when the yeast grows on a medium containing a specific initial ethanol concentration.

Esters of low molecular weight are responsible for fruity odors and consist of acids and their derived compounds such as acetates, propionates, and butyrates. Some examples are ethyl butyrate and isoamyl acetate, which are found in strawberry and banana (Macedo and Pastore 1997).

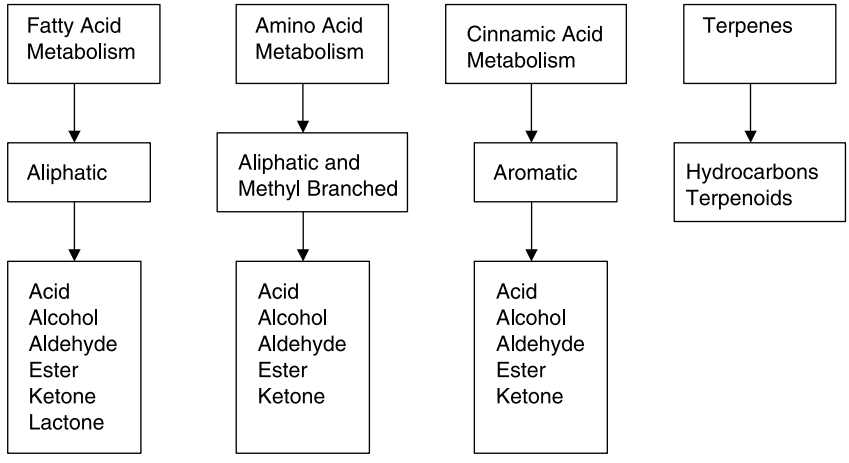

Figure 9.1 *Flavor compounds produced by microorganisms.*

The presence of esters such as ethyl acetate and butyric acetate in the culture medium can eventually describe a detoxification mechanism by which the microorganism avoids the accumulation of toxic compounds. The production of acetates occurs in order to detoxify the medium by converting acetic acid and high alcohols (Latrasse and others 1985).

TABLE 9.2 Microbial Production of Flavor Compounds.

Compounds	Microorganism
Diacetyl	*Saccharomyces lactis, Leuconostoc dextranicum*
Esters	
Geranyl acetate	*Ceratocystis virescens,C. variospora*
Ethyl butyrate and ethyl hexanoate	*Pseudomonas fragi*, *Streptococcus lactis*, *S. cremoris S. diacetylactis*, *L. casei*
Ethyl isovalerate	*Pseudomonas fragi*
2-Phenyl ethanol	*Erwinia carotovora*
Lactones	
α-Decalactone	*C. moniliformis*
t-Decalactone	*Sporobolomyces odorus*
s-Decalactone	*Saccharomyces cereviseae*, *Candida pseudotropicali*, *Sarcina lutea*
L-Menthol from menthyl esters	*Saccharomyces*, *Bacillus*, *Trichoderma*, *Candida*, *Rhizopus*
Pyrazynes	
2-Methoxy-3-isopropyl pyrazine	*Pseudomonas perolenes*, *Streptomyces*, *Streptococcus lactis*
Tetramethyl pyrazine	*B. subtilis*, *Corynebacterium glutanicum*
Terpenes	
Linalool	*Ceratocystic variospora*, *C. moniliformis*, *Phellinus igniarus*, *Kluveromyces lactis*, *Asocoidea, Lentinus lepidus*
Citronellol	*Ceratocystic variospora*, *C. moniliformis*, *K. lactis*, *Trametes odorate*
Geranyl acetate	*Ceratocystic virescens*
Citronemmyl acetate	*Ceratocystic coarulescens*

Source: Modified from Berger (1995) and Singhal and Kulkarni (1999).

Two metabolic pathways can be followed in the formation of esters: alcoholysis of acyl-CoA compounds and the direct esterification of an organic acid. Yeasts follow predominantly the first pathway, and filamentous fungi and bacteria prefer the second (Welsh and others 1989).

Some ramified amino acids are important precursors of flavor compounds and are related to fruit maturation. The initial reaction is called the enzymatic Strecker degradation. Several microorganisms including yeasts and bacteria such as *Streptococcus lactis* can modify the majority of the amino acid structures. Even if alcohols are related to fruit maturation, esters have a dominant role. Ethyl acetate comprises, with other compounds, banana flavor. 2-Methyl-ethyl-butyrate has a great impact on characteristic apple flavor (Fennema 1993).

Janssens and others (1987) found and quantified the fruity banana flavor produced by the yeast *Hansenula mrakii* and by the fungus *Geotrichum penicilliatum* in submerged fermentation using a synthetic medium. In the study using the yeast it was concluded that the fruity aroma occurred due to the biosynthesis of esters and alcohols. Seventeen compounds were identified in concentrations greater than 50 μL/L, including ethanol, ethyl acetate, isobutanol, ethyl propionate, isobutyl acetate, and isopenthyl acetate. Alcohols were formed in the exponential growth phase, but the esters were formed in the stationary phase. Ethyl acetate was the main product. In both studies, some precursors of fruity esters were added, such as vanillin, leucine, isoleucine, and phenylalanine. Thirty-three compounds were identified in concentrations greater than 50 μL/L: ethanol, ethyl acetate, ethyl propionate, and others. Ethyl acetate was produced in the highest concentrations (9924.1 μL/L).

Inoue and others (1994) reported the tolerance of *Hansenula mrakii* to ethyl acetate, which can be used as a sole carbon source. In this study, the esters formed during the production of sake by *Hansenula mrakii* and *Saccharomyces cerevisae*, were compared. Ethyl acetate, isobutyl acetate, and isoamyl acetate were preferably formed, determining the beverage quality. The formation of these compounds was catalyzed by the enzyme acetyl transferase from isoamylic acid and acetyl CoA.

Ethyl acetate, ethanol, acetic acid, and acetaldehyde were also produced by submerged fermentation and identified in the glucose metabolism of *Hansenula anomala*. In this case, the production of ethyl acetate was recognized as an aerobic process (Davies and others 1951).

Strains of *Ceratocystis* were also identified as ester producers. Lanza and others (1976) studied the production of acetates with different carbon (glucose, galactose, and glycerol) and nitrogen (urea and leucine) sources. They concluded that the type of flavor compounds produced depended on both sources (carbon and nitrogen), which is different for other microorganisms such as *Trichoderma viride*.

Collins and Morgan (1961) identified esters synthesized by different species of *Ceratocystis* (*C. moliniformis*, *C. major*, *C. coerulescens*, and *C. fimbriata*) during submerged fermentation in a dextrose potato medium. Ethyl acetate and ethanol were found in higher concentrations, except for *C. fimbriata*, which had isobutanol as the main compound. A strong banana flavor was detected when using dextrose and urea due to the presence of isoamyl acetate, which was also identified in leucine- or isoleucine-based media. For the combination galactose–urea, the main flavor was citric due to the formation of terpenes.

The yeast *Kluyveromyces marxianus* produced some compounds with characteristic fruity flavor, with ethyl acetate found in higher concentrations in solid-state fermentation

of different agro-industrial residues such as cassava bagasse (Medeiros and others 2000, 2001).

Banana flavor has also been identified in some plants when microorganisms (*Erwinia caratovora* ssp atroseptica) infected them. Spinnler and Dijian (1991) identified the volatiles formed in a synthetic medium, similar to the ones in the infected plant. From 13 isolated microorganisms with capacity to produce esters and alcohols, several compounds were identified; including ethyl acetate, 2-methyl-1-propanol, and propyl acetate. In this study, different media were tested with different nitrogen and carbon sources. Better results were found with glucose, fructose, sucrose, and asparagine. The addition of leucine led to the production of isoamyl acetate, corresponding to the degradation of the amino acid following the Erlich route.

Beiju is made of naturally fermented cassava, with fruity characteristics. It was used by the indians in Maranhão, North of Brazil, in order to produce a typical alcoholic beverage, *tiquira*. The microbiological population was identified and quantified by Park and others (1982), with counts between 6×10^5 and 1.9×10^6 CFU, as being predominantly *Aspergillus niger* and *Pecylomyces* sp.

Yoshizawa and others (1988) identified some volatiles produced by a strain of *Neurospora*, isolated by Park and others (1982), in submerged fermentation. These include ethanol, ethyl acetate, isoamyl acetate, ethyl hexanoate, and acetoine.

9.3.2.2 Aldehydes. Aliphatic, aromatic, and terpenoid aldehydes are important contributors to the flavor of fermented dairy products. They are synthesized by microorganisms as intermediates in the formation of alcohols from keto acids. An example is the bioconversion of ethanol to acetaldehyde by *Candida utilis.*

Flavor production using immobilized lipase from the yeast *Candida cylindracea* in a nonaqueous system has been studied for producing a broad range of esters including ethyl butyrate, isoamyl acetate, and isobutyl acetate. Ethyl butyrate has a pineapple–banana flavor, which has a large market demand, and sells at a price of US$150/kg upwards. This process has shown a great stability of the enzyme (more than a month) if kept hydrated intermittently (Singhal and Kulkarni 1999).

9.3.2.3 Alcohols. Alcohols do not contribute as a flavor component unless present in high concentration. They are formed as a primary metabolite from microorganisms' activity or due to the reduction of a carbonyl. Fusel alcohols can be formed from either carbohydrate or amino-acid metabolism and are the predominant volatiles of all fermented beverages, in addition to ethanol.

Different alcohols can be found in the culture of yeasts such as ethanol, propanol, isobutanol, and phenyl ethyl alcohol. In filamentous fungi it is possible to find methyl-3-buthanol, butanol, isobutanol, pentanol, hexanol, octanol-3, and phenyl ethanol from the metabolism of amino acids such as leucine, valine, isoleucine, and phenylalanine (Welsh and others 1989).

9.3.2.4 Carbonyls. Among the ketones, odd-numbered 2-alkanones from five to eleven carbons, along with free fatty acids and 2-alkanols, determine the flavor of *Penicillium*-ripened cheese and have received much attention. Bacteria such as *Aureobasidium*, yeasts, and higher fungi produce 2-alkanones, but only *Penicillium* has been used industrially.

9.3.2.5 Terpenes. Terpenes are the most important natural components of essential oils to be used as flavors. Microorganisms are able not only to synthesize but also to degrade or transform terpenes. Fungi are the main microorganisms responsible for terpene production, but bacteria are capable of synthesizing a few volatile terpenoids, such as geosmin and cadin-4-ene-1-ol (Berger 1995).

The synthesis of monoterpenes by *Ceratocystis variospora* has been studied by Collins and Halim (1972). Numerous other microorganisms are able to synthesize monoterpenes, among them *Ceratocystis moniliformis*, *Kluyveromyces lactis*, *Sporobolomyces odorus*, *Trametes odorata* and *Trichoderma viridae*.

Microbial bioconversion of terpenes has been studied by several authors. Monoterpenoid compounds like citronellal, citral, limonene, and menthol (acetates) can be biotransformed in citronellol, geranic acid, carveol and 1-menthol, respectively.

Valencene is a sesquiterpenoid available from orange oil and has little commercial use. Some bacteria are capable of transforming valencene to nootkatone, a main flavoring component of grapefruit.

From the economic point of view, the development of biotechnological processes for the production of terpenes is not viable due to the low yields obtained and the abundance of vegetable sources available. The real meaning of these studies is the understanding of the steps of the catabolism of terpenes.

9.3.2.6 Lactones. Lactones are associated with fruity, coconut, buttery, sweet, or nutty flavors. *Trichoderma viridae*, a soil fungus, generates a characteristic coconut flavor due to the production of 6-pentyl-2-pyrone. The main component of peach flavor, 4-decalactone, can be synthesized by *Sporobolomyces odorus. Aspergillus niger* can transform β-ionone into a complex mixture resembling tobacco flavor. Lactones make a significant contribution to the flavor of several fermented foods like dairy products and alcoholic beverages.

Some microorganisms such as *Ceratocystic moniliformis*, *Trichoderma viride*, *Sporobolomyces odorus*, and some species of *Candida* have been reported as lactone producers. However, the production is not very significant and has low yields (mg/L), except for the *in situ* production of lactones from dairy products.

Lee and Chou (1994) verified that the addition of 3% castor oil to the culture medium raised the production of lactones by *Sporobolomyces odorus*, with a yield of 8.62 mg/L.

Among lactones, 6-pentyl-α-pyrone (6-PP) presents the most interesting flavor properties. It is a molecule with a strong coconut flavor and is also present in the aroma of peaches and nectarines. The production of 6-PP by *Trichoderma harzanium* with sugar cane bagasse by solid-state fermentation was studied by Sarhy-Bagnon (1999) as an alternative for the production by submerged fermentation, giving a six fold raise in concentration.

9.3.2.7 Pyrazines. Pyrazines are typical flavor components of heated foodstuffs. They give the roasted or nutty flavors characteristic of roasted nuts, coffee, and cocoa beans, and baked and meat products. Microwave foods need the addition of pyrazines because they do not develop a characteristic nonenzymatic browning flavor during cooking. *Bacillus subtilis* was the first organism found to produce pyrazine. Pyrazines were also identified in cultures of *Septoria nodorum* and *Aspergillus parasiticus*.

9.3.3 Enzymatic Technology

Enzymatic processes that are used to obtain flavors can be described by the hydrolysis of some compounds without microbial growth. The majority of the enzymes used in food processing are hydrolases, such as amylases, proteases, pectinases, cellulases, pentonases, invertase, and lactase. They are used, for example, in cheesemaking (lipases, proteases), wine and juice production (pectinases), lactose reduction (lactase). Immobilization techniques, such as gel inclusion, microcapsules entrapment, and covalent or adsorptive binding onto solid supports has improved technical aspects such as handling, recycling, and long-term stability. Microbial enzymes have become an integrated part of processes in the food industry, So it is natural to see their use for the generation of flavor compounds (Whitaker 1991).

Filamentous fungi are capable of producing enzymes that are used to hydrolyze plant cell wall and liberate its content. However, the enzymatic extraction needs a thermal treatment, which sometimes can destroy or change flavor compounds.

Pectinase, cellulase, and hemicellulase of *Aspergillus*, *Penicillium*, *Rhizopus*, and *Trichoderma* are enzymes more commonly used to increase extraction efficiency during fruit, vegetable, cereal, or juice processing (Armstrong and others 1989).

Lipases often show complex patterns after isoeletric focusing, but this heterogeneity is due to the varying degrees and positions of glycosilation of the protein core. Pre-pro-lipase and pro-peptides are now studied in detail by genetic engineering (Berger 1995).

Microbial hydrolases have been reported to improve the sensory quality of food by the synergistic action of mono-, oligo-, and poly-glucanases. Various carbohydrases have been purified and characterized, among them, β-glucosidase from *Aspergillus niger* and α-glucosidase, with maltase properties from banana pulp. Carbohydrases have contributed to the assessment of the identity and origin of plant products, to the understanding of changes during processing and maturation, and to the selection of flavor-rich cultivars. Stability and selectivity data will be decisive for sensory changes in a food and thus for the future application of new enzymes in food processing (Berger 1995). Smaller peptides and free amino acids, which are end products of various proteases, contribute to the non-volatile flavor fraction and act as precursors of volatiles.

Cheese treated with enzymes to enhance flavor, or a significant portion of the flavor profile, is considered to be enzyme-modified cheese (EMC). It provides the food manufacturer with a strong cheese note in a cost-effective, nutritious, and natural way (Moskowitz and Noelck 1987). Such EMCs are ideal in frozen cheese, because proteins from natural cheese tend to coagulate and produce a grainy texture, but the proteins in EMCs have been hydrolyzed to more soluble peptides and amino acids, overcoming these problems (Missel 1996).

EMC flavors available commercially include Cheddar, Muzzarela, Romano, Provolone, Feta, Parmesan, Blue, Gouda, Swiss, Emmental, Gruyere, Colby, and Brick. These cheese flavors have a wide range of applications in salad dressings, dips, soups, sauces, snacks, crisps, pasta products, cheese analogs, frozen foods, microwave meals, ready-made meals, canned foods, crackers, cake mixes, biscuits, quiches, gratins, cheese spreads, low-fat and no-fat cheese products, and cheese substitutes (Buhler 1996).

The basis of EMC technology is the use of specific enzymes acting at optimum conditions to produce typical cheese flavors from suitable substrates. These enzymes consist of proteinases, peptidases, lipases, and esterases. EMCs can be used in food recipes to fulfill several roles, for example, as the sole source of cheese flavor in a product, to

intensify an existing cheesy taste, or to give a specific cheese character to a more bland-tasting cheese product (Anon 1993). They have approximately 15 to 30 times the flavor intensity of natural cheese and are available as pastes or spray-dried powders (Freund 1995). The production of EMC is an important industrial activity, which has grown due to a greater demand for convenience foods, together with the health-related concerns regarding the amounts of fat, cholesterol, and cholesterol-producing saturated fat in traditional dairy products. EMC has been included in no-fat and low-fat products, replacing the functional and flavor characteristics of fats previously derived from natural cheese (Anon 1993; Freund 1995). The addition of EMC creates the desired flavor without increasing fat content. It can be added at levels of 0.1% (w/w) and contribute less than 0.07% fat or 2.28 calories per 100 g. Most new applications are targeted at texture and provide rich mellow tones, pleasant flavor-enhancing effects, fatty mouth-feel, flavor masking, rounding-off of sharp spicy notes, and harmonization of other flavor ingredients (Buhler 1996).

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10

Flavor Production by Solid and Liquid Fermentation

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10.1 INTRODUCTION

Microbial fermentation is a promising biotechnological technique for the production of natural flavors. Although many biotechnological processes have been reported, most have not yet been applied in industrial production. The major reason for this is the low yield. Microbial flavors are often present in low concentrations in fermentation broths,

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1.06

Technologies Used for Microbial Production of Food Ingredients

Anthony L. Pometto III and Ali Demirci

CONTENTS

6.1 INTRODUCTION

The goal of this chapter is to supplement the industrial microbiology components in the first edition of *Food Biotechnology* by Knorr (1) and to present an updated overview of various technologies currently under investigation and employed by the food industry for the production of microbial food ingredients. For the detailed description of industrial microbiology principals, Demain and Davies *Manual of Industrial Microbiology and Biotechnology*, 2nd edition (2) is highly recommended.

For the food industry, the bottom line is cost. They are selling commodity products (food) with various levels of preconsumer processing. The goal is always to produce the most nutritious and safe product at the lowest possible cost.

For centuries microorganisms have been employed for the production of fermented food products (i.e., cheese, soy sauce, sauerkraut, wine, and bread). The consumption of some live microbial cultures (probiotics) has proven to provide a health benefit to humans and animals (3,4). Some foods containing live cultures are yogurt, buttermilk, and acidophilus milk. These microbial fermented food products also have an extended shelf-life

compared to the perishable starting raw material. Thus, microorganisms not only provide a nutritional benefit to humans but act to extend the shelf-life of the food supply.

Microorganisms employed by the food industry include bacteria, yeasts, and molds. These microorganisms have several morphological and physiological differences. Morphologically bacteria are small and difficult to remove, yeasts are larger and will sometime settle out of solution, whereas molds are filamentous and are typically removed by filtration. Physiologically they differ in pH preferences (yeasts and molds prefer a lower pH than bacteria), nutrient requirements (different concentrations and types of nitrogen and other trace elements), growth rates (bacteria grow much faster than yeasts and molds), and more. Thus, different culture media, fermentation methods, and product recovery methods are required depending on the microbial system being cultured.

6.2 MICROORGANISM SELECTION AND DEVELOPMENT

Microorganisms are the biocatalysts that produce and maintain a host of enzymatic pathways that are used to produce the food component of interest. The characteristics of a good industrial microorganism for the production of food ingredients are (1) it must be effective in producing large quantities of a single product, (2) it can be efficiently isolated and purified, (3) it is easy to maintain and cultivate, (4) it is genetically stable, (5) it grows best in an inexpensive culture medium, and (6) it is safe for human consumption. The first step is to isolate the hardiest starter culture possible, then to begin strain improvements via classical mutagenesis or genetic engineering.

A classic example would be the production of L-phenylalanine for the artificial sweetener aspartame (NutraSweet® J.W. Childs Equity Partners II L.P.), which is a dipeptide of L-phenylalanine and L-aspartic acid. When NutraSweet first entered the market in 1981, the L-phenylalanine supply became the bottleneck for production. L-Phenylalanine, L-tyrosine, and L-tryptophan are produced via the shikimic acid pathway in all organisms. To develop a bacterium which over produced L-phenylalanine, first classical chemical mutagenesis of an L-tyrosine auxotroph of *Corynebacterium glutamicum* was employed using -phenylalanine analog resistance in an effort to reduce end product inhibition, and L-tyrosine production (5). Analogs such as *p*-aminophenylalanine, *p*- and *m*-fluorophenylalanine, and β-2-thienylalanine were incorporated into the cellular protein thus poisoning the cell. To combat this poison, surviving mutants must overproduce L-phenylalanine, thus neutralizing the toxic effects of the analogs. This process was repeated several times with mutants resistant to increasing concentrations of analogs. The final analog resistant bacterium selected by Hagino and Nakayama (5) produced 9.5 g/L of L-phenylalanine.

The over producing bacterium was then transformed with plasmids containing L-phenylalanine analog resistant chorismate mutase and prephenate dehydratase genes (6). These are two key enzymes in the shikimic acid pathway for L-phenylalanine production. Except for constitutive enzymes, most enzymes in the cell have a short half life in the cell. Thus, an increase in key enzyme concentrations and residence time in the cell will also increase production. Ozaki et al. (6) transformates produced 19.0 g/L of -Lphenylalanine, thus, illustrating how classical mutagenesis and molecular genetic techniques are employed to further enhance production of some desired metabolites for the food industry.

Another alternative method was whole bioconversion developed by Yamada et al. (7) which produced L-phenylalanine from trans-cinnamic acid via L-phenylalanine ammonia lyase (PAL) reversal in *Rhodotorula glutinis*. In the presence of concentrated ammonium hydroxide, the PAL reversal demonstrated a 70% conversion yield which produced 17.5 g/L of L-phenylalanine. By utilizing a whole cell bioconversion process, no enzyme

purification step was needed and the enzyme proved to be more stable within the yeast under the harsh conditions employed. This process was used to produce some of the initial L-phenylalanine used for the production of NutraSweet. Eventually, however, production by the genetically engineered bacterium exceeded the levels in fed-batch fermentation, which did not involve caustic chemicals, and thus, became the method of choice. L-Phenylalanine purification is performed by ion exchange chromatography for all methods.

The genetic stability of cultures requires minimum culture transfers and long term storage capabilities. Fermentation media are inoculated from working cultures which are produced every few months from master cultures depending on the microorganism. The most common procedures for long term storage are freeze drying ($<-18^{\circ}C$) and ultra-low temperature storage ($-70 - -80^{\circ}C$). Freeze drying requires a cryoprotectant, such as sterile skim milk, followed by freeze drying and vial sealing under a vacuum (2). Sealing under nitrogen gas can also help to stabilize the culture and extend the shelf life. Ultra-low temperature storage is in a rich culture medium with 20% sterile glycerol. Some cultures are sensitive to freeze drying, thus, ultra-low is the most common method employed today, because of long-term culture viability. The risk is loss of electrical power and refrigeration problems.

Suspended cell cultures or spore suspensions are used as inocula for these industrial scale fermentations. Purity is constantly checked until inoculation. For suspended cell inoculation the sequence employed would be culture slant, shake-flask culture, benchtop fermentor, pilot-scale fermentation, then into full scale fermentation. Many fungal fermentations, such as citric acid and soy sauce fermentations, required a suspension of viable fungal spores as the inoculum. These spore suspensions are generated on large agar trays, and then are aseptically transferred into culture bottles suspended in sterile water or saline (2).

Microbial systems frequently constitute efficient mechanisms for the production of nutritionally important ingredients at a relatively low cost, for example, the production of selenomethione in yeast. A slight modification in the yeasts culture medium will force the yeast to substitute the sulfur group in methionine and cysteine with a selenium in standard fed-batch fermentation (8,9). To identify this medium change, Demirci and Pometto (8) developed a gradient delivery unit producing a gradient of sodium selenite or sodium selenate in a continuous bioreactor (Figure 6.1). It has been shown that selenium has several health benefits (10) including a cancer-protective effect (11), and a profound effect on the survival of HIV-infected patients (12).

Furthermore, microbial systems are ideal for the production of essential micronutrients such as amino acids, vitamins, and enzymes, and bulk ingredients such as organic acids and alcohols, whole cell flavor enhancers, and polysaccharides.

6.3 CULTURE MEDIA AND UPSTREAM COMPONENTS

The ideal culture medium will use inexpensive components to supply their complex nutrient requirements. Miller and Churchill (13) provide an excellent summary of inexpensive media components and their makeup. These ingredients are crop, animal, marine or yeast based components. The culture medium alone can represent 30 to 70% of the fermentation production costs. Slight changes in medium micronutrients can have a major impact on the fermentation (14,15). Thus, the food industry demands a consistent product from suppliers of these complex components. Failure to provide a consistent product will eliminate the commercial use. What decides the culture medium makeup? Essentially, it is the nutritional requirements of the microorganism of choice and its ability to biosynthesize essential elements such as amino acids, vitamins, lipids, and carbohydrates. For example, bacteria and yeast are high in protein (40–50%), whereas molds are not (10–25%). Yeast

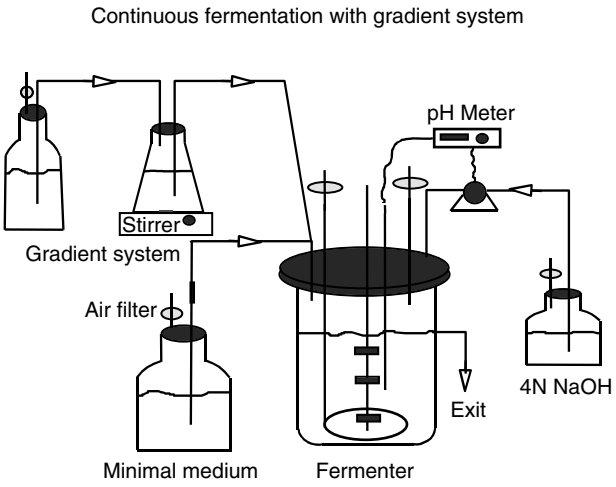

Figure 6.1 Continuous bioreactor gradient delivery system design used for developing the production protocols for selenomethionine production in yeast.

can generally grow in a minimal medium, whereas lactic acid bacteria, essential for the fermented dairy products, require a host of micronutrients to grow.

Each microorganism has different micronutrient requirements. Typically the exact micronutrient which benefits the fermentation that is being supplied by the complex nutrient employed is unknown. Generally it involves specific amino acids, vitamins, trace elements, and lipids. Furthermore, the concentration ratio of carbohydrate to nitrogen and phosphorous has a dramatic impact on microbial growth. Carbon–nitrogen imbalances can result in the production of other byproducts such as extracellular polysaccharide, and fermentation end products such as ethanol. In the case of ethanol production in yeast, excess glucose in the presence of oxygen will direct yeast to produce ethanol. This is called the Crabtree effect (16,17). Yeast cells typically consist of 48% carbon and 8% nitrogen on a dry weight basis. When the C:N ratio is 10:1, yeasts grow aerobically consuming little substrate while producing maximum cell mass, CO_2 and H_2O, but when the C:N ratio is 50:1 yeasts grow anaerobically, consuming much substrate while producing little cell mass, and much CO_2 and ethanol. This difference in yeast growth is also called the Pasteur effect (18).

Why the increase in industrial microbiology fermentation processes over the past 20 years? It is the result of the corn syrup sweetener industry and computer technology. Corn is 70% starch, and when dried to $<$19%, it can be stored for more than two years. Annually 10 billion bushels of corn are produced in the Unites States of America. Thus, the liquefaction of corn starch to glucose syrups for the production of high fructose corn sweeteners represents a consistent, low cost supply of substrate for most industrial microbiology fermentations. Not only is glucose produced, but customized substrates can be also produced. For example, corn syrups containing 19% dextrose, 14% maltose, 12% maltotriose, and 55% higher saccharides are used to control microbial growth rates and biological heat production in many fermentations. Thus, glucose is the platform chemical used for the microbial production of organic acids, amino acids, vitamins, and more. In fact some food grade fermentation facilities have located adjacent to a corn sweetener facility to permit glucose syrup to be piped directly to their fermentors. Glucose corn syrup is also shipped via truck or rail cars as liquid or dried product.

Furthermore, the advent of computer process controls of industrial scale fermentors has removed many of the fears associated with commercial scale fermentation by providing

reliable and easy to operate dissolved oxygen, pH, foam, temperature, and sterilization controls of the process. Sirakaya et al. (19) described fermentation software to monitor and control the utilization fermentation process.

6.4 BIOREACTOR MONITORING SYSTEMS AND DESIGN

The stirred tank bioreactor design is the most common fermentor and consists of agitator, baffles, aeration sparger for aerobic culture growth, sterilizable monitoring probes for pH, dissolved oxygen, temperature, and antifoam, filling and draining ports, and often culture medium sterilization capabilities in the reactor tank. Reactor agitation is essential for temperature control, pH adjustments, oxygen absorption into the liquid medium, overall culture health, and mixing of any required additions of substrate, and nutrients. Typical commercial reactor working volumes for food grade ingredients are 5,000 to 40,000 gallons.

Fermentation health requires real time monitoring system. Microbial growth can be monitored via several methods. The most common method is indirect measurement of biomass by absorbance of the fermentation broth at 620 nm by using a spectrophotometer. The measured absorbance values can then be used to estimate biomass concentrations by using a standard curve. Standard curves are developed by collecting fresh log phase cells via centrifugation, washing cells with water or 0.1 M ammonium acetate pH 7.0 buffers, then serially diluting the pellet (20). Absorbance for each dilution is then determined spectrophotometrically at 620 nm. The actual dry weight biomass (g/l) is determined for each dilution via direct biomass measurement after oven drying of each dilution in preweighed boats at 70°C for 24–36 hr. This needs to be performed in at least replicates of three. By washing biomass with water, any influence of culture medium on dry weights can be eliminated or minimized. Finally, a standard curve can be constructed by plotting absorbance versus actual dry biomass weight (g/l). This method allows for quick, reliable, and easy conversion of absorbance to dry biomass (g/L).

For determining microbial health, viable cell counts can be rapidly performed by using an EPICS XL-MCL flow cytometer (Beckman-Coulter, Miami, FL) in conjunction with the Live/Dead BacLight™ bacterial viability test kit (Molecular Probes, Eugene, OR) (21). BacLight uses a mixture of Syto 9 fluorescence, which is measured as a log FL1 (525 nm) signal, and the propidium iodide fluorescence which is measured as a log FL4 (675 nm) signal. A two color histogram is collected with gating on the bacteria only population from the two parameter light scatter distribution and is used for the analysis of green only (live bacteria), red only (dead bacteria), and both colors (stressed bacteria).

Substrate consumption and product formation rates can be monitored by high pressure liquid chromatography (HPLC) or by membrane bound enzymes biosensors, which requires 20 and 1 min to run, respectively. HPLC analysis is time consuming, but the concentration of multiple metabolites can be monitor simultaneously. HPLC does not provide real time feedback on the health of the fermentation, and it has long sample preparation and run times. In contrast, membrane bound enzyme biosensors such as YSI 2700 select analyzer (Yellow Springs Instruments, Yellow Springs, OH) can analyze a sample in 1 min. However, these units are restricted by the availability of substrate specific oxidases which generate H_2O_2, the measurable product by their electrode. Some compounds currently measurable are glucose, ethanol, maltose, lactic acid, and lysine. Sample preparation is simply filtration (0.45 :m) and dilution with water if the value falls outside the instrument window.

Organic acid production can be continuously monitored via alkali addition rates for pH control. Alkali consumption can be easily monitored by feeding alkali solution from a sterile burette (22). Microbial respiration for aerobic cultures can be continuously

monitored via dissolved oxygen probe or the concentration of CO_2 in the exit gas, which can be monitored via off gas analyzers or simply via alkali (4 N NaOH) traps followed by pH titration. However, more technologies are needed to acquire real time measurements of microbial growth, ensuring optimal fermentation time, and product formation in the shortest time possible.

For rapid analysis of biomass, substrate and product concentrations without any sample preparation, Fourier transform mid infrared (FT-MIR) spectroscopy has been successfully utilized for lactic acid (23) and ethanol (24) fermentation. Calibration models have been developed by using principle least square (PLS) and principle component regression (PCR) on suitable spectral wavenumber regions. This calibration model is then used for unknowns. The advantage of this system is that not only no sample preparation required, but it also provides analysis for substrate, product and biomass at the same time. This method can be used for online, real time analysis for monitoring and process control purposes.

For aerobic culture fermentation, house air is generally supplied under pressure. Oxygen transfer into the culture medium depends on the air bubble residence time in the culture medium and bubble size. The smaller the air bubbles the greater the O_2 transfer. Thus, all stirred tank reactors have aeration spargers that generate bubbles right beneath the first agitator blade. The exiting air bubble collides with the standard flat Rashton turbine agitator blade which strikes the bubbles hard and fast as they leave the sparger generating smaller air bubbles for improved oxygen transfer. Many commercial fermentations then follow up the agitator shaft with a series of down draft marine agitator blades, which look like a motor boat propeller (Figure 6.2). This series of down draft marine blades push the air bubble back down as it migrates up the reactor. This increases gas bubble residence time in the liquid medium before exiting out the top. In some fermentations, the rate limiting

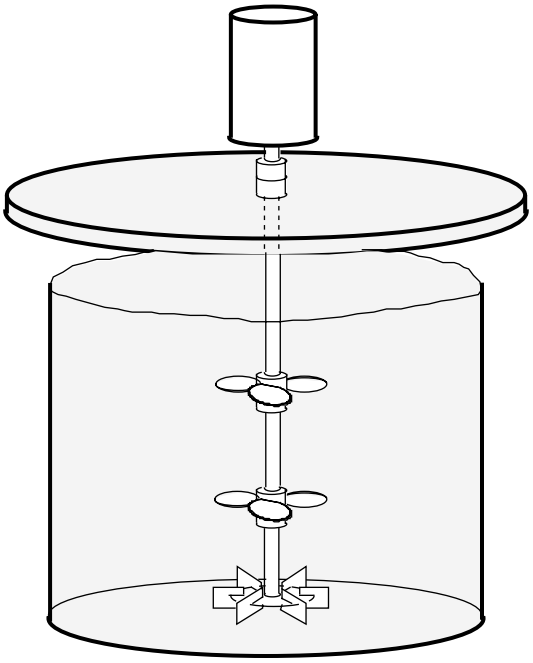

Figure 6.2 Diagram of stirred tank reactor with Rashton turbine agitator blade at the reactor bottom to break up air bubbles followed by a series of down draft marine blades to increase bubble residence time in the culture medium.

substrate will be oxygen. Thus, along with the series of down draft marine agitator blades a supply of pure oxygen may be needed. Pilot scale recombinant *Pichia* fermentations often require pure oxygen supplements to ensure optimal growth.

It is obvious that each microorganism differs in oxygen uptake rate (OUR). Therefore, oxygen transfer rates (OTR) (the rate of oxygen transfer from bubble to fermentation medium) must be equal to or higher than OUR. Maximum OTR can be calculated by using the following equation:

$$\text{OTR (mg } O_2\text{/min)} = k_L a\, C_L^*\, V_L$$

where C_L^* = Equilibrium dissolved oxygen level (mg O_2/L), $k_L a$ = Mass transfer coefficient (min−1), and V_L = Liquid volume in tank (L).

Thus, $k_L a$ is a critical parameter affecting OTR and the desired dissolved oxygen concentration in a fermentation broth during fermentation, and $k_L a$ can be determined experimentally. Briefly, the percentage dissolved oxygen (%DO) level is reduced to almost zero by sparging nitrogen into the culture medium. Then, air or oxygen gas is sparged at the desired temperature, agitation, and aeration conditions. Percent DO values are recorded over time until %DO reaches to saturation level. After converting %DO values into dissolved oxygen concentrations (C_L), plotting ln ($C_L^* - C_L$) versus time gives a straight line with a negative slope which is equal to "$-k_L a$". By knowing $k_L a$, one can calculate OTR with the given aeration and agitation. If OTR is less than OUR, some changes can be implemented to improve OTR, such as increasing aeration rate or agitation. Even utilization of various types of propellers can be compared by calculating OTR under each condition.

Agitation and aeration of stirred tank fermentors may also generate foam, which is the entrapment of gas in lipid, polysaccharide, or protein matrix (25). If not controlled, a foam buildup can literally empty the reactor. Mechanical foam breakers are like giant fans which physically break the foam and blow it back down. Mechanical foam breakers have their limitations and ultimately food grade antifoams are employed to control culture broth foam. A list of common antifoams can be found in Hall et al. (25).

Modification to this basic bioreactor design occurs from specific microbial needs. For example, if your production microorganism is sensitive to agitator shear then an air lift bioreactor is employed. Air lift bioreactors consists of a central or an external draft tube whereby air bubbles passing up these tubes generate convection mixing of the medium (26). Many fungal fermentations require the production of fungal balls for maximum product formation. These fungal balls are very sensitive to the shear caused by the agitator blades. For example, *Aspergillus niger* citric acid fermentation requires a defined medium with specific concentrations of trace elements (i.e., copper, manganese, magnesium, iron, zinc, and molybdenum). The bioreactor is typically lined with glass to prevent the addition of any stray trace elements. A fungal spore suspension is used as an inoculum, and mycelium, for optimal production rates, consists of very small solid pellets, or spheres which require a bioreactor with minimum shear. Throughout the entire fermentation period, the minimum dissolved oxygen concentration of 20–25% of saturation is required (27). Thus, tall air lift fermentors are the bioreactors of choice for these types of fermentations.

Biological heat can also be a problem. Bacteria, yeast, and molds will generate different levels of biological heat because their growth rates are so different. All bioreactors require some kind of jacketed cooling and heating system. Also, the time of year and location of the facility will also dictate the level of cooling required. Biological heat is directly related to growth rates. The faster the growth rate the more heat generated. Thus, a rate limiting substrate can be used to control microbial growth. One example is the use of a substrate containing mono, di, oligo, and poly saccharides. For example, a liquefied corn starch described above containing 19% dextrose, 14% maltose, 12% maltotriose, and 55%

higher saccharides is commonly used to control microbial growth rates. Enzymatic hydrolyses of the di, tri, and oligo saccharides will dictate the level of available glucose in the fermentor, and thus, control microbial growth rates.

6.5 FERMENTATION TYPES EMPLOYED: THE ACTUAL PRODUCTION PROCESS

The work horse of the industry is batch and fed-batch fermentation. Batch fermentations are closed fermentations (28). The fermentation sequence starts with medium sterilization, reactor inoculation [1 to 2% (v/v) typically], incubation for complete microbial growth cycle with lag, log, and stationary phases, fermentation termination, draining the reactor for product recovery down stream, cleanup of the reactor, and starting over. Percentage yield is calculated by taking the concentration of the product formed (g/l) divided by the concentration of substrate consumed (g/l) times 100%, whereas productivity is a measure of the product formation rates. It is calculated by dividing the product concentration (g/l) by the fermentation time (hours); thus, it is presented in g/l/hr.

In fed-batch fermentation, additional carbohydrate is supplied to the batch fermentation during the run (28). High carbohydrate concentrations in the initial culture medium are toxic to many microorganisms. Thus, an optimal carbohydrate concentration is employed initially, which permits maximum culture growth to late log phase. When the carbohydrate concentration is reduced to almost zero, additional sterile carbohydrate is injected into the bioreactor to bring the carbohydrate concentration back to the starting level. When this is consumed, the process is repeated until end product inhibition forces the whole bioconversion to stop. Ideally, at the end of the fermentation you will have a product concentration which is three to four times greater than single batch fermentation with no residual carbohydrate. This will generate the highest yield possible. Also, due to increased end product accumulation with each carbohydrate addition, culture production rates will decrease. Thus, the decision as to how many fed-batch fermentations to perform before harvest is based on the desired final product concentration and the optimal fermentor use time. For example, in lactic acid fermentations a final product concentration $>$120 g/l is desired to enhance product recovery (29). For *Lactobacillus casei* this concentration can only be achieved via fed batch fermentation for a total fermentor run time of eight days.

Continuous fermentations are open fermentations, whereby fresh medium is continually added to the bioreactor, while spent culture medium, cells, and product are continually leaving (28). This fermentation is desired by the industry, because the reactor volume is 10 to 100 times smaller than batch fermentations, a steady stream of fermentation product is produced which will optimize downstream processes, bioconversion rates are always at maximum, operation costs are less, and the system can be fully automated and computer controlled to the point where only two operators are needed to manage the fermentation each day. However, it requires a continuous supply of sterilized or pasteurized culture medium, dilution rates are linked to microbial growth rates and the operational speed of downstream recovery process. Startup is slow, so any facility shut downs have an impact on production, and you are constantly fighting contamination (30). Thus, not all fermentations can be operated this way. The best example is ethanol production for gasohol, which is commonly a continuous fermentation with a four bioreactor train with increasing working volumes in each bioreactor. Thus, the dilution rate is decreasing in each bioreactor over the course of the fermentation. This dilution gradient in the train is critical, because as ethanol builds up in the culture medium, the yeast growth rate slows. Specific growth rate equals dilution rate. Finally a holding tank at the end is used to ensure

Figure 6.3 Example of *Lactobacillus casei* biofilm development on PCS tubes mounted on the agitator shaft for repeat batch fermentation. PCS blend employed contained 50% (w/w) polypropylene, 35% (w/w) ground soybean hulls, 5% (w/w) bovine albumin, 5% (w/w) Ardamine Z yeast extract, and 5% (w/w) soybean flour.

complete bioconversion of any residual sugars to ethanol. The CO_2 is collected and concentrated, then sold as another valuable byproduct. Fermented beverages (i.e., wine and beer) are still performed in batch.

For some continuous fermentations, an increased concentration of biomass in the reactor is required. This can be achieved by cell recycling, immobilized cell, and biofilm fermentations. Cell recycling reactors employ a filtration unit that allows for the constant bleeding of culture supernatant while retaining biomass (26). However, filtration unit fouling is a problem and must be constantly monitored. This type of operation has found use in the treatment of food processing from some starchy food waste streams (26).

6.6 NOVEL BIOREACTOR DESIGNS

One of the most common forms of immobilized cell bioreactor is entrapment, where high concentrations of cells are trapped in a polymer matrix such as alginate and κ-carrageenin (28,31). Thus, a high cell density is continuously retained in the fermentor while substrate is continuously converted to product. This higher concentration of biocatalysts in the reactor results in higher productivities and yields. The disadvantages of this method are migration of substrate through the matrix to the cell and the migration of product out, potentially high concentrations of product around the cells causing end product inhibition, cell leakage from the polymer matrix due to cell growth, and bead swelling and disintegration over time causing the whole fermentation to be stopped, cleaned, and restarted.

Biofilms are a natural form of cell immobilization in which microorganisms are attached to a solid surface (32). In this bioreactor, cells are continually growing, and sloughing off. Thus, the reactor is a mixture of immobilized and suspended cells. Continuous biofilm fermentations are truly open immobilized cell bioreactors (30). Their operation is equivalent to a suspended cell continuous fermentation with the added advantage of increase biomass concentrations in the bioreactor. Biofilms are typically resistant to harsh conditions, and can tolerate changes in the fermentation feed and conditions. However, not all microorganisms form biofilms. Filamentous microorganisms such as fungi and actinomycetes are natural biofilm formers. For nonfilamentous bacteria to form a biofilm, an extracellular polysaccharide needs to be generated by the bacterium (32).

Some bacteria will form biofilms on any surface such as metal, plastic, and glass. However, certain bacteria, such as lactobacilli, require something to stimulate this biofilm development (33). Plastic composite support (PCS) developed at Iowa State University has proven to stimulate biofilm development of *Lactobacillus casei* (22,34,36), *Zymomonas mobilis* (37,38), *Saccharomyces cerevisiae* (37,38,39), and *Actinobacillus succinogenes* (40). PCS is a high temperature extruded material consisting of at least 50% polypropylene, plus ground soybean hulls, bovine albumin and various culture micronutrients. Soybean hulls keep the extruded product porous due to the release of steam as the PCS leaves the extruder die. Bovine albumin performs as a natural plastizer which protects the temperature sensitive micronutrients. Micronutrients are selected based on the specific cultural requirements for amino acids, vitamins, and lipids. Monosaccharides are avoided due to poor PCS production. For example, the PCS blend for lactobacilli contains 50% (w/w) polypropylene, 35% (w/w) ground dried soybean hulls, 5% (w/w) bovine albumin, 5% (w/w) yeast extract, 5% (w/w) soybean flour, and mineral salts (35). PCS have been evaluated in batch (22), fed-batch (29), and continuous (30) lactic acid fermentations (Figure 6.3). In every application the percentage yields and productivity rates were significantly higher than suspended cell lactic acid fermentations. Furthermore, repeat batch fermentations have operated for more than 1.5 years with virtually no change in percentage yields and productivities. This longevity is attributed to the fact that once a biofilm has established on these customized materials, it will continue to grow as a biofilm. This is supported by the fact that a PCS biofilm reactor washed with concentrated ammonium hydroxide, rinsed with mineral salts solution, and then reinoculated with a fresh culture and medium will reestablish itself overnight. Commercially, the quick vinegar process is the most common biofilm process in current operation which uses wood chips for supports and *Acetobacter aceti* for production (27).

Solid substrate fermentation is when a substrate such as soybeans is ground, inoculated with *Aspergillus oryzae*, then incubated for three days for the production of soy sauce (27). It is a simple fermentation process and commonly used for aerobic fermentation due to its large surface area. Thus, oxygen concentration is high without using any mechanical forced air systems. Solid substrate fermentations require large areas or incubation space. A temperature controlled environment, intermittent monitoring for contamination and quality of starting material is essential for success.

6.7 FUTURE RESEARCH

Research is still needed for isolation of new microbial strains with improved production efficiencies and higher yields. More real time measurements are needed for culture conditions and metabolite formation. Recovery will continue to be the key factor associated with final product purity and cost. As an industry we cannot rely solely on genetic engineering

as our method of improving current fermentations. As we have illustrated, there are many other techniques which can be employed to improve productivity and yield, including new inexpensive medium ingredients, more continuous fermentation processes, and new exotic microbial reservoirs in nature and in the food industry.

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1.07

Production of Carotenoids by Gene Combination in *Escherichia coli*

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CONTENTS

7.1 CAROTENOIDS: PROPERTIES, COMMERCIAL ASPECTS, AND BIOLOGICAL FUNCTION IN HUMAN HEALTH

Carotenoids are water-soluble natural pigments of 30–50 carbon atoms. Even shorter structures called apocarotenoids result from their oxidative cleavage. Carotenoids are synthesized de novo by archaea, bacteria, fungi, and higher and lower plants. Animals are supplied with carotenoids from their food and are able to further modify the chemical structure. More than 600 carotenoids are known as intermediates or end products of different biosynthetic branches in various organisms. Most carotenoids consist of 40 carbon atoms; possess an acyclic chain; or carry β-ionone, ε-ionone, or aromatic end groups. Acyclic carotenoids are often modified at C-1 and C-2, e.g., by 1-HO, 1-CH_3O, or 2-keto groups. Typical substitutions of a β-ionone end are 3-hydroxy, 4-keto, and 5,6-epoxy moieties. The 3-hydroxy group can participate in glycosilation or formation of fatty acid esters. The most prominent feature of a carotenoid molecule is the polyene chain. Delocalization of the Π-electrons is responsible for their color and their antioxidative potential. Carotenoids can interact with radical chain reactions and are capable of energy dissipation from photosensitizers as heat. In photosynthesis, carotenoids function as light-harvesting antenna, transferring light