AN ABSTRACT OF THE THESIS OF

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Title: CHEMISTRY OF FRUITY FLAVORS PRODUCED BY PSEUDOMONAS FRAGI

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Cultures of Pseudomonas fragi isolated from fruity flavored cottage cheese and pasteurized milk were grown in sterile homogenized milk supplemented with 0.2% ethanol at 21°C. After a typical fruity aroma developed, the head space volatile constituents in the cultures were examined by gas-liquid chromatography. Ethyl acetate, ethyl propionate, ethyl butyrate, ethyl isovalerate and ethyl hexanoate were tentatively identified on the basis of sensory evaluation of the effluent gas from the column and the retention time.

Volatile constituents from a typically fruity culture were isolated with a cold finger condensor similar to the apparatus described by Lea and Swoboda (1962). Analysis of the ether extracts of a culture distillate by coupled gas-liquid chromatography-mass spectrometry yielded positive identifications for ethyl acetate, ethyl butyrate, and ethyl hexanoate. A weak mass spectrum indicative of ethyl

formate was also obtained. The most abundant esters with fruity odors were ethyl butyrate and ethyl hexanoate. In a culture with a strong fruity aroma, the concentration of these two esters was 0.35 and 0.50 ppm, respectively.

Quantitative gas chromatographic determinations of ethyl butyrate and ethyl hexanoate in media supplemented with 0.2% ethanol showed definite increases in the concentrations of the two esters in comparison to media not supplemented with ethanol. Supplementation with butyric acid in addition to ethanol generally elevated the ethyl butyrate concentration and usually depressed the cell count slightly. Aeration of any of the media during growth tended to reduce the cell population slightly. A relationship between increase in cell number and increase in concentration of esters during the growth of the culture was observed. Media containing high concentrations of ethanol plus milkfat or low molecular weight fatty acids were conducive to the production of a fruity aroma by P. fragi.

The microbial interaction in the mixed cultures of Streptococcus lactis and P. fragi and its influence on the ester levels were studied. The concentrations of ethyl butyrate and ethyl hexanoate in the mixed cultures were about five times greater, compared to the single-strain cultures of P. fragi.

The possibility of coenzyme A participation in the mechanism of ester formation was tested by growing P. fragi in milk media

containing increasing concentrations of $NaAsO_2$, a CoA inhibitor. However, results obtained in this study did not indicate the involvement of CoA in ester formation in P. fragi cultures.

Esterase activity of sonicated cell free extracts, whole cell suspensions and supernatant fluid of P. fragi cultures were assayed with a Gilson differential respirometer. The distribution of the esterase in P. fragi was found to be intracellular. The esterolytic activity of the enzyme preparation was analyzed by gel electrophoresis. Six bands of esterase activity with different rates of mobility and substrate specificity were separated. These were: band 1 with slow mobility, bands 2, 3, 4 with moderate mobility and bands 5 and 6 with rapid mobility. All six bands were active with α-naphthyl acetate, four bands with α-naphthyl propionate and five bands with α-naphthyl butyrate. In general these esterases appeared to be more active on aromatic esters than aliphatic esters and triglycerides.

Since P. fragi cultures contain esters and low concentrations of many other flavor components, the occurrence of synergistic flavor interactions among some of these compounds was investigated. Flavor threshold values were determined for ethyl acetate, ethyl butyrate, ethyl hexanoate, ethyl isovalerate, butyric acid, diacetyl, and dimethyl sulfide in homogenized milk. In addition, flavor threshold values were determined for two component mixtures of the above compounds in milk. Synergistic interactions ($P \leq 0.01$) were

observed in the mixtures of ethyl hexanoate-ethyl butyrate, ethyl butyrate-butyric acid, ethyl butyrate-ethyl acetate and ethyl hexanoate-ethyl isovalerate. Diacetyl-ethyl hexanoate and dimethyl sulfide-ethyl hexanoate mixtures did not show significant flavor interactions.

Chemistry of Fruity Flavors Produced by *Pseudomonas fragi*

by

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INTRODUCTION

The dairy products, milk, cottage cheese and butter, develop a fruity flavor on prolonged storage under refrigeration conditions. This fruity aroma has been described as "ester-like," "apple-like," or pineapple-like," and is usually accompanied by rancidity. Fruity flavor production has been primarily attributed to Pseudomonas fragi. This organism is of special interest to dairy microbiologists because of its ability to grow even at refrigeration temperatures. The presence of these bacteria in milk and milk products is objectionable since they are versatile spoilage agents with pronounced biochemical activity especially on fats and proteins.

Limited information is available concerning the nature of the chemical compounds involved in the ester-like aroma produced by P. fragi. Recent investigations have indicated that the organisms can produce ethyl alcohol and some esters in milk medium. The ability of P. fragi to produce lipase capable of hydrolyzing milk fat has been demonstrated. High concentrations of ethyl alcohol have been shown to enhance fruity flavor production by P. fragi. However, a determined effort has not been made to identify the chemical compounds responsible for this fruity flavor, or the mechanism of

formation. Advances in analytical instrumentation have made detailed studies of microbial metabolism of volatile compounds possible, and thus have permitted a more thorough understanding of the mechanisms of the flavor formation.

The objectives of this investigation were to isolate and identify the chemical components responsible for the fruity flavor and to study the mechanism of their formation in P. fragi cultures. This study was extended to determine the influence of various factors on the ester levels in P. fragi cultures., and the synergistic flavor interaction of esters, and some chemical components of fruity cultures. The results obtained in this investigation should lead to a better understanding of the fruity flavor defect by P. fragi in dairy products.

REVIEW OF LITERATURE

Importance of Pseudomonas fragi to the Dairy Industry

Pseudomonas fragi is considered as a versatile spoilage organism with pronounced biochemical activity. Spoilage by this organism can occur in a variety of ways. This organism can hydrolyse fat and protein, and grow well at refrigeration temperatures. The fruity flavor defect in milk, cottage cheese and butter has been attributed primarily to P. fragi. The organism also produces a white gelatinous film on the cottage cheese curd (Foster et al., 1957, p. 28). Since the species is destroyed by proper pasteurization, its presence in dairy products indicates post pasteurization contamination.

Taxonomy

Pseudomonas fragi is classified under the Pseudomonadeceae family. Pseudomonads can be isolated easily by virtue of the fact that they can grow at temperatures as low as $0^{\circ}C$ (Foster et al., 1957, p. 28). The organisms are gram negative, asporogenous rods, and when motile, possess a single polar flagellum or a tuft of flagella. These organisms can be characterized by their ability to liquify gelatin in agar medium. P. fragi can be easily differentiated from the other members of the genus by its growth in litmus milk. P. fragi produces an acid ring followed by coagulation at the surface.

A typical characteristic of these organisms is their ability to produce fruity aroma in milk cultures.

Fruity Flavor Defect in Dairy Products

The fruity flavor defect in dairy products has been attributed to the biological activity of microorganism. Eichholz (1902) was the first to isolate P. fragi from milk possessing a strawberry-like odor. Hussong, Long and Hammer (1937) reported that the colonies of P. fragi produced a characteristic ester-like odor similar to the aroma of May apples. In cottage cheese, a fruity odor and a flat, rancid bitter flavor has been observed as a result of the growth of P. fragi. In butter, this defect has been observed before rancidity is detected (Foster et al., 1957, p. 43). Pereira and Morgan (1958) demonstrated that pure cultures of P. fragi could produce a fruity aroma in sterile milk media.

Another type of fruity flavor in dairy products has been shown to be caused by the lactic streptococci. Perry (1961) reported that three strains of Streptococcus Lactis produced an off-flavor in the cheese which was described as dirty or fruity. This flavor defect was said to become more pronounced as the cheese aged. Vedamuthu (1965) manufactured cheddar cheese using mixed strains of starter cultures. He observed a consistant production of a fruity flavor defect in cheddar cheese manufactured by using mixtures of S. Lacti,

streptococcus cremoris strains and S. lactis, S. cremoris, streptococcus diacetilactis strains.

Harrison (1927) isolated several yeasts from cheese samples and found that they were able to produce fruity odors when grown in milk. The ability of yeast to produce esters in fermented beverages has been shown by Nordstrom (1964).

Chemistry of the Fruity Flavor

Omelianski (1923) reported that isoamyl isovalerate was responsible for the ester-like aroma in P. fragi cultures. However, sufficient data were not available to adequately support his findings. Using the paper chromatography, Pereira and Morgan (1958) indentified ethyl formate, acetate, propionate and isovalerate esters in steam distillates of milk cultures of P. fragi incubated at 8°C for two weeks. They also detected formic, acetic, propionic, butyric and isovaleric acids. These workers indicated that the major component of the fruity aroma was ethyl isovalerate. P. fragi has been shown to produce ethanol on extended periods of incubation. They speculated that leucine in milk served as a precursor of isovaleric acid which in turn was involved in the formation of isovalerate esters.

Bills et al. (1965) found high concentrations of ethanol, ethyl butyrate and ethyl hexanoate in cheddar cheese displaying the fruity flavor defect. Ethyl acetate, ethyl butyrate, ethyl hexanoate, methyl

hexanoate, ethyl octanoate, 2-butyl acetate, 2-butyl butyrate and 2-butyl hexanoate are few of the esters identified by Day and Libbey (1964) from cheddar cheese. Some of the same esters have also been reported by Suzuki, Hastings, and Hart (1910) and McGugan and Howsam (1962). However, limited information is available concerning the nature of chemical compounds responsible for the ester-like aroma produced by P. fragi.

Lipolysis by Pseudomonads

Considering the ability of P. fragi to produce esters, the lipolytic activity of this organism should be reviewed briefly. As early as 1937, Hussong et al. reported an increase in total acids accompanied by an increase in volatile acids in butter inoculated with Pseudomonads. However, there was no close correlation between these increases. Richards and El-Sadek (1949) studied microbial hydrolysis of fats using a stable, finely divided butter fat emulsion. They stated that butyric acid and hexanoic acid were the major volatile fatty acids produced by the growth of Pseudomonads on a butter oil medium. Goldman and Rayman (1952) tested the lipolytic activity of different microorganisms in a fat emulsion broth. They reported that Pseudomonads hydrolyzed fats possessing widely varying fatty acid compositions. They further noticed wide variations in both rate and extent of hydrolysis among different strains and species of

the Pseudomonads studied.

Using titrimetric analysis Alford and Elliot (1960) demonstrated a decrease in the production of lipase enzymes at higher temperatures of incubation. However, the temperature at which the enzyme was produced did not affect the optimum temperature for the activity of the enzyme. It has been shown by Alford, Pierce and Suggs (1964) that the lipase of P. fragi attacks the di- and monoglycerides at a much slower rate than it does the triglycerides. This was similar to the activity of pancreatic lipase. The rate of fatty acid liberation by this microbial lipase decreased with time, but the ratios of the fatty acids liberated remained constant, at least until about one third of the fatty acids have been liberated (1961).

Using Nile-blue sulfate as an indicator, Collins and Hammer (1934) studied the lipolytic activity of a number of bacterial cultures. These workers reported that the number of cultures that could hydrolyze the triglycerides decreased with increase in molecular weight and degree of saturation of fatty acids in the triglycerides. Tripropionin was hydrolyzed by 98.3 percent of the cultures, and tristearin by none of them. Hydrogenation of unsaturated fats did not significantly influence the action of bacteria.

Factors Influencing Biological Activity of Pseudomonads

The factors affecting the growth and lipolytic activity of

Pseudomonads sp. have been studied by various workers. Richards and El-Sadek (1949) observed a decrease in the count of Pseudomonas fluorescens cultures near the end of a 40 day incubation period. In all cases, the number of bacteria was less after incubation than that of the inoculum. The report did not indicate whether there was a gradual decrease in the count from the time of inoculation, or there was an increase to a maximum first. However, in another study of Pseudomonas sp. Goldman and Rayman (1952) observed a sharp rise in the count which attained a maximum in approximately five days. As the pH dropped from 6.8 to a level of 5.0 in 21 days, the bacterial count decreased to a point below the initial count made at the time of inoculation. These workers also reported that the concentration of fat in the medium influenced the degree of hydrolysis. The efficiency of lipolysis was greater when the fat concentration was below ten gm per 100 ml of medium than at higher concentrations. A wide difference in the lipolytic activity was noticed among the individual Pseudomonas species and between strains of the same species.

It has long been known that the physical properties of fats influence the degree of their dispersion in emulsions. The importance of finely divided and well dispersed fat emulsions to study the lipolytic activity of microorganisms or their enzymes has been recognized by many workers (Richards and El-Sadek, 1949; Goldman and

Rayman, 1952; Alford and Pierce, 1963). Emulsifying agents, such as soy phospholipid, and/or homogenization have been used to obtain finely divided and well dispersed stable emulsions.

Effect of Temperature

The effect of temperature on biological activities of microorganisms has been generally realized. Nashif and Nelson (1953b) investigated various factors that influence lipase production by P. fragi. Their results indicated that at higher temperatures both maximum population and enzyme activity was reached in a considerably shorter time than at low temperature. However, the level of enzyme produced per cell was higher at low temperatures. These workers observed that shaking the medium twice daily during incubation lowered the cell population and lipase production. However, higher counts and greater lipase activity per unit volume were observed when large surface areas were provided. The enzyme activity was highest when the available oxygen was limited. Periera and Morgan (1958) could not detect esters in milk cultures of P. fragi unless the milk was inoculated with cells grown in milk at 8°C. Morgan et al. (1967) further reported that P. fragi did not produce detectable amounts of esters when incubated at 22°C. They speculated that the enzyme systems responsible for either the production of ethanol or actual esterification were favored at low temperature

incubation.

Effect of Medium

The composition of the medium has been shown to have a definite influence on growth and the biological activity of P. fragi. Nashif and Nelson (1953b) tested various commercial protein digests and hydrolysates as media for P. fragi for lipase production. A peptone broth (one percent) was shown to be best of the media evaluated for maximum lipase production in three days at 15°C. Several chemically defined media also were tested by these workers. None of these media improved lipase production. However, the addition of L-leucine alone, or a combination of L-leucine, DL-isoleucine and DL-valine appreciably increased lipase production. Although P. fragi elaborated lipase in media devoid of any lipid substrate, the presence of simple triglycerides, such as tricaprylin, in the medium markedly increased the amount of lipase produced. However, triglycerides higher than trilaurin and, also, butter fat were inhibitory to lipase production. From this the above workers concluded that it was not the triglyceride structure of tricaprylin that was responsible for the increased amount of lipase production. They proposed that the fatty acid component of the tricaprylin was responsible for the stimulation of lipase elaboration. A proportional increase in lipase production was noticed with increased counts.

Goldman and Rayman (1952) observed a decrease in lipolytic activity in cultures of Pseudomonas sp. when large amounts of nitrogenous supplements were added to an emulsion media. Alford and Price (1963) also studied the effect of the medium composition on the production of lipase by P. fragi. They observed good cell growth in several synthetic media but no lipase production. The results obtained indicated that the nutritional requirements to produce lipase vary considerably with different organisms. Since P. fragi is similar to P. fluorescens in its pattern of lipolysis, the differences in nutrient requirements for lipase synthesis probably are not caused by a basic difference in the two enzymes, but rather a difference in synthetic pathways.

The effect of temperature and nutrition on growth and proteases production in Pseudomonas sp. has been studied by Juffs, Hayward and Doelle (1968). They have indicated that the proteolytic activity of Pseudomonas sp. is highly dependent upon the medium employed, and is not necessarily associated with good growth. They have demonstrated maximum protease activity as the cells entered the logarithmic phase indicating a high rate of enzyme elaboration as soon as active division commenced. For Pseudomonas aeruginosa two protease components were found. They suggested the second fraction was due to an intracellular enzyme released by autolysis during the stationary growth phase. Production of the enzyme was

initiated in the presence of organic nitrogen in the form of amino acids or peptides. Protease enzymes were not elaborated in a mineral salt medium containing inorganic nitrogen, or even casein. They speculated that protease synthesis was repressed by glucose or other carbon sources, and induced by end-products in Pseudomonads, particularly in P. aeruginosa.

Omelianski(1923) reported that presence of nitrogenous organic matter in the medium is required by P. fragi to produce a fruity aroma. Periera and Morgan (1957) noticed some fruity flavor production by P. fragi in a basal salt medium containing leucine, alanine or glutamic acid as the carbon source, or in a salt-glucose medium containing threonine as a nitrogen source. Ester production by P. fragi was stimulated by supplementation of the milk culture with ethanol. However, methanol did not have any effect on ester production. Ethanol liberated into the milk by the cow after feeding on certain feeds can contribute to increased ester production (Bassette, Turner and Ward, 1966).

Nordstrom (1964) observed in yeast cultures that addition of acids and alcohols to the medium increased the formation of the corresponding esters. The addition of an acid and an alcohol also caused a decrease in the formation of all other esters except those corresponding to the added components. A close correlation was observed between the formation of esters and yeast growth; that is,

when the latter is inhibited by some deficiency in the medium, the former decreases. However, ester formation is stimulated more by ammonium ions than by amino acids. Ester production by yeast was also affected by genetic factors and ultra-violet light treatment. Washam (1967) reported a decrease in the number of esterases in cells of P. aeruginosa when treated with quaternary ammonium compounds.

Enzyme Systems in Pseudomonas sp.

The lipases and proteases of Pseudomonas sp. have been studied more thoroughly than other enzyme systems.

Lipases

Nashif and Nelson (1953a) were among the first to isolate and characterize the lipase enzyme in P. fragi. They encountered some difficulties in removing the viable cells from the supernatant fluid. By using 0.1 percent formaldehyde as a bacteriostatic agent, they demonstrated that the lipase in P. fragi cultures is extracellular. In the identification of this enzyme, emulsions of different oils and fats were used as the test material. The enzyme was isolated by salting it out from peptone broth, and the maximum activity has been observed at pH 7.2. The optimum temperature was determined to be $40^{\circ}C$, and the enzyme was completely inactivated by heating at

99°C for 20 minutes. However, Söhngen (1911) observed the production of a lipase by P. fluorescens that could withstand heating at 100°C for five minutes. Nashif and Nelson (1953c) demonstrated also that the lipase of P. fragi is active in cream between pH 4.9 to 8.2 with an optimum between pH 5.7 and 6.6 at 36°C incubation. Over 50 percent of the lipase was not inactivated by pasteurizing the cream at 71.5°C for 30 minutes.

Alford et al. (1960, 1961) observed a qualitative effect on the lipase produced when the incubation temperature of the culture is increased. These workers noted a small but consistant increase in the percentage of unsaturated fatty acids liberated by the enzyme produced at 28°C, compared to that produced at 20°C, with a slight decrease in the total saturated acids, particularly stearic acid. In more recent publications, Mencher and Alford (1967) reported that the optimum pH for the purified lipase of P. fragi was 8.6 to 8.7 at 25°C. The enzyme was purified 75 to 100 fold with an over all recovery of about 20 percent. They noted a complete loss of activity of the purified enzyme in ten minutes when exposed to 40°C. Lipase activity was assayed by a titrimetric measurement of fatty acids released from the substrate.

The available information indicates that the lipases of P. fragi and P. fluorescens are extracellular (Nashif and Nelson, 1953a; Alford et al., 1961; Mencher, Ng and Alford, 1965). This lipolytic

activity has been shown to be similar to pancreatic lipase in that it attacks the α and α' positions of triglycerides (Alford et al., 1961; 1964). Alford et al. (1961) noticed a reduction in the rate of fatty acid liberation by these lipases with time; however, the ratios of the fatty acids liberated remained constant, at least until about one third of the fatty acids had been liberated.

Proteases

Proteolytic reactions may be brought about by a variety of organisms. Pseudomonas sp. are active in many dairy products stored under refrigeration. They can attain enormous numbers after a period of storage at low temperatures, and may initiate proteolytic changes which culminate in the production of very objectional odors and flavors (Foster et al., 1957, p. 47). The Pseudomonas species commonly associated with dairy products which possess proteolytic ability are P. fragi, Pseudomonas putrefaciens, and Pseudomonas nigrificans (Breed et al., 1957, p. 110, 112 and 117). The presence of extracellular proteases has been reported also for P. fluorescens (Peterson, 1960) and P. aeruginosa (Morihara, 1963).

Although proteases have been detected in cultures of these organisms by many workers, few of these enzymes have been studied in detail. Van Der Zant (1957) isolated an extracellular proteolytic

enzyme from a P. putrefaciens culture, and found its optimum pH to be at 7.0-7.5. This enzyme was shown to be quite specific to α and β casein fractions of the milk protein. Several other peptidases also have been shown to be present in cell free extracts of P. putrifaciens (Camp et al., 1957). The maximum activity of these peptidases was between pH 7.0 to 8.0. An extracellular protease produced by P. myxogenes (Hagihara, 1960, p. 204 and 205) has been crystallized and studied in some detail. This enzyme has a molecular weight of about 77,000 and an isoelectric point between pH 5.5 and 6.0. The optimum pH was 7.0 to 8.5. It was suggested that this enzyme is similar to papain in its proteolytic activity.

In a recent report Juffs and Doelle (1968) showed that the maximum activity of the extracellular protease of P. aeruginosa was at pH 7 to 8. The enzymes retained a high portion of their activity after simulated pasteurization and are, therefore, relatively heat stable. However, the enzyme treated at 72°C for 15 seconds was six times less active than that heated at 63°C for 30 minutes. Although P. aeruginosa did not grow at 2°C, the enzymes were not denatured at this temperature. The casein substrate was hydrolyzed to the same degree at 2°C as at 30°C, however, the rate of hydrolysis was low.

The following review of literature cites some other enzymes that have been observed in Pseudomonads. Using gas-liquid

chromatography for end-product analysis, Keenan et al. (1967) demonstrated dehydrogenase activity in Pseudomonas sp. Seitz et al. (1963) reported that Pseudomonas sp. possessed diacetyl reductase activity, and suggested that reduction of carbonyls to their corresponding less volatile alcohols could be one of the ways of spoilage of foods by Pseudomonas sp. A semialdehyde dehydrogenase enzyme has been isolated by Nirenberg and Jokaby (1960) from a species of Pseudomonas which can use DPN or TPN as the hydrogen acceptor.

Multiple Enzyme Systems

Washam (1967) demonstrated by using gel electrophoresis that a P. aeruginosa susceptible to quaternary ammonium detergents possessed two esterases, and the resistant strain had only one esterase. Lawrence (1967) in his review of microbial lipases and related esterases referred to a term "isoenzyme" which has been described by the Standing Committee on Enzymes of the International Union of Biochemistry, as the multiple enzyme forms occurring in a single species. Multiple esterases have been separated using gel electrophoresis by Stock, Uriel, and Giraber (1961), Baillie and Norris (1963), and Lund (1965). Sierra (1957b) distinguished three esterases in an unpurified preparation from P. aeruginosa, based on differences in substrate specificity, thermolability and behavior

towards inhibitors. Morichi et al. (1968) demonstrated that many of the esterases of lactobacilli showed activity with α-naphthyl acetate, butyrate and caprylate in decreasing order. The esterases of several of these lactic acid bacteria were inactivated at 60°C and 65°C in ten minutes.

Mechanism of Ester Formation

It is generally accepted that P. fragi is responsible for ester-like aroma in many dairy products. A limited amount of research work has been done on the identification of some of the flavor compounds responsible for the fruity flavor defect. However, no effort has been expended in understanding the possible mechanisms involved in the ester production.

Ester production is a common phenomenon mediated by the living cells. Fat synthesis involves esterification of fatty acids and glycerol which is a natural way of preservation of energy by members of the plant and animal kingdoms. Esters of mono-carboxylic acids and aliphatic n-alcohols are generally accepted as important flavor compounds, however, very little exact knowledge relative to the mechanism of their formation has been gained. A considerable amount of research work has been devoted to the identification and quantitation of esters found in the fruits of higher plants. Although some information is available concerning ester production by

microorganisms used in the brewing industry, more research is needed to understand the flavor changes in foods caused by esters produced by microorganisms.

Ester formation can be accomplished by direct esterification in both non-enzymatic and enzymatic systems.

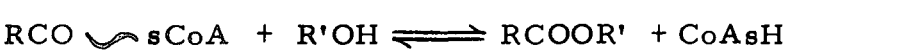
$$RCOOH + R'OH \rightleftharpoons RCOOR' + H_2O$$

The non-enzymic reaction rate is, however, 1000 times slower than the rate of enzymatic esterification at physiological pH-values (Nordstrom, 1964). Taussig and Petreanu (1957) reported that the equilibrium constant for this type of reaction is about three to six for esters of lower alcohols and lower fatty acids.

Some yeasts are capable of producing enormous amounts of volatile esters, especially ethyl acetate. Peel (1951) demonstrated that the concentration of ethyl acetate formed in an aerobic yeast culture was 100 times greater than that which would have resulted from the equilibrium between the reactants and the product. This finding suggested that a mechanism other than simple esterification was responsible for the production of ethyl acetate in these cultures.

Nordstrom (1964) conducted an extensive study of the mechanisms involved in the formation of esters in brewers yeast fermentations. The possibility of ester formation by direct esterification at physiological pH values has been ruled out for several reasons.

First, the non-enzymic reaction is too slow to account for the amounts of ethyl acetate formed. At lower pH values and higher concentrations of ethanol and acetic acid, however, this non-enzymatic reaction is recognized as an important factor in the formation of fatty acid esters. Second, the possibility that direct esterification was accelerated by the presence of an esterase was also ruled out, since there was no increase in the formation of ethyl acetate when acetic acid was added. There was also no correlation between the amount of acetic acid produced and the amount of ethyl acetate formed. Third, the amounts of ethyl acetate formed from added ethyl alcohol exceeded the equilibrium concentration of simple esterification by about 100 times. Further experiments by Canterarelli (as cited by Nordstrom, 1964) showed increased ester formation in the culture with added pantothenic acid. In addition to the above report, Nordstrom (1962) observed inhibition of ester formation with sodium arsenite, a substance known to inhibit coenzyme A (CoA) activity, at concentrations that did not affect growth. These observations together with many other experiments led to the conclusion that CoA is an essential cofactor for enzymatic ester formation by yeast. The manner in which the CoA was believed to participate in the esterification reaction is as follows:



Since this is an important reaction in many stages of metabolism, ester formation was thought to be associated with essential metabolic pathways in the cell. The proposed scheme showing the relationship of fatty acid activation, energy requirement, ester formation and other metabolic pathways is presented in Figure 1. Activation of fatty acids in higher animals is believed to be performed by different enzymes. However, Nordstrom (1964) reported only one acetyl synthetase enzyme in yeast for butyric through undecanoic acids. Formic, propionic, and the C_4 and C_5 isoacids were not utilized as substrates by the yeast in ester formation. Esterification velocity decreased with increasing molecular weight of the alcohol. Secondary and tertiary alcohols did not give rise to esters.

Newsome and Rottoray (1965) demonstrated that pancreatin could catalyze the esterification of ethanol with long chain fatty acids. The esterification appeared to be governed by solubility factors with the rate of esterification decreasing after the point at which a definite turbidity was produced by the dispersed fatty acids. This indicates that the reaction was favored by esterase activity in the soluble state rather than a lipase activity at the interface between the emulsified acid and the aqueous phase. These authors further reported (1966) that ethyl oleate was the main ester produced when ethanol and oleic acid were added to rat plasma. Although plasma contains endogenous fatty acid donor molecules, none

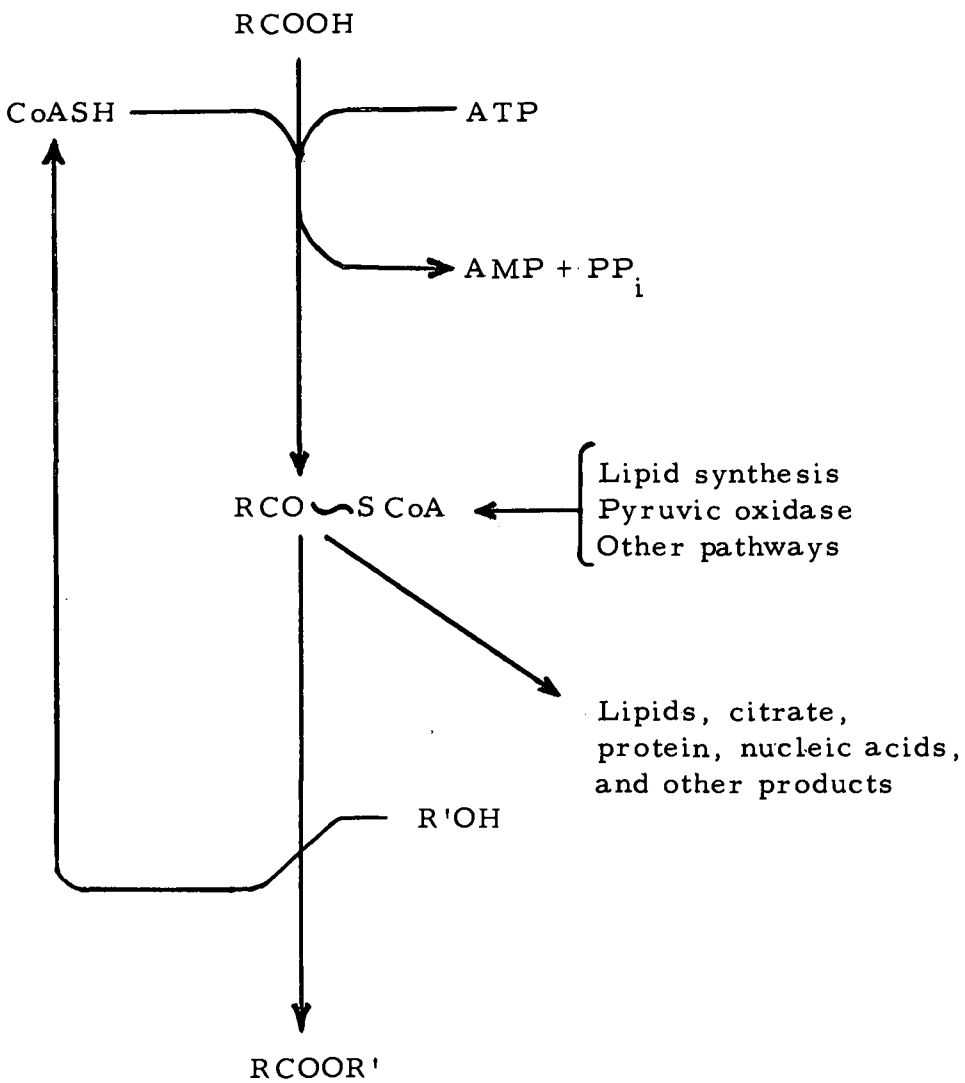


Figure 1. The relationship of fatty acid activation, energy requirement, ester formation, and other metabolic pathways in certain yeasts as outlined by Nordström (1964).

of these molecules were used in esterification which is in contrast to the transesterification mechanism. Hence, they suggested that synthesis of ethyl esters in plasma involves primarily a direct esterification of fatty acid rather than a transesterification requiring a fatty acyl donor.

Pereira and Morgan (1958) identified ethyl esters of isovaleric and acetic acids in a fruity milk culture of P. fragi. Since the addition of isovaleric acid and ethanol to sterile milk did not result in detectable ester formation over the incubation period required by growing cells to produce esters, it was concluded that esterification was actually mediated by the organism.

Synergistic Interaction of Flavor Compounds

Importance in Foods

The importance of synergistic interactions between flavor compounds at subthreshold concentrations has been recognized in recent years. In some foods the flavors may result from an additive interaction of flavor compounds, none of which exist at their threshold concentration.

Experimental Evidence

Lillard and Day (1961) reported that in most cases the carbonyl

compounds occurred at marginal or subthreshold concentrations in oxidized milk fat at its flavor threshold value. Based on these results they suggested that at the flavor threshold value of oxidized flavor, the flavor was due to additive effects of carbonyl compounds and the complete spectrum of compounds seemed necessary for oxidized flavor. In a later report Day, Lillard, and Mongomery (1963) demonstrated synergistic flavor interactions between carbonyl compounds resulting from lipid oxidation. Homologus series of aldehydes were used in their study. In some mixtures of two compounds the flavor judges could detect a flavor at subthreshold concentrations of individual compounds, and in such mixtures the threshold levels of individual compounds were comparable. Similarly, Langler and Day (1964) reported a synergistic flavor interaction of methyl ketones which arise when milk fat is heated. These workers noticed that the flavor threshold values of ketones followed the pattern observed for aldehydes by Lillard and Day (1961). The flavor potency first increased with a corresponding increase in carbon chain length until it reached a maximum, then the flavor potency declines as the length of carbon chain increases. They reported that the individual compounds occurred at their subthreshold concentrations at the point of the average flavor threshold level of the mixture, indicating a synergistic interaction of the ketones. However, limited information is available concerning the interactive

behavior of the flavor compounds with different molecular structure and functional groups.

Individual flavor compounds can yield characteristic flavor notes in some instances, as butyric acid in rancid flavor, isoamyl acetate in banana flavor, and either ethyl butyrate or ethyl hexanoate in some fruity flavors. In most cases, more than one compound is required to approach completeness in flavor. Shabibi et al. (1964) reported that caproic, caprylic, capric and lauric acids were associated with rancidity. Scanlan, Sather, and Day (1965) demonstrated that rancid flavor is a combined effect of fatty acids in the butyric to lauric acid series, and no single acid was predominant over the other in its contribution to rancid flavor. In an attempt to determine the importance of free fatty acids in butter flavor, McDaniel, Sather, and Lindsay (1969) observed that the threshold values decreased from octanoic acid through dodecanoic acid. They used student t-values to indicate synergistic interaction of the compounds. Different t-values were obtained for different mixtures of the fatty acids studied. This indicated that chemical compounds vary in their interaction in mixtures with different compounds. The t-value for butyric acid in a mixture with octanoic acid was -4.5 whereas with decanoic acid it was -2.7. Lindsay, Day, and Sather (1967) found that diacetyl was the predominant flavor note in the culture formulations; however, if the diacetyl concentration was above 2 ppm,

reduction in acetaldhyde content caused a loss of full culture flavor, and a somewhat harsh diacetyl flavor. Their observations also suggest that a mixture of flavor compounds is necessary to impart a well balanced flavor to foods.

Theories of Flavor Perception

A brief review of the different theories of flavor perception is presented below which might aid understanding of the concept of synergistic flavor interactions. There are numerous theories concerning flavor perception, but few of them have experimental evidence. Amoore has proposed a structural theory of olfaction. Based on the molecular shape and size Amoore (1962a, 1962b) classified odors into seven primary groups. By studying the molecular models of chemical compounds belonging to these groups he has demonstrated that each group has different molecular shape, and there is agreement between molecular shape and odor. However, there are limitations to this theory; there are some small compounds which do not fulfill the size requirement, and at least ten molecules have odors not fitting their shape requirements (Gould, 1966, p. 34).

Recently Davies (1965) proposed interface-absorption and membrane-puncturing theory of olfaction. This theory considers molecular size, shape, polarity, location and type of functional

groups. However, with regards to odor quality, the Davies absorption theory has not yet been thoroughly investigated. In an attempt to correlate particular vibrational frequencies of a molecule in the infrared or Raman region with the characteristic odor of a compound, Wright proposed a vibrational theory (Schultz et al., 1967, p. 136). The probability level reached, in correlation of electromagnetic radiation and the odor of chemical compounds, is less than that achieved by the stereochemical theory.

In a recent paper, Kurt Kulka (1967) explained the manner in which the functional groups and the proximity of the functional groups may influence odor perception. He reported that styrene glycol, because of the vicinal configuration of the two hydroxyl groups, is odorless in spite of the aromatic ring. In contrast, benzyl alcohol has a faint but definite aromatic odor. However, the aromatic ring in styrene glycol apparently imparts a bitter taste to it. Calcium and sodium cyclamate both have a sweet taste. On the other hand, the corresponding acid cyclohexane sulfamic acid has a sweet-sour taste. The synergistic effect of these synthetic sweeteners, e.g., mixtures of saccharin and cyclamate are widely utilized in industry.

In a recent publication (Dastoli and Price, 1966) an entirely different mechanism of taste perception has been proposed. In this, taste perception was attributed to a protein complexing with a flavor compound. The protein which complexed with bitter substances was

thought to be different from that which complexed with sweet substances.

The importance of the size, shape and functional groups of odorivector molecules is now generally approved (Gould, 1966, p. 32). Considering the above statement it is conceivable that compounds with similar structure, shape or functional groups exhibit synergistic interaction in mixtures.

EXPERIMENTAL

Isolation and Identification of P. fragi

Commercial cottage cheese and pasteurized homogenized milk samples with a typical fruity aroma developed during refrigerated storage were plated and incubated at 7°C for 10 to 12 days. Three cultures (one from cottage cheese and two from milk) that developed the most pronounced fruity aroma were selected for use in this study. P. fragi ATCC No. 4973 obtained from American Type Culture Collection was also used in this investigation.

The proteolytic activity, which is characteristic of P. fragi, was determined by gelatin liquification. All four cultures were streaked on 0.4 percent gelatin-nutrient agar medium and incubated at 21°C and 7°C for 3 days and 12 days, respectively. At the end of the incubation period, the plates were flooded with acid mercuric chloride solution. Clear zones formed along the edge of the growth of the organism indicated proteolytic activity. The cultures were also tested for acid ring and fuity odor production in milk media.

Preparation of Culture Media

One hundred-milliliter quantities of pasteurized homogenized milk were dispensed into 250-ml Erlenmeyer flasks which were then

plugged with cotton and autoclaved at 121°C for 10 to 12 minutes. Since the addition of ethanol to milk medium enhanced the production of fruity aroma by P. fragi (Periera and Morgan, 1958), 0.2 percent ethanol was added to the sterile milk to be used in the studies of the identification of flavor compounds responsible for the ester-like aroma of P. fragi. The main advantage gained by addition of ethanol was an increase in the level of the fruity compounds which permitted an easier isolation and mass spectrometric identification. Some cultures were grown without added ethanol to observe possible qualitative differences in the production of fruity compounds due to addition of ethanol. A one percent inoculum of a P. fragi culture grown at 7°C for six to nine days was added to the flasks of milk media. Incubation until a definite fruity aroma developed was at either 21°C for 48 to 56 hours or at 7°C for eight days.

Analysis of Head Space Vapors of Fruity Cultures

Identification of Flavor Components

The gas-liquid chromatographic (GLC) technique developed by Morgan and Day (1965) was used as a primary means of evaluating the volatile components in the mature cultures. Figure 2 represents the entrainment assembly described by the authors. This technique principally consists of passing a stream of nitrogen through the

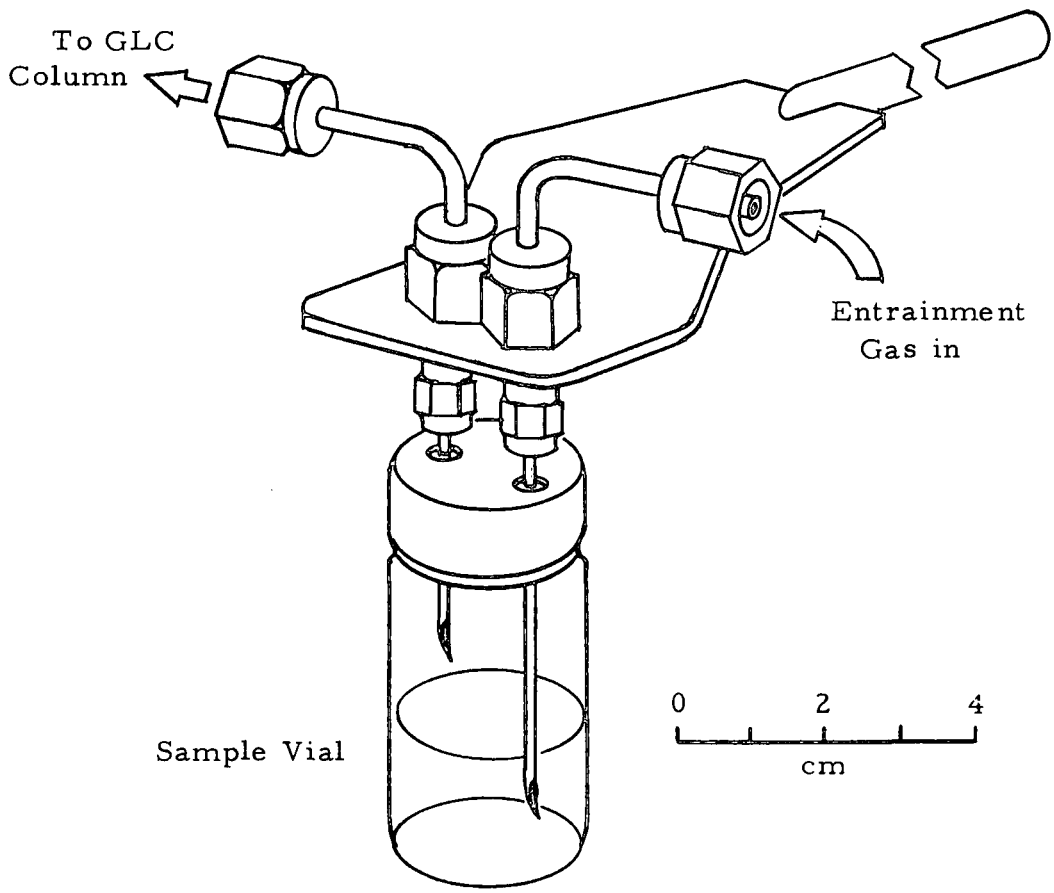


Figure 2. The head space gas entrainment assembly used in the analysis of volatile constituents from cultures.

sample contained in a screw-capped vial (Kimble no. G0957, size no. 1) by means of a hypodermic needle. The hypodermic needle was inserted through one of two holes drilled through the cap, which was lined with 1/8-inch thick silicone rubber. A second shorter needle was inserted through the second hole in the cap to provide an outlet for the nitrogen stream and the entrained volatiles. The volatiles were trapped in a U-shaped bend at the head of the GLC column which was temporarily withdrawn from the oven of a Varian Aerograph model 1200 instrument. The U-shaped portion of the column was immersed in a mixture of dry-ice and 2-methoxy ethanol. After the collection period, the head of the column was placed back in the instrument and the carrier gas flow resumed. During collection periods, a water bath was used to warm and stabilize the temperature of the vial containing the sample to improve the efficiency of removal of volatile materials.

For the analysis of cultures, nitrogen was bubbled at a rate of ten ml per min through four ml of culture made up to eight ml with 0.5 ppm n-butanol or 0.2 ppm 2-butanol in water. The contents of the vial also include sodium sulfate and approximately ten mg of 1-tetradecanol. The water bath in which the vials containing samples were immersed, was maintained at 60 $\pm$ 2°C.

The parameters of the columns used for the analyses are presented in Table 1. The higher temperature in both the instances

Table 1. Components of the columns used for GLC analysis

Column Parameters	Column No. 1	Column No. 2	Column No. 3
Dimensions	0.316 cm od[1], 3m long stainless steel tubing	0.316 cm od, 3m long stainless steel tubing	0.316 cm od, 3m long stainless steel tubing
Packing material	80-100 mesh alkali and acid washed Celite 545 coated with 20% 1,2,3-tris (2-cyano-ethoxy) propane	60-80 mesh Chromosorb W coated with 20% Carbowax 1500	60-80 mesh Chromosorb W coated with 20% 1,2,4-butanetriol.
Carrier gas	19 ml per min	19 ml per min	30 ml per min
Oven temperature	60 and 80°C	60 and 70°C	60°C

[1] od = outer diameter

were employed to obtain more rapid elution of ethyl butyrate and ethyl hexanoate. The flavor components separated by this system were tentatively identified by coincidence of retention times with those of authentic samples.

Isolation and Identification of Volatile Constituents from Cultures

Sample Preparation

Volatile compounds from a typically fruity culture were isolated by means of a cold-finger condenser similar to the apparatus described by Lea and Swoboda (1962). A 150 ml sample of the culture was placed in the flask, and the cold-finger was cooled by liquid nitrogen. After the pressure had been gradually reduced to about 20 mm Hg, the flask was isolated from the vacuum pump by means of a stopcock. The stopcock was opened occasionally to maintain a vacuum within the flask until a visible amount of distillate had collected on the cold-finger. The material condensed on the cold-finger was scraped directly into ethyl ether. The procedure was repeated four times with fresh samples. The aqueous layer was saturated with sodium chloride and allowed to stand for eight hours at 21^{o}C after which the ether layer was decanted from the aqueous layer. The aqueous layer was then exhaustively extracted with ether by continuous liquid-liquid extraction. The final total ether

extract was treated with anhydrous sodium sulfate to remove traces of water. The ether extract was first concentrated by fractional distillation, and finally the volume was brought to 0.3 ml by evaporation under a stream of nitrogen.

Analysis of Ether Extracts

The concentrated sample was analyzed by GLC equipped with flame ionization detector and a 0.316 cm od, and 3 m long stainless steel column packed with 80 to 100 mesh Chromosorb W, HMDS treated and coated with 20 percent Carbowax 20M. The column was operated isothermally at 80°C for six min and then programmed to 175°C at a rate of 6°C per min. Identity of the critical flavor components separated by this system was established by coincidence of retention times.

A portion of the ether extract obtained from the distillate of a P. fragi culture was fractionated by GLC under conditions permitting the olfactory evaluation of each component eluting from the column. For this purpose, a splitting device was attached to the detector end of the column. The splitter routed the effluent to the flame ionization detector and to the atmosphere through a heated outlet in a ratio of 1:5. Two components were found to have definitely fruity odors.

Positive identification of the most abundant volatile esters produced in a P. fragi milk culture was accomplished by mass

spectrometry. A gas Chromatograph, F & M model 810 was used in conjunction with an Atlas CH-4 Nier-type (a 9-inch, 60-degree sector) single focusing mass spectrometer. The mass spectrometer was equipped with a double ion source into which the total effluent was directed from the gas chromatograph. A 91.4 m long and 0.0254 cm id capillary column coated with Carbowax 20 M was used for separation. The column was operated isothermally at 80°C for 6 min and then programmed to 175°C at a rate of 6°C per min. The 20ev source was used as a GC detector. The 70ev source provided the ionization used to obtain mass spectra.

Quantitation of Few Volatile Constituents From Fruity Cultures

Preparation of Standard Curves

The stock solutions of ethyl butyrate and ethyl hexanoate were prepared in pasteurized milk containing 3.6 percent fat. Each ester was weighed into a capillary tube and the entire tube was carefully transferred into a volumetric flask. The compound was mixed thoroughly into the milk by constant stirring for 12 hours with a magnetic stirrer. From these stock solutions, dilutions ranging from 0.05 ppm to 1.0 ppm of ethyl butyrate and 0.05 ppm to 2 ppm of ethyl hexanoate were prepared in pasteurized milk with 3.6 percent fat. The solutions were analyzed by GLC according to the

on-column trapping technique described before.

The peak heights in chromatograms of each concentration of the authentic compound were adjusted by establishing an average recorder response to the internal standard solutions. The differences in sensitivity of the instrument during the sampling periods could be eliminated in this manner.

The quantity of ethyl butyrate and ethyl hexanoate present in P. fragi cultures was determined by GLC using standard curves. This method for the quantitative analysis of volatile compounds has been previously reported (Bills, 1966; Reddy, 1967). The only major modification consisted of the addition of 2-butanol and n-butanol as internal standards for the analysis of ethyl butyrate and ethyl hexanoate, respectively.

Factors Effecting Ester Production by P. fragi

Treatment of the Media

Reconstituted skim milk (Matrix Mother Culture Media, Galloway-West Company, Fond Du Lac, Wisconsin) containing less than 0.1 percent fat and homogenized milk containing 3.6 percent fat were used as the basic milk media. One hundred milliliter quantities of the media were dispensed into 250 ml Erlenmeyer flasks which were then plugged with cotton and autoclaved at 121°C

for 10 to 12 min. Other test media consisted of the above reconstituted skim milk and homogenized milk supplemented with 0.2 percent ethanol or 0.2 percent ethanol and 0.25 percent butyric acid.

Two strains of P. fragi, 9165 and 5986, isolated from commercial cottage cheese and homogenized milk, respectively, were used in this investigation. A 1 percent inoculum from one lot of these cultures grown in milk at 7°C for nine days was added to all test media. Uninoculated controls were also maintained. All cultures were incubated at 21°C quiescently or under agitation on a mechanical shaker at the rate of 96 oscillations per minute. The incubation time required by a majority of the cultures to develop a fruity aroma was 48 to 52 hours. The cultures were then plated in nutrient agar to determine the cell count.

Comparison of Volatile Constituents of Cultures

The qualitative and quantitative changes resulting from various treatments of the cultures were determined by evaluating the volatile components in the cultures using the GLC on-column trapping technique of Morgan and Day (1965). A stainless steel column, 0.316 cm od and 3 m long packed with 80 to 100 mesh, acid and alkali washed Celite 545 coated with 20 percent 1,2,3-tris (2-cyanoethoxy) propane was used. Samples were chromatographed at 80°C. Nitrogen was used as the carrier gas at a flow rate of 19 ml per min.

The quantities of ethyl butyrate and ethyl hexanoate were estimated from the standard curves.

Larger volumes of cultures were used for studies made at intervals during growth. For this purpose, 500 ml quantities of homogenized milk were placed in 1000 ml Erlenmeyer flasks which were then plugged with cotton and autoclaved at 121°C for 10 to 12 minutes. The media were supplemented with 0.2 percent ethanol and inoculated with 1 percent of P. fragi cultures grown at 7°C for nine days. The cultures were incubated at 21°C quiescently or under agitation as described above. Culture aliquots were taken at intervals over a period of 72 hours and analyzed for pH and cell count. Variations in the concentration of esters produced during a period of 72 hours incubation were studied using the GLC technique described in the previous paragraph.

Microbial Interaction

Streptococcus lactis strain LI (Bills, 1966) and P. fragi strain 9165 were grown together in milk to study the inter-relationship between the two organisms. Pasteurized milk with 3.6 percent fat was dispensed in 200 ml quantities into 500 ml Erlenmeyer flasks which were then plugged with cotton and autoclaved at 121°C for 12 minutes. A one percent inoculum of S. lactis was added to two lots of test media which were incubated at 22° C for 36 hours. The

cultures were neutralized with calcium carbonate to approximately pH 6.2. A third lot of test medium was first acidified with lactic acid to approximately pH 4.6, and then was brought up to approximately pH 6.2. All three test media were inoculated with one percent inoculum of P. fragi. A fourth test medium was inoculated with P. fragi culture alone. All four cultures were incubated at 7°C for eight days. An uninoculated medium was also maintained as control. At the end of the incubation period the cultures were analyzed by sensory and GLC methods for esters.

Demonstration of the Presence of Esterase Enzyme Systems in P. fragi

The procedure described by Nordstrom (1962) was applied to determine whether the ester production in P. fragi is due to an enzyme catalyzed reaction or coenzyme A activation phenomenon. It essentially consists of the addition of 2 mM, 4 mM, 6 mM and 8 mM of sodium arsenite to 100 ml aliquots of milk media inoculated with one percent P. fragi. The cultures were incubated at 22°C for four days. The concentration of ethyl butyrate and ethyl hexanoate in the resulting cultures was determined by GLC. The cultures were also analyzed for cell count, short chain free fatty acids and total free fatty acids. Steam distillation (Keenan, Bodyfelt and Lindsay, 1968) was used to determine the volatile short chain fatty acids. The

distillates were titrated to a phenolphthalein end point with 0.1 N NaOH. The results are expressed as milliliters of base to reach the end point. To determine the total fatty acids, fat was extracted from the culture media by treating it with BDI reagent. The acid degree value of the fat was then determined by titrating with 0.1 N alcoholic potassium hydroxide (Thomas, Nielsen, and Olson, 1955).

Evaluation of Esterases in P. fragi

Culturing Procedure

P. fragi strains 9165 and ATCC No. 4973 were used in this investigation. One percent Bactopeptone broth was dispensed in two liter quantities into 4000 ml Erlenmeyer flasks which were then plugged with cotton and autoclaved at 121°C for 10 to 12 minutes. A 0.5 percent inoculum from the two cultures grown in one percent peptone broth at 7°C for nine days was added to the two liter broth aliquots. Both cultures were incubated at 21°C quiescently for four days.

Preparation of Cell Free-Extract

The bacteria were harvested by centrifugation in a cold room (5°C) at 7700 x g for 20 minutes in a Super-speed Servall Centrifuge. The supernatant fluid was saved for tests to determine the presence

of esterases. The cells were washed two times with chilled 0.025 M potassium phosphate buffer (pH 6.8) and suspended in 12 ml of the buffer. The container was immersed in an ice bath while the bacteria were disintegrated for six to eight minutes using Bronwill Biosonic III with an intermediate tip. The extracts were clarified by centrifugation at 100,000 x g for one hour in a Beckman Model L-Z Ultra-Centrifuge. The cell free extract and the supernatant fluid were freeze-dried at 50°F, and subsequently assayed for esterase activity using a Gilson differential respirometer.

Preparation of Substrated for Assay of Esterase Activity

The substrates used for the determination of esterase distribution in P. fragi cultures were ethyl butyrate[1], triacetin[1] and phenyl acetate[1]. The concentrations of these compounds were 1 M. All substrates were prepared in three percent (w/v) Triton X-155[2] and 0.1 percent gum arabic[3]. The esters and triglycerides were homogenized for two minutes in a tap-water cooled microblender.

Assay Procedure

Three separate P. fragi culture fractions were assayed for

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[1] Eastman Organic Chemicals

[2] Rohm and Haas Company

[3] Matheson, Coleman and Bell

esterase activity. These were (1) washed whole cell suspensions, (2) culture supernatant fluid, and (3) cell-free extracts of sonicated P. fragi cells. The method used was a manometric technique similar to that described by Norgaard (1968) and Veerabhadrappa (1968) using a Gilson differential respirometer. The volume of CO_2 liberated from the bicarbonate buffer by the acid produced after substrate hydrolysis was used as the measurement of activity. The main compartment of the respirometer flask contained 1.5 ml of 1.04×10^{-2} M $NaHCO_3$ buffer, one ml of enzyme, and one ml of distilled water or inhibitor preparation, depending upon the experiment. The side arm of each flask contained 0.1 ml of 2.23×10^{-2} M $NaHCO_3$ and 0.4 ml substrate. A mixture of 95 percent N_2 and 5 percent CO_2 was passed through flasks for ten minutes. After gassing, the side arms were closed and the system equilibrated for ten minutes at 25°C and 770 mm Hg pressure. The contents of the side arms were tipped into the main compartment and the readings were taken at 10 minute intervals for 50 minutes. The observations were made in duplicate. Esterase activity was measured in units. One unit of esterase activity was defined as the volume of CO_2 in microliters released per minute per ml of the enzyme preparation. Corrections for room temperature differences were calculated according to Formula D of Gregory and Winter (1965).

Electrophoresis of Esterases

The cell free extract, prepared according to the procedure described above, was used in the electrophoretic study. The

supernatant fluid was not analyzed by electrophoresis because the esterase activity was observed only in the sonicated cell preparation.

Preparation of Substrates for Gel Electrophoresis

The ester substrates used in this study were: α-naphthyl acetate[4], α-naphthyl propionate[4], and α-naphthyl butyrate[4]. Forty mg. of the ester was dissolved in two ml of acetone before mixing with 100 ml of buffer.

Electrophoretic Procedure

Vertical gel electrophoresis was performed in a plexiglass cell (Model EC-470, E-C Apparatus Corporation) with a companion power supply (Model EC-454). Seven percent Cyanogum-41[5] was prepared in 150 ml of 0.017 M Tris-0.2 N HCl buffer (pH 7.5) and filtered through Whatman No. 1 paper. After adding a small drop of Tween 80, 0.15 ml n, n, n', n'-tetramethylethylenediamine[4] (TMED) and 0.15 gm ammonium persulfate[5] were added to catalyze gel polymerization. The gel solution was immediately poured into the space between the cooling plates of the electrophoretic cell and the slot-former was inserted before polymerization occurred. The cell was maintained in a horizontal position for at least 40 minutes to allow the formation

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[4] Sigma Chemical Company

[5] E-C Apparatus Corporation

of a colorless gel. In this study a slot-former containing four slots was used. This permitted a maximum sample volume of approximately 200 µl per slot.

After polymerization the remaining electrophoretic procedure was carried out in the cold room at 5°C. The cell was placed in a vertical position and the electrode buffer (0.03 M boric acid 0.1 N NaOH, pH 8.7) was poured into the upper compartment until overflow covered the platinum wire electrode in the lower compartment by two to three cm. Cold water (5°C) circulation through the cooling channels of the cell was maintained during the run. The excess gel above the slot former was excised, and the slot former was removed by gently sliding upward. The buffer was circulated at least for 20 min before sample application to allow equilibration of the gel-buffer system.

Each sample was prepared with ten percent sucrose and contained a small quantity of bromphenol blue as a marker. The buffer circulation was stopped before application of the sample. Approximately 150 µl of the sample was applied with a one cc tuberculin syringe directly onto the slots at the top of the gel to form a layer under the buffer. Buffer circulation was started after the samples were completely impregnated into the gel (ca. 15 min after voltage was applied). Electrophoresis was performed at a constant potential of 300 volts for 1.5 to 2.0 hours.

Zymogram Development

On completion of electrophoresis, the gel was removed from the cell and cut into four strips. The gel strips were placed in 100 ml of 0.025 M potassium phosphate buffer (pH 6.8) containing 70 mg of Fast Blue RR salt and 40 mg of the ester dissolved in two ml of acetone. The time of incubation at 25°C ranged from 45 minutes to 2 hours depending on the substrate used.

Synergistic Interaction of Esters

Flavor interactions were studied in the mixture of ethyl hexanoate-ethyl butyrate, ethyl butyrate-butyric acid, ethyl butyrate-ethyl acetate and ethyl hexanoate-ethyl isovalarate, diacetyl-ethyl hexanoate and dimethyl sulfide-ethyl hexanoate. The flavor threshold values (FTVs) of individual compounds were determined prior to the study of synergistic interactions of mixtures.

Chemicals

Ethyl butyrate[6], ethyl isovalerate[6], ethyl hexanoate[6] and ethyl acetate[6] were purified by preparative gas chromatography. The instrument used was an Aerograph Model A-700 (Autoprep) fitted

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[6] K & K Laboratories

with a 183 cm long and 0.4 cm id column with 15 percent diethylene glycol succinate (DEGS) on Chromosorb G. Redistilled methyl sulfide[7], butyric acid[7] and diacetyl[7] were used. All compounds were examined by high-sensitivity gas chromatography and were found to be at least 99.9 percent pure.

Preparation of the Dilutions

Stock solutions (100 ppm concentration) of the individual compounds were prepared in sterile homogenized milk. Since ethyl butyrate, ethyl hexanoate and ethyl isovalerate are not readily soluble in an aqueous system, stock solutions were agitated constantly in tightly sealed volumetric flasks by means of a magnetic stirrer.

Dilutions were made in homogenized milk from the stock solutions in appropriate ranges for FTV determinations. The range of these dilutions varied with different compounds, but always included concentrations below and above the flavor threshold.

Threshold Panel Tests

The flavor threshold values (FTVs) were determined by a method similar to that described by Wyatt and Day (1965) and was the 50 percent positive response. The panel consisted of ten

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[7] Eastman Distillation Products

experienced flavor judges. A "yes-no" type ballot similar to that described by Wyatt and Day (1965) was used. The individual FTVs for a pair of compounds and their mixture threshold values were determined on the same day using the same panelists. Fresh homogenized milk and a sample containing a high concentration of the test compounds were labeled and presented with each tray as references. The dilutions for the mixtures were based on the FTV of each individual compound determined during the same day. Five dilutions were made by mixing compounds in ratios proportional to their concentration at their individual FTVs. For example, in the ethyl butyrate and ethyl acetate mixture, the first dilution was prepared by combining ethyl butyrate at 25 percent of its FTV (i.e., 3.75 ppb) and ethyl acetate at 25 percent of its FTV (i.e., 1.18 ppm). The dilutions of the mixtures prepared covered a range from 25 to 125 percent of the FTVs of the individual compounds. Fresh dilutions were prepared for each evaluation. All dilutions were warmed to room temperature before serving to the panel in random coded glasses. To determine whether combinations of the compounds exhibit an additive interaction, the concentration of each compound at the FTV of the mixture was compared to the flavor threshold of the individual compounds.

Statistical Analysis

The data from replicate panels consisting of the ten judges were statistically analyzed. The flavor threshold value (FTV) of an individual compound was calculated from the FTVs obtained for the compound in a number of independent threshold tests. The FTVs were transformed by dividing the FTV of each compound in the mixture by the FTV of the compound determined when it was evaluated by itself. This transformation resulted in an approximate common variance for all compounds. A pooled estimate of this variance was used in the t-tests Student's t-tests were used to test for significant synergistic flavor interactions. Negative t-values indicate participation in a synergistic flavor interaction. The 95 percent confidence limits were calculated for the FTVs of individual compounds.

RESULTS AND DISCUSSION

Organoleptic Evaluation of Cultures

The development of the fruity flavor was enhanced at higher temperatures, as the cultures of P. fragi incubated at 22°C developed a stronger fruity odor than those incubated at 7°C. The strains of P. fragi used in this investigation varied in their ability to produce a fruity flavor. Sensory evaluation of the cultures revealed that the time required for the development of detectable fruity flavor was approximately 36-48 hours at the higher temperature and seven to eight days at the lower temperature of incubation. These findings emphasize the importance of proper refrigeration in extending the shelf life of certain dairy products.

Compounds Responsible for the Fruity Flavor

The direct gas-liquid chromatographic analysis of the volatiles trapped from the P. fragi cultures showed a number of peaks which could be tentatively identified on the basis of their retention times. Typical chromatograms of the highly volatile constituents of the culture non-supplemented with ethanol and culture added with 0.2% are presented in Figure 3. Peaks corresponding to acetaldehyde, dimethyl sulfide, acetone, ethyl acetate, ethanol, ethyl butyrate,

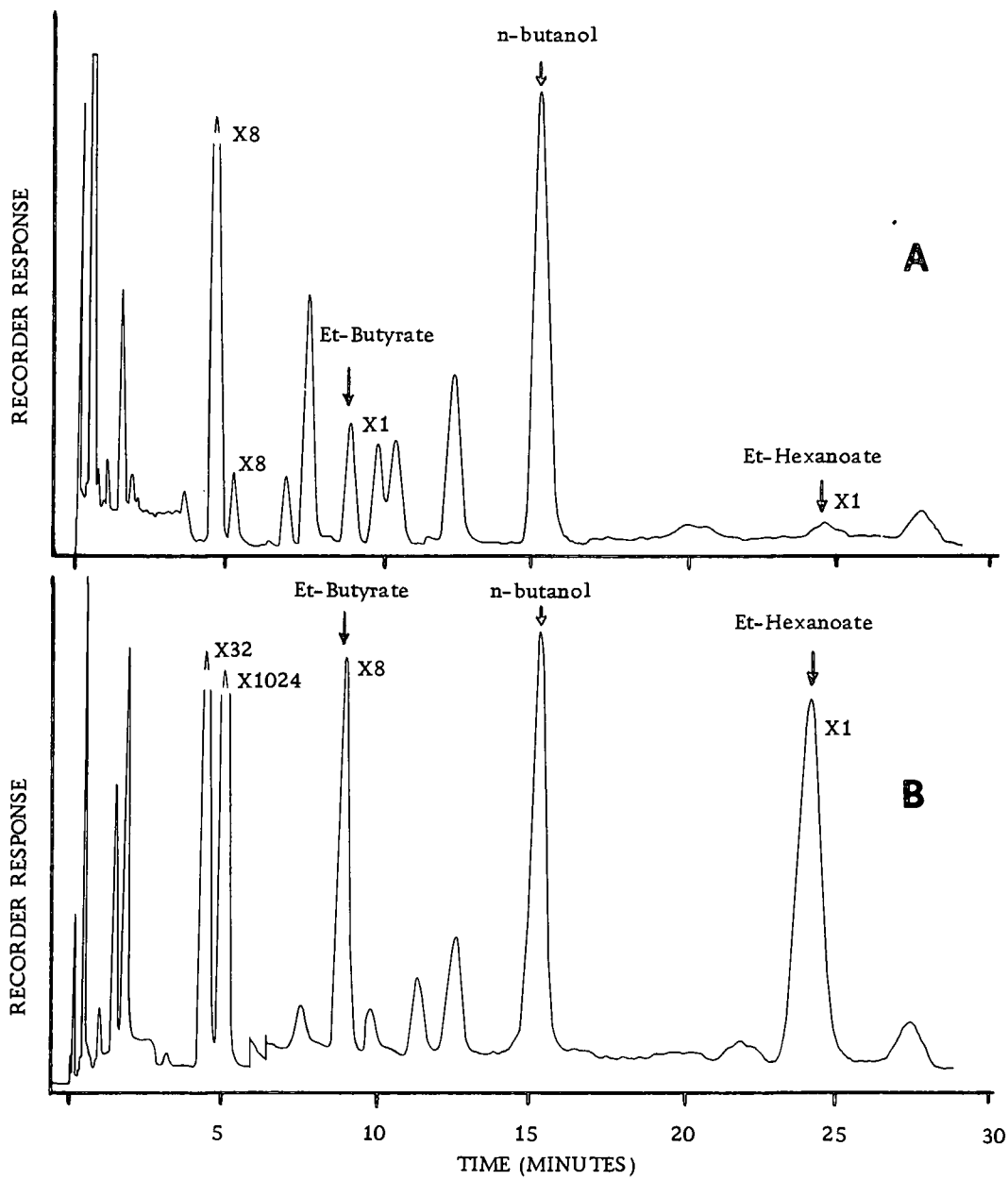

Figure 3. Chromatograms of the volatile components of P. fragi culture; A, non-supplemented with ethanol; B, supplemented with 0.2% ethanol.

ethyl isovalerate and ethyl hexanoate were detected with the 1, 2, 3-tris (2-cyanoethyoxy) propane column. However, two extra peaks corresponding to ethyl propionate and isopropanol were observed with the Carbowax 1500 column. The retention times of ethanol and isopropanol on the former column were such that the peak for isopropanol was obscured in chromatograms of the volatiles from the cultures fortified with ethanol. However, these alcohols were well separated on the 1, 2, 4-butanetriol column. The retention times for the compounds separated on the 1, 2, 3-tris (2-cyanoethoxy) propane column as well as the corresponding authentic compounds are presented in Table 2.

Of the compounds listed, acetaldehyde, dimethyl sulfide and acetone are always present in milk (Bills and Day, 1966). During the course of this study great variations were observed in the concentrations of ethanol in control milks. Its occurrence as a trace to major component of the volatiles undoubtedly related to the feed of the animals from which the milk was drawn (Bassette et al., 1966).

Peaks tentatively identified as ethyl acetate, ethyl propionate, ethyl butyrate and ethyl hexanoate esters were detected in the volatiles from cultures fortified with ethanol and incubated at 22°C, and in unfortified cultures incubated at 7°C. The ratio of the concentrations of these esters was approximately 40:1:20:15, respectively. This indicated that the propionate ester was a minor component

compared to the other esters. The peak tentatively identified as ethyl isovalerate was not observed in all the cultures analyzed, and when observed was very small compared to ethyl butyrate and ethyl hexanoate peaks. From the standpoint of the fruity aroma produced by this organism, the large peaks tentatively identified as ethyl butyrate and ethyl hexanoate appeared to be particularly important. Evaluation of the odor of authentic compounds indicated that ethyl acetate has a sharp, solvent-like odor, while ethyl butyrate and ethyl hexanoate are quite fruity. As can be observed from the Figure 3, the levels of ethyl butyrate and ethyl hexanoate were found to be higher in the culture supplemented with ethanol than in the non-supplemented culture.

Table 2. Tentative identification assigned to volatile constituents of P. fragi culture.

Peak No.	Compound	t_R/t_R n-butanol	
		Unknown	Known
1	dimethyl sulfide	0.13	0.13
2	acetaldehyde	0.16	0.16
3	unknown	0.24	
4	ethyl acetate	0.30	0.29
5	acetone	0.32	0.32
6	ethanol	0.36	0.36
7	unknown	0.51	
8	ethyl butyrate	0.60	0.60
9	unknown	0.84	
10	n-butanol	1.00	1.00
11	unknown	1.36	
12	ethyl hexanoate	1.60	1.59
13	unknown	1.80	

Mass Spectral Analysis

The identity of ethyl acetate, ethyl butyrate and ethyl hexanoate was confirmed by mass spectrometry. Fragmentation peaks in the mass spectra of these components were compared to the data obtained by Sharkey et al. (1959) for aliphatic esters, and the Atlas of Mass Spectral Data (Stenhagen, Abrahansson and McLafferty, 1969). All significant fragmentation peaks required for identification were found in the mass spectra of the three esters. A weak spectrum indicating the presence of ethyl formate was also obtained.

Quantitation of the Principle Esters in Fruity Cultures

Standard curves obtained by plotting the peak heights for various concentrations of ethyl butyrate and ethyl hexanoate in milk are shown in Figure 4. Based on these standard curves, the concentrations of ethyl butyrate and ethyl hexanoate in a culture of P. fragi grown at 22°C in homogenized milk fortified with 0.2 percent ethanol were found to be 0.35 and 0.5 ppm, respectively. In unfortified cultures, the level of these esters was eight to ten times lower. The addition of 0.35 ppm of ethyl butyrate and 0.5 ppm of ethyl hexanoate to homogenized and autoclaved milk resulted in a product with a fruity aroma very similar to that of a mature P. fragi culture. This observation supports the conclusion that these two components are

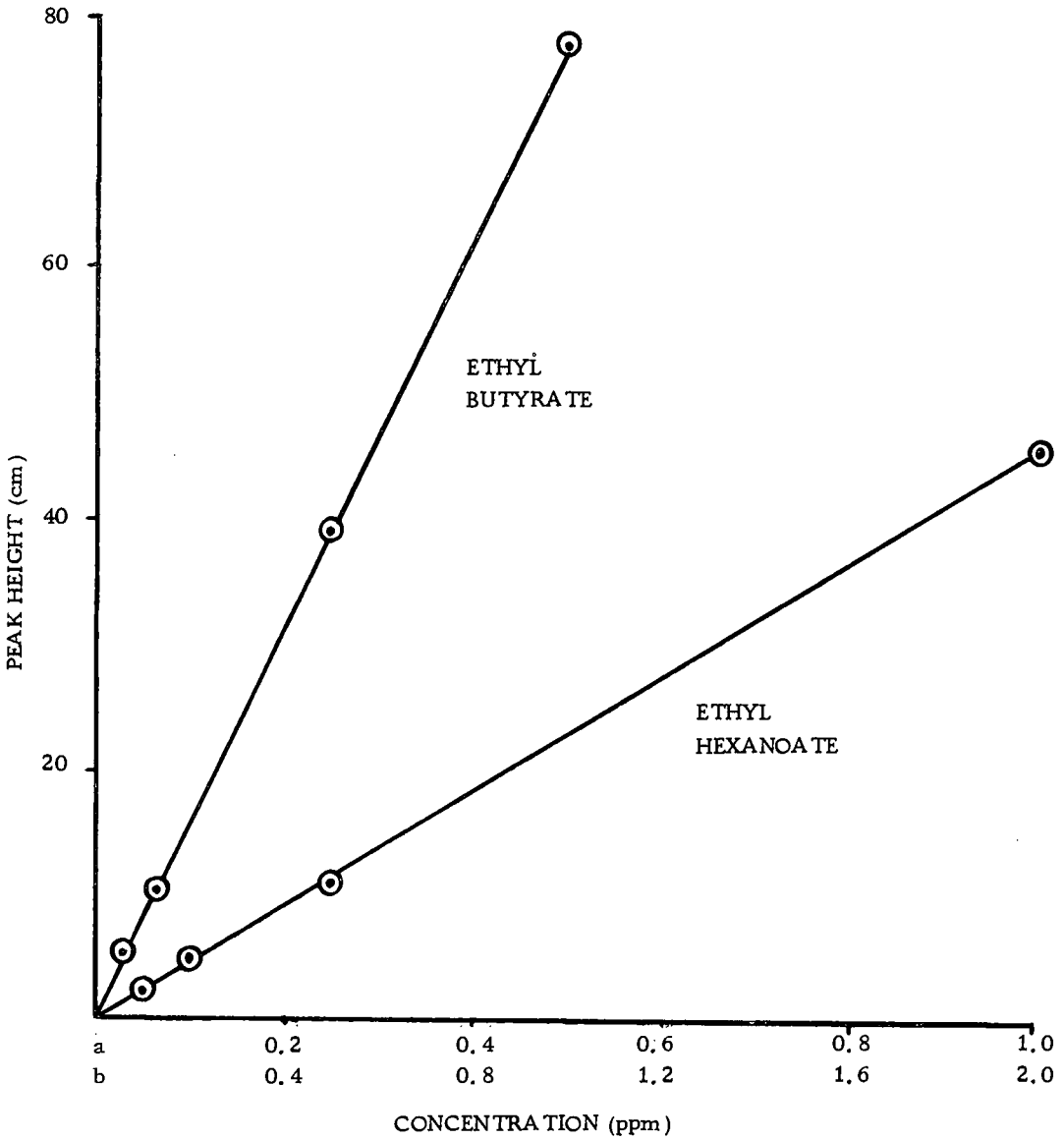

Figure 4. Recorder response obtained with various concentrations of ethyl butyrate and ethyl hexanoate.
a = ethyl butyrate concentration.
b = ethyl hexanoate concentration.

essentially responsible for the fruity flavor. Considering the demonstrated lipolytic ability of P. fragi (Nashif and Nelson, 1953) and free fatty acids resulting from the hydrolysis of milk triglycerides (Richards and El-Sadek, 1949), the presence of ethyl butyrate and ethyl hexanoate as principle esters in fruity cultures of P. fragi can be justified.

Although there is some evidence for the production of ethyl isovalerate by one strain of P. fragi included in this study, it was found in much lower concentration than the ethyl butyrate and ethyl hexanoate esters. Previously, Periera and Morgan (1958) reported ethyl isovalerate in P. fragi cultures in quantities that led to the conclusion that it was one of the more abundant esters. Probably some strains of the organism are less lipolytic and tend to produce less butyric and hexanoic acids as substrates for esterification. Ethyl isovalerate might then become one of the predominant esters produced by such cultures.

Various Treatments of P. fragi Cultures

Considerable variations were observed in the concentrations of ethyl butyrate and ethyl hexanoate esters produced by P. fragi in different media. The results presented in Tables 3 and 4 summarize the effect of aeration and medium on ester production and the cell number.

Table 3. Effect of aeration and medium on ester production and cell count in homogenized milk culture of P. fragi.

Culture	Type of media (Aerated)			Type of media (Non-aerated)		
	Milk	Milk+ethanol	Milk+ethanol + C_4 acid	Milk	Milk+ethanol	Milk+ethanol + C_4 acid
9165 (1st trial)						
cell count[a]	5.0	9.6	7.2	13.2	28.2	9.0
Ethyl butyrate[b]	0.01	0.68	0.84	ND[c]	0.16	0.25
Ethyl hexanoate[b]	0.14	0.81	0.43	ND	0.40	0.35
9165 (2nd trial)						
cell count[a]	15.4	15.6	13.8	36.0	44.0	32.0
Ethyl butyrate[b]	0.02	0.20	0.61	0.02	0.60	0.72
Ethyl hexanoate[b]	< 0.01	0.08	0.11	0.01	0.28	0.22
5986 (1st trial)						
cell count[a]	8.3	10.1	6.2	11.9	12.9	10.2
Ethyl butyrate[b]	0.04	0.16	0.18	0.03	0.43	0.39
Ethyl hexanoate[b]	0.10	0.11	0.20	0.05	1.80	1.60
5986 (2nd trial)						
cell count[a]	6.0	9.7	4.9	24.0	28.0	21.0
Ethyl butyrate[b]	0.01	0.08	0.14	0.05	0.41	0.80
Ethyl hexanoate[b]	ND	< 0.01	< 0.01	< 0.01	0.09	0.03

[a] Count x 10^8/ml

[b] Ester concentrations in parts per million

[c] ND = not detected

Table 4. Effect of aeration and medium on ester production by P. fragi in reconstituted skim milk

Culture	Type of media (Aerated)			Type of media (Non-aerated)		
	Skim milk	Skim milk + ethanol	Skim milk + ethanol + C_4 acid	Skim milk	Skim milk + ethanol	Skim milk + ethanol + C_4 acid
9165						
Cell count[a]	10.2	12.6	5.7	25.8	28.3	21.4
Ethyl butyrate[b]	< 0.01	0.45	1.80	< 0.01	0.07	0.48
Ethyl hexanoate[b]	< 0.01	0.01	0.03	ND[c]	0.01	< 0.01
5986 (1st trial)						
Cell count[a]	9.7	7.8	6.6	14.0	13.1	9.8
Ethyl butyrate[b]	0.01	0.18	0.40	< 0.01	0.14	0.28
Ethyl hexanoate[b]	ND	0.24	0.27	ND	0.2	0.44
5986 (2nd trial)						
Cell count[a]	5.0	1.7	3.4	32.0	29.0	14.0
Ethyl butyrate[b]	ND	0.05	1.04	0.02	0.26	1.42
Ethyl hexanoate[b]	ND	ND	< 0.01	ND	0.10	0.04

[a] Count x 10^8/ml

[b] Ester concentrations in parts per million

[c] ND = not detected

Effect on Culture Growth

In all the treatments of both homogenized milk culture and skim milk culture a definite decrease in cell number was noticed as a result of aeration. Nashif and Nelson (1953b) also observed similar decrease in cell population caused by constantly shaking the medium. They suggested that since P. fragi is aerobic, it usually forms a pellicle on the surface of the undisturbed liquid medium and thus allows greater contact of the surface growth with air.

The results revealed a general decrease in cell number in cultures supplemented with 0.2 percent ethanol and 0.25 percent butyric acid compared to the cultures supplemented with ethanol alone. This seems logical since the addition of butyric acid changes the pH of the medium, and this might not be conducive for rapid growth of P. fragi. A similar effect brought about by increased acidity was reported by Goldman and Rayman (1952) when they grew P. fragi in butter oil. It would appear that the cell count is generally higher in media supplemented with ethanol than in non-supplemented media. This could result from the delay of inhibitor effect on growth caused by acids in the medium through the removal of the acids by esterification.

Effect on Quantity of Esters Formed

The addition of ethanol to the culture medium did not appear to qualitatively influence ester production. There was no evidence for ester formation in uninoculated controls. The addition of both butyric acid and ethanol to either the homogenized milk or reconstituted skim milk media markedly enhanced the final concentration of ethyl butyrate, but the effect was more pronounced in reconstituted skim milk media. Further, the addition of butyric acid and ethanol resulted in a lower ethyl hexanoate concentration than in media supplemented with ethanol alone (Table 3). The decreased production of ethyl hexanoate in cultures supplemented with butyric acid is comparable to the observation of Nordstrom (1964), who found that the addition of hexanoic acid to a culture of yeast increased the yield of hexanoate esters but reduced the amount of acetate esters formed.

The results presented in Tables 3 and 4 revealed that the concentrations of esters in cultures supplemented with either ethanol alone or ethanol and butyric acid were higher than those in non-supplemented cultures. The observation that the addition of ethanol to the culture medium increases the concentration of ester formed is in agreement with the earlier work of Periera and Morgan (1958). This is conceivable, since the ability of P. fragi to readily hydrolyze butterfat has been demonstrated by Goldman and Rayman (1952). The

liberated short-chain fatty acids likely serve as the acid substrate for esterification. Hence, the addition of ethanol to the medium would be expected to result in a definite increase in the concentration of esters if the ethanol substrate concentration is the limiting factor. The concentration ratio of ethyl butyrate in non-supplemented homogenized milk media to that in the homogenized milk media supplemented with ethanol was 1:17, and the ratio of ethyl hexanoate was 1:11. In reconstituted skim milk the concentration ratio of ethyl butyrate between the above two treatments was 1:20 and that of ethyl hexanoate was approximately 1:70. In homogenized milk supplemented with ethanol, higher cell number was associated with higher ester concentration. However, this trend was not observed in the trials conducted with skim milk.

The highest concentration of ethyl butyrate was noticed in the cultures supplemented with both butyric acid and ethanol. The average concentration of ethyl butyrate in such media was 1.5 times greater in homogenized milk and 5.0 times greater in reconstituted skim milk than in media supplemented with ethanol alone.

The results concerning the effect of media on ester production are presented in Table 5. With one exception, the mean concentrations of ethyl butyrate and ethyl hexanoate in homogenized milk media were higher than those in reconstituted skim milk media. The relatively high concentration of the two esters in homogenized milk medium

Table 5. Effect of media on mean concentration of esters for all trials

Compound	Homogenized milk media			Reconstituted skim milk media		
	Milk	Milk + ethanol	Milk + ethanol + C_4 acid	Skim Milk	Skim milk + ethanol	Skim milk + ethanol + C_4 acid
Ethyl butyrate[a]	0.02	0.34	0.50	<0.01	0.20	0.90
Ethyl hexanoate[a]	0.04	0.45	0.37	<0.01	0.10	0.12

[a] Ester concentrations in parts per million.

supplemented with ethanol over skim milk medium subjected to similar treatment could be due to the presence of more fat in the homogenized milk medium. The fat could be hydrolyzed by P. fragi into free fatty acids which in turn act as substrates in ester production.

Relationship Between Ester Formation and Cell Growth

Typical time-dependent patterns for ester formation in both aerated and nonaerated media inoculated with the same strain of P. fragi are presented in Figures 5 and 6. These results show a general tendency of the ester concentration to increase with the increase in cell population. This observation is in agreement with that of Nordstrom (1964), who indicated that, in case of brewers' yeast, an increased growth in most cases was associated with an increased formation of the esters. However, Nashif and Nelson (1953b) did not observe any close relation between cell count and lipase production at different pH levels. Figures 5 and 6 further illustrate that the ethyl butyrate formation in nonaerated cultures was delayed by 24 hours compared to the aerated cultures. The formation of ethyl butyrate was faster than ethyl hexanoate formation in both the cultures. In general aeration reduced the cell count. This agrees with the findings of Nashif and Nelson (1953b).

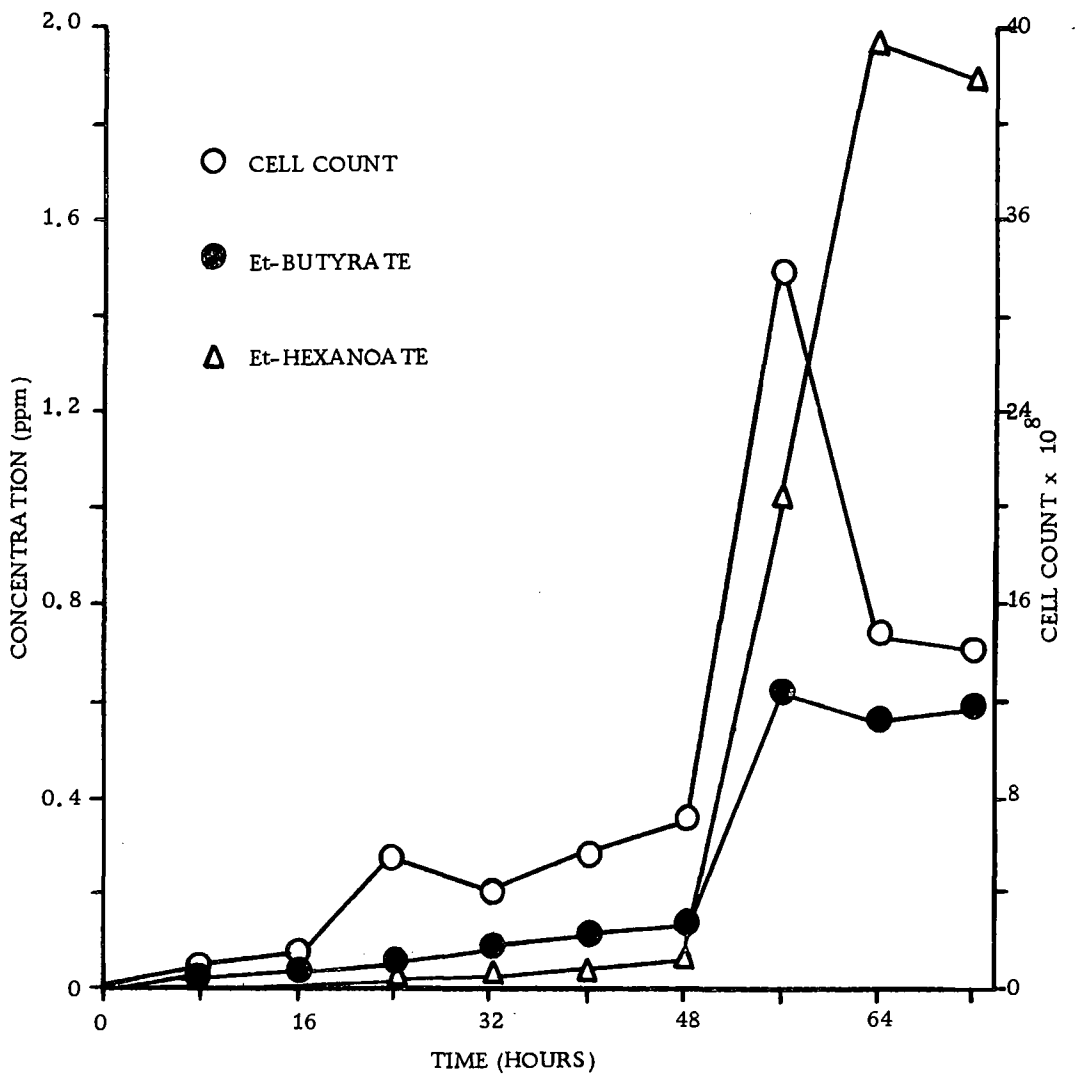

Figure 5. Typical time-dependent pattern for ester production by P. fragi in aerated reconstituted skim milk medium supplemented with 0.2% ethyl alcohol.

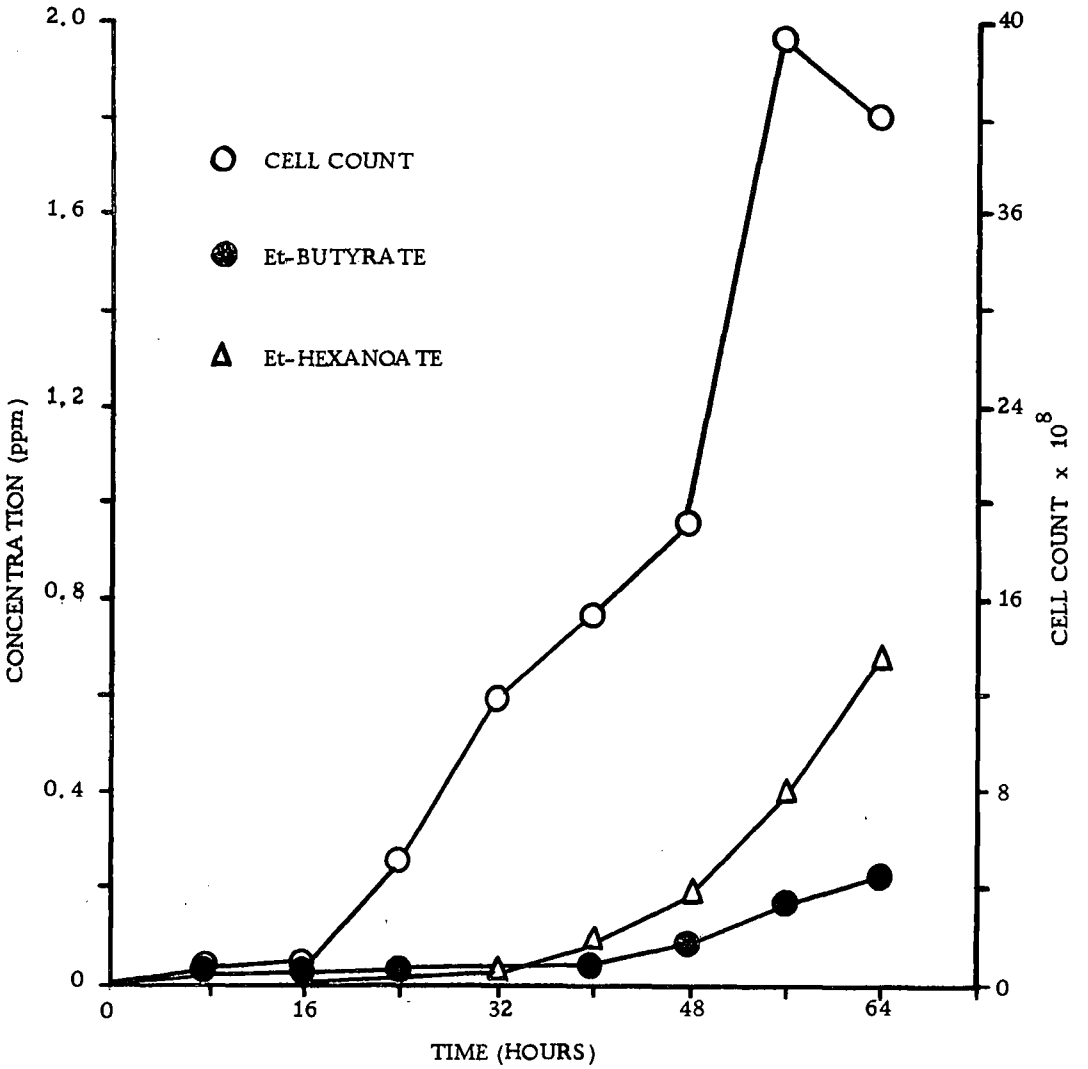

Figure 6. Typical time-dependent pattern for ester production by P. fragi in nonaerated reconstituted skim milk medium supplemented with 0.2% ethyl alcohol.

Microbial Interaction in Milk Cultures

The results presented in Table 6 show the quantities of ethyl butyrate and ethyl hexanoate esters produced by P. fragi grown in combination with S. lactis in milk cultures. This S. lactis strain has been shown to produce approximately 12 ppm of ethanol at 21°C in 36 hours of incubation (Bills, 1966).

Table 6. Effect of S. lactis on ester production by P. fragi.

Compound	S. lactis	P. fragi plus S. lactis	P. fragi	Control
Ethyl butyrate[a]	ND[b]	0.38	0.08	ND
Ethyl hexanoate[a]	ND	0.05	0.02	ND

[a] Ester concentration in parts per million.

[b] ND = not detected.

When S. lactis was grown alone at 22°C for 48 hours the pH was lowered to 4.6. Calcium carbonate, heat treated at 171°C for one hour, was added to raise the pH to 6.2 and facilitate the growth of P. fragi. Organoleptic evaluation of the cultures revealed fruity aroma for both P. fragi cultures and S. lactis plus P. fragi cultures after six days of incubation at 7°C. However, the concentration of ethyl butyrate in the mixed culture of S. lactis and P. fragi was almost five times more than that present in medium inoculated with P. fragi alone (Table 6). This could be due to availability of the ethanol

produced by S. lactis which acts as a substrate for production of esters by P. fragi. Considering that P. fragi can hydrolyze fat at 10°C when grown in combination with S. lactis (Haromon and Nelson, 1955), the higher ester levels in mixed cultures of S. lactis and P. fragi probably result from a synergistic production of esterification reactants. This observation could have a significant bearing on the development of methods to reduce or eliminate fruity flavor defects in products such as cottage cheese.

Effect of Sodium Arsenite on Ester Production by P. fragi

Sodium arsenite ($NaAsO_2$) has been shown to inhibit the ester production by yeast due to its toxic effect on the CoA system; the inhibition is accompanied by an increase in acid formed (Nordstrom, 1962). However, from the data presented in Figure 7 it appears doubtful that $NaAsO_2$ has a similar effect on ester production by P. fragi. The data presented indicate that the addition of low concentrations (less than 4 mM) of $NaAsO_2$ to P. fragi cultures caused a slight decrease in cell growth and ethyl hexanoate concentration, and a marked decrease in the concentration of ethyl butyrate and the acid degree value. At concentrations above 4.0 mM of $NaAsO_2$ the cell count was markedly reduced. In general, it appears that the decrease in ester concentrations is due to a reduction in the number of cells rather than a poisoning of the CoA system by $NaAsO_2$.

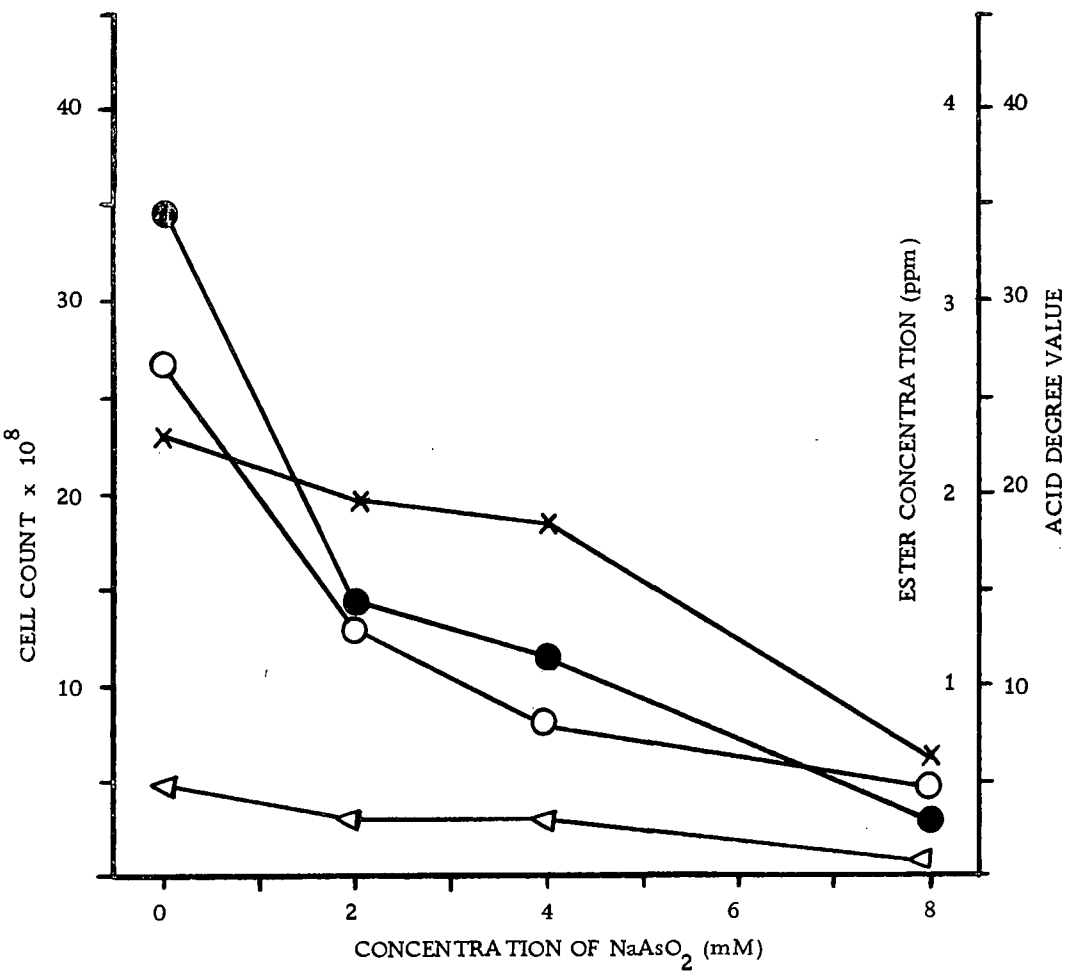

Figure 7. Effect of addition of sodium arsenite on formation of volatile acids (●), ethyl butyrate (o), cell count (X) and ethyl hexonoate (Δ) in P. fragi culture.

Study of the Esterase Enzyme System

Assay of the Esterases in P. fragi

The distribution of the esterase enzymes was established by assaying the esterase activity in different fractions of the culture. The unit of activity of the enzyme was defined as μl of CO_2 liberated per minute per ml of the enzyme preparation. The data presented in Figure 8 indicates that the greatest esterase activity was in sonicated cell preparations. There was an equal amount of cellular material in the sonicated cell fraction and in the whole cell suspension. However, the esterase activity observed for whole cell suspension is only about 25% of the activity exhibited by the sonicated cell preparation. The enzyme activity of the whole cell suspension could be partially due to some ruptured cells. The esterase activity shown by the culture supernatant fluid is negligible when compared to the activity of the sonicated cell fraction. The results indicated that the esterases are intracellular in P. fragi.

Substrate Specificity Observed by Respirometer Assay

The substrate specificity of esterases of P. fragi was tested by using phenyl acetate, ethyl butyrate and triacetin. The volume of gas liberated during 40 min incubation by one ml of the sonicated cell

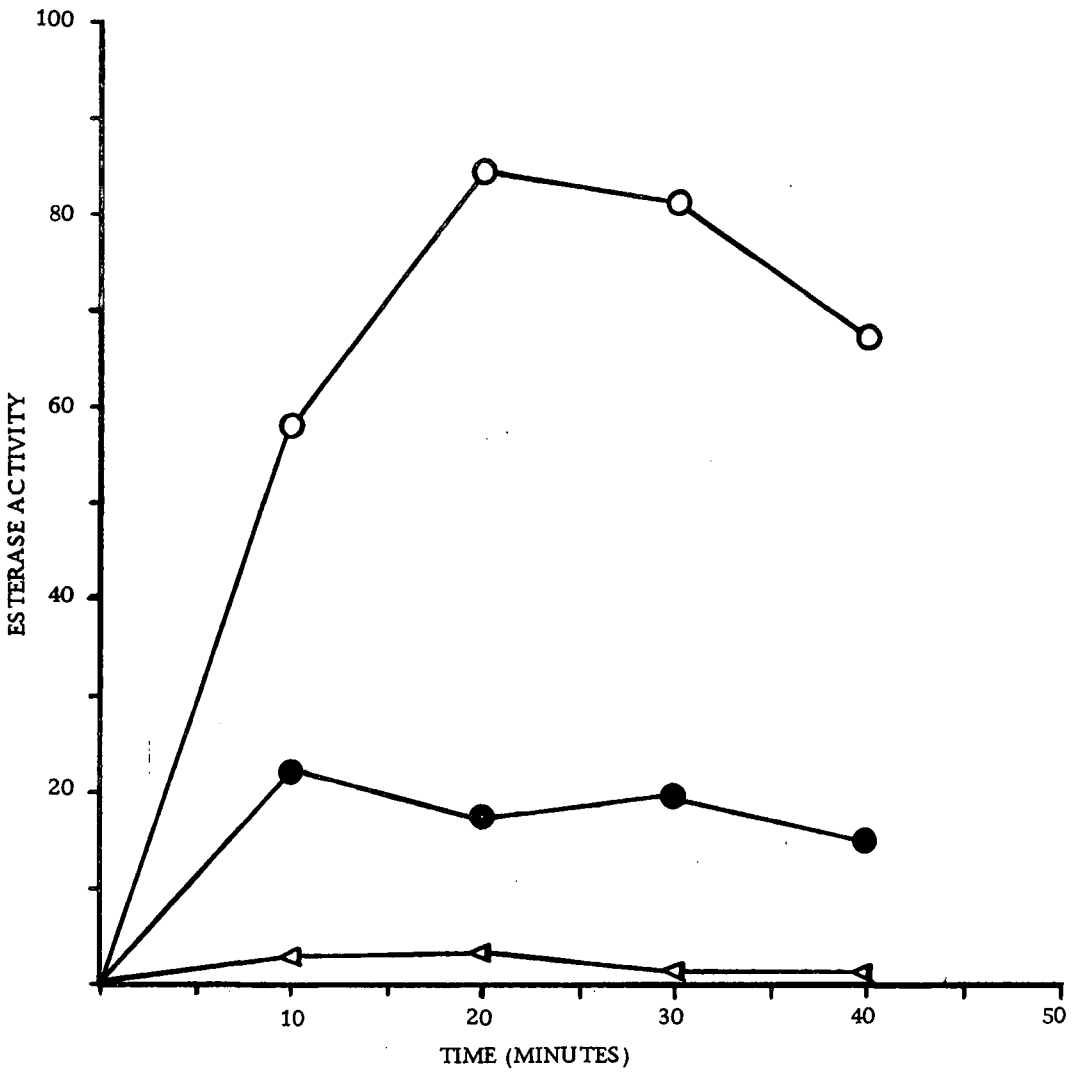

Figure 8. Esterase activity of (o) sonicated cell preparation, (●) whole cell suspension, and (Δ) culture supernatant fluid of P. fragi.

preparation was 290 µl when phenyl acetate was the substrate, 35 µl when ethyl butyrate was the substrate, and 47 µl when triacetin was the substrate. The enzymes appeared to be more specific for aromatic esters than for aliphatic esters. A 12 hour incubation period was required to liberate 250 µl of CO_2 by one ml of sonicated cell preparation with ethyl butyrate as the substrate. The decreased activity of this enzyme system for ethyl butyrate and triacetin could have resulted from their poor solubility in aqueous systems. The esterases, unlike lipases, can hydrolyze water soluble esters.

Electrophoretic Pattern of P. fragi Esterases

A typical zymogram of replicate analyses of sonicated cell preparations of a P. fragi strain 9165 is presented in Figure 9. Evidence for the presence of six esterase enzymes was provided by using α-napthyl acetate as the substrate. The bands with esterolytic activity had quite different electrophoretic mobility. Band 1 had slow mobility, bands 2, 3 and 4 showed intermediate mobility, and bands 5 and 6 were fast-moving.

Substrate Specificity Observed in Gel Electrophoresis

The reactivity of the esterases was studied with α-naphthyl propionate and α-naphthyl butyrate in addition to α-naphthyl acetate. Examination of the zymograms indicated that when α-naphthyl acetate was

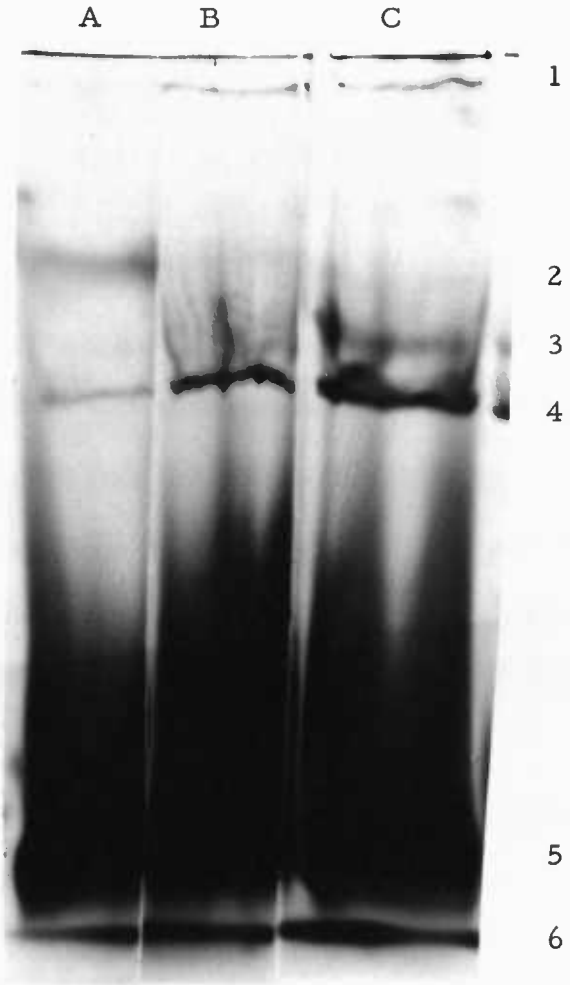

Figure 9. Esterase zymogram of P. fragi stained with: A, α-naphthyl butyrate; B, α-naphthyl propionate; C, α-naphthyl acetate.

used as the substrate, all six bands were well developed in 50 min of incubation. However, with α-naphthyl propionate only bands 1, 4, 5, and 6 were stained. When α-naphthyl butyrate was used, two hours of incubation were required for the development of bands 1, 2, 4, 5 and 6.

Bands marked 5 and 6 were active with all substrates tested. Bands 1 and 4 reacted equally well with α-naphthyl acetate and propionate, but their activity was weak with α-naphthyl butyrate. Band 3 did not react with α-naphthyl propionate or butyrate. The activity of band 2 was moderate with α-naphthyl butyrate, weak with α-naphthyl acetate and nil with α-naphthyl propionate. These variations in the enzyme activity with different substrates is at least partially due to their specificity to certain substrates. However, another factor to be considered in the case of gel electrophoresis is substrate diffusibility. The enzyme is located in the polyacrylamide gel and larger substrate molecules might not gain contact with the enzyme. However, in the present investigation the influence of this factor has been minimized, since there is not a considerable difference in the molecular size of the substrates used.

Synergistic Flavor Interaction of Esters

In the preliminary investigations the sensory evaluation of the cultures revealed that the cultures containing subthreshold concentrations of ethyl butyrate and ethyl hexanoate had stronger fruity aroma

than cultures containing either of these compounds at considerably higher concentration. This observation posed the possibility that synergistic flavor interactions were occurring among flavor compounds present in the cultures. Hence, a study of flavor interactions among esters, and esters and unrelated compounds was initiated.

Flavor Threshold Values of Individual Compounds

The flavor threshold values (FTVs) of individual compounds and their mixtures are presented in Table 7. The FTVs of individual compounds are averages of at least duplicate panel evaluations of each compound. However, ethyl hexanoate and ethyl butyrate threshold tests were repeated nine and seven times, respectively, and the variabilities are presented in Table 7. The FTV of ethyl butyrate, 0.015 ppm, obtained in this study is comparable with the value of 0.025 ppm reported by Honkanen et al. (1964). There is a considerable difference between the FTV of ethyl hexanoate, 0.021 ppm, determined in the current work and the value 0.075 ppm, reported by Honkanen et al. (1964), which is about equal to the FTV obtained during our preliminary study with unpurified ethyl hexanoate. The threshold value of 29 ppb for diacetyl is in reasonably close agreement with the value of 19ppb reported by Hempenius et al. (1966). A difference was observed between the FTV, 9 ppb, of dimethyl sulfide determined in this study and the threshold value of 19 ppb reported

Table 7. Average flavor threshold values and t-values for flavor compounds in homogenized milk

Mixture	Average flavor threshold[a]		Mixture Composition	Calculated t-value
	Of individual compounds	Of mixture		
	(ppm)			
Ethyl hexanoate, Ethyl butyrate		0.0163		
Ethyl hexanoate	0.021 ± 0.026		0.0098	-3.823[b]
Ethyl butyrate	0.015 ± 0.0020		0.0065	-3.838[b]
Ethyl butyrate, Butyric acid		4.3		
Ethyl butyrate	0.015 ± 0.002		0.0042	-4.020[b]
Butyric acid	12.9 ± 3.28		4.2957	-3.148[b]
Ethyl butyrate, Ethyl acetate		1.2750		
Ethyl butyrate	0.015 ±0.002		0.0048	-3.981[b]
Ethyl acetate	4.7 ±1.196		1.2702	-3.443[b]
Ethyl hexanoate, Ethyl isovalerate		0.0055		
Ethyl hexanoate	0.021 ±0.0026		0.0051	-4.618[b]
Ethyl isovalerate	0.002 ±0.0005		0.0004	-3.702[b]
Ethyl hexanoate, Diacetyl		0.040		
Ethyl hexanoate	0.021 ±0.0026		0.0174	-1.085
Diacetyl	0.029 ±0.0074		0.0226	-1.014
Ethyl hexanoate, Dimethyl sulfide		0.0186		
Ethyl hexanoate	0.021 ±0.0026		0.0126	-2.451[c]
Dimethyl sulfide	0.009 ±0.0021		0.0060	-1.387

[a] Average flavor threshold value with 95% confidence limits.
[b] $P \leq 0.01$ ($t = -2.457$ for 30 d.f.)
[c] $P \leq 0.05$ ($t = -1.697$ for 30 d.f.)

by Reddy et al. (1967). The variability in the FTV of added dimethyl sulfide is undoubtedly due in part to the variation in the level of indigenous dimethyl sulfide in milk as reported by Reddy et al. (1967) and Dunham et al. (1968).

The FTVs of unpurified ethyl hexanoate and ethyl butyrate were found to be much higher than those of the purified esters. However, it would not appear to be due to a suppressor effect of the acid dissociated from the ester since a highly significant synergistic enhancement interaction was observed in the acid-ester mixture.

Flavor Threshold Values of the Mixtures of Compounds

The results of the evaluation of six two component mixtures are presented in Table 7. These results indicated that the concentration of individual compounds at the FTV of their mixture was below their flavor threshold level. For example in the ethyl hexanoate-ethyl isovalerate mixture, the concentration of ethyl hexanoate was only approximately 25% of its concentration at its individual FTV and that of ethyl isovalerate was still lower, which is indicative of synergistic flavor interaction.

In this study, only two compounds were evaluated per mixture. If the number of compounds with similar molecular shape, size or functional groups were increased in a mixture, it is probable that additional synergistic flavor interactions would result.

Statistical Analysis

The results of the statistical analysis for significant flavor interactions are presented in Table 7. The student t-test used to test for significant synergistic flavor interactions compared the average flavor threshold of each individual compound alone with the average flavor threshold of each compound in the mixture. This type of statistical evaluation indicated significant flavor interactions. For example, the calculated t-values for the ethyl hexanoate and ethyl butyrate mixture were -3.823 and -3.838 respectively. These relatively large negative t-values reveal that the concentrations of both the esters at the FTV of the mixture are significantly lower than the concentration of each at its individual average flavor threshold. The data presented in Table 7 indicate significant flavor interactions ($P \leq 0.01$) in the mixtures of esters and the mixtures containing an ester and butyric acid. The t-values for the ethyl hexanoate-diacetyl mixture, and for dimethyl sulfide in the ethyl hexanoate-dimethyl sulfide mixture are not significant at either $P \leq 0.05$ or $P \leq 0.01$.

SUMMARY AND CONCLUSIONS

Three strains of P. fragi were isolated from milk and cottage cheese. The volatile flavor components of fruity milk cultures of P. fragi were separated by GLC. Ethyl butyrate and ethyl hexanoate were tentatively identified as chemical components with a fruity aroma by monitoring the odor of the effluent from the column, and coincidence of their retention times with authentic compounds. The fruity flavor compounds were positively identified by mass spectral analysis. The concentrations of ethyl butyrate and ethyl hexanoate in media supplemented with ethanol were compared with the concentrations in non-supplemented media. The influence of certain factors on ester levels and bacterial population in P. fragi cultures was investigated. A relationship between the pattern of ester production and cell number has been established. The interaction between S. lactis and P. fragi and its effect on the concentration of the esters has been studied.

The information available in the literature indicated that coenzyme A activation of the acid moiety is important for esterification in yeast cultures. Using $NaAsO_2$ as a CoA inhibitor, the importance of CoA activation in ester production by P. fragi was investigated. The sonicated cell free extracts, whole cell suspension and culture supernatant fluid were assayed for their esterolytic activity, using a Gilson differential respirometer. The sonicated cell free extract was

subjected to vertical gel electrophoresis on polyacrylamide gel. Evidence for the presence of six bands of esterase activity was noticed on the zymogram. Variations were observed in the substrate specificity of these esterases.

The importance of synergistic interaction of flavor components has been recognized in flavor research. Since P. fragi can produce esters in milk, the occurrence of synergistic interactions with mixture of esters and with esters and other compounds in milk was investigated.

The following conclusions were drawn from the results of the investigation.

1. Ethyl butyrate and ethyl hexanoate are the principal esters produced by P. fragi. The production of these esters results in the fruity flavor of dairy products. The addition of 0.35 ppm ethyl butyrate and 0.5 ppm ethyl hexanoate to homogenized and autoclaved milk resulted in a fruity aroma very similar to that of fruity cultures.

2. Ester production by P. fragi can be influenced considerably by the medium. Aeration did not increase the cell number. However, the concentration of the esters was generally raised. The addition of ethanol, ethanol and butyric acid enhanced ester formation. An additional conclusion of equal significance is that ester production

does not remain constant for a given strain of the organism after a series of subcultures have been made in the laboratory.

3. A correlation between the increase in concentration of esters and increase in bacterial population was observed.

4. The concentrations of ethyl butyrate and ethyl hexanoate in mixed cultures of S. lactis and P. fragi were about five times greater than the concentrations found in the P. fragi cultures grown singly.

5. The addition of $NaAsO_2$ did not specifically affect ester formation. This observation along with the observation that only small quantities of esters are formed in media supplemented with acids and ethanol, nearly rules out the involvement of CoA in ester production by P. fragi.

6. The assay of the esterase activity of various fractions of P. fragi cultures indicated a maximum activity in sonicated cell free preparations with phenyl acetate as the substrate. The gel-electrophoretic separation of the esterases present in the sonicated cell free preparations revealed six, four and five bands of esterolytic activity with substrates of α-naphthyl acetate, α-naphthyl propionate and α-butyrate, respectively. These results substantiate the conclusions that P. fragi esterases are intracellular, and they vary in their substrate specificity.

7. The synergistic flavor interactions among the esters and

esters and butyric acid studied in this investigation are highly significant ($P \leq 0.01$).

The conclusion can be made that concentrations of esters below the FTV of the individual compounds can be important to flavor when they occur in combination.

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