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Single-strain cultures of Streptococcus cremoris, Streptococcus lactis, Streptococcus diacetilactis, and Leuconostoc citrovorum produced little or no acetone and no dimethyl sulfide when grown in milk culture. These organisms had little or no ability to decarboxylate an exogenous source of acetoacetic acid nor were they capable of producing dimethyl sulfide from methyl methionine sulfonium chloride. The dimethyl sulfide content of milk was increased by heating which indicated that a heat labile dimethyl sulfide precursor was present in milk. The precursor remained in the skimmilk fraction and was dialyzable. The precursor was identified as a methyl methionine sulfonium salt on the basis of its thin-layer chromatographic mobility and the heat instability of the compound. Heating of samples caused the disappearance of the precursor compound with a subsequent increase in the content of homoserine and dimethyl sulfide.

Single strain cultures of Pseudomonas fragi, Pseudomonas

fluorescens, Pseudomonas putrefaciens, and two marine Pseudomonas species reduced acetaldehyde, propionaldehyde, and butyraldehyde to the corresponding alcohols at 21° C. All species studied reduced propionaldehyde at 6° C. P. fragi and the marine species reducted butanone and/or acetone at both 6 and 21° C. Under aerobic conditions a strain of P. fragi quantitatively reduced added propionaldehyde to n-propanol.

The quantities of acetaldehyde and ethanol produced by single-strain cultures of Lactobacillus brevis, Lactobacillus casei, Lactolactis, and Lactobacillus plantarum differed significantly both between species and between strains of a species on incubation at both their optimum growth temperature and 8° C. Growth and production of these compounds were very slow at 8° C. All organisms studied were capable of reducing acetaldehyde and propionaldehyde to the corresponding alcohol. L. brevis strains alone reduced added butanone to 2-butanol. A strain of L. brevis produced n-propanol as a normal metabolite when grown in milk culture.

Single-strain cultures of L. casei and L. plantarum accumulated diacetyl when grown in milk culture at both 8 and 30° C, but strains of L. lactis and L. brevis did not. Diacetyl reductase activity was demonstrated in single-strain cultures of L. casei, L. brevis, and L. lactis. Diacetyl reductase could be induced in L. plantarum by growth in the presence of citrate. Growth in milk medium

supplemented with citrate resulted in a stimulation of diacetyl reductase activity with L. casei.

Metabolism of Volatile Compounds by Microorganisms

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METABOLISM OF VOLATILE COMPOUNDS BY MICROORGANISMS

INTRODUCTION

Recent advances in gas chromatography have permitted investigations of minor changes in concentration of important flavor compounds. These methods have made possible detailed studies of microbial metabolism of volatile compounds and thus have led to a more thorough understanding of the involvement of microorganisms in flavor formation and deterioration.

In the case of cultured dairy products, much is already known about the role of lactic acid bacteria in the development of flavor. However, there is still much controversy over the ability of these organisms to produce certain volatile compounds. The role of psychrophilic organisms in decomposition of dairy products has been extensively investigated, but little is known as yet about the ability of these bacteria to attack key flavor compounds. Another gap in the existing knowledge is the contribution of the lactobacilli and other organisms to development of flavor in Cheddar cheese.

The objectives of this investigation were to apply recent advances in analytical gas chromatography to a study of microbial production and utilization of some important flavor compounds. The results should lead to a better understanding of microbial involvement

in flavor formation and deterioration of dairy products and encourage further investigations of the role of microorganisms in production and utilization of compounds important in food flavors.

REVIEW OF LITERATURE

Lactic Streptococci and Leuconostoc Species

Importance to the Dairy Industry

One of the major uses of these organisms to the dairy industry is in butter and cheese cultures. The microbial species incorporated into butter cultures can be grouped into three categories (111): 1. the lactic acid producing streptococci, Streptococcus lactis and Streptococcus cremoris; 2. the citrate fermenting aroma bacteria, Leuconostoc citrovorum and Leuconostoc dextranicum; and 3. the lactic acid and aroma producing strains of Streptococcus diacetilactis. It is an established fact that some of the metabolic products which occur from the associative growth of these organisms impart the normal flavor and aroma to mixed-strain butter cultures.

S. lactis, S. cremoris, and S. diacetilactis, either singly or in combination, have been used conventionally as starter cultures for Cheddar cheese. Other streptococci, such as Streptococcus durans, Streptococcus thermophilus, and Streptococcus faecalis, have been used experimentally as starter organisms, but they have never been adopted for commercial cheese manufacturing. The role of starter organisms in the development of the typical body and flavor of Cheddar cheese is still somewhat unclear. Organisms are

conventionally selected for their ability to produce lactic acid at a convenient rate for the initial manufacturing process.

Volatile Compounds Produced

Much of the early research on these organisms was concerned with the organic acid production by butter cultures. Lactic acid is the major metabolic product of homofermentative lactic streptococci. Pure lactic acid is odorless and non-volatile, thus it does not contribute to the odor, but is considered to be largely responsible for the acid taste of butter cultures and Cheddar cheese (14, 51, 13, p. 14). In milk cultures of these organisms, acetic acid comprises the major portion of the volatile acid fraction (52). Other volatile organic acids which have been identified in milk cultures are formic, propionic, butyric, and valeric acids (52, 71, 29, p. 126, and 77, p. 154). Friedman (41), and Platt and Foster (106) have shown that formic acid is an end-product of lactose and glucose metabolism in non-milk media by S. cremoris, S. lactis, and L. dextranicum. The amounts of formic acid were small compared to the amount of acetic acid produced. The volatile acids are considered to be important to the flavor of both butter cultures and Cheddar cheese (51, 77, p. 206, and 101).

Production and utilization of acetaldehyde by lactic streptococci and Leuconostoc species has been the subject of several

investigations. A small amount of this compound is necessary to impart a balanced flavor to butter cultures, but in high concentrations it causes a flavor defect described as green or yogurt-like (6, vol B, p. 199-208, 77, p. 175, and 79). Harvey (56) reported that all strains of S. lactis, S. cremoris, and S. diacetilactis studied in his laboratory produced significant quantities of acetaldehyde when grown in milk media. Keenan et al. (68) have found that acetaldehyde production by these streptococci parallels culture growth and that the levels of this compound which accumulate vary widely both between species and between strains within a species. These findings have been confirmed by others (8, 15).

Acetaldehyde utilization by S. lactis, S. cremoris, S. diacetilactis, L. citrovorum, and L. dextranicum has been reported by several workers (6, vol B, p. 191-208, 67, 68, and 79). At least part of the acetaldehyde utilized by these organisms is converted to ethanol, suggesting that it serves as a terminal hydrogen acceptor (15, 68). Bills and Day (15) demonstrated dehydrogenase activity by these organisms and found that they are capable of reducing acetaldehyde and propionaldehyde, but not acetone.

Since the recognition that diacetyl is a principal component of butter culture flavor by van Niel, Kluyuer, and Drex (128), many investigations have been carried out on its production by thse organisms. It is now known that S. diacetilactis and L. citrovorum

produce significant amounts of this compound (43, vol D, p. 153, and 115, p. 44-59). The biosynthesis of diacetyl will be covered in the discussion of citric acid fermentation. The partial reduction products of diacetyl, acetoin and 2, 3-butanediol, have received considerable attention, but these have no odor and are probably never present in concentrations high enough to affect the taste of cultures (49). Seitz et al. (116) have reported diacetyl reductase, an enzyme which catalyzes the irreversible reaction of diacetyl to acetoin, to be present in several strains of S. diacetilactis and Leuconostoc species.

There are several contradictory reports on the ability of lactic acid bacteria to produce acetone when grown in milk cultures. Harvey (56) reported that small quantities of acetone were produced by seven out of 11 strains of S. cremoris and S. lactis. Keenan et al. (68) also reported the production of acetone by these organisms and by a strain of S. diacetilactis. Vedamuthu, Sandine, and Elliker (130) reported acetone production in two mixed-strain lactic starter cultures. In contrast, Bassette and Claydon (9) found that a strain of S. lactis and a strain of S. diacetilactis did not produce detectable amounts of acetone. Harvey (56) found that a strain of S. diacetilactis utilized acetone present in milk medium. This result was not confirmed by later work (15).

Dimethyl sulfide has been isolated from bulk butter cultures by Day, Lindsay, and Forss (35). These workers observed that

dimethyl sulfide smooths out the harsh flavor of diacetyl and acids associated with culture flavor. At the present time there is some controversy over the ability of the lactic streptococci to produce dimethyl sulfide. It has been variously detected in milk cultures of these organisms, but it normally occurs in milk as well as other dairy products (35, 62, 102, 137).

Ethanol production by single-strain cultures of S. lactis, S. cremoris, and L. dextranicum in non-milk media has been demonstrated by Friedman (41) and Platt and Foster (106). It has also been identified in single-strain milk cultures of S. lactis, S. cremoris, S. diacetilactis and L. citrovorum, and in mixed-strain butter cultures grown in milk media (6, 8, 15, 29, 68, 80). Palladina (99) found that S. lactis decomposed ethanol but that S. cremoris did not.

With the exception of carbon dioxide, no volatile compounds other than those discussed above have been identified as products of the metabolism of lactic streptococci. In view of the extensive work with these organisms, it appears that production of many other volatile compounds is not a general feature of these organisms.

Bacteriology

Taxonomy and Classification

The microorganisms used in this study are common to cultures

used for making cultured butter and buttermilk, Cottage cheese and Cheddar cheese. In the manufacture of these products, cultures are added for either or both of two purposes: to produce lactic acid or to produce a desirable aroma.

The taxonomy of lactic acid bacteria has been discussed in detail in reviews by several authors (32, 51, 109, and 43, vol D, p. 143-158). The classification and nomenclature of some of the organisms involved is still a controversial topic. The three species of lactic streptococci generally recognized as being common to mixed-strain butter and cheese cultures are S. cremoris, S. diacetilactis, and S. lactis (32, 109). Many criteria have been used to classify these bacteria, but reactions may vary within a species and lead to results which are difficult to interpret. The most common Leuconostoc found in lactic cultures is L. citrovorum. The classification L. dextranicum is used to some extent, but as Garvie (45) has pointed out, this organism is rare and many of the organisms classified as L. dextranicum are actually strains of Pediococcus cerevisiae, an organism very similar to L. dextranicum. As pointed out by Keenan, Lindsay, and Day (67), L. citrovorum is preferable to L. dextranicum both for diacetyl production and acetaldehyde utilization. It now appears that L. citrovorum is the organism of choice for formulating butter cultures.

S. cremoris, S. diacetilactis, and S. lactis are all species of

the group N streptococci (109). Sandine, Elliker, and Anderson (113) have made use of well-known and rapid methods to characterize these organisms. Lactic streptococci were differentiated from Leuconostoc organisms by the former's ability to produce sufficient acid in 48 hr at 30° C to coagulate and reduce litmus milk. S. diacetilacitis and S. lactis were differentiated from S. cremoris on the basis of the arginine hydrolysis test of Niven et al. (96). S. diacetilactis was differentiated from S. lactis on the basis of the ability of the former to produce diacetyl and acetoin, these compounds being detected by the modified creatine test of King (69). The ability of S. diacetilactis to produce high levels of carbon dioxide from citrate was also used to differentiate it from S. lactis and S. cremoris, which lack the ability to ferment citrate.

Because of some instances where non-citrate fermenting strains of S. cremoris and S. lactis give a slightly positive creatine test, some doubt has been expressed as to the validity of this test to differentiate these organisms from S. diacetilactis (43, vol. D, p. 144). Sandine, Elliker, and Anderson (113) favor the classification of these organisms as S. lactis or S. cremoris variety aromaticus. Harvey and Collins (57) have shown that S. diacetilactis organisms may lose their ability to produce citrate permease, and would thus give negative creatine tests. False negative creatine tests can be obtained because of the reduction of acetoin to 2,3-butanediol by some

bacteria. This ability has been reported for S. diacetilactis cultivated in cream (76) and several common adventitious organisms in dairy products (109). However, by avoiding contamination and testing the culture at various stages of incubation, the creatine test can be used with confidence. The need for testing at various stages of incubation is that S. diacetilactis has relatively mild reducing properties and cultures will usually give positive creatine tests for incubation periods up to several days (109).

The citrate fermenting Leuconostoc species can be differentiated from the lactic streptococci by their inability to acidify litmus milk incubated at 21° and 30° C (113). L. dextranicum can be differentiated from L. citrovorum by the former's ability to produce dextran (slimey colonies) when streaked on a sucrose enriched agar medium (113).

Collins (32) has discussed the many different names in use for the genus Leuconostoc and the various species within this genus. As an example, he states that all of the following names are used to refer to a single species: Streptococcus citrovorus, Betacoccus cremoris, Leuconostoc cremoris, and L. citrovorum. Since L. citrovorum is the name used in Bergey's Manual (12, p. 532), it will be used here. Sandine, Elliker, and Anderson (113) recommend the designation Leuconostoc mesenteroides for those organisms which do not produce diacetyl or acetoin when grown in non-fat

milk but do produce dextran on sucrose medium. They recommend that so-called "Leuconostoc" organisms having neither of these properties be placed in the genus Pediococcus.

Biochemistry

The metabolism of mixed-strain cultures is complex because it encompasses the utilization of many metabolites and the formation of many products. Furthermore, the mechanisms involved in the associative growth of microorganisms in a complex biological fluid such as milk are difficult to study. These difficulties have led many investigators to the use of synthetic media for their studies. In these cases, care must be taken in relating results obtained in these media to those observed in milk media because the inductible enzymes of the cell may be affected. However, much information has been gathered by employing both of these approaches.

Fermentation of Lactose

Although trace amounts of glucose, galactose, and other sugars are present, lactose is the only sugar present in milk in significant quantities, and as such serves as the principal substrate for lactic bacteria (61, p. 73). The initial step in the fermentation of lactose is hydrolysis to yield glucose and galactose. Glucose is immediately susceptible to further catabolic processes, but galactose must first

be converted to glucose-1-phosphate as outlined by Kandler (65).

1. Galactose $\xrightarrow{\text{Galactokinase}}$ Galactose-1-PO_4
   + ATP + ADP
2. Galactose-1-PO_4 $\xrightarrow[\text{uridyl transferase}]{\text{Phosphogalactose}}$ UDP-Galactose
   + UDP-Glucose + Glucose-1-PO_4
3. UDP-Galactose $\xrightarrow[\text{galactose epimerase}]{\text{Uridine diphosphate}}$ UDP-glucose

Citti, Sandine, and Elliker (31) have presented evidence which suggests that lactose uptake by a strain of S. lactis is accomplished by a β-galactoside permease and that lactose induces the specific synthesis of this permease.

Lactose Fermentation by Homofermentative Lactic Acid Bacteria. By definition, homofermentative microorganisms are those which utilize glucose via the well-known Embden-Meyerhof-Parnes (EMP) pathway and produce lactic acid almost exclusively as a terminal product of the fermentation. These organisms produce L (+)-lactic acid, a fact which serves to distinguish them from the heterofermentative lactic acid bacteria (43, vol. D, p. 144). Organisms found in lactic cultures which would be classified as homofermentative are S. lactis, S. cremoris, and S. diacetilactis (38, p. 17).

Van Slyke and Bosworth (129) found that S. lactis grown in skimmilk at 32° C fermented only 20 percent of the available lactose in 96 hr. The percentage of fermented lactose converted to lactic acid varied from 70-90 percent. Some of the fermented lactose which is

not accounted for in lactic acid undoubtedly is utilized in the formation of the volatile products discussed previously.

Marth (85) has summarized the reactions which pyruvic acid, a key intermediate in the fermentation of lactose, can undergo. The predominant reaction is the reduction of pyruvate to lactate. Some other compounds which arise from pyruvate are alanine, malic acid, oxalacetic acid, acetaldehyde, alpha-acetolactic acid, and acetoin. Oxalacetic acid can arise from malic acid or from the carboxylation of pyruvate to phosphoenol-pyruvate which can then be converted to oxalacetic acid. The formation of oxalacetic acid, an important precursor of amino acids, by these routes is necessary since the lactic acid bacteria are unable to utilize the citric acid cycle for the formation of this acid. The production of oxalacetic acid involves carboxylation of pyruvic acid, and this is undoubtedly part of reason for the carbon dioxide requirement for growth of the lactic streptococci (118, 133).

Homofermentative bacteria apparently have enzymes which oxidize and decarboxylate glucose-6-phosphate to ribulose-5-phosphate (65). This could account for the formation of compounds other than lactic acid by these organisms. As mentioned by Reiter and Møller-Madsen (109), there is evidence that some of the homofermentative lactic streptococci are also able to utilize glucose via the Entner-Doudoroff pathway, shown in Figure 1. This pathway

provides another route to acetaldehyde, ethanol, and carbon dioxide.

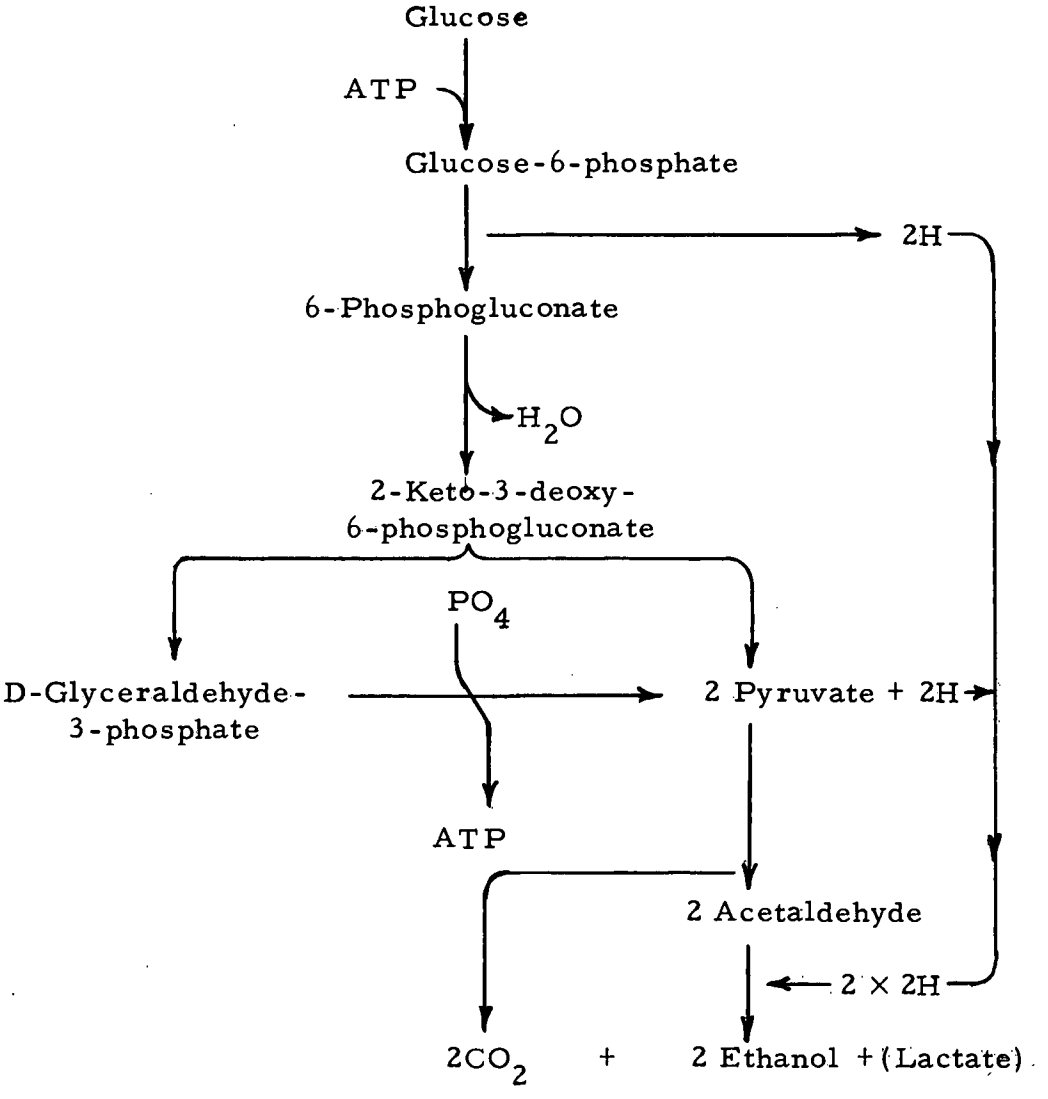


Figure 1. The Entner-Doudoroff pathway (139, p. 59).

Lactose Fermentation by Heterofermentative Lactic Acid Bacteria. The microorganisms of the Leuconostoc genus are classified as heterofermentative because they produce several catabolic products from glucose. They produce small amounts of D (-)-lactic

acid, which distinguishes them from the lactic streptococci (43, vol D, p. 144). Kandler (65) has outlined the pathway used by heterofermentative bacteria for the utilization of glucose. This pathway (shown in Figure 2) partly involves the hexosemonophosphate shunt (HMP). Heterofermentative organisms utilize this route rather than the EMP pathway because they lack aldolase, an enzyme which catalyzes the conversion of fructose-1, 6-diphosphate to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate.

Galesloot (43, vol. D, p. 149) has stated that the reduction of acetylphosphate to ethanol is a waste of considerable energy, but the Leuconostoc organisms must do this to regenerate oxidized pyridine nucleotides. Under optimum conditions these organisms convert acetylphosphate to acetic acid rather than ethanol.

Reiter and Møller-Madsen (109) have suggested a third class of lactic acid bacteria, the facultatively homofermentative organisms. Organisms in this class have the enzymes necessary for glucose metabolism via either the EMP or HMP scheme, but utilize the EMP pathway nearly exclusively. Some evidence has accumulated that at least some strains of S. lactis and S. cremoris have the ability to utilize the HMP pathway. Shahani and co-workers (117, 118, 119) have demonstrated key enzymes involved in both schemes for one strain of S. lactis.

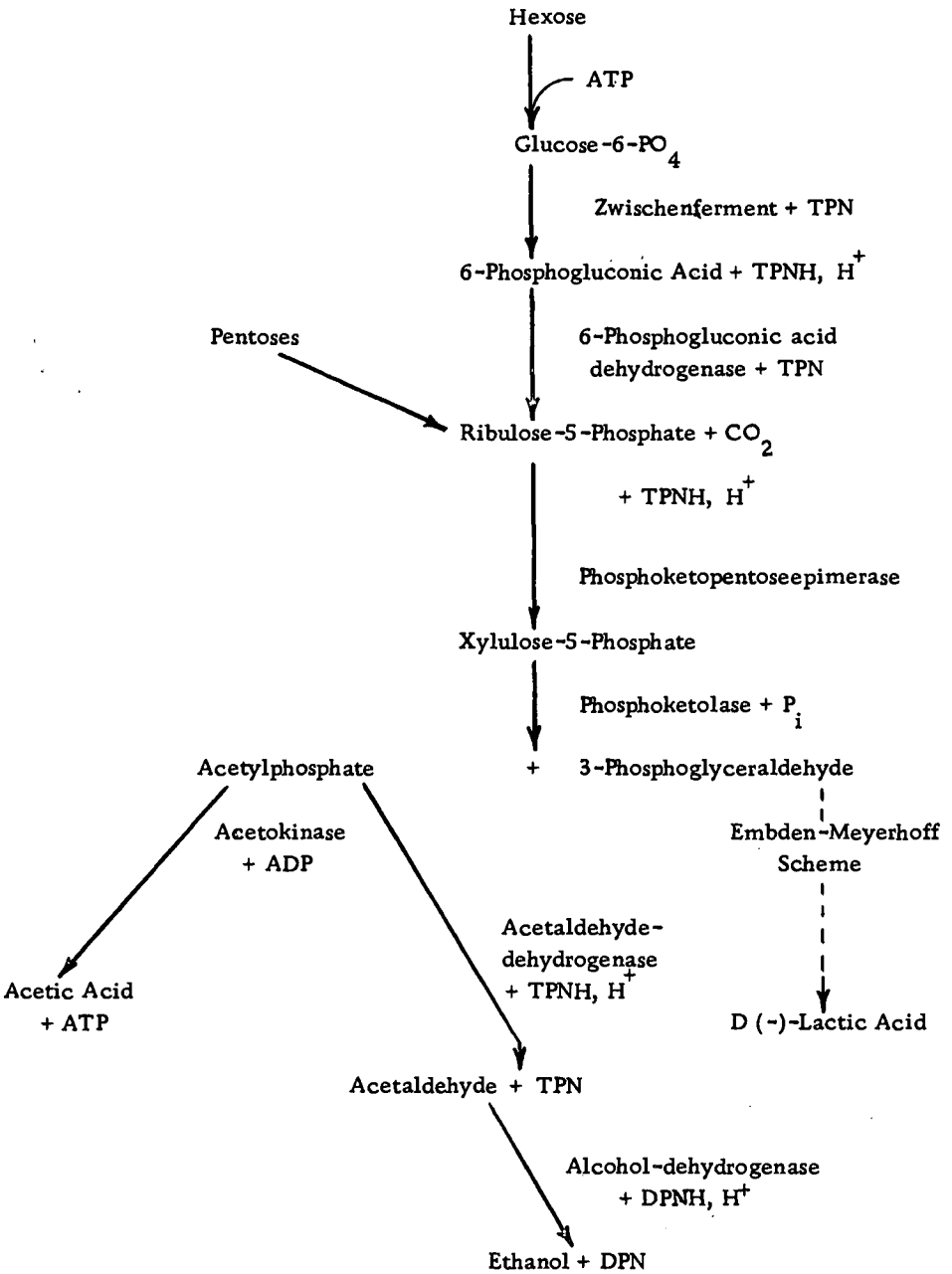
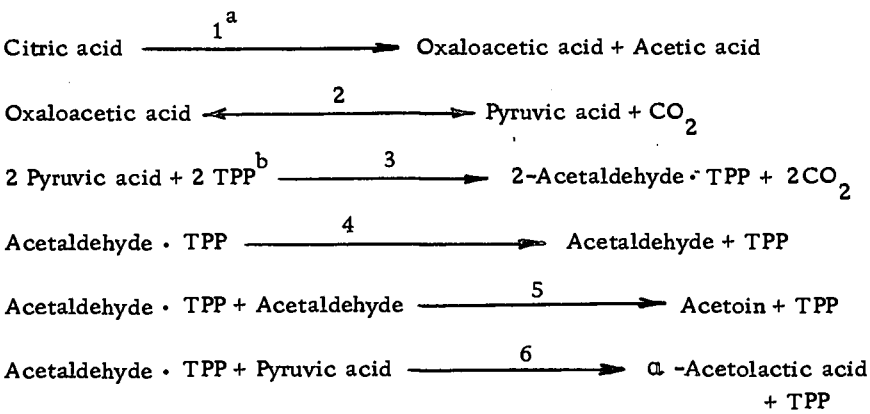


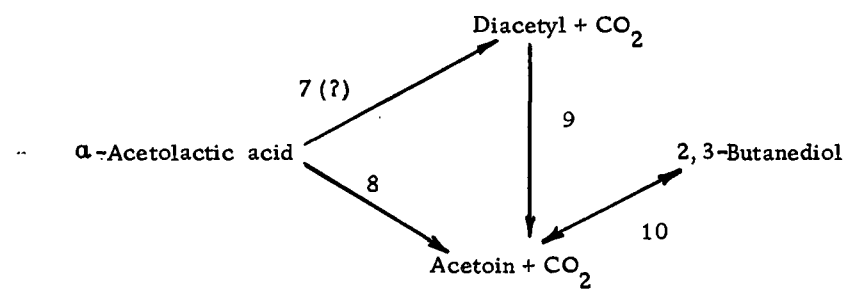
Figure 2. Carbohydrate metabolism of heterofermentative lactic acid bacteria. From Kandler (65, p. 524).

Citric Acid Fermentation

The fermentation of citric acid provides a route to diacetyl, an important flavor compound in many cultured dairy products. Although widely investigated, citric acid fermentation is still the subject of much confusion.

The pathways for enzymatic conversion of citric acid by S. diacetilactis have been summarized by Seitz (115, p. 91-96) and are shown in Figure 3. Galesloot (43, vol. D, p. 153) summarized results which indicate that the mechanisms for citrate utilization by L. citrovorum appear to the the same as those given in Figure 3. These pathways were widely accepted until the recent reports of Speckman and Collins (124, 125). These workers found that acetoin is produced stoichiometrically from pyruvate and alpha-acetolactate, but that diacetyl is not produced from either of these substrates or from acetoin, even when NAD^+ is added to the incubation system. The same results were obtained for both a strain of S. diacetilactis and a strain of L. citrovorum. These workers found that cell free extracts of these organisms formed diacetyl and acetoin when acetyl-CoA was added to a test system of pyruvate, thiamine pyrophosphate (TPP), and manganese. Their data substantiate the conclusion that, in these organisms, diacetyl is formed by attack of acetaldehyde-TPP on the carbonyl carbon of acetyl-CoA.





[a]Enzymes catalyzing each reaction:

1. Citritase
2. Oxaloacetate decarboxylase
3. Pyruvate decarboxylase
4. Non-enzymatic
5. Acetoin synthetase
6. α-Acetolactate synthetase
7. α-Acetolactate oxidase
8. α-Acetolactate decarboxylase
9. Diacetyl reductase
10. 2, 3-butanediol dehydrogenase

[b]Thiamine pyrophosphate

Figure 3. Pathways for enzymatic conversion of citric acid by S. diacetilactis. From Seitz (115, p. 95).

The most widely accepted beliefs concerning diacetyl and acetoin production are that no aroma is produced from sugar alone, but is produced from citrate alone and from a mixture of sugar and citrate. Harvey and Collins (57) have observed a citrate transport system in S. diacetilactis. These workers reported that this system can be induced and permits greatest entry of citrate into cells at pH values below pH 6.0. Marth (86) has summarized the generally accepted ideas on the production of acetoin and diacetyl. Pyruvic acid is the key intermediate in the fermentation of lactose and citrate to acetoin and diacetyl. He has further stated that in the fermentation of lactose to pyruvate in the EMP scheme, sufficient NADH is produced to reduce pyruvate to lactate, but that the fermentation of citrate results in the production of pyruvate without a simultaneous supply of reduced NAD and, thus, products other than lactic acid are formed. Galesloot (43, vol. D, p. 152-153) has pointed out that at pH values near 5.0 the sugar fermentation slows and when aroma production starts the pyruvate pool is fed mainly by citrate fermentation.

DeMann and Galesloot (36) have reported that active Leuconostoc starters produce little diacetyl aroma. These Leuconostocs tend to avoid the reduction of acetylphosphate to ethanol and prefer to convert the former to acetic acid with the simultaneous gain of one ATP. The reduced pyridine nucleotides generated during sugar fermentation

appear to be utilized in the reduction of diacetyl to acetoin and 2,3-butanediol. It has been suggested that when the Leuconostoc organisms cannot complete their metabolic processes because of low numbers or a low pH, diacetyl tends to accumulate rather than being reduced (43, vol. D, p. 153).

Proteolysis and Lipolysis

Fatty acids and amino acids are important in the flavor of many cultured dairy products. Studies of the origin of these compounds have led to investigations of the proteolytic and lipolytic ability of starter streptococci.

Proteolytic ability of lactic streptococci has been clearly demonstrated by several authors. Morgan (87) reported that S. lactis produced a considerable amount of free amino acids when grown in skimmilk. The intracellular proteinases of S. lactis were investigated by Baribo and Foster (7). These workers found that proteinases active at pH 5.0 - 5.5 as well as at pH 7.0 were present in the cells. Czulak and Shimmin (34) compared the proteolytic ability of several strains of S. cremoris and concluded that all strains tested were able to hydrolyze casein, but some strains were less efficient in completing the hydrolysis of peptides to amino acids.

Lipolytic activity of lactic streptococci was observed in the early work of Long and Hammer (82) and Wolf (136). In a recent,

extensive study, Fryer, Reiter, and Lawrence (42) found that weak lipolytic activity was a general feature of lactic streptococci. They concluded that although this activity was weak, it could be significant in the flavor development of Cheddar cheese.

Lactobacilli of Cheddar Cheese

Importance to the Dairy Industry

Lactobacilli occur in many different habitats. They are found in dairy products such as milk and cheese, in plant matter such as fruit juices, silage, grain, grass, beer, and wine, and in the saliva and alimentary tracts of man and animals (121). In short, lactobacilli may be found wherever sugar, protein breakdown products, and vitamins occur (121). Lactobacilli constitute part of the natural microflora of raw milk. Species which have been enumerated in milk include Lactobacillus casei, Lactobacillus plantarum, Lactobacillus lactis, Lactobacillus fermenti, Lactobacillus acidophilus, Lactobacillus bulgaricus, and Lactobacillus buchneri (63, 105, 120).

Lactobacilli are used in starter cultures for preparation of a number of dairy products. According to Sharpe (120), L. bulgaricus, Lactobacillus helviticus, and L. lactis have been used as starters for the manufacture of Emmental, Gruyère, and Swiss cheeses. L. bulgaricus is used in the manufacture of yoghurt and L. acidophilus

is used for culturing acidophilus milk (120).

The largest proportion of the work done on enumeration of these organisms has been concerned with enumerating those lactobacilli common to Cheddar cheese. Only under experimental conditions are lactobacilli intentionally added to the cheese milk. Nevertheless, species of this genus are almost always found in large numbers in Cheddar cheese and are believed to be important in the development of the typical Cheddar flavor. According to Johns and Cole (64), the numbers of lactobacilli are low in newly-made Cheddar cheese, but there is a steady rise in numbers for the first few months of ripening, followed by a gradual decline in numbers after several months. This finding has been confirmed by Naylor and Sharpe (93, 94). Species which have been frequently identified in the microflora of Cheddar cheese include L. casei, L. brevis, L. plantarum, and, to a lesser extent, L. lactis (37, 39, 93, 94, 95, 120, 123). Other lactobacilli which have been isolated from Cheddar cheese are L. bulgaricus and L. fermenti (58, 105). Judging from the literature, the latter two organisms occur sporadically and are probably not common to the microflora of Cheddar cheese.

Volatile Compounds Produced

The coincident growth of large numbers of lactobacilli with the development of typical Cheddar flavor has led many investigators to speculate on the role of these organisms in Cheddar cheese ripening.

Some workers have shown that the addition of certain strains of lactobacilli to cheese milk improved the flavor of the resultant cheese, while other strains caused off-flavors (120). According to Sharpe (120), the presence of lactobacilli may enhance the Cheddar flavor, but is unlikely to be the main cause of the flavor. At the present time there are few reports on the volatile compounds produced and utilized by these organisms.

Kristoffersen and Nelson (72) were among the first to study production of volatile fatty acids by lactobacilli. These workers found that L. casei produces a deaminase which released ammonia and produced fatty acids from serine, cysteine, and asparagine. In recent extensive studies, Nakae and Elliot (91, 92) found that two Lactobacillus species isolated from Cheddar cheese produced varying amounts of volatile fatty acids from casein hydrolysates and from individual amino acids. These workers were able to demonstrate production of acetic, propionic, butyric, isobutyric, valeric, and caproic acids from casein hydrolysates (91). They also demonstrated the production of acetic acid from pyruvate and of the fatty acids listed above from individual amino acids. Their results led them to postulate that amino acids were converted to fatty acids by oxidative deamination followed by decarboxylation of the intermediate keto acid (92).

Bassette, Bawdon, and Claydon (8) have detected small amounts

of diacetyl and ethanol in milk cultures of L. casei. They did not detect acetaldehyde or methyl sulfide in these cultures. Christensen and Pederson (30) have shown that extensive growth of L. brevis and L. plantarum during the concentration of fruit juice causes spoilage by production of diacetyl from the citrate present in the fruit juice.

The production of hydrogen sulfide by strains of L. casei has been documented by Kristoffersen and Nelson (73, 74) and by Sharpe and Franklin (122). The former authors noted that in the Cheddar cheeses studied, at least one strain of hydrogen sulfide producing L. casei was isolated from each cheese with a nearly perfect flavor score.

Hart et al. (55) investigated the formation of esters by bacteria as early as 1914. This worker reported that a strain of Bacterium casei (probably L. casei), isolated from aging cheese, produced esters. This investigator ruled out the possibility that ester formation was merely due to contact of alcohol and acid.

The volatile compounds discussed above apparently are the only ones which have been reported as products of the metabolism of lactobacilli common to Cheddar cheese. Because of the speculated importance of these organisms in Cheddar cheese ripening, this area deserves further attention.

Bacteriology

Taxonomy and Classification

The lactobacilli are all species of the family Lactobacillaceae and are one of the more clearly defined groups of bacteria (12, p. 541-544, 121). According to Sharpe (121) these organisms are gram-positive, catalase negative, non-spore forming rods. They are non-motile, non-pigmented, and do not reduce nitrates. Their nutritional requirements are complex; they need to be supplied with amino acids, peptides, vitamins, and fatty acids.

Although not valid according to the Bacteriological Code of Nomenclature, there are three useful subdivisions of the genus Lactobacillus (121). These are (1) Thermobacterium, a group containing homofermentative strains growing at high temperatures, (2) Streptobacterium, whose members are homofermentative strains growing at low temperatures, and (3) Betabacterium, whose members are heterofermentative. L. lactis is classified as a Thermobacterium, L. casei and L. plantarum are classified as Streptobacterium, and L. brevis is a species of the Betabacterium group.

L. brevis can be differentiated from the homofermentative species by two tests: (1) the ability to produce CO_2 from glucose, using Gibson and Abd-el-Malek's (46) method, and (2) the ability to

produce ammonia from arginine (20, 121). L. lactis can be differentiated from the other lactobacilli of Cheddar cheese on the basis of its ability to grow well at 45° C and by its inability to grow at 15° C. This organism also produces exclusively D (-) - lactic acid, which distinguishes it from the other three species (112).

The two species of the sub-genus Streptobacterium, L. casei and L. plantarum can be distinguished in a number of ways. L. plantarum produces only a small amount of acid in milk whereas L. casei produces up to 1.2%. They are also differentiated by the type of lactic acid formed, L. plantarum forming DL - and L. casei L (+) - lactic acid. In addition, L. plantarum is able to ferment melibiose and to grow in 0.4% Teepol, whereas L. casei cannot (121).

By paper chromatography of the amino acids and peptides extracted from cells with dilute acetic acid, Cheeseman (28) has been able to differentiate the Lactobacillus species of Cheddar cheese. Although a rigidly standardized technique for growing the organisms is necessary, the work of Cheeseman (28) and others indicates some potential usefulness of this technique for the differentiation of lactobacilli (121).

Biochemistry

Lactobacilli are very similar to the lactic streptococci and have similar nutritional requirements (120). One of the most

striking differences between these organisms and the lactic streptococci is that lactobacilli are all aciduric, some growing at pH's as low as 3.5 (120).

Fermentation of Lactose

As with the lactic streptococci, the first step in catabolism of lactose is hydrolysis to yield glucose and galactose. Galactose apparently is converted to glucose-1-phosphate via the route outlined previously (109).

Lactose Fermentation by Homofermentative Lactobacilli. The major product of glucose fermentation by these organisms is lactic acid. Glucose is utilized via the EMP pathway. The lactobacilli associated with Cheddar cheese which would be classified as homofermentative are L. casei, L. lactis, and L. plantarum. As with the lactic streptococci, L. casei produces L (+) - lactic acid. In contrast, L. plantarum produces a racemic mixture of lactic acid, and L. lactis produces D (-) - lactic acid (121). L. plantarum also differs from L. casei and the homofermentative lactic streptococci in that only some freshly isolated strains produce sufficient acid to coagulate milk (12, p. 549).

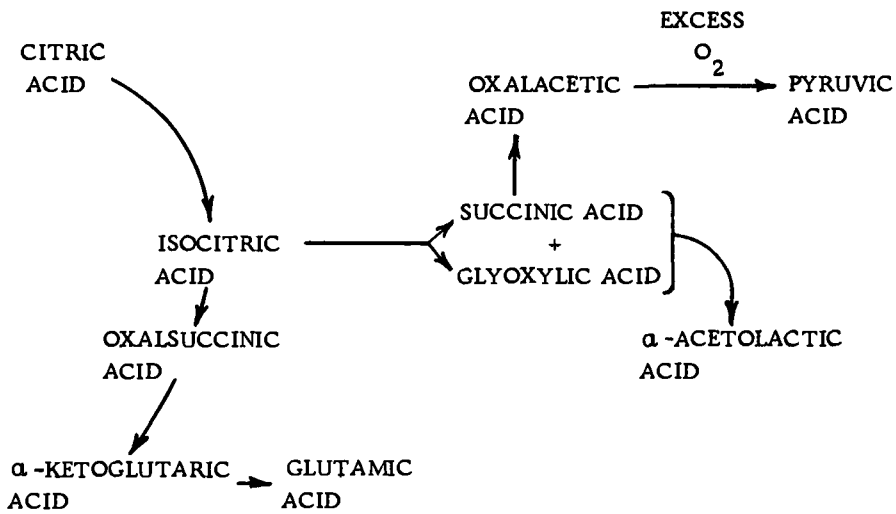
As summarized by Reiter and Møller-Madsen (109), there is some evidence to indicate that, with low glucose concentrations, L. casei passes proportionally more glucose along the HMP pathway

(shown in Figure 2), and that growing cells metabolize more glucose via this pathway than by way of the EMP pathway. Utilization of this pathway accounts for some of the products other than lactic acid which have been detected in cultures of this organism.

Lactose Fermentation by Heterofermentative Lactobacilli. Utilization of lactose by heterofermentative organisms results in a considerable amount of products other than lactic acid. Glucose is utilized by these organisms by way of the HMP pathway (Figure 2). L. brevis is the typical heterofermentative lactobacilli associated with Cheddar cheese. This organism usually produces optically inactive lactic acid (12, p. 549). Use of the HMP shunt by this organism explains its production of acetic acid and carbon dioxide.

Citric Acid Fermentation

The formation of various acidic carbonyl compounds from citric acid by L. lactis has recently been demonstrated by Harper (54). Oxalsuccinic, alpha-ketoglutaric, alpha-acetolactic, glyoxylic and oxalacetic acids are produced from citrate by this organism. Harper (54) also found that L. lactis produced isocitric, succinic, glutamic and aspartic acids and leucine from citrate. On the basis of this and other evidence, this worker proposed the following scheme for citric acid metabolism by L. lactis:



Christensen and Pederson (30) found that L. brevis and L. plantarum produce diacetyl in fruit juice by fermentation of citrate. Other than this, there has been little work done on citric acid fermentation by lactobacilli.

Proteolysis and Lipolysis

Lactobacilli in general are both weakly proteolytic and lipolytic (121). Proteolytic enzymes have been isolated from cells of L. casei by Baribo and Foster (7). These authors reported maximum enzyme activity near pH 7.0. Brandsaeter and Nelson (18, 19) reported the isolation from L. casei of a proteinase active in the pH range 5.5 to 6.5 and a peptidase with maximum activity near pH 7.0. The proteolytic activity of L. casei in Cheddar cheese is well documented (21, 75).

Fryer, Reiter, and Lawrence (42) examined a total of 25

strains of L. casei, L. brevis, and L. plantarum and found them all to be weakly lipolytic. Their lipolytic activity was comparable to that of the lactic streptococci.

Pseudomonas Species

Importance to the Dairy Industry

Species of this genus that are important to the dairy microbiologist are Pseudomonas fluorescens, Pseudomonas fragi, Pseudomonas nigrifaciens, Pseudomonas putrefaciens, and Pseudomonas vicosa (38, p. 28). The presence of these bacteria in milk and its products is almost always objectionable, since they are versatile spoilage organisms with pronounced biochemical activity, particularly on proteins and fats.

Taxonomy and Classification

This genus is classified under the very broad Pseudomonadaceae family. The genus itself is quite large; at least 149 species have been characterized as members of the genus Pseudomonas (12, p. 90-96). Fortunately, for the purpose of isolation, the species common to to dairy products are psychrophilic, many of them being capable of growth at temperatures as low as 0° C (38, p. 28). This greatly simplifies their isolation and characterization.

Of the species reported to be important in dairy product spoilage, one, P. viscosa is not recognized by Bergey's Manual (12, p. 90-99). The other four species can be differentiated by simple tests. Of these four species, P. fluorescens is unique in its ability to produce a reddish gray pigment when grown on gelatin or agar (12, p. 105). This microorganism will not coagulate litmus milk. In fact, the medium becomes alkaline on extended incubation. P. fragi, P. putrefaciens, and P. nigrifaciens can all be differentiated on the basis of their growth in litmus milk. P. fragi initially produces an acid ring followed by coagulation at the surface. There is complete coagulation within two to three weeks (12, p. 111). With P. putrefaciens there is rapid reduction of litmus milk and proteolysis is pronounced (12, p. 112). In litmus milk P. nigrifaciens produces a black ring after about three days at 15° C. There is an alkaline reaction and no coagulation. Litmus is reduced (12, p. 117).

Stanier, Palleroni, and Doudoroff (126) recently completed a very extensive taxonomic study on the aerobic Pseudomonads. These workers studied the nutritional requirements of a large number of species in detail. Many of the species studied could be characterized on the basis of their results. Unfortunately, of the species of importance in the dairy industry, only P. fluorescens was studied. Mandel (84) has found that many Pseudomonas species can be characterized by their deoxyribonucleic acid base composition. Of the organisms

of interest here, only P. fluorescens was included in Mandel's study.

Role of Pseudomonads in Food Spoilage

Pseudomonas species are implicated in the spoilage of many foods. Spoilage by these organisms can occur in a variety of ways. Various Pseudomonas species can cause proteolytic and lipolytic decomposition. P. fragi and P. fluorescens can produce a fruity flavor defect in various foods (44, 104). Some species have been shown to be the causative agent in the development of slime in foods such as Cottage cheese (38, p. 358). Still another form of spoilage in the prevention of the development of aroma in foods by some species of this genus (100).

Proteolysis. Pseudomonads 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 with the production of very objectionable odors and flavors (38, p. 47). The Pseudomonads commonly associated with dairy products which possess proteolytic ability are P. fragi, P. putrefaciens, and P. nigrificans (12, pp. 110, 112, and 117).

As summarized by Hagihara (50, p. 204) the presence of extracellular proteases in the culture medium has been reported for a large number of Pseudomonas species. 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 (127) recovered an extra-cellular proteolytic enzyme from a culture of P. putrefaciens and found that it showed maximum activity against casein at pH 7.0 to 7.5. Of the milk protein fractions tested, this enzyme showed highest activity against alpha- and beta-casein. Camp and Van Der Zant (25) have also shown the presence of several peptidases in a cell-free extract of P. putrefaciens. The maximum activity of these peptidases was found between pH 7.0 to 8.0. The rate of hydrolysis decreased sharply at pH values below 6.0 and above 9.0. An extracellular protease produced by Pseudomonas myxogenes has been crystallized and studied in some detail (50, p. 204-205). This enzyme has a molecular weight of about 77,000 and an isoelectric point between pH 5.5 and 6.0. Activity is optimum at pH 7 to 8.5. On the basis of analyses of terminal amino acid residues of the peptides in the digestion mixture of gelatin, it has been suggested that this enzyme is similar to papain (50, p. 205).

Although not well documented, hydrolysis of protein by Pseudomonas species does not appear to be random. With P. fragi and P. putrefaciens, it has been found that tyrosine is rapidly liberated from casein and from synthetic peptides (25, 53).

Lipolysis. Since the early work of Collins and Hammer (33), in which they showed that lipolytic bacteria of the "Pseudomonas-Achromobacter" groups are common in dairy products, the lipolytic

activity of various Pseudomonas species has been extensively investigated. Goldman and Rayman (47) were among the first to study the bacterial hydrolysis of fats using a stable, finely divided fat globule emulsion. These workers found that with the Pseudomonas species tested, substantially the same degree of cleavage was achieved with fats possessing widely varying fatty acid composition. Lipolytic activities of different strains and species of Pseudomonas were found to vary widely in both rate and extent of hydrolysis. With P. fluorescens, Goldman and Rayman (47) found that the degree of hydrolysis was greater at fat concentrations below 10 g per 100 ml of medium than at higher concentrations.

Alford and co-workers (1, 4) observed a great reduction in the amount of lipase produced by several Pseudomonas species when the incubation temperature was raised from 20 to 28° C. There also appeared to be a qualitative effect on the lipase when the production temperature was increased. These workers noted a small but consistent increase in the percentage of unsaturated fatty acids liberated by the enzyme produced at 28° C, with a slight decrease in the total saturated acids, particularly stearic acid.

Alford and Price (2) have studied the effect of composition of the medium on the production of lipase by P. fragi. They found the nutritive requirements for production of lipase were variable and also that there was good cell growth but no lipase production in several

synthetic media. P. fluorescens produced very little lipase in any of the media examined. Since P. fragi is similar to P. fluorescens in its pattern of lipolysis (4), 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 (2).

The available evidence indicates that the lipases of P. fragi and P. fluorescens are extracellular (4). This lipolytic activity has been shown to be similar to pancreatic lipase in that it attacks the alpha, alpha' positions of triglycerides (3, 4). The rate of fatty acid liberation by these lipases decreases with time, but the ratios of the fatty acids liberated remain constant for some time (4).

Ester Production. The fruity aroma defect occurring in dairy products is caused primarily by P. fragi. Hussong, Long, and Hammer (60) likened the odor of cultures of this organism to that of the flower of the May apple. Pereira and Morgan (104) found that this odor was due to production of esters by P. fragi. Using paper chromatography, the latter workers reported that isovalerate and acetate esters were the principal esters in steam distillates of a milk culture of this organism. It was suggested that the major component of the fruity aroma was ethyl isovalerate. P. fragi has been shown to produce small amounts of ethanol on extended incubation and to convert leucine to isovaleric acid. Based on this, Pereira and Morgan (104) concluded that leucine in milk serves as the source of

acid and that ethanol production is limiting in the formation of ethyl isovalerate. Using more refined techniques, Reddy et al. (107) were not able to detect ethyl isovalerate in milk cultures of P. fragi. The esters positively identified in this study were ethyl butyrate and ethyl caproate. It was found that the addition of ethanol and aeration of cultures increased production of these esters.

Incubation at 8° C has been shown to be more favorable for development of a fruity aroma than incubation at 20° C (104, 107). This serves to explain the development of a fruity flavor defect in products such as Cottage cheese under refrigeration conditions. Not all strains of P. fragi produce a fruity aroma and strains lose their ability to produce esters under ill-defined conditions (88).

Lack of Aroma Development. This is a frequent defect of Cottage cheese and can sometimes result from growth of P. fragi in the cheese (38, p. 328). Parker and Elliker (100) have shown that P. fragi does this by reducing diacetyl to acetoin. This has been confirmed by Wales and Harmon (131), who also found P. fluorescens to be capable of carrying out this conversion. Seitz (116) has demonstrated the presence of an intracellular diacetyl reductase in P. fragi and P. fluorescens.

As yet, studies on lack of aroma have not been extended to the reduction of carbonyls other than diacetyl. The work of Payne and others (103, 135) indicates that this may also be involved in food

spoilage. These workers found that a particular Pseudomonas species was capable of growing on and metabolizing several even- and odd-carbon chain length alcohols. They observed constitutive alcohol dehydrogenase activity in cell-free extracts with 6-, 8-, and 10-carbon linear primary alcohols. In addition, a dehydrogenase which was active with the 12-carbon linear primary alcohol could be induced by growth on sodium dodecyl sulfate.

EXPERIMENTAL

Cultures and Culturing Conditions

Single-strain cultures were used exclusively in this study. Microorganisms utilized in the various studies and their source are listed in Table 1. With the exception of Pseudomonas species, all cultures were maintained in a sterile, reconstituted, 11% solids non-fat milk medium. Pseudomonas species were maintained in brain-heart infusion broth (Difco). Cultures were transferred every fourth day, using from one to three percent inoculum. L. lactis was incubated at 40° C and all other cultures were incubated at 21° or 30° C, except as otherwise noted.

Gas Chromatographic Analysis

Culture samples were analyzed for volatile constituents by the on-column trapping, gas-liquid chromatographic (GLC) technique developed by Morgan and Day (89). In essence, this technique consisted of passing a stream of nitrogen through a sample contained in a screw-capped vial (Kimble no. 60957, size no. 1) by means of the modified needle described by Bills (13, p. 52). The needle was inserted through one of two holes drilled through the cap, the original liner of the cap having been replaced by a 1/8-inch-thick silicone

Table 1. Microorganisms and their source.

Culture	Strain	Source[a]
S. cremoris	459	Mb culture collection
S. cremoris	799	Mb culture collection
S. diacetilactis	M21-35	Mb culture collection
S. diacetilactis	DRC-1	Mb culture collection
S. lactis	C2-F	Mb culture collection
S. lactis	2-AH	M. E. Morgan, University of Connecticut
L. citrovorum	Foster	Mb culture collection
L. citrovorum	91404	Mb culture collection
P. fragi	4973	ATCC
P. fragi	T-2	FST culture collection
P. fluorescens	13525	ATCC
P. fluorescens	T-5	FST culture collection
P. putrefaciens	T-6	FST culture collection
Pseudomonas type I	153	NCMB
Pseudomonas type II	133	NCMB
L. brevis	4006	ATCC
L. brevis	14434	ATCC
L. casei	334	ATCC
L. casei	393	ATCC
L. lactis	11061	ATCC
L. lactis	13215	ATCC
L. plantarum	4008	ATCC
L. plantarum	10776	ATCC

[a]Mb refers to the Department of Microbiology, and FST refers to the Department of Food Science and Technology; Oregon State University. ATCC refers to the American Type Culture Collection. NCMB refers to the National Collection of Marine Bacteria, Torry Research Station, Aberdeen, Scotland.

rubber septum. A second shorter modified 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 an F and M Model 810 instrument equipped with a flame ionization detector. The U-shaped portion of the column was immersed in a mixture of dry ice and 2-methoxyethanol. After the collection period, the head of the column was placed back in the instrument and the carrier gas flow resumed. A water bath was used to warm the vial containing the sample during trapping to improve the efficiency of removal of volatile materials. The entrainment assembly for this on-column trapping, gas-liquid chromatographic (OCT-GLC) technique is shown in Figure 4.

For analysis of cultures, nitrogen was bubbled at a rate of 10 ml per min for 5 min through 4 ml of culture made up to 8 ml with distilled water and mixed with 5 g anhydrous sodium sulfate and approximately 1 mg 1-tetradecanol. The water bath in which the sample vial was immersed was held at either 50 or 60° C. A 1/8-inch by 12 foot column packed with 20% 1, 2, 3-tris(2-cyanoethoxy)-propane on 80-100 mesh Celite 545 was used throughout this study. Depending on the volatile compound of interest, the oven temperature was maintained at 50, 60, or 80° C. In some cases an internal standard was added to the vial. Quantitative data were obtained by

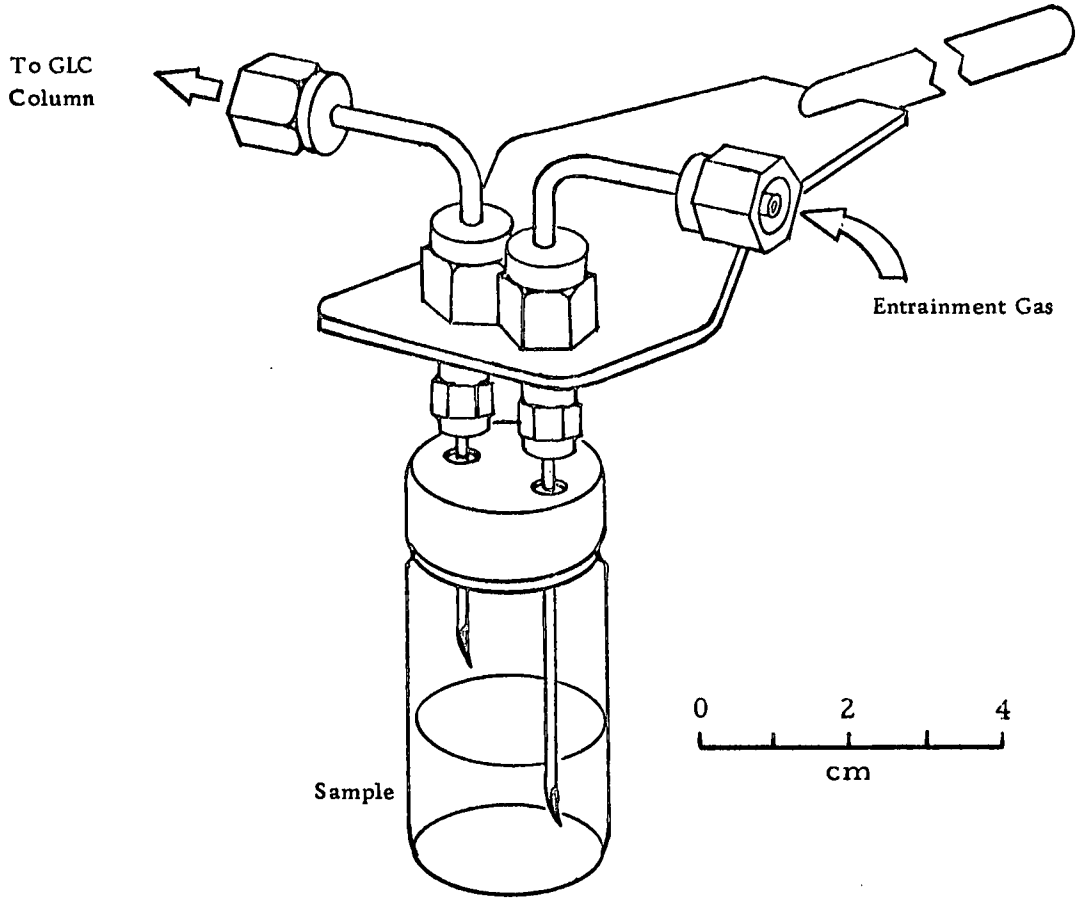


Figure 4. The entrainment assembly used in the analysis of volatiles.

calibrating recorder response for graduated concentrations of the constituent of interest in either milk or distilled water. The reproducibility of this technique has been previously demonstrated (15).

Preparation of Sterile Solutions

Various concentrations of acetaldehyde, acetone, ethyl acetate, and ethanol were autoclaved for 15 min in 15 cm culture tubes with Teflon-lined screw caps (Kimble no. 45066-A; Owens-Illinois Glass Co., Toledo, Ohio). Solutions were prepared, divided into two portions, and dispensed into culture tubes. One portion was reserved as a control and the other portion was autoclaved. The culture tubes were filled two-thirds full and the Teflon-lined caps screwed on tightly. Immediately after autoclaving the tubes were immersed in an ice water bath for rapid cooling.

Glass ampoules containing a solution of ethyl acetate were also prepared and autoclaved. Ampoules were constructed of thick-wall 0.75 cm i.d. pyrex tubing 18 cm in length. After adding 5 ml of the prepared solution, the ampoules were placed in an ice water bath, and the tops were sealed with a flame at a level approximately 5 cm above the solution. Half of the ampoules were autoclaved and half were reserved as controls.

The concentration of each compound in both the control and autoclaved solutions was determined by the OCT-GLC technique.

Both the column and water bath were maintained at 60 ° C and a nitrogen carrier gas flow rate of 25 ml per min was used. A known amount of propionaldehyde in aqueous solution, sufficient to yield a response similar to that of the compound being analyzed, was added just before analysis. Propionaldehyde thus served as an internal standard in each determination and compensated for minor variations in sample size and instrument sensitivity between analyses. Quantification was based on the ratio between internal standard peak height and compound peak height between the heated and unheated solutions. Reproduction of retention time was verified before peak height measurement in all cases.

Based on results obtained in this experiment, all sterile solutions used throughout this study were prepared by autoclaving concentrated aqueous solutions in the culture tubes described above.

Acetone in Milk Cultures of Lactic Organisms

Acetone levels were examined in milk cultures of S. cremoris, S. lactis , S. diacetilactis, and L. citrovorum using the OCT-GLC technique. Organisms were cultured in skimmilk heated in a steam bath for one hour and in skimmilk autoclaved for 12 min at 121 ° C. Milk medium was inoculated with 2% of the respective single-strain culture and incubated at 21 ° C for 24 hr or 30 ° C for 16 hr in culture tubes sealed with Teflon-lined caps. For the analysis, 4.0 ml of

culture was diluted to a total volume of 8.0 ml with distilled water and equilibrated at 50° C. The oven temperature was maintained at 50° C. Quantitative data were obtained by calibrating recorder response for known concentrations of acetone in distilled water.

To simplify the determination of the acetone content of cultures, reconstituted non-fat milk was treated by a modification of the technique of Bassette and Claydon (9) to reduce acetone content. The milk was heated to 65° C and purged with nitrogen under vacuum (28 mm Hg) for three hours, after which it was dispensed in tubes and autoclaved. To eliminate variations in GLC analyses, propionaldehyde was added as an internal standard to the diluted culture to give a final concentration of 0.5 ppm. Recorder response for acetone was corrected for variations in recorder response for propionaldehyde.

To investigate the capability of these organisms to convert the most logical immediate precursor to acetone, one strain of each species studied was grown for 24 hr at 21° C in a milk medium containing 100 ppm acetoacetic acid, prepared from ethyl acetoacetate (11, p. 630). After incubation, cultures were analyzed for acetone content by the GLC technique.

Dimethyl Sulfide Production by Lactic Organisms

Single-strain cultures of S. cremoris, S. diacetilactis, S. lactis, and L. citrovorum were grown in non-fat milk devolatilized by the procedure outlined in the previous section. The medium was inoculated with 2% of the respective culture and incubated at 21 °C for 24 hr or at 30 °C for 16 hr. At the end of the incubation period, cultures were analyzed for dimethyl sulfide (Me_2S) content by the OCT-GLC technique. The oven and water bath temperatures were maintained at 50 °C. A known amount of propionaldehyde was added as an internal standard to 4.0 ml of culture and the mixture was made up to 8.0 ml with distilled water. To obtain quantitative data, recorder response was calibrated for known concentrations of Me_2S in control milk medium.

To determine if these organisms are capable of converting the Me_2S precursor of milk to dimethyl sulfide, one strain of each species was incubated in milk medium containing 161 ppm methyl methionine sulfonium chloride (calculated to yield a total potential of 50 ppm Me_2S). Incubation was carried out for 24 hr at 21 °C and then the cultures were analyzed for Me_2S content by the OCT-GLC technique. Methyl acetate was used as an internal standard in this determination.

Dimethyl Sulfide Precursor in Milk

To determine if there was a heat-labile Me_2S precursor in milk, samples of raw whole milk were heated individually at 60, 80, and 100° C for 30 min and at 121° C for 15 min in tubes sealed with Teflon-lined caps and then analyzed for Me_2S content by the OCT-GLC technique. Conditions of the analysis were identical with those described in the above section, except that the sample size was 7.0 ml.

To gain an insight as to the nature of the precursor, raw whole milk was separated by centrifugation at 12,000 × g for 10 min at 5° C. Portions of the skimmilk and cream obtained were autoclaved for 15 min in tubes sealed with Teflon-lined caps and then analyzed for Me_2S content. A portion of the skimmilk was dialyzed at 5° C against three changes of distilled water. The dialyzed skimmilk was then autoclaved for 15 min and analyzed for Me_2S content. The dialysate was lyophilized and the solids obtained were redissolved in a volume of distilled water equivalent to the original volume of skimmilk. A portion of this solution was autoclaved for 15 min and analyzed for Me_2S content. Identity of the Me_2S peak was established on the basis of its retention time and by reaction with mercuric chloride by the method of Bassette and Whitnah (10).

To isolate the Me_2S precursor, raw skimmilk was dialyzed

against three changes of distilled water at 5° C. The dialysate was lyophilized and minimal volumes of methanol were used to dissolve the solids; the insoluble material was discarded. Isopropanol was added to the methanolic solution five to one and any precipitate formed after 8 hr at 5° C was filtered off and discarded. The alcoholic solution was taken to dryness in vacuo without heating and the solids obtained were redissolved in minimal volumes of water. This solution was applied directly to thin-layer chromatographic (TLC) plates.

TLC plates were coated with a 250 μ layer of silica gel G as previously described (66). The aqueous sample was either applied directly to the plate or turned just alkaline with 1 N NaOH, autoclaved for 20 min and then applied to the plate. Solvent systems used for development of TLC plates were n-propanol:30% ammonia in a ratio of 60:40, ethanol:30% ammonia in a ratio of 50:50, and dimethyl sulfoxide:dimethyl formamide:30% ammonia in a ratio of 10:10:20. Amino acids were detected by spraying plates with 0.5% ninhydrin in 95% ethanol, drying, and heating at 70° for five minutes. In some cases compounds were recovered from plates as previously reported (66).

To determine the total Me_2S potential of milk, raw skimmilk was autoclaved in tubes sealed with Teflon-lined caps for periods up to two hours and then analyzed for Me_2S content. To determine the

optimum pH for Me_2S formation, the pH of raw skimmilk was adjusted to 7.5, 8.5, and 9.5 with 1 N NaOH and the resulting milk was autoclaved for ten minutes and analyzed for Me_2S content.

Carbonyl Reduction by Pseudomonas Species

The ability of single-strain Pseudomonas cultures to reduce carbonyls to the corresponding alcohols was evaluated by adding 1.0 ml of a sterile carbonyl solution to 19.0 ml of the sterile non-fat milk to yield a final concentration of 100 ppm of carbonyl in the resulting medium. After inoculation with 2% of the respective stock culture, cultures were incubated at 21° C for 48 hours. Carbonyls used in this study were acetaldehyde, propionaldehyde, butyraldehyde, acetone, and butanone. All were gas chromatographically pure in the concentrations used under the conditions of analysis.

At the end of the incubation period cultures were analyzed for alcohol content by the OCT-GLC technique. The water bath was maintained at 60° C and the column was operated at 50° C to determine reduction of acetone, at 60° C to evaluate reduction of acetaldehyde, propionaldehyde, and butanone, and at 80° C to determine butyraldehyde reduction. To estimate the amount of alcohol produced, recorder response was related to alcohol concentration.

Based on results at 21° C, cultures were incubated individually with 100 ppm propionaldehyde or butanone at 6° C for seven days and

then analyzed for alcohol content. To determine if carbonyl reduction parallels the increase of cell numbers, P. fragi 4973 was incubated with 100 ppm propionaldehyde for 48 hours at 21° C in milk medium. The volume of the medium was 400 ml, and incubation was carried out in a two-liter Erlenmeyer flask to provide a large surface area and improve the aerobic environment required for optimal growth. Samples were withdrawn at various intervals during incubation, plated to determine cell numbers, and analyzed for propionaldehyde and n-propanol content. All cell counts reported in conjunction with Pseudomonas work were made by plating dilutions of cultures in standard plate count agar (Difco).

Acetaldehyde and Ethanol Production by Lactobacillus Species

Sterile non-fat milk medium was inoculated individually with single-strain cultures of L. brevis, L. casei, L. lactis, and L. plantarum. Cultures were analyzed for acetaldehyde and ethanol content by the OCT-GLC technique after 24 and 48 hours incubation at their optimum growth temperature (12, p. 544-551) and after ten days incubation at 8° C, a temperature near that used in the ripening of Cheddar cheese. The water bath and column were both maintained at 60° C. The recorder response was determined for graduated concentrations of acetaldehyde and ethanol in milk medium. Methyl

acetate was used as an internal standard in all GLC analyses involving lactobacilli.

Compounds detected by the GLC analysis was tentatively identified on the basis of retention time. Identity of the acetaldehyde peak was confirmed by splitting the GLC effluent 10:1 and bubbling the effluent of the acetaldehyde peak from a representative culture into the 3-methyl-2-benzothiazolone hydrazone hydrochloride reagent prepared in the manner described by Lindsay and Day (78). Absorbance maxima were determined with a Beckman Model DK-1 recording spectrophotometer.

The identity of the ethanol and n-propanol peaks were confirmed by trapping these compounds from the packed GLC column and obtaining their mass spectra by the method described by Scanlan, Arnold and Lindsay (114). A 0.01 inch i.d. by 300 foot Carbowax 20 M capillary column was used in conjunction with the mass spectrometer.

Carbonyl Reduction by Lactobacillus Species

Ability of single-strain cultures to reduce carbonyls to the corresponding alcohols was evaluated by adding 1.0 ml of a sterile carbonyl solution to 19.0 ml of sterile non-fat milk, to yield a concentration of 100 ppm in the resulting medium. Carbonyls used were acetaldehyde, propionaldehyde, and butanone. After inoculation,

cultures were incubated at their optimum growth temperature for 48 hours and 8° C for ten days. In some cases, cultures of L. brevis and L. plantarum were acidified to pH 5.4 with a sterile 20% phosphoric acid solution before incubation. Cultures without added carbonyl were concurrently incubated to ascertain the amount of each alcohol produced through normal fermentation. Controls consisted of the sterile milk medium plus carbonyl and of milk acidified to pH 4.5 with sterile 20% phosphoric acid plus carbonyl. Quantitative data were obtained as outlined above, again using methyl acetate as an internal standard. All plate counts reported in conjunction with the Lactobacillus work were made by plating dilutions of cultures in lactic agar (Difco).

Diacetyl Production and Utilization by Lactobacillus Species

Milk cultures of each of the Lactobacillus strains were analyzed for diacetyl content after 48 hours incubation at their optimum growth temperature (12, p. 544-551). Diacetyl content was determined by the colorimetric method described by Pack et al. (98), except that aliquots of cultures were weighed rather than measured volumetrically. A Beckman Model DU spectrophotometer was used for the colorimetric analyses. Based on results of this initial study, sterile milk medium was inoculated with L. casei and then split into two

equal portions. A small amount of a sterile sodium citrate solution of sufficient concentration to yield 0.2% citrate in the resultant medium was added to one portion of each culture. Incubation was carried out for 60 hours at 30° C and diacetyl content, pH, and microbial numbers were determined at 12 hour intervals. The same type study was made with incubation at 8° C, except that citrate was not added to cultures. Analyses were made at weekly intervals for four weeks.

To determine if these organisms are capable of utilizing diacetyl, an amount of a sterile diacetyl solution was added to sterile milk and the resultant medium was divided into several equal portions. One portion was held as a control and the others inoculated with the respective single-strain culture. L. brevis and L. plantarum cultures were incubated along with a control at 30° C for 48 hours and then analyzed for diacetyl content. L. lactis strains were incubated at 40° C for 48 hours. The 40° C control was acidified to pH 4.5 with sterile 20% phosphoric acid before incubation. This study was repeated with incubation at 8° C for two weeks.

All cultures were assayed for diacetyl reductase activity by a modification of the method used by Seitz et al. (116). Cells were grown for 48-50 hours at their optimum growth temperature in one liter volumes of lactic broth (Difco) and in lactic broth containing 0.2% added citrate. The enzyme source was prepared as follows:

cells were harvested by centrifugation, resuspended in 40 ml of pH 7.2 phosphate buffer (48, p. 144), and disrupted in a Raytheon 10 KC sonic oscillator for 15 minutes. Cell debris was removed by centrifugation at 16,000 × g for 20 minutes. The supernatant obtained served as the enzyme source and was frozen and held at -20° C until used. Protein determinations were made by the Biuret method (49), using crystalline bovine serum albumin as the standard.

Diacetyl reductase activity was measured by following the decrease in absorbancy at 340 mμ caused by the oxidation of NADH with a Beckman Model DK-1 recording spectrophotometer. The assay system contained a 2 μM NADH, 10 μM diacetyl, and 1 ml of the crude enzyme preparation made up to a total volume of 3.0 ml.

RESULTS AND DISCUSSION

Method for Preparing Sterile Solutions of Volatile Organic Compounds

Because of the need for sterile solutions of highly volatile compounds throughout this study, attempts were made to find a rapid method for preparation of these solutions. Sealed glass ampoules provide leak-proof containers in which volatile compounds can be autoclaved, but they are time consuming to prepare and difficult to work with. In this study various concentrations of selected volatile compounds were autoclaved as described in the experimental section.

Table 2 outlines the results of this study. Recoveries in the autoclaved culture tubes were 90% or greater in all cases. Recoveries were generally higher when the concentration of the compound was higher. Little variation was found when recovery between duplicate culture tubes was determined. Surprisingly, the recovery of ethyl acetate was nearly as low in the sealed glass ampoules as in the culture tubes (92.4 and 91.8 percent recovery, respectively, for a 100 part per million solution). The apparent loss of ethyl acetate from a completely sealed system is difficult to explain. Since ethanol was not observed in the chromatograms of autoclaved ethyl acetate solutions, it is doubtful that the ester was hydrolyzed to any

extent. Walradt (132, p. 36) has observed similar decreases with time in the concentration of isopropanol or acetone in unheated aqueous solutions. This phenomenon was observed in a variety of tightly-closed containers including sealed glass ampoules. Perhaps adsorption of certain organic compounds to the glass walls of the container or to residual material remaining in the distilled water becomes significant in very dilute solutions.

Table 2. Recovery of volatile compounds following autoclaving.

Compound	Original Concentration[a]	% Recovery
Acetaldehyde	100 ppm	100.0
	10 ppm	96.0
Acetone	100 ppm	97.5
	10 ppm	98.1
Ethyl acetate	100 ppt	97.5
	100 ppm	91.8
	100 ppm[b]	92.4
	10 ppm	90.8
Ethanol	100 ppt	94.5
	100 ppm	92.4
	10 ppm	90.0

[a] ppm = parts per million
ppt = parts per thousand

[b] This sample autoclaved in a sealed glass ampoule.

For many experiments the recoveries found in this study should be satisfactory. The similar recovery of ethyl acetate in sealed

ampoules and culture tubes indicates that there is probably little advantage in using sealed ampoules for compounds similar to those included in this study. When it is necessary to know more precisely the concentration of a volatile compound in a sterile solution, the GLC method outlined herein provides rapid and accurate results. The use of an internal standard resulted in duplicate analyses which were generally within ± 1 percent of their mean value.

Acetone in Milk Cultures of Lactic Organisms

In view of the controversy which exists on the ability of these organisms to produce acetone, attempts were made to determine whether the organisms common to lactic dairy cultures were capable of producing acetone when grown in milk culture. Acetone concentrations were measured in single-strain cultures grown in skimmilk heated for one hour and in skimmilk autoclaved for 12 minutes at 121° C. The identity of the acetone peak had been previously confirmed by mass spectrometry (90). The results presented in Table 3, although somewhat variable, demonstrated that if these organisms produced acetone, it was at levels of less than 0.5 ppm. It was difficult to draw any conclusions about the production of lesser quantities due to the relatively high levels of indigenous acetone (0.54 ppm in autoclaved milk and 0.86 ppm in steamed milk).

To simplify the determination of the acetone content of cultures,

reconstituted non-fat milk was treated to reduce acetone content and an internal standard was used in the analysis. Results (Table 4) show little evidence for acetone production by any of the organisms at 21° C (standard deviation of nine analyses = 0.016 ppm). At 30° C, S. cremoris 459 produced 0.25 ppm acetone and S. cremoris 799 produced 0.1 ppm acetone, but the other organisms appeared to accumulate only very small amounts, if any, of this compound. Apparently there are differences in the metabolism of these organisms at these different incubation temperatures. The results with S. cremoris are in agreement with the work of Harvey (56). However, in contrast to Harvey's results, S. diacetilactis DRC-1 did not utilize acetone under similar incubation conditions. In fact, none of the organisms studied utilized acetone present in the medium. This latter result is in agreement with the work of Bills and Day (15), who found that these organisms reduce acetaldehyde and propionaldehyde, but not acetone.

To investigate the capability of these organisms to convert the most logical immediate precursor to acetone, one strain of each species studied was grown for 24 hours at 21° C in a milk medium containing 100 ppm acetoacetic acid. Analysis of these cultures revealed small amounts of acetone production by two of the organisms from acetoacetic acid (Table 5). Thus these organisms apparently cannot readily decarboxylate an exogenous source of

Table 3. Acetone in autoclaved and steamed milk cultures.

Culture	ppm Acetone	
	Autoclaved[1]	Steamed[2]
S. cremoris 459	0	0.14
S. cremoris 799	0	0.03
S. diacetilactis M21-35	0	0
S. diacetilactis DRC-1	0.21	0.14
S. lactis C2-F	0	0.19
S. lactis 2-AH	0.06	0.42
L. citrovorum Foster	0	0.38
L. citrovorum 91404	0.24	0.22

[1]Corrected for amount of acetone in control milk, 0.54 ppm.

[2]Corrected for amount of acetone in control milk, 0.86 ppm.

Table 4. Acetone in cultures grown in autoclaved milk treated to reduce acetone levels.

Culture	ppm Acetone, 21°C[1]	ppm Acetone, 30°C[2]
S. cremoris 459	0.02	0.25
S. cremoris 799	0	0.10
S. diacetilactis M21-35	0.01	0.06
S. diacetilactis DRC-1	0	0.05
S. lactis C2-F	0	0.06
S. lactis 2-AH	0	0.04
L. citrovorum Foster	0	0.04
L. citrovorum 91404	0	0

[1]Corrected for amount of acetone in control milk, 0.14 ppm.

[2]Corrected for amount of acetone in control milk, 0.10 ppm.

acetoacetate, suggesting that they cannot readily utilize the aceto-acetate normally present in milk (59).

Table 5. Acetone production by cultures grown in autoclaved milk containing 100 ppm acetoacetic acid.

	ppm Acetone	
	Control[1]	100 ppm Acetoacetic Acid[2]
S. cremoris 459	0.01	0
S. diacetilactis DRC-1	0	0.36
S. lactis C2-F	0.05	0.75
L. citrovorum 91404	0.05	0

[1] Corrected for amount of acetone in control milk, 0.23 ppm.

[2] Corrected for amount of acetone in control milk, 6.8 ppm.

The foregoing results suggest that production of significant amounts of acetone is not a general feature of the Streptococcus and Leuconostoc species studied. The levels of acetone encountered in cultures and in both raw and heated milk were generally below one ppm. The odor threshold of this compound is 500 ppm in distilled water (134). Thus the levels of acetone normally encountered in cultured milk products would have no effect on flavor.

Dimethyl Sulfide in Milk Cultures of Lactic Organisms

Although Me_2S is important in the flavor of many cultured dairy products, there is some controversy over the ability of the culture

organisms to produce this compound (81). Data presented in Table 6 show that these organisms do not produce Me_2S at either 21 or 30° C when grown in milk culture. S. cremoris 459 and S. lactis C2-F appeared to produce small amounts of this compound at 30° C, but in levels below 0.5 ppb. These data are representative of many observations made in this laboratory. The flavor threshold of Me_2S in milk, 19-21 ppb, is quite low, and this compound normally occurs in milk and cultured products in levels near the threshold level (35, 102, 108).

The presence of heat-labile Me_2S precursors in milk has been suggested, but never demonstrated (35, 108). Results presented in Table 7 indicate that there is indeed a heat-labile Me_2S precursor in milk. Me_2S was generated by heating the milk at 100° C for 30 minutes and by autoclaving for 15 minutes. Identity of the Me_2S peak was confirmed by the technique of Bassette and Whitnah (10). The milk sample used in this study was produced during the winter, and this explains the low Me_2S concentration in the raw milk sample. However, Me_2S content was observed to increase on heating in milk samples surveyed monthly from January to May, inclusive.

The data of Table 7 also give an indication of the nature of the precursor. On separation the precursor stayed in the skimmilk fraction and was dialyzable, indicating that it was a polar, low molecular weight compound. A search of the literature revealed

Table 6. Dimethyl sulfide in cultures grown in autoclaved milk treated to reduce dimethyl sulfide levels.

Culture	ppb Me_2S, 21°C[1]	ppb Me_2S, 30°C[2]
S. cremoris 459	0	0.2
S. cremoris 799	0	0
S. lactis C2-F	0	0.1
S. lactis 2-AH	0	0
S. diacetilactis M21-35	0	0
S. diacetilactis DRC-1	0	0
L. citrovorum Foster	0	0
L. citrovorum 91404	0	0

[1]Corrected for amount of Me_2S in control milk, 7.0 ppb.

[2]Corrected for amount of Me_2S in control milk, 3.2 ppb.

Table 7. Dimethyl sulfide in treated milk.[1]

Sample	Me_2S (ppb)
Raw Whole Milk	< 1
60°C Whole Milk	< 1
80°C Whole Milk	< 1
100°C Whole Milk	6
121°C Whole Milk	10
121°C Whole Milk	10
121°C Skim Milk	12
121°C Cream	< 1
121°C Dialyzed Skim Milk	< 1
121°C Dialysate	10

[1]See experimental section for description of treatments.

that S-methyl methionine sulfonium salts (MMS) are widespread in plant materials. These salts, which decompose on heating to yield homoserine and Me_2S, are important in flavor development of various foods. MMS salts have been isolated from a variety of plants, including seaweed (27), bracken (22), asparagus (26), cabbage (83), green tea (70), and tomato (137). It seemed an attractive possibility that an MMS salt is the Me_2S precursor in milk. This possibility has been suggested by others (35).

A portion of the sample prepared from milk as described in the experimental section was spotted on a TLC plate. Another portion of this sample was made alkaline and autoclaved before application to the TLC plate. Authentic MMS chloride and homoserine were also spotted on the plate. The TLC plate was developed in an n-propanol: 30% ammonia (60:40) solvent system. Results (Figure 5) revealed a compound with an R_f identical with known MMS chloride in the unheated sample. In the autoclaved sample this compound was not detected and the spot corresponding to homoserine in R_f was more intense. The compound corresponding in R_f with MMS chloride, when recovered from the TLC plate and heated gave off Me_2S (detected by GLC). As additional evidence for the occurrence of an MMS salt in milk, the sample was applied to TLC plates and these were developed in three different solvent systems. All three solvent systems separated a ninhydrin positive compound corresponding in

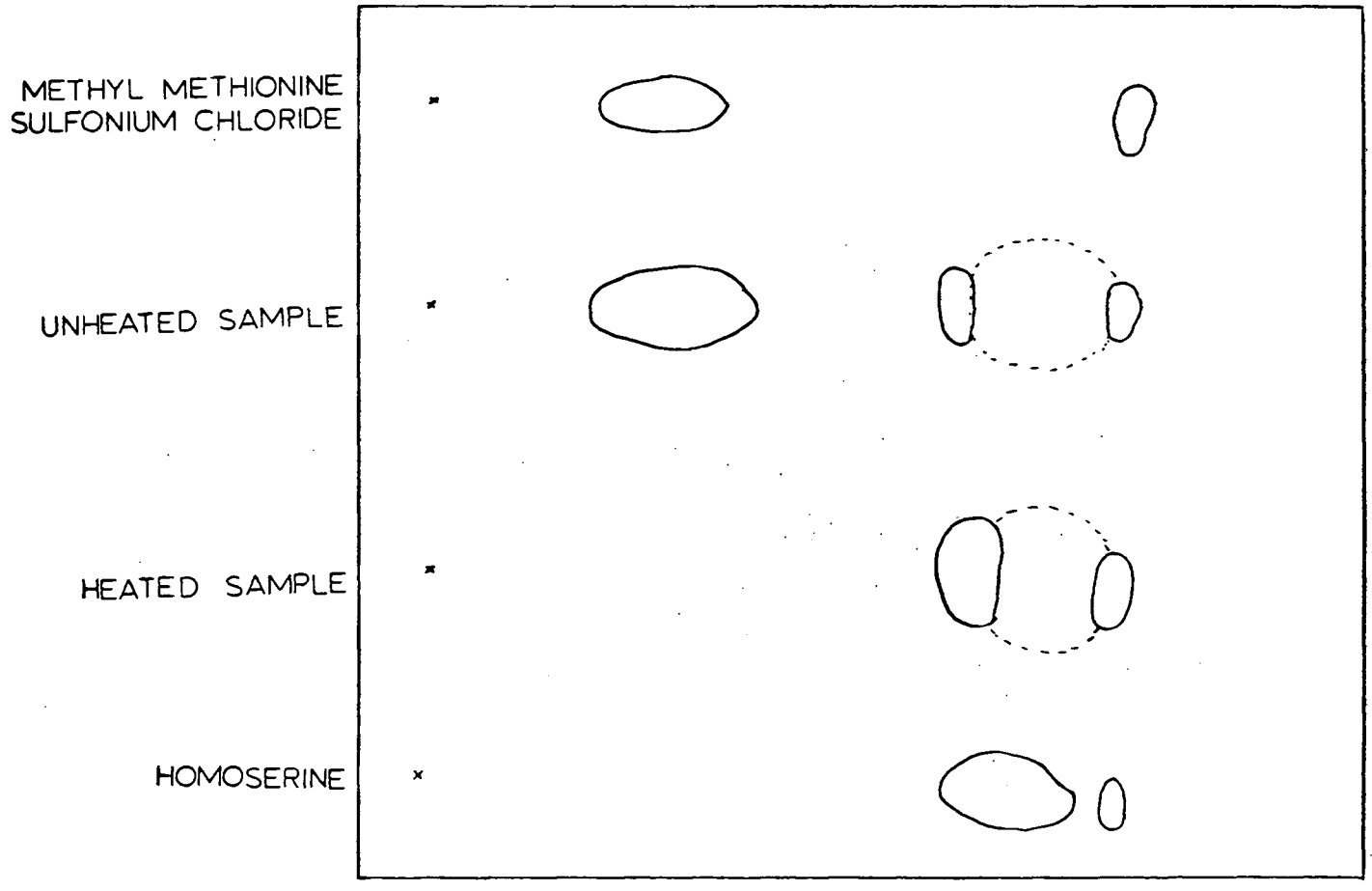


Figure 5. Trace of thin-layer chromatogram of dimethyl sulfide precursor fraction isolated from milk.

R_f to authentic MMS chloride (Table 8).

Table 8. R_f's of isolated compound and authentic MMS chloride.

Solvent	Ratio	R_f	
		Unknown	Authentic MMS Chloride
n-Propanol 30% Ammonia	60 40	0.24	0.24
Dimethyl Sulfoxide Dimethyl Formamide 30% Ammonia	10 10 20	0.31	0.29
95% Ethanol 30% Ammonia	50 50	0.34	0.32

Based on the results obtained, it was tentatively concluded that this Me_2S precursor was a MMS salt. Repeated attempts to gain enough of this compound of sufficient purity to obtain an infrared spectrum were unsuccessful. The occurrence of this compound was documented both by TLC and heat generation of Me_2S in three separate lots of milk, indicating that its presence in the original sample was not an isolated incident.

To gain an insight into the total Me_2S potential of milk, raw skimmilk was autoclaved for periods up to two hours. Results obtained (Figure 6) indicated that autoclaving for as little as five minutes generates essentially all the Me_2S possible. Figure 6 also shows that raising the pH of milk does not increase Me_2S production. It has been previously reported that MMS salts decompose to

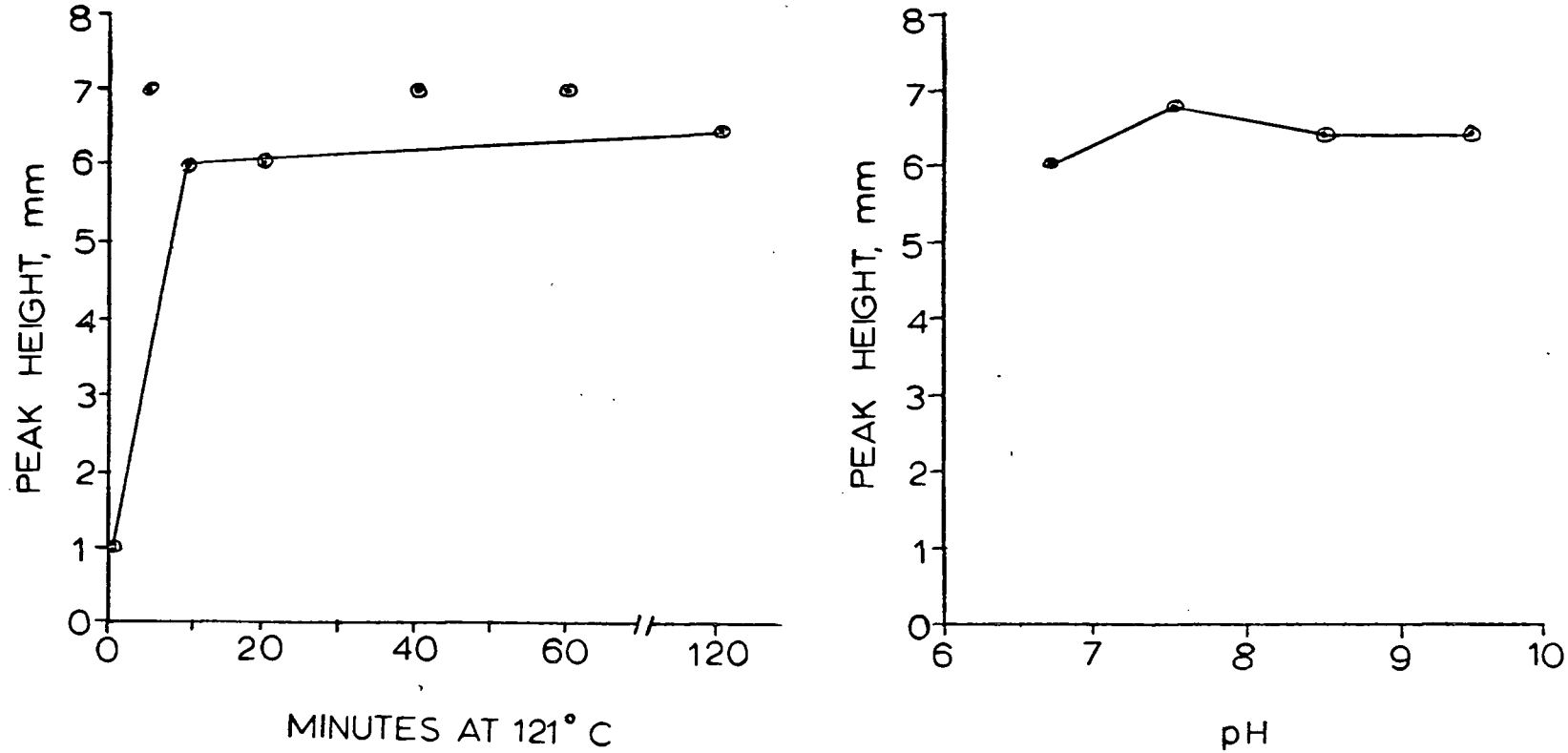


Figure 6. Effect of heating time and pH on dimethyl sulfide production in milk.

Me_2S on heating more rapidly at pH 8 and higher (137).

To determine if the common lactic dairy organisms are capable of converting MMS salts to Me_2S, S. diacetilactis DRC-1, S. lactis C2-F, S. cremoris 459, and L. citrovorum 91404 were cultured for 24 hours at 21° C in milk containing MMS chloride. Analysis of these cultures revealed no evidence for Me_2S production by any of these organisms, suggesting that those organisms common to lactic dairy cultures cannot produce Me_2S from the MMS salt of milk.

Dehydrogenase Activity of Pseudomonas Species

As discussed in the review of literature, these organisms can cause spoilage of foods in a variety of ways. Parker and Elliker (100) found that P. fragi can prevent aroma development in some products by reduction of diacetyl to acetoin. Observations in this labroatory have indicated that cultured products contaminated with Pseudomonas species show subtle flavor changes before signs of gross decomposition become evident. This, coupled with the known involvement of carbonyls in the flavor of these products, prompted a study to determine if species of this genus are capable of reducing exogenous volatile carbonyls to their corresponding alcohols. In addition to lesser volatility, the alcohols are markedly different from carbonyls in their flavor properties. This study was expanded to include two marine Pseudomonas species in addition to those

implicated in the spoilage of dairy products.

The extent of carbonyl reduction at 21° C by the species studied is shown in Table 9. Cultures without added carbonyl were incubated and analyzed concurrently with the samples. None of the control cultures produced detectable amounts of any of the alcohols listed in Table 9. Both strains of P. fragi and both marine Pseudomonas species reduced methyl ketones as well as aldehydes. P. fragi T-2 and Pseudomonas type I 153 apparently reduced butanone but not acetone. However, levels of isopropanol below 1 ppm would not have been detected by this technique. In all cases each of the three homologous aldehydes were reduced to some extent. With the exception of Pseudomonas type I 153, propionaldehyde was always reduced in greater quantities than was acetaldehyde. With all organisms except P. putrefaciens T-6, butyraldehyde was always reduced to a lesser extent than propionaldehyde. Plate counts on controls at the initiation and at the end of the 48 hours incubation period revealed few parallels between cell numbers and dehydrogenase activity.

To determine if the dehydrogenase activity was functional under refrigeration conditions, each of the cultures was incubated separately with 100 ppm of propionaldehyde or butanone at 6° C for seven days. Results (Table 10) indicate that all of these organisms are capable of reducing propionaldehyde under these conditions. In addition to P. fragi and the marine Pseudomonas species, which also reduced

Table 9. Alcohol production from added carbonyls[a] by Pseudomonas species in 48 hr at 21° C.

Culture	Alcohol Concentration, ppm					Plate Count x 10^6[b]	
	Ethanol	n-Propanol	n-Butanol	Isopropanol	2-Butanol	0 hr	48 hr
P. fragi 4973	20	45	17	4	5	18	560
P. fragi T-2	20	28	21.5	0	1	23	510
P. fluorescens 13525	12	16.5	12	0	0	37	530
P. fluorescens T-5	7.5	19	11	0	0	6.7	130
P. putrefaciens T-6	1	7	8.5	0	0	1.4	62
Pseudomonas type I 153	27	21	14	0	0.6	19	600
Pseudomonas type II 133	21.5	37	22.5	5	10	11	620

[a]100 ppm of each carbonyl added to individual cultures.

[b]Plate counts on control cultures without added carbonyl.

Table 10. Alcohol production from added carbonyls[a] by Pseudomonas species in seven days at 6°C.

Culture	Alcohol Concentration, ppm		Plate Count x 10^6[b]	
	n-Propanol	2-Butanol	0 hr	7 day
P. fragi 4973	20	5.5	34	130
P. fragi T-2	29.5	0.6	40	46
P. fluorescens 13525	11	0.9	40	15
P. fluorescens T-5	7	0	11	11
P. putrefaciens T-6	11	0	21	<1
Pseudomonas type I 153	19.5	0.5	150	51
Pseudomonas type II 133	21	8.0	23	320

[a]100 ppm of each carbonyl added to individual cultures.

[b]Plate counts on control cultures without added carbonyl.

butanone at 21° C, P. fluorescens 13525 produced a small amount of 2-butanol at 6° C. Chromatograms obtained showed that Pseudomonas type II 133 utilized nearly all of the added propionaldehyde at 6° C. The amount of n-propanol produced accounts for only about 20 percent of the added carbonyl. Apparently these organisms are capable of metabolizing propionaldehyde or n-propanol by means of other biosynthetic pathways in addition to using this carbonyl as a terminal hydrogen acceptor. This is not unusual, since some aldehydes are known to undergo several biosynthetic reactions. Amerine (5) has summarized the known microbial conversions of aldehydes. These reactions involve oxidation, reduction, and a number of condensations. Nordstrom (97) considers acetaldehyde to be a metabolic branching point in the metabolism of Saccharomyces cerevisiae, since this yeast both oxidizes and reduces acetaldehyde in alcoholic fermentation.

The reduction of propionaldehyde to n-propanol was essentially quantitative, however, in the 48 hour at 21° C study involving P. fragi 4973. Figure 7 graphically depicts the results. Propionaldehyde reduction was slow during the first few hours of incubation, after which the concentration of this compound decreased rapidly. Propionaldehyde was reduced to levels below 1 ppm in 36 hours.

The role of carbonyl reduction in the metabolism of Pseudomonas species is not known. It seems strange that these aerobic

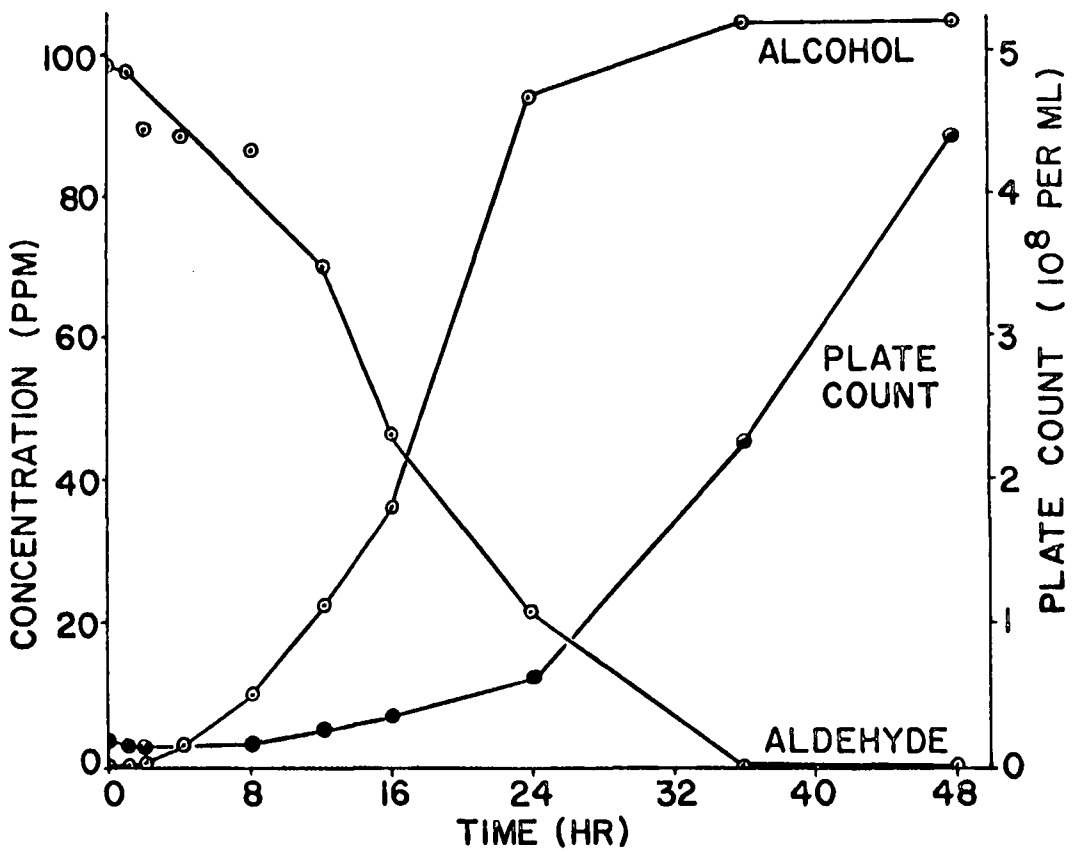


Figure 7. Reduction of propionaldehyde to n-propanol by P. fragi 4973.

organisms would reduce carbonyls when it is so much more favorable energetically to transfer the hydrogen to oxygen. It is possible that certain carbonyls are toxic to the Pseudomonas species studied.

The dehydrogenase activity displayed by the organisms studied is great enough to play a role in the deterioration of food flavor during storage. This, coupled with the previous demonstration of diacetyl reductase activity of Pseudomonas species suggests that reduction of carbonyls to their corresponding less volatile alcohols is an additional role played by these organisms in food spoilage.(116). In addition, this dehydrogenase activity is significant in that it can provide a readily available source of alcohol for ester formation by P. fragi.

Dehydrogenase Activity of Lactobacillus Species

Although many workers have speculated on the importance of lactobacilli in the development of Cheddar cheese flavor, little is known about the production of volatile compounds by these organisms. As an extension of earlier work (15) on the origin of alcohols in Cheddar cheese, the research reported herein was designed to determine the qualitative and quantitative aspects of acetaldehyde and ethanol production by the Lactobacillus species common to Cheddar cheese and to provide evidence for the presence of alcohol dehydrogenase activity. Some other volatile compounds produced

by these organisms were also identified.

Amounts of acetaldehyde and ethanol accumulated in milk cultures after 24 and 48 hours incubation at their optimum growth temperatures are given in Table 11. With the exception of L. lactis strains, the amount of acetaldehyde accumulated was less than 2 ppm in all cases. With the exception of L. casei 334, all organisms accumulated detectable amounts of ethanol in 48 hours. The L. casei and L. plantarum strains produced only small amounts of ethanol. The amount of acetaldehyde in cultures of L. brevis was always less than the amount in the control medium. L. brevis 14434 produced relatively large amounts of ethanol, whereas L. brevis 4006 produced much smaller quantities of this compound. L. casei and L. plantarum appear to be quite similar in regard to produciton of acetaldehyde and ethanol. There appears to be little correlation between cell numbers and production of these compounds.

Identification of the acetaldehyde peak was confirmed by forming the 3-methyl-2-benzothiazolone hydrazone hydrochloride derivative and determining its absorption maximum. This derivative exhibited a distinct maximum at 666 mμ and a shoulder at 635 mμ, which is in agreement with previously reported values (78). The mass spectrum obtained for ethanol was in close agreement with that obtained by Friedel, Schultz, and Sharkey (40). In addition to acetaldehyde and ethanol, both cultures of L. brevis produced small

Table 11. Acetaldehyde and ethanol production by Lactobacillus species at their optimum growth temperature.

Culture	Incubation Temperature, °C	Acetaldehyde, ppm		Ethanol, ppm		Plate Count		
		24 hr	48 hr	24 hr	48 hr	0 hr	24 hr	48 hr
L. brevis 4006	30	LTC*	LTC	0.5	4.7	28×10^4	13×10^6	25×10^6
L. brevis 14434	30	LTC	LTC	77.0	104.6	49×10^4	28×10^6	17×10^6
L. plantarum 4008	30	0.13	0.4	0	0.8	97×10^4	32×10^6	71×10^6
L. plantarum 10776	30	0.4	0.7	0	0.2	37×10^4	33×10^6	24×10^6
L. casei 393	30	LTC	LTC	0.4	2.0	68×10^4	44×10^7	12×10^8
L. casei 334	30	1.6	1.0	0	0	18×10^6	70×10^7	11×10^8
L. lactis 11061	40	16.0	12.8	0.5	0.5	24×10^4	65×10^5	4×10^5
L. lactis 12315	40	4.8	0.5	0.1	0.2	13×10^6	77×10^7	12×10^8

*LTC = Less than amount in control medium.

amounts of isopropanol and 2-butanol (tentatively identified on the basis of retention time). In these cultures it was also apparent that the indigenous concentrations of acetone and 2-butanone were decreased. L. brevis 4006 produced a compound identified as n-propanol by its retention time and mass spectrum in levels of approximately 2.5 ppm in 48 hours at 30° C. There was no evidence for production of n-propanol by L. brevis 14434. Other than these compounds and diacetyl, which will be discussed later, there was little evidence for the production of other volatile compounds by these organisms.

Results obtained for acetaldehyde and ethanol production when these cultures were incubated for ten days at 8° C indicate that only very small amounts of these compounds accumulate (Table 12). At this temperature, which is near that used to ripen Cheddar cheese, the lactobacilli apparently metabolize very slowly. This conclusion is substantiated by the small decreases in plate counts over this incubation period. There is evidence, summarized by Sharpe (120), that these organisms multiply slowly in Cheddar cheese for the first few months of ripening and that this is followed by a gradual decline in numbers after several months.

The ability of these organisms to reduce added carbonyls to the corresponding alcohols is shown in Table 13. All organisms studied reduced both acetaldehyde and propionaldehyde. The extent

Table 12. Acetaldehyde and ethanol production by Lactobacillus species in ten days at 8°C.

Culture	Acetaldehyde, ppm	Ethanol, ppm	Plate Count	
			0	10 day
L. brevis 4006	0	0	31×10^4	55×10^4
L. brevis 14434	LTC*	1.5	47×10^4	38×10^4
L. plantarum 4008	0.1	0	95×10^4	76×10^4
L. plantarum 10776	0.1	0	69×10^4	32×10^4
L. casei 393	0	0	30×10^6	29×10^6
L. casei 334	0.2	0	16×10^6	27×10^6
L. lactis 11061	0.15	0	20×10^3	60×10^2
L. lactis 12315	0.15	0	10×10^6	62×10^5

*LTC = Less than amount in control medium.

Table 13. Alcohol dehydrogenase activity of Lactobacillus species at their optimum growth temperature.

Culture	Incubation Temperature, °C	Alcohol Concentration, ppm[a]		
		Ethanol[b]	n-Propanol[b]	2-Butanol[b]
L. brevis 4006	30	18.5	32.0	6.3
L. brevis 14434	30	70.8	25.5	1.2
L. plantarum 4008	30	3.5	2.5	0
L. plantarum 10776	30	4.4	4.5	0
L. casei 393	30	8.5	14.0	0
L. casei 334	30	1.5	6.5	0
L. lactis 11061	40	1.2	6.0	0
L. lactis 12315	40	21.5	6.0	0

[a]100 ppm of respective carbonyl added.

[b]Corrected for amount of alcohol in control culture.

of reduction varied widely among species and also among strains of a species. Both strains of the heterofermentative L. brevis produced 2-butanol from butanone, but none of the other species were capable of carrying out this transformation. As mentioned previously, L. brevis reduced the indigenous acetone to isopropanol.

Results shown in Table 14 indicate that these organisms produce only small amounts of alcohols from added carbonyls in ten days at 8° C, again indicating that they metabolize very slowly at temperatures near those used for ripening Cheddar cheese. These results do show that L. casei and L. brevis produce ethanol and n-propanol under these conditions and it can be concluded that these organisms contribute some of the alcohols found in Cheddar cheese (16).

Table 14. Alcohol dehydrogenase activity of Lactobacillus species at 8° C.

Culture	Alcohol Concentration, ppm[a]		
	Ethanol[b]	n-Propanol[b]	2-Butanol[b]
L. brevis 4006	0	0.2	0.1
L. brevis 14434	2.0	3.0	0
L. plantarum 4008	0	0	0
L. plantarum 10776	0	0	0
L. casei 393	0.2	0.5	0
L. casei 334	0.1	0.2	0
L. lactis 11061	0	0	0
L. lactis 12315	0	0	0

[a] 100 ppm of respective carbonyl added.
[b] Corrected for amount of alcohol in control culture.

Since neither L. brevis nor L. plantarum was observed to lower the pH of the milk medium, cultures of these organisms were incubated for ten days at 8° C in sterile milk acidified to pH 5.4 with sterile 20% phosphoric acid. Results (Table 15) showed that at this pH, which is near that of Cheddar cheese, there was little difference in production of acetaldehyde and ethanol or in the reduction of carbonyls between these cultures and those grown in non-acidified medium.

The results presented herein indicate that, with the exception of L. brevis, the lactobacilli common to Cheddar cheese are very similar to the lactic streptococci in the volatile compounds they produce and in dehydrogenase activity (15). The results obtained with L. brevis indicate a possible origin of secondary alcohols in Cheddar cheese.

In view of the incrimination of esters in the fruity flavor defect of Cheddar cheese, it was of interest to determine whether these organisms were capable of forming volatile esters when provided with the appropriate substrates (17). A strain of each Lactobacillus species used in this study was incubated with 100 ppm ethanol and either 100 ppm butyric or caproic acid at their optimum growth temperature. Contrary to the results of Hart et al. (55), no evidence for ester formation was found, suggesting that these organisms are not directly responsible for the volatile esters found in

Table 15. Acetaldehyde and ethanol production and dehydrogenase activity of Lactobacillus species in acidified medium at 8°C.

Culture	Control		Alcohol Concentration, ppm[a]			Plate Counts on Controls	
	Acetaldehyde, ppm	Ethanol, ppm	Ethanol[b]	n-Propanol[b]	2-Butanol[b]	Initial	10 day
L. brevis 4006	0.1	0	0	1.1	1.5	31×10^4	42×10^4
L. brevis 14434	LTC[c]	2.0	1.0	10.7	0	47×10^4	38×10^4
L. plantarum 4008	0.1	0	0	0	0	95×10^4	91×10^4
L. plantarum 10776	0.1	0	0	0	0	69×10^4	64×10^4

[a] 100 ppm of respective carbonyl added.

[b] Corrected for amount of alcohol in control culture.

[c] LTC = less than amount in control medium.

Cheddar cheese (16, 17).

Diacetyl Production and Utilization by Lactobacillus Species

There have been few reports on the ability of lactobacilli to produce diacetyl. Bassette, Bawdon, and Claydon (8) reported diacetyl production by L. casei when grown in milk culture. Christensen and Pederson (30) have shown that extensive growth of L. brevis and L. plantarum during the concentration of fruit juice causes spoilage by production of diacetyl from the citrate present in the juice. Since diacetyl is produced in Cheddar cheese and reportedly contributes to the typical Cheddar flavor in low levels (23, 24), it was desirable to determine the qualitative and quantitative aspects of diacetyl production and utilization by those Lactobacillus species common to Cheddar cheese.

Neither of the two strains of L. brevis nor L. lactis studied accumulated detectable amounts of diacetyl on incubation for 48 hours at their optimum growth temperature. Both strains of L. plantarum accumulated diacetyl, but in levels below 1 ppm under similar incubation conditions. L. casei strains produced relatively large amounts of diacetyl and were studied more fully.

Duplicate cultures of each L. casei strain were grown in milk medium and in milk medium supplemented with 0.2% citrate. Data presented in Table 16 show that L. casei 393 accumulated 7 ppm

Table 16. Diacetyl production by L. casei at 30° C in milk and milk fortified with 0.2% citrate.

Organism	Incubation Time, hr	pH		Diacetyl (ppm)		Plate Counts	
		Control	Citrate[a]	Control	Citrate[a]	Control	Citrate[a]
L. casei 393							
	0	6.30	6.30	0.11	0.11	67×10^4	67×10^4
	12	6.10	6.20	1.00	0.96	44×10^6	-------
	24	5.80	5.88	2.32	2.04	16×10^7	26×10^7
	36	5.38	5.38	3.92	3.74	38×10^7	69×10^7
	48	5.01	4.92	6.00	3.64	13×10^8	17×10^8
	60	4.70	4.58	7.00	1.08	13×10^8	14×10^8
L. casei 334							
	0	6.30	6.30	0.06	0.06	14×10^6	14×10^6
	12	6.11	6.20	0.14	0.14	12×10^7	-------
	24	5.80	5.91	0.41	0.50	37×10^7	34×10^7
	36	5.42	5.48	0.41	0.83	70×10^7	72×10^7
	48	5.13	5.13	2.19	1.96	79×10^7	87×10^7
	60	4.82	4.80	2.23	1.76	81×10^7	12×10^8

[a] 0.2% citrate added to milk medium.

diacetyl in 60 hours at 30° C and L. casei 334 accumulated 2.23 ppm in a similar period. Diacetyl content of the cultures increased with increasing cell population. The data of Table 16 show that when these organisms were grown in milk medium supplemented with citrate, diacetyl utilization was stimulated. With L. casei 393, 7 ppm diacetyl accumulated in the milk culture without added citrate in 60 hours and there was no evidence of diacetyl utilization. This is in contrast to the decrease in diacetyl content from an apparent high of 3.74 ppm after 36 hours to 1.08 ppm after 60 hours in the citrated culture. Although not as pronounced, this citrate effect was also evident with L. casei 334. These trends were confirmed by repeated analyses of fresh cultures. Plate counts on cultures grown in citrated milk were generally higher during later stages of incubation, demonstrating that this effect was not due to a lower microbial population.

This citrate effect is surprising, especially in view of the fact that citrate is known to be stimulatory to diacetyl production by organisms such as L. citrovorum. However, as Galesloot (43, vol. D, p. 152-153) has indicated, in later stages of growth when diacetyl production is rapid, there also exists highly reducing conditions and this tends to accelerate the reduction of diacetyl.

Table 17 presents data showing that both L. casei strains produce diacetyl at 8° C, a temperature near that used to age Cheddar

cheese. At these conditions, L. casei 393 again accumulated more diacetyl than did L. casei 334. These results indicate that L. casei probably contributes to the production of diacetyl normally found in Cheddar cheese and partially explains the increase in diacetyl content sometimes noted during manufacture of Cheddar cheese (23, 24). The pH and plate count values indicate that the metabolism and reproduction of these organisms is slow under these conditions.

Results presented in Table 18 show that both strains of L. brevis and L. lactis utilize added diacetyl when incubated at their optimum growth temperatures. Both strains of L. plantarum increased the diacetyl content of the medium somewhat, with L. plantarum 10776 producing more diacetyl than L. plantarum 4008. At 8° C L. brevis 14434 and L. lactis 11061 utilized small amounts of added diacetyl in two weeks, but there was no evidence for diacetyl utilization by L. brevis 4006 and L. lactis 12315 under these conditions. Both strains of L. plantarum increased the diacetyl content of the medium at 8° C, suggesting that these organisms can also contribute to the diacetyl content of Cheddar cheese.

To determine the mechanism of diacetyl utilization, these lactobacilli were assayed for diacetyl reductase activity. Results (Table 19) show that, with the exception of L. plantarum 10776, there was a measurable amount of diacetyl reductase in all organisms. Seitz et al. (116) have shown that diacetyl reductase is widely

Table 17. Diacetyl production by L. casei at 8° C.

Organism	Incubation Time, Weeks	pH	Diacetyl (ppm)	Plate Count ($\times 10^6$)
L. casei 393	0	6.31	0.14	22
	1	6.21	1.50	33
	2	6.21	3.01	38
	3	6.05	4.05	32
	4	6.01	4.03	34
L. casei 334	0	6.30	0.07	15
	1	6.21	0.71	20
	2	6.20	0.97	32
	3	6.19	1.18	36
	4	6.15	1.27	68

Table 18. Diacetyl utilization by Lactobacillus species.

Organism	Diacetyl (ppm)	
	Incubation at Optimum Temp.[a]	Incubation at 8C[b]
Control	1.42[c]	2.50
L. brevis 4006	0	2.50
L. brevis 14434	0.55	1.87
L. plantarum 4008	1.55	3.18
L. plantarum 10776	2.00	2.84
L. lactis 11061	0	2.23
L. lactis 12315	0	2.50

[a] 48 hr incubation at optimum growth temperature; 40C for L. lactis, 30C for L. brevis and L. plantarum.

[b] Incubated for two weeks.

[c] Values for 30 and 40C uninoculated controls were identical.

distributed among bacteria. He found that the reaction required NADH and was irreversible. The specific activities found for these lactobacilli compare favorably with the values Seitz et al. obtained for lactic streptococci. These data also help to explain the citrate effect discussed previously. Cultures of L. casei 393, L. plantarum 4008 and L. plantarum 10776 showed a significantly greater specific activity when grown in citrated lactic broth compared to cultures grown in the plain broth. This effect was greatest with strains of L. plantarum. To confirm this, both L. plantarum strains were incubated in sterile milk and in milk medium with 0.2% added citrate. Diacetyl was detected in the controls but not in the citrated cultures at the end of this incubation period. To determine if citrate stimulated enzymatic activity, 1 μM of citric acid was added to the assay mixture, and the decrease in absorbance of NADH was measured as described. The results provided no evidence for stimulation of diacetyl reductase. Thus it can be concluded that with L. casei 393 and both L. plantarum strains, growth in the presence of citrate stimulates the formation of diacetyl reductase. In view of the work of Harper (54), who found that some lactobacilli metabolize citrate, it cannot be concluded that citrate itself is responsible for this increase in diacetyl reductase activity.

The results presented herein indicate that L. casei and L. plantarum strains can contribute to the diacetyl content of Cheddar

cheese and that some Lactobacillus species are undoubtedly involved in the gradual decrease in the amount of diacetyl noted in Cheddar cheese during curing (23, 24). If higher levels of diacetyl are indeed undesirable in Cheddar cheese (23), a profitable approach, at least on an experimental basis, would be to add citrate to the cheese milk.

These lactobacilli produce few of the volatile compounds which have been identified in Cheddar cheese when grown in single-strain cultures. However, when grown in combination with other organisms, other compounds may be produced. It has been previously shown that extracts of S. cremoris and S. lactis stimultate the development of L. casei. Studying these lactobacilli in combinations with the other microorganisms prevalent in Cheddar cheese would be a formidable task. However, in view of the time and effort already invested into attempts to define Cheddar flavor, this may well prove to be a productive approach.

Table 19. Diacetyl reductase activity of Lactobacillus species.

Organism	Specific Activity[a]	
	Control	Citrate[b]
L. casei 393	16	24
L. casei 334	16	17
L. brevis 4006	3	4
L. brevis 14434	8	8
L. plantarum 4008	< 1	31
L. plantarum 10776	< 1	33
L. lactis 11061	5	5
L. lactis 12315	5	4

[a]Expressed as units per mg of protein, where one unit was defined as a 0.001 change in absorbance at 340 mμ per mg protein during the second 30 sec time interval after addition of NADH.

[b]Organisms grown in lactic broth fortified with 0.2% citrate.

SUMMARY AND CONCLUSIONS

A method for rapid preparation of sterile solutions of volatile organic compounds was developed. For compounds similar to those included in this study this method is nearly as good as the use of sealed glass ampoules. The GLC method outlined provides rapid and accurate information on the concentration of these solutions.

Using a GLC method, it was possible to show that those lactic streptococci common to butter and cheese cultures produce only negligible amounts of acetone in milk medium. These organisms were found to have little or no ability to decarboxylate an exogenous source of acetoacetic acid. It was also found that these lactic organisms do not produce Me_2S when grown in milk culture, nor are they able to produce Me_2S from the Me_2S precursor identified in milk. The Me_2S precursor of milk was identified as a MMS salt on the basis of its TLC mobility and decomposition to Me_2S and homoserine on heating.

Several Pseudomonas species commonly implicated in food spoilage as well as two marine Pseudomonas species were found to be capable of reducing added aldehydes to the corresponding alcohols at both 8 and 21 °C. Some species were capable of reducing acetone and butanone under these conditions. Carbonyl reduction was carried out to an extent that would be significant in food spoilage.

The quantities of acetaldehyde and ethanol produced by the Lactobacillus species common to Cheddar cheese were determined after incubation at both their optimum growth temperatures and at 8° C. Significant differences were noted both between species and between strains within a species. Growth and production of these compounds were very slow at 8° C. All species studied were capable of reducing acetaldehyde and propionaldehyde to the corresponding alcohol. In addition, L. brevis strains reduced butanone to 2-butanol. A strain of L. brevis was found to produce n-propanol as a normal metabolite when grown in milk cultures.

Single-strain cultures of L. casei and L. plantarum accumulated diacetyl when grown in milk culture at both 8 and 30° C, but strains of L. brevis and L. lactis did not. Significant differences in the production of diacetyl were noted between strains of a species. Diacetyl reductase activity was demonstrated in cultures of L. casei, L. brevis, and L. lactis. Diacetyl reductase could be induced in L. plantarum by growth in the presence of citrate. Growth in a milk medium supplemented with citrate resulted in a stimulation of diacetyl reductase activity with L. casei.

The following conclusions were drawn from the results of this investigation:

1. Dilute solutions of volatile organic compounds could be sterilized with acceptable recoveries by autoclaving them in culture

tubes sealed with Teflon-lined screw caps.

2. There was little or no acetone produced by the lactic acid bacteria studied when grown in milk culture. These organisms cannot readily decarboxylate an exogenous source of acetoacetate.

3. The lactic acid bacteria studied did not produce Me_2S when grown in milk culture. These organisms were unable to convert MMS chloride to dimethyl sulfide.

4. A heat-labile Me_2S precursor was isolated from raw milk and identified as a MMS salt on the basis of its thin-layer chromatographic mobility and decomposition to homoserine and Me_2S.

5. Single-strain cultures of P. fragi, P. fluorescens, P. putrefaciens and two marine Pseudomonas species reduced acetaldehyde, propionaldehyde, and butyraldehyde to the corresponding alcohols at 21° C. All species reduced propionaldehyde at 6° C. P fragi and the marine Pseudomonads reduced methyl ketones. Under aerobic conditions P. fragi reduced added propionaldehyde to n-propanol quantitatively at 21° C.

6. The quantities of acetaldehyde and ethanol produced by single-strain cultures of L. brevis, L. casei, L. lactis, and L. plantarum differed significantly both between species and between strains within a species. Growth and production of these compounds were slow at 8° C.

7. All Lactobacillus species studied were capable of reducing acetaldehyde and propionaldehyde to the corresponding alcohol. L. brevis strains reduced added butanone to 2-butanol.
8. A strain of L. brevis produced n-propanol as a normal metabolite when grown in milk culture.
9. L. casei and L. plantarum accumulated diacetyl when grown in milk culture at both 8 and 30° C. L. lactis and L. brevis did not accumulate this compound.
10. L. casei, L. brevis, and L. lactis reduced diacetyl. Diacetyl reductase activity can be induced in L. plantarum by growth in the presence of citrate. Growth in the presence of citrate stimulated diacetyl reduction by L. casei.

BIBLIOGRAPHY

1. Alford, J. A. and L. E. Elliot. Lipolytic activity of microorganisms at low and intermediate temperatures. I. Action of Pseudomonas fluorescens on lard. Food Research 25:296-303. 1960.

2. Alford, J. A. and D. A. Pierce. Production of lipase by Pseudomonas fragi in a synthetic medium. Journal of Bacteriology 86:24-29. 1963.

3. Alford, J. A., D. A. Pierce and F. G. Suggs. Activity of microbial lipases on natural fats and synthetic triglycerides. Journal of Lipid Research 5:390-394. 1964.

4. Alfrod, J. A. et al. Lipolytic activity of microrganisms at low and intermediate temperatures. II. Fatty acids released as determined by gas chromatography. Journal of Food Science 26:234-238. 1961.

5. Amerine, N. A. Acetaldehyde and related compounds in foods. Journal of Food Science and Technology 1:87-98. 1964.

6. Badings, H. T. and Th. E. Galesloot. Studies on the flavour of different types of butter starters with reference to the defect "yoghurt flavour" in butter. In: Proceedings of the Sixteenth International Dairy Congress, København, 1962. Vol. B. Odense, Andelsbogtrykkeriet, 1962. p. 199-208.

7. Baribo, L. E. and E. M. Foster. The intracellular proteinases of certain organisms from cheese and their relationship to the proteinases in cheese. Journal of Dairy Science 35:149-160. 1952.

8. Bassette, R., R. E. Bawdon and T. J. Claydon. Production of volatile materials in milk by some species of bacteria. Journal of Dairy Science 50:167-171. 1967.

9. Bassette, R. and T. J. Claydon. Characterization of some bacteria by gas chromatographic analysis of head space vapors from milk cultures. (Abstract) Journal of Dairy Science 48: 775. 1965.

10. Bassette, R. and C. H. Whitnah. Removal and identification of organic compounds by chemical reaction, in chromatographic analysis. Analytical Chemistry 32:1098-1100. 1960.

11. Beilstein's handbuck der organischen chemie. Band 3. Ed. by B. Prager and P. Jacobson. Berlin, Julius Springer, 1931. p. 630.

12. Bergey's manual of determinative bacteriology, ed. by Robert S. Breed et al. 7th ed. Baltimore, Williams and Wilkins, 1957. 1094 p.

13. Bills, D. D. The role of ethanol and certain ethyl esters in the fruity flavor defect of cheddar cheese. Ph. D. thesis. Corvallis, Oregon State University. 1966. 123 numb. leaves.

14. Bills, D. D. and E. A. Day. Determination of the major free fatty acids of cheddar cheese. Journal of Dairy Science 47: 733-738. 1964.

15. _____ Dehydrogenase activity of lactic streptococci. Journal of Dairy Science 49:1473-1477. 1966.

16. Bills, D. D., R. E. Willits and E. A. Day. Determination of some volatile constituents in ten commercial cheeses. Journal of Dairy Science 49:681-684. 1966.

17. Bills, D. D. et al. Identification of compounds responsible for fruity flavor defect of experimental cheeses. Journal of Dairy Science 48:1168-1173. 1965.

18. Brandsaeter, E. and F. E. Nelson. Proteolysis by Lactobacillus casei. I. Proteinase activity. Journal of Bacteriology 72: 68-72. 1956.

19. _____ Proteolysis by Lactobacillus casei. II. Peptidase activity. Journal of Bacteriology 72:73-78. 1956.

20. Briggs, Mary. The classification of lactobacilli by means of physiological tests. Journal of General Microbiology 9:234-248. 1953.

21. Bullock, D. H. and O. R. Irvine. A chromatographic study of cheddar cheese ripening. Journal of Dairy Science 39:1229-1235. 1956.

22. Bywood, R. et al. The evolution of dimethyl sulfide from bracken, 'horse's tail' and other plants on treatment with sodium hydroxide. Biochemical Journal 48:xxx-xxxi. 1951.

23. Calbert, Harold E. and Walter V. Price. A study of the diacetyl in cheese. I. Diacetyl content and flavor of cheddar cheese. Journal of Dairy Science 32:515-520. 1949.

24. _____ A study of the diacetyl in cheese. II. The changes in diacetyl content of cheddar cheese during manufacturing and curing. Journal of Dairy Science 32:521-526. 1949.

25. Camp, B. J. and W. C. Van Der Zant. Proteolytic enzymes from Pseudomonas putrefaciens. II. Characterization of an endocellular proteolytic enzyme system. Food Research 22: 158-163. 1957.

26. Callenger, F. and B. J. Haywood. The occurrence of a methyl sulphonium derivative of methionine in asparagus. Chemistry and Industry (London), June 19, 1954, p. 729-730.

27. Challenger, F. and M. I. Simpson. Studies on biological methylation. Part XII. A precursor of the dimethyl 2-carboxyethylsulphonium hydroxide and its salts. Journal of the Chemical Society, 1948, p. 1591-1597.

28. Cheeseman, G. C. Identification of lactobacilli from cheddar cheese by chromatography. Journal of Dairy Research 27:33-39. 1960.

29. Chou, Tsung Chu. The chemical nature of the characteristic flavor of cultured buttermilk. Ph. D. thesis. Columbus, Ohio State University, 1962. 161 numb. leaves. (Microfilm)

30. Christensen, M. D. and C. S. Pederson. Factors affecting diacetyl production by lactic acid bacteria. Applied Microbiology 6:319-322. 1958.

31. Citti, J. E., W. E. Sandine and P. R. Elliker. Lactose and maltose uptake by Streptococcus lactis. Journal of Dairy Science 50:485-487. 1967.

32. Collins, E. B. Symposium on lactic starter cultures. I. Culture identity and selection. Journal of Dairy Science 45:1263-1266. 1962.

33. Collins, M. A. and B. W. Hammer. Types of lipolysis brought about by bacteria as shown by Nile-blue sulfate. Journal of Bacteriology 27:487-496. 1934.

34. Czulak, J. and P. D. Shimmin. Further notes on bitter flavour in cheese. Australian Journal of Dairy Technology 16:96-98. 1961.

35. Day, E. A., R. C. Lindsay and D. A. Forss. Dimethyl sulfide and the flavor of butter. Journal of Dairy Science 47:197-199. 1964.

36. DeMann, J. C. and Th. E. Galesloot. The effect of the addition of manganese to milk upon the growth of starter bacteria. Netherlands Milk and Dairy Journal 16:19-23. 1962.

37. Evans, A. C., E. G. Hastings and E. B. Hart. Bacteria concerned in the production of the characteristic flavor in cheese of the cheddar type. Journal of Agricultural Research 2:167-192. 1914.

38. Foster, Edwin M. et al. Dairy microbiology. Englewood Cliffs, Prentice-Hall. 1957. 492 p.

39. Franklin, J. G. and M. Elisabeth Sharpe. The incidence of bacteria in cheese milk and cheddar cheese and their association with flavour. Journal of Dairy Research 30:87-99. 1963.

40. Friedel, R. A., J. L. Schultz and A. G. Sharkey. Mass spectra of alcohols. Analytical Chemistry 28:926-934. 1956.

41. Friedman, T. E. The carbohydrate metabolism of streptococci. Journal of Biological Chemistry 130:757-761. 1939.

42. Fryer, T. F., B. Reiter and R. C. Lawrence. Lipolytic activity of lactic acid bacteria. Journal of Dairy Science 50:388-389. 1967.

43. Galesloot, Th. E. The bacteriology and biochemistry of starters and ripened cream. In: Proceedings of the Sixteenth International Dairy Congress, København, 1962. Vol. D. Odense, Andelsbogtrykkereit, 1962. p. 143-167.

44. Garrison, E. R. and B. W. Hammer. Fluorescent bacteria in dairy products. Iowa State College Journal of Science 16: 363-377. 1942.

45. Garvie, E. I. The genus Leuconostoc and its nomenclature. Journal of Dairy Research 27:283-292. 1960.

46. Gibson, T. and Y. Abd-El-Malek. The formation of carbon dioxide by lactic acid bacteria and Bacillus licheniformis and a cultural method of detecting the process. Journal of Dairy Research 14:35-44. 1945.

47. Goldman, M. L. and M. M. Rayman. Hydrolysis of fats by bacteria of the Pseudomonas genus. Food Research 17:326-337. 1952.

48. Gomori, G. Preparation of buffers for use in enzyme studies. In: Methods in enzymology, ed. by S. P. Colowick and N. O. Kaplan. New York, Academic, 1955. pp. 138-146.

49. Gornall, A. G., C. J. Bardawill and M. M. David. Determination of serum proteins by means of the Biuret reaction. Journal of Biological Chemistry 177:751-766. 1948.

50. Hagihara, B. Bacterial and mold proteases. In: The enzymes, ed. by P. D. Boyer, H. Lardy and K. Myrbäck. Vol. 4, 2nd ed. New York, Academic, 1960. p. 193-213.

51. Hammer, B. W. and F. J. Babel. Bacteriology of butter cultures. A review. Journal of Dairy Science 26:83-168. 1943.

52. Hammer, B. W. and F. F. Sherwood. The volatile acids produced by starters and by organisms isolated from them. Ames, Iowa. 1923. 15 p. (Iowa Agricultural Experiment Station. Research Bulletin no. 80)

53. Harmon, L. G. and F. E. Nelson. Interrelationships of microorganisms in cream. I. Streptococcus lactis, Pseudomonas fragi, and Geotrichum candidum. Journal of Dairy Science 38: 1189-1198. 1955.

54. Harper, W. J. The metabolism of some carbon-14 labeled substrates in certain cheeses and in cultures of lactic acid bacteria. Milchwissenschaft 20:354-357. 1965.

55. Hart, E. B. et al. Relation of the action of certain bacteria to the ripening of cheese of the cheddar type. Journal of Agricultural Research 2:193-216. 1914.

56. Harvey, R. J. Production of acetaldehyde and acetone by lactic streptococci. Journal of Dairy Research 27:41-45. 1960.

57. Harvey, R. J. and E. B. Collins. Citrate transport system of Streptococcus diacetilactis. Journal of Bacteriology 83: 1005-1009. 1962.

58. Hastings, E. G., Alice C. Evans and E. B. Hart. The bacteriology of cheddar cheese. Washington, D. C. 1912. 52 p. (U. S. Department of Agriculture. Bureau of Animal Industry. Bulletin 150)

59. Hawke, J. C. Reviews of the progress of dairy science. The formation and metabolism of methyl ketones and related compounds. Journal of Dairy Research 33:225-243. 1966.

60. Hussong, R. V., H. F. Long and B. W. Hammer. Classification of the organisms important in dairy products. II. Pseudomonas fragi. Ames, Iowa. 1937. 136 p. (Iowa Agricultural Experiment Station. Research Bulletin no. 225)

61. Jenness, Robert and Stuart Patton. Principles of dairy chemistry. New York, Wiley, 1959. 446 p.

62. Jennings, W. G., S. Viljhalmsson and W. L. Dunkley. Direct gas chromatography of milk vapors. Journal of Food Science 27:306-308. 1962.

63. Jensen, R. G. and J. E. Edmondson. The characterization of some lactobacilli found in milk. Journal of Dairy Science 40: 180-186. 1957.

64. Johns, C. K. and S. E. Cole. Lactobacilli in cheddar cheese. Journal of Dairy Research 26:157-161. 1959.

65. Kandler, O. Stoffwechsel der Säureweckerorganismen. Milchwissenschaft 16:523-581. 1961.

66. Keenan, T. W. and R. C. Lindsay. Rapid resolution of methyl methionine sulfonium salts and homoserine by thin-layer chromatography. Journal of Chromatography, In press.

67. Keenan, T. W., R. C. Lindsay and E. A. Day. Acetaldehyde utilization by Leuconostoc species. Applied Microbiology 14: 802-806. 1966.

68. Keenan, T. W. et al. Acetaldehyde production by single-strain lactic streptococci. Journal of Dairy Science 49:10-14. 1966.

69. King, N. Modification of the Voges-Proskauer test for rapid colorimetric determination of acetylmethylcarbinol plus diacetyl in butter cultures. Dairy Industries 13:860-861. 1948.

70. Kiribuchi, T. and T. Yamanishi. Studies on the flavor of green tea. Part IV. Dimethyl sulfide and its precursor. Agricultural and Biological Chemistry (Japan) 27:56-59. 1963.

71. Knusden, Søncke. Starters. Journal of Dairy Research 2:137-163. 1931.

72. Kristoffersen, T. and F. E. Nelson. Degradation of amino acids by Lactobacillus casei and some factors influencing deamination of serine. Applied Microbiology 3:268-273. 1955.

73. _____ Metabolism of amino acids by Lactobacillus casei. (Abstract) Journal of Dairy Science 37:635. 1954.

74. _____ The relationship of serine deamination and hydrogen sulfide production by Lactobacillus casei to cheddar cheese flavor. Journal of Dairy Science 38:1319-1325. 1955.

75. Lane, C. B. and B. W. Hammer. Bacteriology of cheese. II. Effect of Lactobacillus casei on the nitrogenous decomposition and flavor development in cheddar cheese made from pasteurized milk. Ames, Iowa. 1935. 30 p. (Iowa Agricultural Experiment Station. Research Bulletin no. 190)

76. Lightbody, G. Diacetyl production in cream by Streptococcus diacetilactis. Australian Journal of Dairy Technology 17:36-40. 1962.

77. Lindsay, Robert Clarence. Flavor chemistry of butter culture. Ph. D. thesis. Corvallis, Oregon State University, 1965. 228 numb. leaves.

78. Lindsay, R. C. and E. A. Day. Rapid quantitative method for determination of acetaldehyde in lactic starter cultures. Journal of Dairy Science 48:665-669. 1965.

79. Lindsay, R. C., E. A. Day and W. E. Sandine. Green flavor defect in lactic starter cultures. Journal of Dairy Science 48: 863-869. 1965.

80. _____ Identification of volatile flavor components of butter culture. Journal of Dairy Science 48:1566-1574. 1965.

81. Lindsay, R. C., E. A. Day and L. A. Sather. Preparation and evaluation of butter culture flavor concentrates. Journal of Dairy Science 50:25-31. 1967.

82. Long, H. F. and B. W. Hammer. Methods for the detection of lipolysis by microorganisms. Iowa State College Journal of Science 11:343-349. 1937.

83. McRorie, Robert A. et al. Isolation and identification of a naturally occurring analog of methionine. Journal of the American Chemical Society 76:115-118. 1954.

84. Mandel, M. Deoxyribonucleic acid base composition in the genus Pseudomonas. Journal of General Microbiology 43: 273-292. 1966.

85. Marth, E. H. Microbiological and chemical aspects of cheddar cheese ripening. A review. Journal of Dairy Science 46: 869-890. 1963.

86. _____ Symposium on lactic starter cultures. III. Certain aspects of starter culture metabolism. Journal of Dairy Science 45:1271-1281. 1962.

87. Morgan, M. E. The distribution of certain amino acids in the soluble nitrogen fraction of milk cultures of Streptococcus lactis. Iowa State College Journal of Science 24:87-89. 1949.

88. _____ Professor, University of Connecticut, Department of Animal Science. Personal communication. Corvallis, Oregon. 1965.

89. Morgan, M. E. and E. A. Day. Simple on-column trapping procedure for gas chromatographic analysis of flavor volatiles. Journal of Dairy Science 48:1382-1384. 1965.

90. Morgan, M. E. et al. Identity of additional aroma constituents in milk cultures of Streptococcus lactis var. Maltigenes. Journal of Dairy Science 49:15-18. 1966.

91. Nakae, T. and J. A. Elliott. Volatile fatty acids produced by some lactic acid bacteria. I. Factors influencing production of volatile fatty acids from casein hydrolysate. Journal of Dairy Science 48:287-292. 1965.

92. _____ Production of volatile fatty acids by some lactic acid bacteria. II. Selective formation of volatile fatty acids by degradation of amino acids. Journal of Dairy Science 48:293-299. 1965.

93. Naylor, Jill and M. Elizabeth Sharpe. Lactobacilli in cheddar cheese. I. The use of selective medium for isolation and of serological typing for identification. Journal of Dairy Research 25:92-103. 1958.

94. _____ Lactobacilli of cheddar cheese. II. Duplicate cheeses. Journal of Dairy Research 25:421-430. 1958.

95. _____ Lactobacilli in cheddar cheese. III. The source of lactobacilli in cheddar cheese. Journal of Dairy Research 25:431-438. 1958.

96. Niven, C. F., Jr., K. L. Smiley and J. M. Sherman. The hydrolysis of arginine by streptococci. Journal of Bacteriology 43:651-660. 1942.

97. Nordstrom, K. Metabolism of acetaldehyde--a metabolic branching point. Acta Chemica Scandinavica 20:474-478. 1966.

98. Pack, M. Y. et al. Use of the Owades and Jacksovac method for diacetyl determination in mixed strain starters. Journal of Dairy Science 47:981-986. 1964.

99. Palladina, O. K. Changes in lactic acid fermentation depending on conditions of the medium. I. Effect of nitrogen balance and vitamin content of the medium on the biochemistry of lactic acid streptococci. Microbiology (U. S. S. R.)8:733-745. 1939. (Abstracted in Chemical Abstracts 35:2554. 1941)

100. Parker, R. B. and P. R. Elliker. Effect of spoilage bacteria on flavor components of cottage cheese. Journal of Dairy Science 35:482-483. 1952.

101. Patton, Stuart. Volatile acids and the aroma of cheddar cheese. Journal of Dairy Science 46:856-858. 1963.

102. Patton, Stuart, D. A. Forss and E. A. Day. Methyl sulfide and the flavor of milk. Journal of Dairy Science 39:1469-1470. 1956.

103. Payne, W. J. Pure culture studies of the degradation of detergent compounds. Biotechnology and Bioengineering 5:355-365. 1963.

104. Pereira, J. N. and M. E. Morgan. Identity of esters produced in milk cultures of Pseudomonas fragi. Journal of Dairy Science 41:1201-1205. 1958.

105. Perry, K. D. and M. Elizabeth Sharpe. Lactobacilli in raw milk and in cheddar cheese. Journal of Dairy Research 27: 267-275. 1960.

106. Platt, T. B. and E. M. Foster. Products of glucose metabolism by homofermentative streptococci under anaerobic conditions. Journal of Bacteriology 75:453-459. 1958.

107. Reddy, M. C. et al. Ester production by Pseudomonas fragi. I. Identification and quantification of some of the esters produced in milk cultures. Submitted to Journal of Dairy Science. 1967.

108. _____. Relationship of methyl sulfide and flavor score of milk. Journal of Dairy Science 50:147-150. 1967.

109. Reiter, B. and A. Møller-Madsen. Reviews on the progress of dairy science. Section B. Cheese and butter starters. Journal of Dairy Research 30:419-456. 1963.

110. Reiter, B. and J. D. Oram. A note on the carbon dioxide requirement of Streptococcus lactis, strain ML 3. Journal of Dairy Research 28:175-176. 1961.

111. Riel, R. R. and C. A. Gibson. The use of starter distillate for flavoring butter. Food Technology 15:137-140. 1961.

112. Rogosa, M. and M. Elizabeth Sharpe. An approach to the classification of the lactobacilli. Journal of Applied Bacteriology 22:329-340. 1959.

113. Sandine, W. E., P. R. Elliker and A. W. Anderson. Taxonomic study of high carbon dioxide-producing lactic acid streptococci isolated from mixed-strain starter cultures. Journal of Dairy Science 42:799-808. 1959.

114. Scanlan, R. A., R. G. Arnold and R. C. Lindsay. Collecting and transferring packed column gas chromatography volatiles to capillary columns for fast-scan mass spectral analysis. Submitted to Analytical Chemistry. 1967.

115. Seitz, Eugene Walter. Studies on diacetyl production by Streptococcus diacetilactis Matuszewski et al. Ph. D. thesis. Corvallis, Oregon State University, 1962. 113 numb. leaves.

116. Seitz, E. W. et al. Distribution of diacetyl reductase among bacteria. Journal of Dairy Science 46:186-189. 1963.

117. Shahani, K. M. Galactokinase and galactowaldenase enzyme activity of Streptococcus lactis. (Abstract) Journal of Dairy Science 43:852. 1960.

118. Shahani, K. M. and W. J. Harper. Differences in the acid production and phosphorus metabolism of oxytetracycline sensitive and resistant organisms. Applied Microbiology 6:9-14. 1958.

119. Shahani, K. M. and J. R. Vakil. Certain enzymes of glycolytic and hexosemonophosphate shunt pathways of Streptococcus lactis. (Abstract) Journal of Dairy Science 45:655. 1962.

120. Sharpe, M. Elizabeth. Enumeration and studies of lactobacilli in food products. Dairy Science Abstracts 24:165-171. 1962.

121. _____ Taxonomy of the lactobacilli. Dairy Science Abstracts 24:109-118. 1962.

122. Sharpe, M. Elizabeth and J. G. Franklin. Production of hydrogen sulfide by lactobacilli with special reference to strains from cheddar cheese. (Abstract) In: Abstracts of the VIII International Congress for Microbiology. Montreal, 1962. p. 46.

123. Smith, R. E. and J. D. Cunningham. The characterization of lactobacilli from cheddar cheese. I. An evaluation of physiological and biochemical tests. Canadian Journal of Microbiology 8:727-735. 1962.

124. Speckman, R. A. and E. B. Collins. Biosynthesis of diacetyl by Leuconostoc citrovorum. (Abstract) Journal of Dairy Science 49:701. 1966.

125. Speckman, R. A. and E. B. Collins. Biosynthesis of diacetyl by Streptococcus diacetilactis. (Abstract) American Society of Microbiology, Bacteriological Proceedings, 1966, no. A30.

126. Stanier, R. Y., N. J. Palleroni and M. Doudoroff. The aerobic pseudomonads: A taxonomic study. Journal of General Microbiology 43:159-271. 1966.

127. Van Der Zant, W. C. Proteolytic enzymes from Pseudomonas putrefaciens. I. Characterization of an extracellular proteolytic enzyme system. Food Research 22:151-157. 1957.

128. Van Niel, C. B., A. J. Kluyuer and H. G. Drex. Uber des Butteraroma. Biochemische Zeitschrift 210:234-251. 1929.

129. Van Slyke, Lucius L. and Alfred W. Bosworth. Chemical changes in the souring of milk. Journal of Biological Chemistry 24:191-202. 1916.

130. Vedamuthu, E. R., W. E. Sandine and P. R. Elliker. Flavor and texture in cheddar cheese. II. Carbonyl compounds produced by mixed-strain lactic starter cultures. Journal of Dairy Science 49:151-157. 1966.

131. Wales, C. S. and L. G. Harmon. Changes in the biacetyl content of creamed cottage cheese caused by organisms associated with spoilage. Food Research 22:170-175. 1957.

132. Walradt, John Pierce. Quantitative gas chromatographic analysis of synthetic dairy culture flavors. Master's thesis. Corvallis, Oregon State University, 1967. 48 numb. leaves.

133. Whitehead, H. R., P. A. Jones and Robertson, P. S. The influence of carbon dioxide on the growth of lactic streptococci. Journal of Dairy Research 25:24-31. 1958.

134. Wick, Emily L. Flavor update. One opinion. Food Technology 20:1549-1554. 1966.

135. Williams, J. P., W. R. Mayberry and W. J. Payne. Metabolism of linear alcohols with various chain lengths by a Pseudomonas species. Applied Microbiology 14:156-160. 1966.

136. Wolf, J. A note on the lipase of some lactic acid organisms. (Abstract) Proceedings of the Society of Agricultural Bacteriologists 1941. p. 21.

137. Wong, Francis F. and J. F. Carson. Isolation of S-methyl methionine sulfonium salt from fresh tomatoes. Journal of Agricultural and Food Chemistry 14:247-249. 1966.

138. Wong, N. P. and Stuart Patton. Identification of some volatile compounds related to the flavor of milk and cream. Journal of Dairy Science 45:724-728. 1962.

139. Wood, W. A. Fermentation of carbohydrates and related compounds. In: The bacteria, ed. by I. G. Gunsalus and R. Y. Stanier. Vol. 2, New York, Academic, 1961. p. 59-149.