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RELATIONS BETWEEN ACIDOGENESIS AND THE UTILIZATION OF LACTATE, SULFATE AND NITRATE DURING ANAEROBIC DIGESTION

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ABSTRACT

Acidogenesis results in accumulation of various substrates, which can be utilized by denitrifying and sulfate reducing bacteria (SRB) if suitable terminal electron acceptors are present. This phenomenon is brought about by a diverse group of predominantly strict anaerobic rather than facultative bacteria, which includes classic fermentative, cellulolytic and homoacetogenic bacteria. In some cases a transitory accumulation of lactic acid has been observed. Among lactate - utilizing bacteria, denitrifiers and especially SRB can play an important role in anaerobic digestion. But it is probable that lactic acid is not important in digesters where H_2 - using methanogens rapidly use the H_2.

Inhibition of methanogens by SRB seems to be due to the interaction of these two groups rather than as a result of sulfate toxicity. One group of SRB partially oxidizes lactic acid into acetic acid while the second group completely oxidizes the lactic/acetic acid. In these two cases, mutualistic relationships between SRB and methanogens can exist on the basis of H_2 interspecies transfer as in cocultures of SRB with syntrophic bacteria.

Nitrate is known to be a negative effector of methanogenesis. This inhibitory effect seems to be due to changes in the Eh rather than substrate competition. The dissimilatory reduction of nitrate to ammonia appears to be the main pathway of nitrate reduction in digesters.

KEYWORDS

Anaerobic digestion, acidogenesis, lactate, fermentative bacteria, sulfate - reducing bacteria, denitrification.

INTRODUCTION

A wide range of biochemical transformations take place during anaerobic digestion, as a result of different trophic groups of microorganisms degrading a wide variety of substrates. Four groups are recognizable (Fig. 1) and include : 1 - hydrolytic bacteria that catabolize biological polymers ; 2 - hydrogen producing acetogenic bacteria that catabolize certain fatty acids and neutral end products ; 3 - homoacetogenic bacteria that catabolize unicarbon compounds or hydrolyse multicarbon compounds to acetic acid ; 4 - methanogenic bacteria that catabolize acetate and one carbon compounds to methane (Zeikus, 1979). The coordinated activity of these groups ensures process stability during anaerobic fermentation.

The former group reduces the chemical potential of the medium and thus provides a redox - potential (Eh) suitable for volatile fatty acids (VFA) formation and methane production. The thermodynamic sequence or ecological succession in methanogenic systems has been proposed by Pohland (1969) as follows : aerobic respiration, nitrate reduction and denitrification, fermentation, sulfate reduction and methane production (Fig. 2).

In the presence of nitrogen oxides and sulfate, acidogenesis can be directly related to the metabolism of denitrifying and sulfate - reducing bacteria (SRB) capable of utilizing lactic acid as energy and carbon sources for their growth.

ACIDOGENESIS

Acidogenesis is the phenomenon by which carbohydrates, and long-chain fatty acids are transformed into organic acids and VFA of low molecular weight (C_1 - C_5 acids). The anaerobic degradation of sugars is relatively well understood in terms of biochemistry and thermodynamics (Thauer and coworkers, 1977). The EMP and HMP are the most important pathways for glucose degradation in anaerobic bacteria (Fig. 3). Pyruvate takes the control position from where alcoholic, lactic acid or VFA fermentations start. The

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energy distribution between the fermentative bacteria and the special group of microorganisms capable of degrading VFA to methane precursors - called syntrophic or obligatory hydrogen producing acetogenic bacteria (O H P A B) (Fig. 1), largely depends upon the nature of the end product of the acidogenic phase (Verstraete and others, 1981).

Propionic acid is the last to disappear from the liquor during anaerobic fermentation and it seems to be a poor substrate for the O H P A bacteria. So acidogenesis results in accumulation of 1) substrates favourable to the growth of denitrifying and SR bacteria, 2) intermediary metabolites (lactate, pyruvate, succinate) and 3) end products (acetate), if suitable terminal electron acceptors are present (fig. 1).

The overproduction of VFA lowers the pH and is a frequent source of inhibition. Most of the bacteria have a fairly narrow pH range i.e. 6.4 - 7.5 within which their metabolic functions are optimal. It is thought by some workers that the VFA are directly toxic to the methanogenic bacteria but not to the acidogenic bacteria. At a pH of 6.2, the acidic conditions exhibit acute toxicity to the methanogenic bacteria. It is interesting to note that this pH does not stop the acid production. The fermentative bacteria will continue to produce acids until the pH drops to 4.5 or 5.0. Control should be exercised when the pH dropes below a value of 6.6. This can be done by the addition of alkali. Acetate, of all the VFA produced in digesters, appears to be less toxic. Propionic acid has been shown particularly to be toxic to methanogenic bacteria. It appears, in some way, to control the rate of its own, as well as, acetate metabolism without drastically altering methane production, at concentrations below toxic level (Stafford and co-workers, 1980).

The fermentative stage is carried out by a widely diverse group of strict anaerobic and facultative bacteria. The numbers, types and species of this group will depend on the qualitative and quantitative composition of the waste fed to the digesters (Hobson and Shaw, 1974). Using strictly anaerobic techniques, it has now been demonstrated that obligate anaerobic bacteria are in fact present in far greater numbers than are facultative anaerobes, and the major hydrolytic activities in digestion appear to depend on these obligate anaerobes. Using digester liquor in all their media to stimulate growth, Toerien and Siebert (1967) found that aerobes and facultative anaerobes formed a minor part of the acid-producing population , while fastidious obligate anaerobes formed the major part of the population, usually in numbers one to ten hundred times greater (Mah and Susman, 1967 ; Toerien and Hattingh, 1969). We have also found comparable results during our studies on microbial ecology of anaerobic digestion (Garcia and others, 1982).

Little good information is available concerning the more numerous fermentative bacteria active in digesters. Among facultative anaerobes, streptococci and members of *Enterobacteriaceae* are numerous in digesting piggery waste and in waste-water sludge respectively (Hobson and Shaw, 1974). We found *Bacillus* spp. dominant in digesters with tannery by-products. Various genera of anaerobic bacteria including *Peptostreptococcus, Propionibacterium, Bacteroides, Micrococcus* and *Clostridium* can generally be isolated from most digesting systems. Despite their critical role, cellulolytic bacteria constitute a small fraction of the total acidogenic population in anaerobic digesters (Scharer and Moo-Young, 1979). Techniques for isolation and characterization of anaerobic cellulase-producing bacteria have been developed by Hungate (1950). The most active cellulolytic species have been shown to be gram negative, short rods (*Bacteroides sp.*) and cocci (*Ruminococcus sp.*). In mixed cultures of sewage sludge, the maximum rate of cellulose hydrolysis is reported to be at pH 7.5, while rumen microflora usually have maxima at more acidic conditions.

The oxidation of hydrogen and reduction of carbon dioxide to acetic acid can be brought about by *Clostridium aceticum* (Braun and others, 1981), *C. formicoaceticum* (Andreesen and co-workers, 1970), *C. thermoautotrophicum* (Wiegel and colleagues, 1981), *Acetobacterium woodii* (Balch and others, 1977) and *Acetogenium kivui* (Leigh and co-workers, 1981) according to the following equation :

$$2\ CO_2 + 4\ H_2 \rightarrow CH_3COOH + 2\ H_2O$$

The exact role of these hydrogen-consuming acetogenic bacteria (i.e. homoacetogenic bacteria) in anaerobic digestion is not yet clear, although populations of $10^5 - 10^6$ par ml have been reported in sewage sludge (Braun and others, 1979). They can play a significant role either as competitors or as syntrophic donors of acetate, hydrogen, and carbon dioxide to methanogens (fig. 1). Recently a sporeforming species has been isolated from sewage digesters that metabolizes $H_2/CO_2/$ methanol to acetate, or methanol and acetate to butyrate (i.e. a homobutyric acid fermentation) (Zeikus and colleagues, 1980).

FB : Fermentative Bacteria
DB : Denitrifying Bacteria
SRB : Sulfate-Reducing Bacteria
HAB : Homoacetogenic Bacteria
SB : Syntrophic Bacteria
AMB : Acetophilic Methanogenic Bacteria
MB : Methanogenic Bacteria

Fig. 1. Scheme of pathways implicated in anaerobic digestion.

LACTATE UTILIZATION

During digestion of agro-alimentary effluents rich in sugars, it seems that the acidogenic stage develops through lactate intermediary. Although this compound is not detected during normal course of fermentation, some experiments permitted to show the transitory accumulation of lactate and its further degradation (fig. 4). These experiments also showed the effect of pH and partial pressure of H_2 on accumulated VFA. At pH below 5.0, soluble sugars are partially degraded to lactate while at pH 5.7 or under, the breakdown of sugars and lactic acid into VFA was observed. These VFA were formed from breakdown of lactate and not directly from carbohydrate (Morfaux and co-workers, 1981).

In a biphasic digestion with separation of acidogenic and methanogenic phases, it would be more interesting to stop the first phase just after the hydrolysis of the substrate to be methanised. This accumulates lactic acid in the case of sugary effluent. Very few gases are produced during the first phase and the whole methane can be recuperated in the second phase where acetogenesis and methanogenesis operate under optimum conditions (Morfaux and others, 1981). For the separation and optimization of the phase of lactic acid production, while using effluents rich in soluble sugars, one has to select 1) a low pH to inhibit acetogenic and methanogenic bacteria, and 2) an inoculum able to realise hydrolysis and homolactic fermentation of polysaccharides. Separation and recycling of the bacteria from the first stage may accelerate the hydrolysis of substrate to be digested. The use of filtration systems on slud-

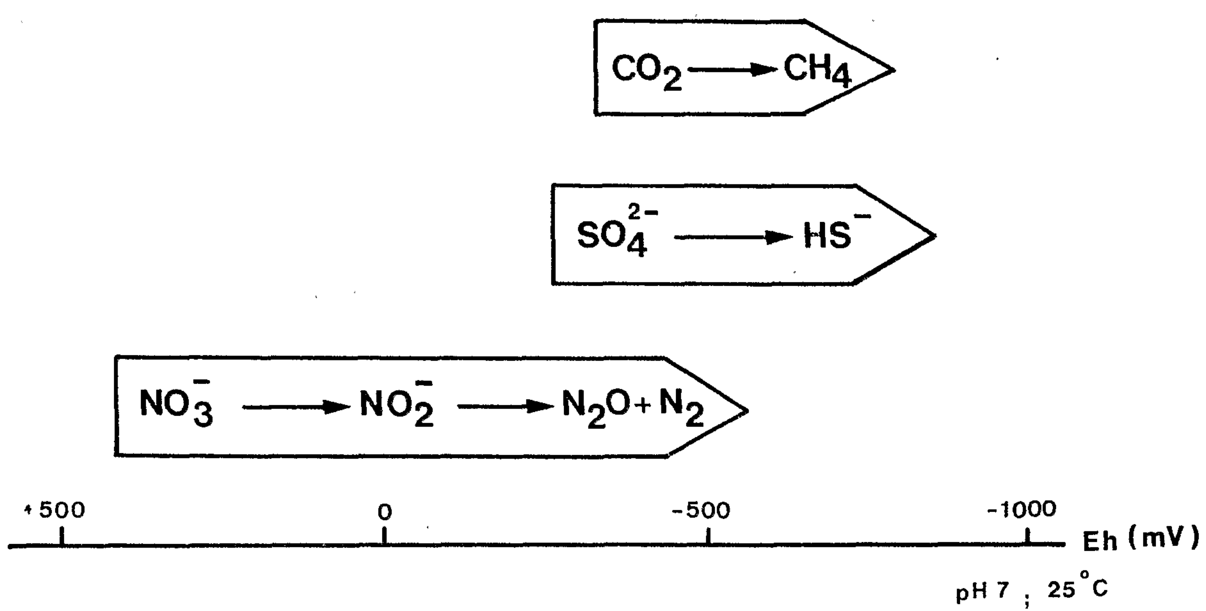


Fig. 2. Sequence of biologically mediated reductions of inorganic compounds as terminal electron acceptors under anaerobic conditions [From Zehnder, 1978].

ge layer preconised by Lettinga and colleagues (1980) seems to be promising to realise the acidogenic step by entrapping the active anaerobic bacterial mass.

According to Morfaux and co-workers (1981), lactic acid-forming bacteria are the dominant species in acidogenic fermentors. Lactate - utilizers are also numerous in digesters (Bryant and others, 1977 ; Ueki and colleagues, 1980 a). According to these authors, the most numerous are SRB, which in media low with sulfate, can play an active role in lactate catabolism in presence of H_2-utilizers - as methanogens. Denitrifying bacteria can also use lactate as electron donor and carbon source to dissimilate nitrate. Bacteria using lactate as carbon and energy sources have been isolated by Touzel and co-workers (1981). They were species from genus *Clostridium*, *Butyribacterium*, *Propionibacterium* and *Megasphaera*. They represented about 10 % of the total microflora of the digester studied. The various fermentative pathways of these strains are summarized in fig. 5. An inhibitory effect of lactate to some bacteria was observed by Ueki and others (1980 a). So the lactate - utilizing bacteria can play an important part in anaerobic digestion by their number and fermentation products.

In mixed cultures, some of the products of cellulolytic bacteria, particularly lactic acid, succinic acid, and ethanol, are rapidly metabolized by non-cellulolytic species (Scharer and Moo-Young, 1979) ; lactic acid is converted primarily to proprionic acid by virtually all the species of *Propionibacterium*. But it is probable that these organic acids are not important in digesters where H_2-using methanogenic bacteria rapidly use the H_2 ; and this allows the fermentative bacteria to produce more H_2 and acetate and less of the other end products such as lactate and ethanol (Wolin, 1976).

SULFATE UTILIZATION

The inhibition of methanogenesis in the presence of sulfate in some natural ecosystems has been shown to occur as a result of 1) an increase in Eh (Mac Gregor and Keeney, 1973) ; 2) competition between methanogens and sulfate reducers for H_2 and acetate, which are methane precursors (Abram and Nedwell, 1978 ; Winfrey and Zeikus, 1977) ; 3) lack of H_2 production (Bryant and colleagues, 1977), and 4) toxicity to methanogens of H_2S which is produced on higher quantities from sulfate by sulfate reducers (Cappenberg, 1974b). Increasing concentrations of sulfate have been shown to inhibit methanogenesis in anaerobic digesters (Mac Gregor and Keeney, 1973 ; Winfrey and Zeikus, 1977). But in this ecosystem where two-thirds of the methane is produced from acetic acid, sulfate reduction may be extensive without apparent effect on performance (Middleton and Lawrence, 1977) and addition of sulfate is sometimes beneficial (Van den Berg and Lentz, 1977). Sulfate inhibition of methanogenesis was reversed by the addition of excess hydrogen or acetate, suggesting that both of these substrates may be competitively used by methanogenic and SR bacteria (Winfrey and Zeikus, 1977).

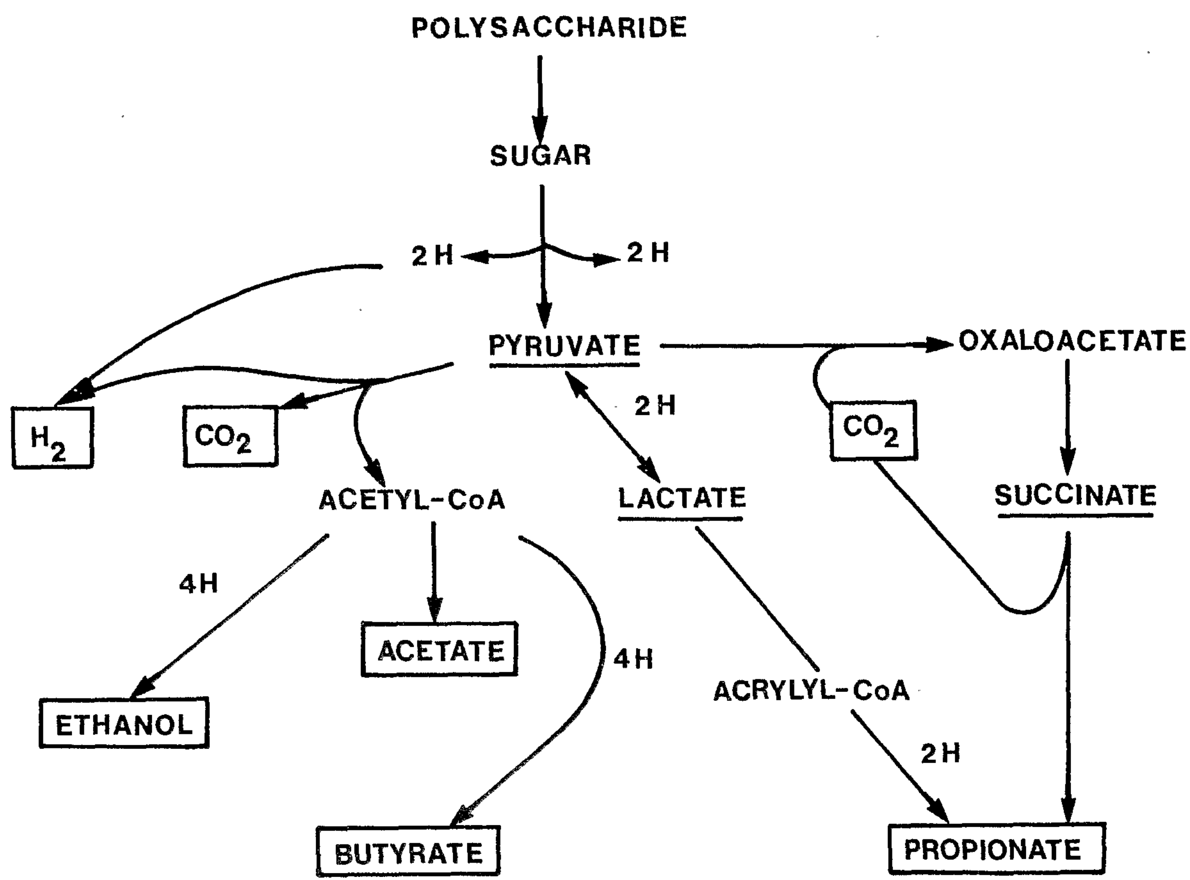


Fig. 3. Pathways of anaerobic degradation of carbohydrates [From Bryant, 1979]

Sulfate and iron interact strongly in stimulating acetic acid conversion to methane (Van der Berg and co-workers, 1980). Sulfate alone is not essential for this stimulation, the maximum of which is obtained at about 0.6 mM in the presence of iron.

In the anaerobic digestion of waste, the presence of sulfides has been shown to precipitate toxic heavy metals such as chromium, cobalt, copper, iron, nickel and zinc (Kugelman and Chin, 1971 ; Lawrence and Mc Carty, 1965).

The complete inhibition of methanogens observed in mixed cultures of sulfate-reducing and methane-producing bacteria appears to be due to their interaction rather than as a result of toxicity of sulfate to methanogens (Patel and Roth, 1978). The results of Patel and co-workers (1978) indicated that although the amount of sulfate used in many synthetic media for isolating and growing methanogens is not completely inhibitory, it is much higher than that necessary for the cultivation of pure cultures of these organisms. For these authors, the involvement of sulfate in the synthesis of coenzyme M which contains a SO_3^{2-} group and is responsible for methyl group transfer reactions in the formation of methane, or in the electron transfer system of methanogens cannot be ruled out. Work on *Methanobacterium ruminantium* and *M. bryantii* has shown that sulfate does not serve as sulfur source for these methanogens (Bryant and colleagues, 1977 b). In recent reports, there are however indications that methane formation is highly dependent on the availability of sulfide, much more than previously anticipated (Khan and Trottier, 1978 ; Mountfort and Asher, 1979 ; Rönnow and Gunnarsson, 1981 ; Wellinger and Wuhrmann, 1977).

However the trophic importance and metabolic activity of sulfate reducing bacteria in active methane digesters (i.e. at low sulfate concentrations) remains to be established. As seen before, lactate is a natural substrate for the SRB. With a limited number of other fermentation products as ethanol, it is used as substrate by the first group of SRB (SRB_1), i.e. species of genus *Desulfovibrio* and *Desulfotomaculum* that accomplish only a partial oxidation of the substrates due to lack of a complete tricarboxylic acid cycle in these bacteria. In mixed continuous culture experiments, acetate is produced during the lactate oxidation and a mutualistic relationship between the SRB_1 and methanogens was postulated on the basis of acetate transfer between the two types of bacteria in media low in SO_4^{2-} (Bryant and co-wokers, 1977 b ; Cappenberg, 1975).

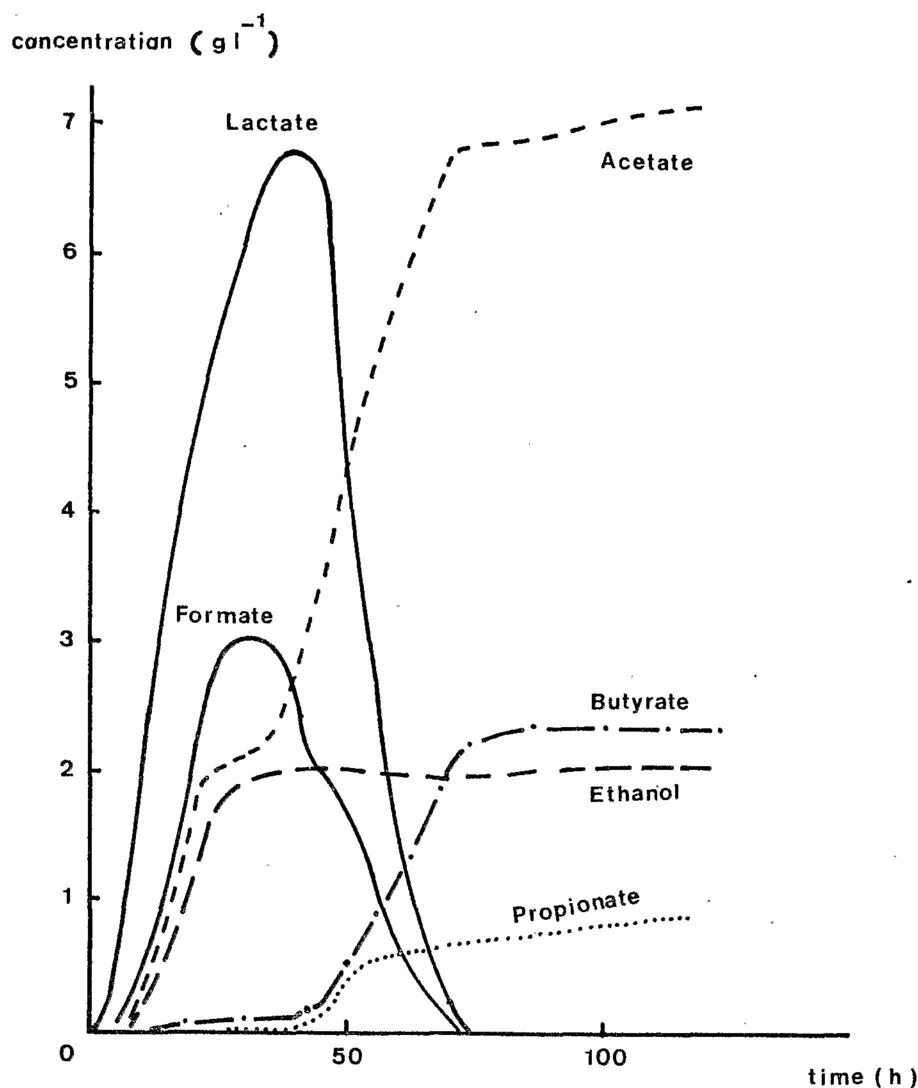


Fig. 4. Intermediary products of anaerobic sucrose fermentation [From Touzel and co-workers, 1981].

Thauer and colleagues (1977) showed that it was thermodynamically possible for SRB to use hydrogen as an electron source and that this reaction yielded more energy than the reduction of carbon dioxide by H_2. During anaerobic digestion, H_2 is produced in the presence of minimal amounts of sulfate. In the presence of adequate amounts of SO_4^{2-}, the preferred route of electron disposal is via sulfate reduction, and hence no H_2 evolution. If the concentration of sulfate is limiting, a coculture of *Desulfovibrio* with a methanogenic bacterium produces CH_4 but not H_2. SO_4^{2-}, above its limiting concentrations, serves as a terminal electron acceptor even in the presence of methanogenic bacteria, and neither CH_4 nor H_2 are produced (Mc Inerney and Bryant, 1981).

It has also been shown that under special conditions (high concentrations of lactate and sulfate), SRB can evolve molecular hydrogen (Hatchikian and co-workers, 1976 ; Traoré, 1981). This H_2 - production is generally considered as an abnormal reaction which occurs when bacteria are devoid of sulfate (Bryant and others, 1977 a ; Postgate, 1952) or when the electron transport pathway is defective (Vosjan, 1975). On this basis, SRB and methanogens were mutualistically linked i.e. SRB give H_2, CO_2 and acetate which in turn are used by methanogens to produce CH_4 in an interspecies H_2 transfer (Bryant and others, 1977 a ; Traoré, 1981).

Laube and Martin (1981) firstly demonstrated a coculture of three bacteria (cellulolytic, sulfate-

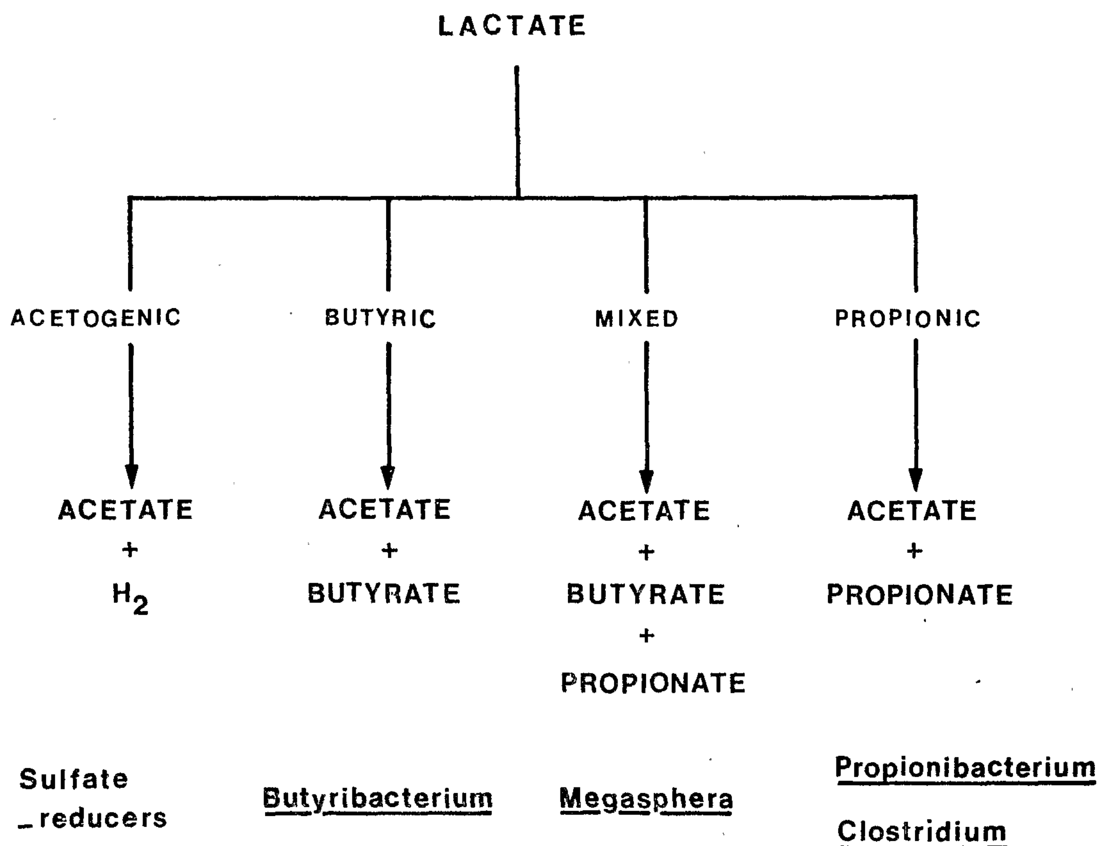
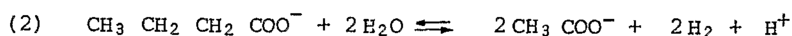


Fig. 5. Fermentative pathways of lactate-utilizing bacteria [From Touzel and co-workers, 1981].

reducing and acetophilic methanogenic bacteria) capable of degrading cellulose to methane and carbon dioxide in less than two weeks. The SRB could have produced either a stimulatory product such as sulfide or removed an inhibitor., e.g., ethanol. High rate of acetate utilization in the triculture could also be a result of H_2 production by *Desulfovibrio sp.* Although H_2 is not required for acetate metabolism, but low levels produced by SRB during ethanol oxidation may have resulted in the more efficient utilization of acetate.

Recently two obligate anaerobic proton-reducing, β - oxidizing saturated monocarboxylic C_4 - C_8 fatty acids with acetate and H_2 as end products (equations 1 and 2) have been isolated and named *Syntrophobacter wolinii* (Boone and Bryant, 1980) and *Syntrophomonas wolfei* (Mc Inerney and co-workers, 1981) with a *Desulfovibrio sp.* as H_2 - utilizing bacterium (equation 3) :

(1) $CH_3\,CH_2\,COO^- + 3\,H_2O \rightleftharpoons CH_3\,COO^- + H\,CO_3^- + H^+ + 3\,H_2$



(2) $CH_3\,CH_2\,CH_2\,COO^- + 2\,H_2O \rightleftharpoons 2\,CH_3\,COO^- + 2\,H_2 + H^+$

(3) $4\,H_2 + SO_4^{2-} + H^+ \rightleftharpoons HS^- + 4\,H_2O$

Growth and degradation of fatty acids occur only in syntrophic association with H_2 - using bacteria as sulfate-reducers or methanogens.

Other sulfate-reducing bacteria (SRB_2) have now been isolated which oxidize acetate and other important fermentation products like C_3 to C_{18} fatty acids, keto acids, alcohols, and aromatic compounds (Pfenning and Widdel, 1981 ; Widdel, 1980 ; Widdel and Pfennig, 1981). Thus, a complete anaerobic oxidation of organic matter by the SRB should be possible. Thus a relationship, competitive, rather than stimulatory, may exist between the SRB and methanogenic bacteria. As for SRB_1, energy conservation must be connected with electron-transport for these new types of SRB e.g. *Desulfobacter, Desulfococcus, Desulfobulbus, Desulfosarcina* and *Desulfonema* (Pfenning and others, 1981). Morphologically different, these new genera can however be considered to be linked as one physiological-ecological group.

The significance of sulfate-reducing bacteria during anaerobic digestion is not yet fully understood. Populations of 10^7 have been seen in our enumerations of effluents from various digesters.Counts of SRB was improved by the supplement with the supernatant of digester fluid (Ueki and co-workers, 1980 b, 1981). Both *Desulfovibrio* and *Desulfotomaculum* species are routinely isolated from digesters. We have also found original facultative SRB capable of fermenting glucose and showing an aerobic growth. SRB of secondtype are researched in such biotop.

NITRATE UTILIZATION

Nitrate is known as negative effector of methanogenesis but its role in anaerobic digester performance and species composition is not well characterized. Denitrifying bacteria are thought to convert substrate energy to ATP by electron transport phosphorylation coupled to reduction of O_2 or nitrogenous oxides. But very little is known about the extent and ecological characteristics of the denitrifying capacity in strictly anaerobic environments.

Studies on the influence of various nitrogen oxides on methanogenesis have been developed in soils and have led to the conclusion that the observed inhibitory effect was due to changes in the oxidation potential and/or substrate competition (Bell, 1969 ; Bollag and Czlonkowski, 1973 ; Laskowski and Moraghan, 1967). Methane was produced with little or no lag under anaerobic conditions by lake sediments (Knowles, 1979). The addition of NO_3^- imposed a 6 - to 10 - days lag, as has been observed by various authors (Balderston and Payne, 1976 ; Chen and others, 1972).

It is possible that denitrifiers are more successful competitors that divert carbon and energy sources from eventual use by methanogenic bacteria when an alternate electron acceptor (nitrate) is supplied. However, the results reported by Balderston and Payne (1976) indicate that substrate competition may not be influential, for H_2 and CO_2 needed for methanogenesis were continually present in excess. It might be argued that CO_2 and H_2 were limiting due to competition by other organisms. In digesters, nitrate has been shown to inhibit methane generation at concentrations exceeding 0.15 g l^{-1} N - NO_3^- (Scharer and Moo - Young, 1979). Inhibition of methane production by nitrate in cell - free extracts of *Methanobacillus omelianskii* was reported, and suggested to be due to the inhibition of enzyme activity (Wolfe and co-workers, 1966). The growth of this syntrophic culture has been reported to be almost completely inhibited at NO_3^- concentration between 0.0111 to 0.0139 % (Barker, 1941).

The most striking result of the recent study of Kaspar and co-workers (1981) was the presence of denitrification capacity in digested sludge, an habitat devoid of nitrate and O_2. This suggests that there may be undiscovered fermenters and (or) fumarate respirers capable of denitrification. This property is not harbored by butyrate and propionate oxidizers or methanogens. There is, therefore, indirect evidence for a reduction of nitrate to nitrogen gases by *Propionibacterium acidi-propionici* (Payne and Balderston, 1978). Recent work by Yoshinari (1980) showed that *Vibrio succinogenes*, a rumen inhabitant, was able to reduce nitrous oxide to N_2 and that acetylene inhibited this reaction.

Compared to denitrification, little research has been done on dissimilatory reduction of nitrate to ammonia. It appears to be negligible in a soil but it was virtually the only mechanism of nitrate reduction in rumen contents (Kaspar and Tiedje, 1981). These authors showed that the potential of digested sludge to reduce nitrate to ammonia was about twice as high as its potential for denitrification. They also concluded that nitrate reduction to ammonia was of a dissimilatory nature. Pasteurization did not elliminate the nitrate-reducing capacity, indicating that organisms involved in this mechanism were heat resistant.

Most of the methods for enumerating denitrifying bacteria are based on the most probable number (MPN) technique but some false positive tubes can occur due to nitrate and nitrite removal by ammonia producing organisms ; false negative tubes also occur due to failure of totally removing the nitrate and nitrite. To avoid these problems, a modified method bas been recommended by Tiedje (1981) using a medium of 5.0 mM nitrate in nutrient broth to reduce the number of false negatives ; to avoid false positives, acetylene is added in tubes to accumulate N_2O at concentrations over 20 %. The use of the acetylene inhibition technique could lead to errors if a significant portion of the N_2O produced was a byproduct of dissimilatory nitrate reduction to ammonia.

Using this technique, we have detected 10^3 to 10^6 nitrate dissimilating bacteria per ml of various digester juices producing nitrous oxide. But only one true denitrifier, *Paracoccus denitrificans*, a common aerobic denitrifying bacteria was isolated. Several strains able to dissimilate nitrate to ammonia were shown to belong to *Bacillus*, *Pseudomonas* and *Enterobacter*.

CONCLUSION

Bacteriological studies of anaerobic digesters have still to be carried out. There have not been sufficient observations to determine normal variations in the bacterial population during steady state of digestion, changes which may occur in the bacterial spectrum before or during digester failure, changes during or after shock loading. Pure and mixed continuous cultures of dominant digester bacteria would also help in the understanding of digester function, but such experiments cannot meaningfully be carried out until the most important species of digester such as methanogenic, hydrolytic, fermentative ,syntrophic, sulfate-reducing and nitrate dissimilating bacteria have been identified.

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