PRELIMINARY ELUCIDATION OF THE METHANOGENIC FERMENTATIONS OF VERATRIC AND SYRINGIC ACIDS BY INTERACTING MICROBIAL ASSOCIATIONS ISOLATED FROM ANOXIC FRESHWATER SEDIMENT

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To Mohammed, Ali, Maha, Nada and my wife Inaam

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ABSTRACT

Lignin is the second most abundant natural polymer after cellulose and since it is semi-recalcitrant in the environment, tends to accumulate. After pretreatment, however, labile molecules may be released from which, for example, methane, value-added products and chemical feedstocks may be generated.

In this study ground pine (Pinus sylvestris) sawdust was used as the source of lignocellulose. Two pretreatment methods, acid and alkali hydrolysis, were examined and the variables of concentration, temperature and time optimised. Biomethanation of the two pretreatment products, hydrolysates and residual solids, in closed cultures by isolated microbial associations, resulted in 8.5-fold increases in total methane generation compared with untreated controls.

Closed culture methods were used to enrich and isolate microbial associations from freshwater sediment capable of catabolising selected monomers under anoxic conditions. In all, 20 associations were isolated of which two, a veratric acid-catabolising and a syringic acid-catabolising culture, were chosen for further study. Although initially both associations dissimilated the respective substrates to the terminal product, methane, the metabolic activity of the veratric acid-catabolising association changed such that catechol accumulated. A second veratric acid-catabolising association was then

isolated which, like the syringic acid-catabolising association, was stable during repeated subcultivations. During open culture cultivations, however, in which increasing dilution rate regimes were applied, species displacement from both associations were apparent.

Closed culture studies demonstrated optimum culture conditions of a temperature of 35°C and a pH of 7.0.

Dissimilations of both substrates were accompanied by concomitant sulphate reduction and methane production as the major terminal product.

Determination of K_s values from both open and closed culture data showed that higher affinities were apparent in the case of the former. The validity of such determinations and comparisons was, however, questioned.

For both associations, inhibitions of methanogenesis by an analogue of coenzyme M, 2-bromoethanesulfonic acid, resulted in the accumulation of metabolic intermediates which were subsequently identified by the use of HPLC, TLC, GLC and UV spectrophotometry. Based on these identifications tentative breakdown pathways were formulated.

Preliminary species isolations indicated that each association contained a minimum of one sulphate reducer, two methanogens and two to three facultative anaerobes.

CONTENTS

CHAPTER ONE

INTRODUCTION

1.1 Sources of Biomass

The term biomass covers a wide spectrum of organic materials of natural origin such as forests, grassland, agricultural crops and aquatic plants, together with wastes collected from sources such as domestic, agricultural, food processing plants and other industries. The annual production of biomass world wide is estimated to be of the order of 2×10^{11} tonnes (dry matter), equivalent to ten times the world's current annual consumption of energy (Cain, 1980; Crawford, 1981; Brown, 1983).

With the exception of municipal solid waste, serious problems may be encountered in the collection of biomass. Waste sources are usually too thinly dispersed and as a consequence the energy is too dilute to be used directly as an energy source. In addition, some processing, which necessitates an energy input such as collection, drying, compaction and transport may be required before it can be used as fuel (Parker, 1983). These wastes are composed of a large percentage of lignocellulosic materials which are composed of three major groups of polymers: cellulose, hemicellulose and lignin. The exact formulation varies from source to

source in terms of both its chemical constituents and their relative ratios with these in turn directed by age, growth environments and physiological conditions. Immature tissues may contain a considerable percentage of other non-structural components such as pectin, alkaloids, phenolics and terpenes which can significantly influence the biodegradability of the material (Kirk, 1983).

Cellulose is a major structural component of plant cell walls, forming an outer support matrix for the cell membrane. Hemicellulose in turn forms within the spaces created by the network of cellulosic strands. As the cell dies, due primarily to the lack of flow of nutrients through the relatively rigid cellulosic wall, many secondary products are secreted. In particular, the material of which lignin is composed appears as a form of cement between the cell walls.

1.1.1 *Cellulose*

Cellulose is the simplest of the polymers of lignocellulosics and is composed of a linear continuous chain of D-glucose molecules linked in the β-1,4 configuration (Figure 1). These chains or micelles may contain more than 10^4 anhydroglucopyranose units giving a molecular mass in excess of 1.5 MDa (Sihtola and Neimo, 1975). Cellulose micelles are bunched together

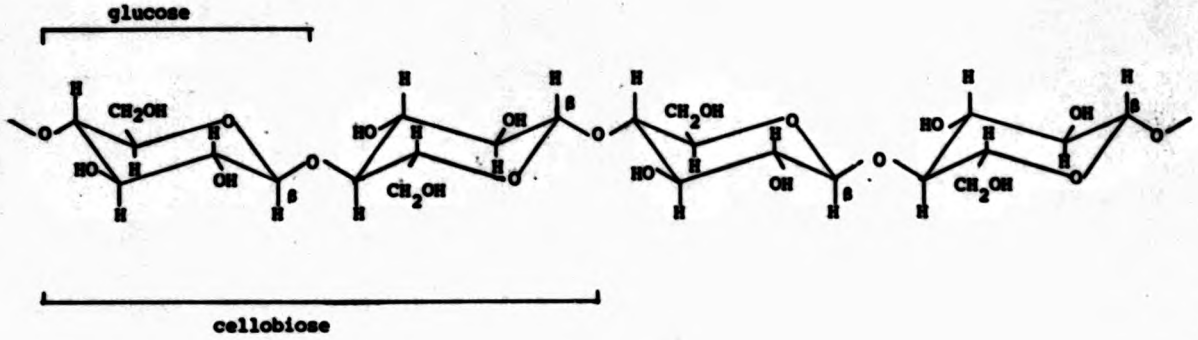


Figure 1. Structure of the Cellulose Molecule

to form thread-like microfibrils, the individual cellulosic polymer strands of which are hydrogen bonded between the ring oxygen of glucose molecules and the hydroxyl groups at position 3. The cellulosic fibrils are composed of highly ordered micelles which possess crystalline structures interspersed with disorderly areas of so-called amorphous cellulose (Cowling and Kirk, 1976; Smith, 1980; Kirk, 1983).

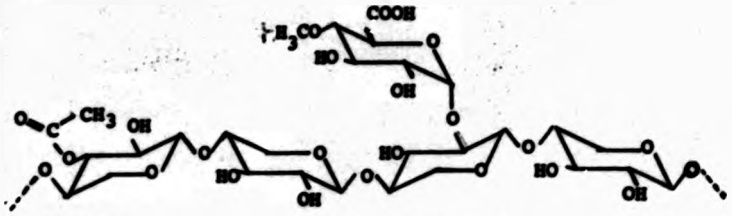
1.1.2 Hemicellulose

Hemicelluloses in wood consist of short, mainly branched heteropolymers of anhydrosugar units, linked by glycosidic bonds. The sugar units include xylose, galactose, mannose and arabinose, as well as uronic acids of glucose and galactose (Cowling and Kirk, 1976; Kirk, 1983). According to Wenzl (1970) three well defined groups can be identified:

(1) xylans, which consist of a basic backbone of poly-β-1,4 xylan with additional side links to arabinose, glucuronic acid and arabinoglucuronic acid;

(2) mannans which are composed of glucomannans and galactomannans; and

(3) galactans which are present as arabinogalactans. The origin of the lignocellulosic material defines the nature of the hemicelluloses (Figure 2).

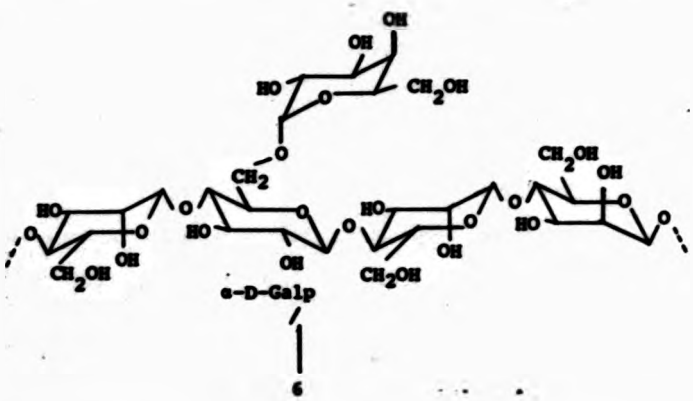


O-Acetyl 4-O-Me-a-D-Glup A

3 2

→4)-ß-D-Xylp-(1→4)-ß-D-Xylp-(1→4)-ß-D-Xylp-(1→4)-ß-D-Xylp-(1→

(a)



→ 4)-ß-D-Manp-(1→4)-ß-D-Glup-(1→4)-ß-D-Manp-(1→4)-ß-D-Manp-(1→

(b)

Figure 2.

a) **Structure of O-acetyl-4-o-methylglucuronoxylan, the Principal Hemicellulose of Angiosperm Tissues.**

b) **Structure of an o-acetyl-galactoglucomannan, the Principal Hemicellulose of Conifer Tissues.**

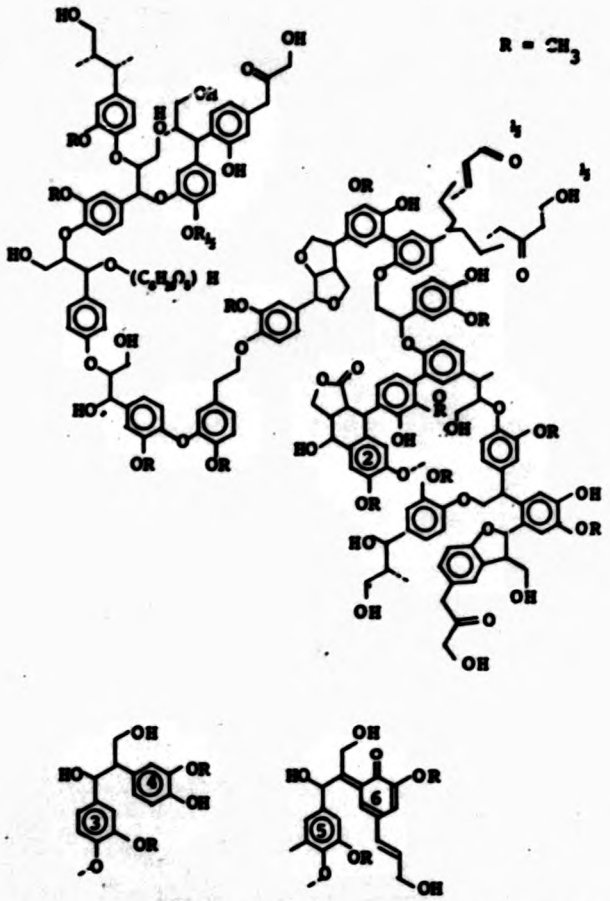
1.1.3 *Lignin*

Lignin is a three dimensional polymer of phenylpropane units, synthesised by the oxidative polymerisation of three major aromatic alcohols (Figure 3): coumaryl, sinapyl and coniferyl (Higuchi, 1980). These three alcohols oxidised in the nascent cell wall by single electron abstraction to give free radical species which couple randomly, with each other and, primarily, with the growing lignin polymer, which contains phenolic residues and is itself oxidised by single electron abstraction (Kirk, 1983). The true formulation of lignin in the natural state can only be deduced from theoretical reconstructions of various degradation products. The proportions of the three precursor alcohols differ between the major angiosperm and gymnosperm lignocelluloses, and also among the various taxa, particularly within the angiosperms. From the various postulated linkages, three major groups of lignin can be identified:

(1) coniferous (gymnosperm lignin) which is formed predominantly from the coniferyl alcohol units;

(2) grass (angiosperm lignin) formed from coniferyl, sinapyl and coumaryl alcohols; and

(3) hardwood lignin which contains a mixed poly-



In some cases, the structures marked '1' and '2' will be replaced by the structures marked '3' and '4' or the structures marked '5' and '6'.

Figure 3. The Structure of Softwood Lignin

mer of coniferyl and sinapyl alcohol (Kirk, 1983).

The actual polymerisation process involves reactive intermediate structures (quinone methides) which can add nucleophiles, including hemicellulose-hydroxyls and -carboxyls. Thus, lignin-hemicellulose ether and ester linkages occur at a frequency of about one linkage per 36 phenylpropane units (Obst, 1982). Lignification of plant cells can be detected by treatment with phloroglucinol and concentrated hydrochloric acid which yields a characteristic cherry-red colour (Weisner reaction) due to the presence of coniferyl aldehyde linked to the remainder of lignin polymer through a phenolic hydroxyl group. The solubilisation and subsequent removal of lignins are major operations in the manufacture of wood pulp for papermaking and are achieved by boiling wood chips in sodium bisulphite solution (for sulphite pulp) or in an alkaline mixture of sodium sulphate and sodium sulphide (for the stronger kraft pulp).. These treatments break down the lignin polymer but, as yet, no economic use has been found for the millions of tons of lignaceous wastes that are produced annually (Walker, 1975).

1.2 The Biosynthesis of Lignin

The groundwork for the elucidation of the bio-

synthetic pathway for lignin was established by Erdtman (1933) who studied the oxidative coupling of phenols, which was shown to proceed by a free radical process. This work was extended by Freudenberg (1965) to consider the polymerisation of coniferyl alcohol when it was shown that a lignin-like dehydrogenative polymer (DHP) could be formed by treatment of an aqueous solution of coniferyl alcohol with a crude preparation of mushroom phenol oxidase. This DHP exhibited very similar properties to spruce millwood lignin (MWL) with respect to degradation products, elemental and functional group analyses and physical characteristics (Freudenberg and Neish, 1968). Also, if reactions were inhibited, soon after inhibition, a number of compounds, known as lignols were formed due to the non-enzymic coupling of coniferyl alcohol radicals to form polymers (Adler, 1977). The central role of this free radical process in lignin biosynthesis ensures that no absolutely definitive structure can be recorded.

In both gymnosperms and angiosperms, with the exception of grass, lignin is synthesized from carbon dioxide via shikimic acid, which in turn is converted to L-phenylalanine (Higuchi, Shimada, Nakatsubo and Tanahashi, 1977; and Higuchi, 1980). Subsequently, L-phenylalanine is transformed by the enzyme phenylalanine layase (PAL) to trans cinnamic acid, and thence

by a series of hydroxylations and methylations to ferulic acid and sinapic acid, which are finally reduced to coniferyl and sinapyl alcohols respectively. Figure 4 shows the complete biosynthetic pathways whereby plants convert CO_2 to lignin (Crawford, 1981).

1.3 Degradation and Conversion of Lignocelluloses

The prominent reactions of lignin polymer degradation are mediated by oxidations and oxidative cleavages in the propyl side chains, demethylations of the methoxy groups, and cleavages in the aromatic rings. These reactions and the enzymes involved are, however, less well understood than those in cellulose degradation (Eriksson, 1981; Kirk, 1983).

An oxidoreductase enzyme, cellobiose: quinone oxidoreductase, which is involved in cellulose ion degradation, also plays a role in lignin degradation. This enzyme reduces quinones and phenoxyradicals in the presence of cellobiose, which is then oxidised to cellobiono-δ-lactone (Smith, 1980; Eriksson, 1981).

Other enzymes which may be involved include laccases and phenol oxidases (Smith, 1980; Eriksson, 1978, 1981; Milgrom, 1985). Work with laccase-minus mutants of Sporotrichum pulverulentum by Eriksson (1978), showed that they were incapable of degrading lignin, thus emphasising the importance of these enzymes.

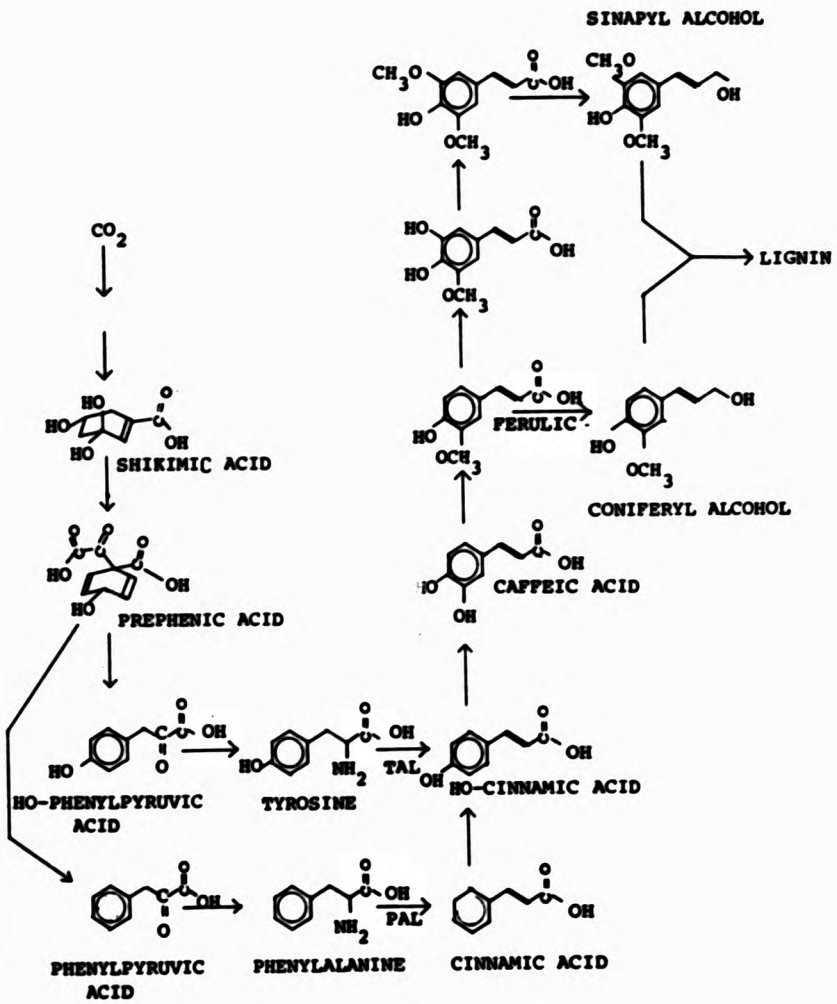


Figure 4. The Biosynthesis of Lignin

Degradation of lignin by S. pulverulentum must, however, be accompanied by the mixotrophic dissimilation of a wood polysaccharide such as cellulose or cellobiose which provides an easily accessible energy source (Eriksson, 1978, 1981; Eriksson, Goring and Lindgren, 1980). The lignolytic system is not induced by lignin but appears constitutively as the cultures enter the secondary metabolism state. Initiation of the system is triggered by conditions of carbohydrate, nitrogen or sulphur limitation (Kirk, 1981).

1.3.1 Pretreatments of lignocelluloses

Woody biomass without pretreatment is generally considered to be recalcitrant to anaerobic decomposition due to the crystalline nature of the component cellulose and the presence of the complex association of carbohydrates with lignin. The recalcitrance of native lignocellulosic materials to biodegradation requires most bioconversion schemes to use chemical or physical pretreatment to separate the more easily fermentable polysaccharide component from the more refractory lignin component (Datta, 1981a). Chandler, Jewell, Gossett, van Soest and Robertson (1980) reported a strong linear relationship between the lignin content and the volatile solids destruction efficiency with a 3% decrease in efficiency for every 1% lignin content increase.

Physical processes

(i) *Hammer milling*

Although hammer milling causes an effective reduction in particle size together with an increase in bulk density it does not enhance susceptibility to degradation (Ghose and Ghosh, 1979).

(ii) *Grinding*

Grinding of lignocellulosic substrates into very small particles markedly enhances their susceptibility to hydrolytic, enzymatic and microbiological attack (Millett, Baker and Satter, 1975, 1976). Han and Callihan (1974) studied the effect of particle size on microbial degradation of sugarcane bagasse. When the particle size was in excess of 60 mesh there was no apparent improvement, although when the size was reduced below 60 mesh, digestibility increased slightly. From these results they concluded that the slight increases in digestibility did not make grinding, on a production scale, economically viable.

(iii) *Ball milling*

This treatment both reduces particle size and also increases susceptibility to microbial attack. Mandels, Hontz and Nystrom (1974) concluded that a ball mill is the most effective device in imparting an increased reactivity at a given interfacial area. Ball milling has in fact been applied to increase the nutrit-

ional value of cellulosics for animal feed. The effectiveness of this method has been demonstrated in the enzymatic saccharification of many waste cellulosics. For example, Pew and Weyna (1962) obtained essentially complete cellulose digestion of spruce and aspen sawdust after ball milling treatment, compared with a figure of less than 10% digestion of the unmilled substrate. Similarly, Kelsey and Shafizadeh (1980) reported a 3 to 10 fold increase in the rate of glucose production after milling during enzymatic hydrolysis of various cellulosics. Unfortunately, ball milling is an expensive process and thus its use may be limited on economic grounds despite its effectiveness (Millett et al, 1976).

Other physical processes

(i) *Irradiation*

Gamma rays or high velocity electrons have been shown to improve the digestibility of straw and wood materials by rumen microorganisms (Millett et al, 1975; Han, 1978). Digestibility of aspen carbohydrate in fact increased quantitatively up to an electron dosage of 10^8 rads, although spruce digestibility did not increase more than 14% at this dosage. Gamma irradiation, at a dosage of 50 M rad or more, significantly increased the susceptibility of corn grain to enzymatic hydrolysis and at the same time decomposed the starch content with

approximately 30% (dry weight) of reducing sugar produced (Han, 1978 ; Han, Timpa and Ziegler, 1981; Han and Ziegler, 1982). Unfortunately these types of treatment are extremely slow in affecting the lignin composition, and this,together with the necessary equipment and quantities of irradiation required, make the process too expensive to be of commercial interest.

(ii) *Photodegradation*

Photodegradation has been used in the production of Aspergillus fumigatus fungal protein from a variety of cellulosic substrates (Millett et al, 1975). The polysaccharide substrates were exposed to high intensity ultra-violet light (3650Å) in the presence of a photosensitizer (dilute sodium nitrate solution) and it was found that a 24h irradiation pretreatment increased the rate of biodegradation by a factor of four (Millett et al, 1975, 1976).

Once again, the high cost of equipment and energy are too great to make this process commercially feasible.

(iii) *High temperature*

An optimum temperature of 200°C, for 32h, has been shown to be required to maximise the rate of dilute acid hydrolysis although only 35% of the cellulose was hydrolysed with the sugar yield increased by 27% (Millett et al, 1975). As a consequence of the modest

benefits, compared with the time involved and energy requirements to maintain the high temperature, the process is not commercially viable (Millett et al, 1975; Buchholz, Puls, Godelmann and Dietrichs, 1980; Knappert, Grethlein and Converse, 1980).

(iv) *Low temperature*

Freezing of cellulosic materials in a water suspension at -75°C has been reported to reduce both the strength and degree of polymerisation of cellulosic material (Millett et al, 1975, 1976). Once again, however, the energy requirements are too great to make this an economically viable pretreatment procedure.

(v) *Pressure*

Compression of cotton hydrocellulose, at pressures from 0 to 8000 kg cm^{-2} for 30 minutes at room temperature, has been shown to be approximately double the quantity of materials dissolved during ethanolysis. For spruce sulphite pulp, repeated compression between cylinder rollers results in increased ethanolysis from 12 to 54% (Millett et al, 1976).

Since this process is a combination of physical and chemical pretreatments both chemical cost and high pressure energy requirements must be considered.

(vi) *Steam*

Steaming of lignocellulosics in a range of temperatures from 150 to 200°C has been shown to facili-

tate cleavage of component acetyl groups and as a consequence result in increased enzymatic digestibility, increased pore size, autohydrolysis and increased moisture to allow enzyme penetration and thus substantially increase bioconversion yields (Meese, Wallick and Harper, 1977; MacDonald and Mathews, 1979; Buchholz et al (1980). Datta (1981b) reported that the actual steam requirement depends on the final moisture content which ranges from 60 to 75%. A biomass conversion of 50% solids to a product with 25 to 40% solids, by steam treatment, at 150 to 200°C would in fact require between 450-300 kg of steam dry ton^{-1} of biomass which is equivalent to 20% of the energy content of the substrate when a 50% heat recovery is assumed. However, difficulties of heat transfer from 60 to 75% wet solids may result in lower heat recovery and thus less efficiency.

Steaming does, however, avoid the disadvantages of chemical pretreatments such as by-product formation, expensive corrosion-proof equipment and environmental pollution problems and for this reason it may be worthy of consideration (Bender, Heaney and Bowden, 1970; Heaney and Bender, 1970; Millett et al, 1976).

Chemical treatments

(i) *Alkali*

Alkali pretreatment of lignocellulosics is

probably the oldest and best known method for the subsequent enhancement of microbial degradation of cellulose. The effects of alkali treatment are multiple. In general, the application of alkali results in the removal of lignin, an extensive swelling and separation of the structural elements of the native cellulose, and an alteration of crystalline and amorphous structure (Tsao and Chiang, 1983). Mercerizing strengths, above 20%, of sodium hydroxide solution have been shown to cause extensive swelling and separation of the structural elements in native cellulose. Thus, the product may undergo acid hydrolysis up to 40 times faster than the untreated cellulose (Millett et al, 1975).

As an alternative, alkali may be used as a pretreatment to increase or upgrade the nutritive value of forage and forest residues for ruminants. This can be accomplished by treatment with low concentrations of alkaline. For example, loosely-baled straw is steeped in 1.0 to 1.5% (w/v) NaOH solution for between 4 and 24h. The liquor is then drained off and supplemented with more alkali for re-use. The result of this treatment is to increase the digestibility to more than 65% of the organic matter in the straw, thus making the digestibility equivalent to that of high quality hays. The process was in fact patented and was used extensively in Europe and Britain during the two World

Wars (Millett *et al*, 1976; Han, 1978; Owen, 1980; Detroy, Lindenfelser, Jullian and Orton 1980).

Dekker and Richards (1973) used three different chemical methods for partial delignification of bagasse, sodium sulphide/hydroxide (kraft process), sodium hydroxide and sodium sulphite. The kraft and alkali treatments each reduced the lignin content by about 55% and as a consequence increased the rumen dry matter digestibility by over 170%.

Hartley, Jones, King and Smith (1974) showed that the most successful modification of beech, oak and poplar sawdusts and barley straw as potential components of animal feed was achieved by prior colonisation with the basidiomycete fungus *Fomes lividus* followed by NaOH treatment. The apparent organic matter digestibility of beech was increased from 5 to 59% and oak from 14 to 56%.

Investigations of the influence of alkali concentration on the *in vitro* digestibility of various hardwoods have indicated that between 5 and 6 g NaOH per 100g of wood are required for maximum effect (Feist, Baker and Tarkow, 1970; Millet *et al*, 1975). This concentration of alkali is essentially equivalent to the combined acetyl and carboxyl content of the woods (Millett *et al*, 1976).

Wood, treated with hot dilute aqueous alkali,

liquefies to form a viscous tar, from which as many as 39 phenols have been separated. Russell, Miller and Molton (1983) found that the aromatic compounds are not only derived from the lignin but are also formed by the condensation and aromatisation of 2- and 3-carbon decomposition products of cellulose. Avgerinos and Wang (1983) showed that a selective solvent pretreatment with an alkaline-ethanol-water mixture partially delignified agricultural residues such as corn stover and wheat straws. By increasing the ethanol concentration, at low temperatures, the alkaline solubilisation of cellulose and hemicellulose was minimised. With corn stovers, approximately 70% of the lignin was extracted and was accompanied by a 5% loss of the pentosan carbohydrates when a solution of 5% (v/v) ethanol in water, which contained 0.2M NaOH, was used for 72h at 25°C.

Toyama, Kazuo and Ishihara (1980) determined that a treatment of 20 volumes of 0.5% (v/v) aqueous NaOH for 20 minutes at 20°C was required to hydrolyse bagasse.

Detroy, Lindenfelser, Sommer and Orton (1981) used a two-stage chemical pretreatment, with an initial NaOH step followed by ethylenediamine (EDA) or NH_4OH, prior to enzymatic saccharification of wheat straw to improve the 75% concentration obtained with NaOH alone.

Datta (1981a) found that the treatment of

acidogenic-fermented corn stover with a mixture of sodium carbonate and lime was both a cheaper alternative and also a more effective pretreatment than sodium hydroxide alone.

Pavlostathis and Gossett (1985) showed that alkaline treatments of wheat straw resulted in both higher anaerobic degradability and elevated methane production than untreated controls. From this study the workers concluded that alkaline treatment has practical advantages over acidic treatment, particularly when the pretreatment is to be followed by anaerobic digestion.

(ii) *Acids*

The acidic treatment of lignocellulosic materials, prior to enzymic hydrolyses, is used as a procedure in the degradation of the polymeric structure for analytical purposes (Knappert et al, 1981; Wilke, Yang, Sciamanna and Freitas, 1981).

Acid hydrolysis has, however, been considered for a number of years as a method in its own right, for producing sugar liquors, particularly for ultimate fermentation to, for example, ethanol (Wenzl, 1970). Although degradation is possible with any acidic solution, work has tended to favour sulphuric acid as the catalyst of the hydrolysis (Grant, Han, Anderson and Frey, 1977) with the use of 70 to 75% H_2SO_4 or fuming

(40%) HCl forming the basis for two of the wood saccharification processes operative in Europe during World War II (Lloyd and Harris, 1963).

Sulphuric acid was also used in the pretreatment of wood by Keith and Daniels (1976) and grass straw (Han and Anderson, 1975) for animal feed productions. Palatability of such treated straw was, however, low, possibly because of the high concentration of sulphate (Israelides, Grant and Han, 1978).

Work by Han and Callihan (1974) focussed on various chemicals for the treatment of sugarcane bagasse. Notable amongst these was the use of concentrated acid (50% v/v H_2SO_4) which was then followed by 0.5, 1, 2 and 10% v/v H_2SO_4 when a maximum sugar yield of 23% was recorded. Unfortunately this process in terms of digestibility, cell yield, substrate loss and production of reducing sugars is less attractive than treatment with other chemicals since the presence of acid, whilst hydrolysing the β-1,4 link in the sugar polymer, rendered the sugar non-fermentable by further modification (Brown, 1983).

Finally, delignification can be achieved by using different methods, viz., oxidising agents, such as, peracetic acid, sulphur dioxide (Dunlap, Thomson and Chiang, 1976), nitrogen dioxide (Wilke, Yang, Sciamanna and Freitas, 1981), or ozone (Binder, Pelloni, and

Fiechter, 1980). Gould (1985) showed that treatment with alkali-hydrogen peroxide is successful in delignification of agricultural waste. All commercial delignification procedures are based on the removal of lignin through the selective action of chemical pulping and bleaching agents. Since none of these processes are truly selective, considerable carbohydrate material commonly accompanies the lignin into the waste stream, reducing the overall yield as well as decreasing the amount of carbohydrate available for enzymatic or microbiological conversion (Millett et al, 1976).

1.3.2 Biodegradation of lignocelluloses and lignin-related compounds

Lignocelluloses, which comprise about 95% of the earth's land-based biomass, are made up of the structural polymers cellulose, hemicellulose and lignin, and is the major repository of photosynthetic energy and renewable organic matter (Amer and Drew, 1980).

As a consequence of the abundance of lignocelluloses, their decomposition is the single most important biodegradative event in the earth's carbon cycle (Cain, 1980; Kirk, 1983). The variety of biodegradative microorganisms, the extracellular conditions under which the catabolisms take place and the products to which lignocelluloses are converted are of practical

scientific interest with respect to their technological applications. Studies of the catabolic processes range from determination of the precise chemical mechanisms to analysis of the microbial associations responsible for the multiplicity of reactions required to convert the heterogeneous lignocellulose molecules to metabolic intermediates and end products (Kirk, 1983).

With a lignin content below 18 to 20% lignocelluloses are degraded to an increasing extent by cellulases and hemicellulases, and consequently by the many bacteria and fungi which secrete these enzymes. Thus fungi, from each of the major classes, and anaerobic, as well as aerobic, bacteria can partially dissimilate and metabolise such substrates. As the accessible polysaccharides are removed, the residual core, high in lignin content, becomes progressively recalcitrant to further degradation, unless the catabolising species can also degrade the lignin (Kirk, 1983).

Fungi

The major degraders of fully-lignified tissues are the filamentous fungi, primarily the Basidiomycetes which are responsible for the white-rot type of wood decay. Gilbertson (1980) estimated that there are between 1600 and 1700 North American species of wood-rotting fungi, of which over 90% are white-rot

fungi. On a global basis more than 2000 species have been recorded (Cain, 1980).

A second group of wood-decaying Basidomycetes which cause brown-rot also degrade fully lignified tissues, but without substantially depleting the lignin content (Kirk, 1983).

The suggestion has been made that the metabolic activities of white-rot fungi generate products which contain one, two, or, at most, three aromatic moieties (Cain, 1980). Analysis of decayed lignins by Kirk (1971) showed that three major changes were apparent:

(1) oxidation of side chains;
(2) oxidation of the α-carbon in the propanoid side chains; and
(3) the cleavage of aromatic rings still attached to the polymers.

Soft rot fungi degrade all major wood chemical components and appear to decompose hardwood lignins more effectively than those in softwood (Zeikus, 1981). Chemical analysis of woods decomposed by six soft rot species has indicated significant weight loss of lignin (as determined by the sulphuric acid method) in red alder and balsam poplar but not in white pine (Eslyn, Kirk and Effland, 1975). Although soft rot fungi seem to have an absolute requirement for a labile carbon source as co-substrate (Drew and Kadam, 1979; Amer and

Drew, 1980), work by Haider and Trojanowski (1975) with ^{14}C-labelled lignin conclusively demonstrated that these species can effect considerable degradation.

Decomposition of native lignin is not restricted to these fungi but is possessed, to varying extents, by a wide range of fungi, including many microscopic genera such as Aspergillus, Penicillium, Chaetomium, Fusarium and Alternaria (Crawford, 1981). Aspergillus fumigatus, for example, has been reported to convert ^{14}C-labelled kraft lignin to $^{14}CO_2$ in the presence of starch as a co-substrate (Drew and Kadam, 1979; Hall, Glasser and Drew, 1980).

Aerobic bacteria and microbial associations

A number of bacteria including Flavobacterium, Micrococcus, Mycobacterium, Pseudomonas, Xanthomonas, Streptomyces and Nocardia have been reported to be capable of lignin degradation (Amer and Drew, 1980; Kirk, 1983). Trojanowski, Haider and Sundman (1977) and Haider, Trojanowski and Sundman (1978) reported that certain monocultures of bacteria, particularly Nocardia spp.,were able to decompose lignin and to assimilate the catabolic products as carbon sources.

Slow degradation of lignin by soil bacteria has been demonstrated (Crawford and Crawford, 1980; Crawford, 1981). Cartwright and Haldom (1973) found that Arthrobacter spp. were capable of utilising lignin

as a sole carbon source.

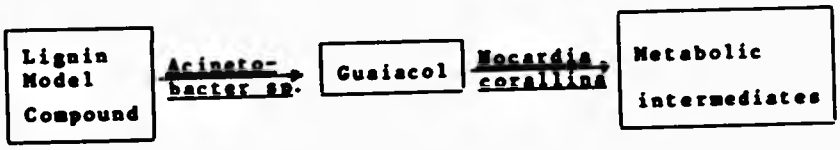
Odier and Monties (1978) similarly showed that Xanthomonas sp., isolated from decomposing wheat straw, was able to utilise dioxane lignin as a sole carbon source when they reported that 77% of the initial lignin, in supplemented minimal medium, was degraded after 15 days of cultivation. In the presence of glucose, however, only 23% of lignin was degraded.

In a previous study, the same authors, Odier and Monties (1978) found that 18 of 85 aerobic bacterial isolates tested could use dioxane-lignin as a sole source of carbon and energy. Of the remainder, 21 degraded lignin in the presence of glucose.

Deschamps, Mahoudeau and Lebeault (1980) found that a strain of Aeromonas sp. was capable of utilising 98% of the lignin present in a fermentation medium, supplemented to a concentration of 1 gl^{-1}, in the first 5 days of incubation.

Crawford (1978) provided proof that Streptomyces spp. can degrade lignin, when he showed an 11 to 12% recovery of ^{14}C as $^{14}CO_2$ after 28 days incubation of different preparations of ^{14}C-kraft lignin and ^{14}C-milled wood lignin from ^{14}C-[lignin]-Douglas Fir. Finally, Bacillus megaterium has been shown to have lignolytic activities (Robinson and Crawford, 1978). Although, as can be seen from the above discuss-

ion a number of studies has been made on the catabolism of lignin by aerobic monocultures of bacteria, reports on interacting microbial associations have been somewhat lacking. Crawford (1975) described a 2-membered microbial association which was able to catabolise a lignin model compound as follows:



In the absence of Nocardia corallina, however, growth of the primary species, Acinetobacter was inhibited.

Anaerobic bacteria

Although aerobic bacterial catabolism of lignin is relatively well documented much less reports have been made on anaerobic degradation. Boruff and Buswell (1934) reported that cornstalk lignin fermented only slowly and incompletely under anaerobic conditions. Later, Levine, Nelson, Anderson and Jacobs (1935) were unsuccessful in their attempts to isolate bacteria capable of decomposing lignin under anoxic conditions. However, although the addition of alkali-treated lignin to an active methanogenic digester inhibited gas production, a similar

addition of cornstalk flour, as a source of natural lignin, resulted in a final degradation of 37%. Similarly, Acharaya (1935) found that 35% of the lignin component of rice straw was degraded under anaerobic conditions. However, all the above data reported on lignin degradation are now considered to be unreliable since the methods used for measuring lignin concentration were inadequate (Sleat and Robinson, 1984). Zeikus, Wellstein and Kirk (1982) in fact reported that lignin is a recalcitrant natural compound in anoxic neutral environments because of its high Mr-value and the inability of in situ anaerobic microbes to significantly depolymerise the substrate. They also suggested that lignin must be chemically modified or depolymerised ($\leq$ Mr 850) before significant decomposition occurs in anoxic ecosystems.

Odier and Monties (1983) used ^{14}C-labelled lignin and reported that after 6 months incubation at 30°C under anaerobic conditions no radioactivity was detected in any organic acid fraction, thus indicating that no fermentative catabolism of lignin had taken place. The workers suggested that the recalcitrance of lignin towards microbial biodegradation resides in its high molecular weight and three-dimensional structure.

Hackett, Connors, Kirk and Zeikus (1977) similarly reported that ^{14}C-dehydropolymers (DHP) were

also recalcitrant in a variety of natural materials incubated under anaerobic conditions.

However, the possibility of limited lignin degradation in the absence of oxygen has been suggested (Federle and Vestal, 1980). Odier and Monties (1978) reported that a strain of Xanthomonas decomposed wheat dioxane-lignin anaerobically, in the presence of nitrate and glucose, when two phenolic compounds with carbonyl groups were detected in the culture supernatant. The suggestion was then made that the low molecular weight fractions of dioxane-lignin were metabolised by the bacteria (Crawford and Crawford, 1980). Benner, MacCubbin and Hodson (1984) reported that only 1.5% of the lignin component and 4.1% of the polysaccharide component of ligninocellulose derived from the hardwood used (Rhizophora mangle) were degraded to gaseous end products after 246 days of incubation anaerobically with anoxic sediments collected from a saltmarsh, a freshwater marsh and a mangrove swamp. Benner and Hodson (1985) also reported that 2 to 4% of synthetic and natural pine lignin were degraded during thermophilic (55°C) anaerobic incubation. Colberg and Young (1985) reported that solubilised lignin, of different molecular size fractions, used as the sole source of carbon in anaerobic enrichment cultures, was mineralised to methane and carbon dioxide. The workers

also found that the production of methane and carbon dioxide was related to the molecular size of the fractions since the smaller the size the more extensive was the degradation to gaseous end products.

Zeikus (1980a) reviewed lignin catabolism and reported that lignin metabolism follows nature's principles regarding the synthesis and depolymerisation of biopolymers. Thus, since lignin evolved in an oxygen-rich atmosphere with its synthesis requiring oxygen then depolymerisation must also require the presence of molecular oxygen.

1.3.3 *Biodegradation of aromatic lignin monomers under anoxic conditions*

Although the lignin polymer is essentially recalcitrant under anoxic conditions the constituent aromatic alcohols are degradable. This fact has been recognised since 1934 when Tarvin and Buswell examined the fermentation of biological materials in sewage sludge and reported that benzoate, phenylacetate, phenylpropionate and cinnamate were completely dissimilated under anoxic conditions. Since then, and particularly during the last two decades, the degradation of aromatic compounds under anoxic conditions has received considerable attention. Table 1 shows a range of the aromatic compounds and the mechanisms of

Table 1. Aromatic Compounds and Their Catabolic Mechanisms Under Anoxic Conditions.

P: photometabolism; N: nitrate-dependent; S: sulphate-dependent; M: methanogenic fermentation

Substrate	Catabolic Mechanism	Reference
Benzoate	P	Scher (1960), Guyer and Hegeman (1969), Dutton and Evans (1967, 1969).
	N	Scher (1960), Williams and Evans (1973), Aftring and Taylor (1981).
	S	Balba and Evans (1980a), Widdel (1980).
	M	Tarvin and Buswell (1934), Clark and Fina (1952), Ferry and Wolfe (1976), Balba and Evans (1977), Sleat and Robinson (1983).
o-Hydroxybenzoate	N	Taylor et al (1970).
	S	Widdel (1980)
	M	Balba et al (1979)
m-Hydroxybenzoate	P	Dutton and Evans (1969).
	N	Taylor et al (1970), Williams and Evans (1975).
	S	Widdel (1980).
p-Hydroxybenzoate	P	Dutton and Evans (1969). Williams and Evans (1975).
	S	Widdel (1980).
	M	Balba et al (1979), Healy and Young (1979)
Protocatechuate	N	Oshima (1965), Taylor et al (1970), Williams and Evans (1975).
	M	Balba et al (1979), Healy and Young (1979).
Catechol	N	Healy and Young (1978, 1979), Balba et al (1979).
Benzaldehyde	N	Williams and Evans (1975).
	M	McCarty et al (1977).
Benzyl alcohol	N	Al-Sarraj (1983).
	N	Williams and Evans (1975).
Phenol	N	Bakker (1977).
	M	Healy and Young (1979), Balba et al (1979).
Phenylacetate	N	Williams and Evans (1975).
	S	Widdel (1980).
	M	Tarvin and Buswell (1934), Balba and Evans (1979).
Hippurate	S	Widdel (1980).
o,m,p-cresol	N	Bakker (1977).
o,m,p-methoxybenzoate	N	Balba et al (1979).
o,m,p-phthalic acid	N	Aftring et al (1981), Aftring and Taylor (1981).
Cinnamic acid	N	Williams and Evans (1975).
	M	Tarvin and Buswell (1934), Healy & Young (1979)
p-Hydroxycinnamate	N	Williams and Evans (1975).
Cinnamyl aldehyde	N	Al-Sarraj (1983).
Caffeic acid	N	Williams and Evans (1975)
Syringic acid	N	Healy and Young (1979). Kaiser and Hanselmann (1982a, b).
Vanillic acid	N	Healy and Young (1979), Zeikus et al (1982).
Syringaldehyde	N	Healy and Young (1979).
Vanillin	N	Healy and Young (1979).
Ferulic acid	N	Healy and Young (1979).
	M	Healy et al (1980), Grbic-Galic & Young (1985)
Gallic acid	N)	Kaiser and Hanselmann (1982a),
Pyrogallol	N)	Schink and Pfennig (1982).
Phloroglucinol	N)	
Hydroxy-hydroquinone	N	Schink and Pfennig (1982).
Anthranilic acid	N	Al-Sarraj (1983).
	N	Braun and Gibson (1984).
Coniferyl alcohol	N	Grbic-Galic (1983).

catabolism under these conditions.

From studies such as these the knowledge gained of the mechanisms involved and the responsible species may allow the development of bacterial cultures capable of improving the process of anaerobic digestion of plant lignocellulosic fractions and thus obviate the need for expensive pretreatments.

1.4 Biochemistry of Anaerobic Catabolism of Aromatic Compounds

The biochemistry of bacterial catabolism of aromatic compounds in anaerobic environments has been reviewed by Evans (1977) and Zeikus (1980a). Essentially, three biochemical mechanisms exist for the catabolism of the aromatic nucleus by mixed or pure bacterial cultures in the absence of oxygen:

(i) anaerobic respiration;

(ii) photometabolism; and

(iii) fermentation.

(i) *Anaerobic respiration*

Anaerobic respiration is the linking of the dehydrogenation reactions of aromatic catabolism to the reduction of external inorganic electron acceptors such as NO_3^- (reduced to N_2 or NH_3), SO_4^{2-} (to S^{2-}) and CO_2 (to CH_4). Anaerobic respiration of aromatic compounds

can be performed by monocultures with the pathways often identical to those of aerobic respiration (Evans, 1977).

Oshima (1965) examined the anaerobic metabolism of aromatic compounds in the presence of nitrate by soil microorganisms in an attempt to determine whether nitrate could replace oxygen in the oxidative decomposition of aromatic compounds by specific facultative anaerobes. The worker found that anaerobic decomposition of protocatechuate occurred only in dual cultures of specific bacteria and not during the growth of monocultures of isolated bacteria under the same conditions. One of the isolates, tentatively identified as a Pseudomonas sp., was able to grow aerobically on protocatechuate or p-hydroxybenzoate and could also reduce nitrate in nutrient broth medium. When this Pseudomonas sp. was inoculated into anoxic nitrate-protocatechuate medium together with a small, non motile, gram-negative, nitrate-reducing rod, cell growth was again observed. In addition, it was found that the Pseudomonas sp. could be replaced by other pseudomonads which were also able to oxidise protocatechuate aerobically and reduce nitrate in nutrient broth in the absence of oxygen. As a consequence of these results it was suggested that the biochemical pathway of protocatechuate metabolism was the same under both anaerobic and aerobic conditions.

The aerobic and anaerobic nitrate-dependent

pathways of aromatic metabolism were further investigated by Williams and Evans (1973). These workers isolated a soil microorganism which was able to grow anaerobically on benzoate in the presence of nitrate. During anaerobic growth none of the enzymes characteristic of the aerobic pathway were detected.

Similarly, Balba and Evans (1980a) found that Desulfovibrio vulgaris was not capable of sulphate-dependent benzoate utilisation. The presence of Pseudomonas aeruginosa in the culture, however, resulted in both benzoate utilisation and sulphate reduction. The pseudomonad grew aerobically on benzoate, but nitrate would not serve as a terminal electron acceptor for anaerobic benzoate dissimilation. It was concluded that Desulfovibrio vulgaris produced small quantities of organic acids which then acted as electron acceptors for the anaerobic dissimilation of benzoate by the Pseudomonas sp. The electron donor facilitating growth of Desulfovibrio sp. was not identified.

Widdel (1980) isolated three distinct genera of sulphate reducers, Desulfococcus, Desulfonema and Desulfosarcina, which dissimilated aromatic compounds. Unfortunately the particular anaerobic pathway which was utilised for the dissimilation by the sulphate-reducing bacteria has, as yet, not been established.

(ii) *Photometabolism*

In photometabolism aromatic substrates are metabolised as a source of reducing equivalents for growth, and this can be accomplished by monocultures. Scher (1960) and Proctor and Scher (1960) demonstrated that several species of the purple non-sulphur Rhodospirillaceae were able to metabolise some aromatic substrates both anaerobically, in the presence of light, by photosynthesis, and aerobically, in the dark, by respiration.

Dutton and Evans (1967) examined a range of aromatic compounds dissimilated by anaerobically-grown Rhodopseudomonas palustris and found that there was no significant oxygen uptake with benzoate, catechol, protocatechuate or the monohydroxybenzoates. The introduction of air into cultures which were anaerobically photometabolising benzoate caused a complete cessation of benzoate utilisation. Subsequently, Dutton and Evans (1969) were able to detect, by isotope dilution methods, a number of intermediates of anaerobic benzoate metabolism by R. palustris, from which they elucidated the reduction pathway. A novel reduction mechanism was identified which resulted in the final product of pimelic acid. The key difference between the aerobic and anaerobic pathways is the reduction of the aromatic nucleus to an aliphatic cyclic acid first,

before ring fission occurs in the case of the former. Benzoate is first reduced to cyclohex-1-ene-1-carboxylic acid and subsequently to the intermediate 2-hydroxycyclohexanecarboxylic acid. Dehydrogenation followed by hydration then results in the formation of pimelic acid. Evans (1977) postulated that during photometabolism activation of benzoate to benzoyl phosphate might be necessitated before ring reduction.

Hutber and Ribbons (1983) demonstrated the involvement of coenzyme A esters in the metabolism of benzoate and cyclohexanecarboxylate by R. palustris. Studies of oxygen uptake by intact bacteria indicated that cyclohexanecarboxylate was metabolised aerobically by a β-oxidation sequence although anaerobically benzoate-grown cultures also possessed this capacity. The enzymes necessary for β-oxidation of cyclohexanecarboxylate were found to be constitutive in both anaerobic and aerobic bacteria, the only exception being an acyl-CoA synthetase which used benzoate and some alicyclic acids as substrates.

(iii) *Fermentation*

In fermentation, no external electron acceptor is required and dehydrogenation reactions of catabolism are linked to hydrogenation of an original portion of the aromatic substrate metabolised. The carbon source is degraded anaerobically by a series of reactions which

release energy by substrate-level phosphorylation. The formation of methane and CO_2 from aromatic natural products was the first observation to be made during the metabolism of benzenoid structures in anoxic environments (Tarvin and Buswell, 1934). Production of methane in this way requires the intervention of at least one bacterium coupled to a methanogenic species. This was clearly demonstrated by Ferry and Wolfe (1976) who observed that o-chlorobenzoate inhibited benzoate degradation without affecting the production of methane from acetate and proposed that benzoate was fermented to methane and CO_2 by a consortium of bacteria in which the methanogenic species served as the terminal organism of the catabolic chain. Subsequently, it was established that the methanogenic fermentation of benzoate takes place in a number of sequential stages. In the initial stage, the benzene nucleus is first reduced and then cleaved to aliphatic acids by Gram-negative facultative anaerobes. In the second stage, degradation of aliphatic acids to acetate, H_2 and CO_2 proceeds provided that a low H_2 partial pressure is maintained (McInerney, Bryant and Pfennig, 1979; McInerney and Bryant, 1981). Finally, acetate and H_2 plus CO_2 are converted to methane.

In the rumen the volatile fatty acids are absorbed by the host animal as a source of carbon and

energy. In other environments the accumulation of these compounds is an indication of the inhibition of methanogenesis (Taylor, 1982). In general, H_2, ethanol and succinate are present in either very low or undetectable concentrations (Hungate, 1967). Similarly, the lactate concentration is also characteristically low with the possible exception of fresh water sediment (Taylor, 1982). These results suggest that these compounds have a very rapid turnover and/or that they are produced *in vivo* only due to modifications of the fermentation pathways (Taylor, 1982). Hydrogen also has a rapid turnover, such that in the rumen, for example, concentrations of the order of 10^{-6} M are common (Hungate, 1967).

Originally it was thought that C_3 to C_6 volatile fatty acids and C_2 to C_6 alcohols were fermented directly to methane (Barker, 1956)) although Wolfe (1971) has since speculated that these fermentations were made by mixed cultures. Subsequently the component members of these have been designated the "hydrogen producing acetogenic bacteria" (Bryant, 1979), which form stable associations with the methanogenic bacteria (Bryant, Wolin, Wolin and Wolfe, 1967; McInerney *et al*, 1979).

The methanogens and, to some extent, the homoacetogens, which are a group of bacteria which are

able to synthesise acetic acid and utilise CO_2 as the sole electron acceptor (Schulman, Ghambeer, Ljungdahl and Wood, 1973; Zeikus, 1980b), together with other H_2 oxidisers and species which convert fermentation intermediates to precursors for the methanogens, play an important role in anaerobic decomposition of organic substrates (Zeikus, 1980b).

Mah (1982) reported that the methanogens perform two principal metabolic roles in mixed culture fermentation systems:

(i) they maintain electron flow towards proton reduction by means of hydrogen oxidation and CO_2 reduction to form methane, thereby creating a shift in reduced to oxidised end products produced by chemoheterotrophic fermentative bacteria, as well as supporting the acetogenic obligate proton reducers; and

(ii) they remove the acetate end product generated by the interspecies H_2 transfer reactions, by an aceticlastic reaction to form methane and CO_2.

1.4.1 *Methanogenic interactions*

The methanogens play a key role in the anaerobic digestion process as a result of their metabolic interactions with other anaerobic bacteria. Many of

these interactions involve an obligate interdependence such that the isolation of monocultures of the organisms involved is not readily possible.

Monocultures of methanogens were first isolated by Schnellen (1947) although many subsequent investigations have involved microbial associations which have included non methanogenic components. These studies have suggested that a variety of substrates such as ethanol, propionate and butyrate together with long-chain fatty acids and alcohols may be used as methanogenic substrates although it is now known that these compounds were in fact metabolised by the non-methanogenic components which in turn supplied the methanogens with the substrates acetate and/or H_2 and CO_2.

An example of a methanogenic association is Methanobacillus omelianskii which was shown to metabolise ethanol and CO_2 to acetate and methane (Barker, 1956). Later Bryant et al (1967) demonstrated that M. omelianskii was, in fact, a syntrophic co-culture of a heterotrophic 'S' organism and a methanogen. The 'S' organism oxidized ethanol to acetate and H_2 and the methanogen, Methanobacterium bryantii, used the H_2 to reduce CO_2 to methane. The basis of the association was that the 'S' organism was unable to ferment ethanol alone, due to inhibition by

the product H_2, whilst the methanogen was dependent on this since it could not use ethanol as a substrate. Thus, although neither organism would grow on ethanol alone, the molecule was readily metabolised to methane by the co-culture.

Since the resolution of the M. omelianskii symbiosis a variety of interacting microbial associations between methanogens and non methanogens has been described (Wolin, 1982; Bryant, 1979; McInerney and Bryant, 1980, 1981; Mah, 1982). These metabolic associations fall into two categories: those which contain fermentative bacterial components and those which contain obligate H_2-producing acetogenic bacteria.

Interactions with fermentative bacteria

This type of interaction normally results in a major shift in the fermentation products formed. In monoculture, fermentative bacteria produce reduced products such as ethanol, propionate, butyrate, lactate and succinate as well as acetate and CO_2 whereas in co-culture, these reduced products are either not detected or are present in low concentrations while significant increases in acetate and H_2 production result. Hydrogen is generally not detected although its production can be calculated from the volume of methane formed since four moles of H_2 are required to produce one mole of methane. Thus, the fermentative bacteria usually produce little

or no ethanol or lactate, considerably less propionate and butyrate, and more acetate, CO_2, and H_2 in a particular ecosystem when the methanogens are effectively utilising H_2 (Winfrey, 1984).

Interactions with obligate hydrogen-producing acetogenic bacteria

The H_2-producing acetogenic bacteria, unlike the fermentative bacteria, form obligate associations with methanogens or other H_2-consuming bacteria. These organisms catabolise various fatty acids and alcohols to acetate, H_2 and, in the case of odd-numbered fatty acids CO_2 (Winfrey, 1984). For example, Desulfovibrio sp. can ferment lactate to acetate, CO_2 and H_2 in the absence of sulphate provided that a methanogen is present (Bryant et al, 1977). Rapid use of H_2 by the methanogen makes this fermentation feasible such that sulphate-reducing bacteria no longer require sulphate as an electron acceptor (Ben-Bassat, Lamed and Zeikus, 1981).

McInerney, Bryant, Hespell and Costerton (1981a) and McInerney, Mackie, and Bryant (1981b) identified a bacterium, Syntrophomonas wolfei, in a butyrate enrichment culture which degraded even-numbered fatty acids such as butyrate, caproate and caprylate to acetate and H_2, and the odd-numbered fatty acids, valerate and heptanoate, to acetate, propionate and H_2. S. wolfei, however, could not be grown in monoculture

and was unable to use any common energy source in the absence of a H_2-oxidising species.

A variety of aromatic compounds are degraded to methane and CO_2 under anaerobic conditions in the absence of molecular oxygen (Evans, 1977). This observation led McInerney and Bryant (1981) to speculate on the existence of a H_2-producing acetogenic bacterium which has subsequently been demonstrated in co-culture with Desulfovibrio sp. and triculture with M. hungatei and Desulfovibrio sp. (Winfrey, 1984).

Unlike the fermentative bacteria, discussed above, interspecies H_2 transfer is required for these organisms to catabolise their growth substrates. Thus, without the removal of H_2 by methanogens or other H_2-oxidising bacteria many of the intermediates formed in anaerobic decomposition would not be subsequently dissimilated. Interspecies H_2 transfer is, therefore, not only beneficial to the anaerobic bacteria but also mandatory for anaerobic decomposition of organic matter.

1.4.2 Factors affecting methanogenesis

Energy sources

A few methanogenic environments, such as the rumen and sewage sludge digesters, provide relatively stable and near optimum conditions for methanogenic

bacteria. For energy generation, methanogenic species are restricted to the production of methane from simple organic compounds, probably no greater than two or three carbons in length. Their known substrates are hydrogen and CO_2, formate, methanol, acetate and mono-, di- and trimethylamines (Mah, 1982), although individual species are usually restricted to one or two of these (McInerney and Bryant, 1981).

Substrate limitation is, however, ecologically advantageous to efficient anaerobic decomposition. Methanogens in fact have a very high affinity for substrates, particularly H_2 (Zehnder, Ingvorsen and Marti, 1982) and their ability to utilise this is vital to anaerobic decomposition. Available H_2 allows efficient decomposition under changing conditions. Limitation by organic substrates enables methanogens, and, therefore, anaerobic decomposition, to respond rapidly to increases in organic substrates and therefore allows sufficient decomposition to occur in the presence of varying organic inputs (Winfrey, Nelson, Klevickis and Zeikus, 1977; Jones, Simon and Gardner, 1982; Strayer and Tiedje, 1978; Tam, Mayfield and Inniss, 1981).

Temperature

Methanogenesis occurs in anaerobic environments at temperatures between 0 and >100°C (Baross, Lilley and Gordon, 1982; Zehnder et al, 1982). Temperature signi-

ficantly affects the rate of methanogenesis *in situ* since increases stimulate formation rates in lake sediments and anaerobic digesters, where the optimal temperature is approximately 40°C (Koyama, 1963; Zeikus and Winfrey, 1976; Kelly and Chynoweth, 1981). Since freshwater sediments are characterised by much lower ambient temperatures, this suggests that *in situ* methanogenesis may be limited. This, however, represents an ecological advantage, since it enables the methanogenic populations to respond to any variations (Winfrey, 1984).

Redox conditions

Methanogens can grow only when oxygen is excluded and the redox potential is maintained at less than -200 mV, in lake sediments (Cappenberg, 1974), or below -330 mV in the rumen (Smith and Hungate, 1958).

Methanogens do, however, vary in their tolerance to oxygen. Zehnder and Wuhrmann (1977), for example, showed that a culture of *Methanobacterium arbophilicus* tolerated oxygen exposure for 4 days and still produced methane after reduced conditions were re-established. This then perhaps explains their widespread distribution in nature despite their dependence on fermentative bacteria to facilitate anaerobiosis and maintain a low redox potential (Hobson, Bousfield and Summers, 1974).

pH

According to McCarty (1964) the optimum pH for methanogenesis in anaerobic digesters is between 7.0 and 7.2. Values below 6.6 slow production rates sharply with complete cessation apparent at values below 6.2 (Winfrey, 1984). The fatty acid-producing bacteria are, however, less sensitive to low pH and as a consequence continue production until the pH drops between 4.5 and 5.0 when "souring" of the digester is apparent, which is characterised by the inhibition of the entire microbial population (Pfeffer, 1980). One exception to the pH sensitivity of methanogenesis in nature is found in acid bogs where acidophilic methanogens continue to generate methane at pH values as low as 3.0 (Kuster, 1978; Zehnder et al, 1982).

Toxic inhibitors

Many environmental pollutants can be inhibitory to methanogenesis. For example, heavy metals, alkali and alkaline earth metals, some aromatic compounds and chlorinated hydrocarbons all inhibit methane generation in anaerobic digesters (Pfeffer, 1980; van Velsen and Lettinga, 1980). Since such compounds are frequently found in wastes they can pose particular problems in anaerobic digestion. Although sulphide is used as a sulphur source by methanogens, it is inhibitory in high concentrations (Zehnder and Wuhrmann, 1977). Similarly,

ammonia, particularly the unionized form, can also inhibit methanogenesis since inhibition begins at concentrations between 100 and 200 mM if the pH is greater than 7.4. At concentrations greater than 200 mM, however, the ammonium ion becomes toxic regardless of the pH (McCarty, 1964).

Nitrate and sulphate

Various electron acceptors are known to inhibit methanogenesis. For example, nitrate and other oxidized nitrogen compounds at concentrations as low as 10 μg l^{-1} (NO_3-) have been shown to inhibit methanogenesis from acetate in sediments ((Winfrey and Zeikus, 1979).

Sulphate has a similar inhibitory effect on methanogenesis. Although freshwater sediments contain little sulphate, the addition of this electron acceptor results in a near complete inhibition of methanogenesis (Winfrey and Zeikus, 1977). Natural concentrations of sulphate also exert an inhibitory effect on methanogenesis in lake sediments where sulphate-reducing and methanogenic bacteria co-exist (Winfrey and Zeikus, 1977).

Competition for acetate and H_2 is mainly responsible for the inhibition of methanogenesis by sulphate-reducing bacteria in high sulphate-containing marine sediments. Acetate and H_2 are both metabolised by sulphate-reducing bacteria in these environments with

methyl carbon and acetate oxidized to CO_2 (Banat, Lindström, Nedwell and Balba, 1981; Winfrey and Ward, 1983).

Retention time

In addition to the factors discussed above methane fermentation in anaerobic digesters must also take into consideration the retention time of the bio-reactor. The retention time of a system expresses the volume of fluids in the reactor per volume of fluids passing into and out of the reactor per day. As the retention time decreases, a smaller percentage of the organic matter is mineralised but the amount of methane produced per volume of reactor is often increased (Varel, Isaacson and Bryant, 1977; McInerney and Bryant, 1981). There is a minimum retention time, however, below which fermentation stops, because of the washout of essential bacterial groups. The acetoclastic methanogens and obligate H_2-producing acetogens have long generation times and would be the first organisms to be washed out of the fermenter maintained under low retention time conditions. McCarty (1971) reported that polymer-degrading bacteria held in a digester at 35°C were active at retention times of as little as one day although fatty acid fermentations did not occur until the retention time was increased to 5 days.

Anaerobic digester designs have now been

developed to eliminate the need for the long retention times required to prevent washout of the slow-growing methanogens. The upflow anaerobic sludge blanket (UASB), anaerobic contact reactor and anaerobic filter all separate the biomass retention time from the wastewater retention time (Lettinga, van Velsen, Hobma, de Zeeuw and Klapwijk, 1980; McCarty, 1982; van den Berg, 1984). The microbial population is in fact retained either in a dense sludge or is attached to an inert support and only the clear treated wastewater is removed. By use of such systems, retention times have been reduced to as little as 4h.

1.5 Enrichment and Isolation of Interacting Microbial Associations

1.5.1 *Enrichment*

Generally, enrichment involves the transfer of soil, sewage or some other suitable inoculum with a large and diverse population of microorganisms into a selective medium followed by incubation of the culture under such conditions of, for example, temperature and pH which favour growth of the desired species.

Several reviews have been made on the wide variety of enrichment systems available to the microbiologist. Notable amongst these have been those of Hutner (1962), who considered nutritional aspects,

Stanier (1953) methods, Veldkamp (1965, 1970, 1977), history, review and chemostat studies, Pochon and Tardieux (1967), methods for soil bacteria, and Parkes (1982), laboratory systems. The majority of enrichment methods have been developed for aerobic species while anaerobes have largely been neglected.

Parkes (1982) considered two types of enrichments: closed and open. In closed enrichments a fixed quantity of enrichment material is added at the begining of the experiment, whereas in open enrichments fresh quantities are continuously added. Veldkamp (1970) pointed out that the enrichments should contain enough water to make proliferation possible, the media should contain the necessary growth elements needed, and, most important, the organism(s) to be enriched should be present in the inoculum.

The type of anaerobic closed enrichment culture most frequently used is the batch flask liquid culture in which the inoculum is taken from an anoxic environment such as an anaerobic digester, aquatic sediment, the rumen or landfill. A number of environmental conditions can be specified in this type of system such as medium composition, pH, Eh, temperature and light. Closed cultures such as these have been successfully used in the past to enrich nitrate reducers, sulphate reducers, aromatic compound-degrading organisms and

methanogens. For example, this approach was used by Otto, Hugenholtz, Koning and Veldkamp (1980), to enrich and isolate the nitrate-reducing bacterium Pseudomonas stutzeri, by Widdel and Pfennig (1977), to isolate the acetate-utilising sulphate-reducer Desulfotomaculum acetoxidans, by Bache and Pfennig (1981), to isolate the demethoxylating bacterium Acetobacterium woodii, and by Kaiser and Hanslemann (1982a, b) to enrich and isolate an anaerobic microbial association capable of degrading syringic acid to methane and CO_2.

Similarly, Balba (1978) used a batch culture with gas collection system to enrich benzoate-metabolising species under anoxic conditions.

In all closed culture experiments nutrients become exhausted and metabolic products accumulate thus competitive selection of microbes in this instance is based solely on their maximum specific growth rate (μ max).

Conversely, in open culture enrichments, since fresh nutrients are continuously supplied and metabolic products continuously removed, a totally different set of conditions are operable. Two types of open enrichment systems have been recognised by Schlegel and Jannasch (1967), the hetero- and homo-continuous culture. An example of the hetero-continuous culture is the refuse column described by Senior and Balba (1984)

which was used to enrich and isolate an interacting microbial association from landfill. A second type of hetero-continuous culture is the gradostat (Wimpenny, Lovitt and Coombs, 1983) although, as yet, this has not been used to study anoxic environments. Two types of homo-continuous culture are the turbidostat and chemostat with the latter having been more commonly used in enrichment studies.

Parkes (1982) listed several advantages of using chemostat enrichments:

1. the enrichment is reproducible, by keeping the conditions throughout constant;
2. the dilution rate of the system determines the specific growth rate of the enrichment organism(s);
3. the constant displacement of metabolites and cells prevents the accumulation of potentially inhibitory products;
4. the system is ideally suited for enrichment of microbial associations which are ubiquitous in the environment; and
5. the method enables the long-term effects of potentially toxic compounds to be investigated.

Chemostat cultures have been used to a limited extent in anaerobic enrichment studies. For example, van den Berg (1977) used acetic acid-limited open

cultures, inoculated with sludge from a pear waste digester, to enrich and isolate methanogenic bacteria. Similarly, Sinerise and Pirt (1977) used this method to enrich and isolate a microbial association, from a sewage sludge digester, which catabolised glucose to methane.

1.5.2 *Isolation of microbial associations and component species*

The diversity of microorganisms encountered in nature illustrates the diversity of ecological niches that harbour these species (Harder and Dijkhuizen, 1982). The mineralisation of organic matter in sediment and sewage sludge is usually the result of a sequence of processes in which the products of one metabolic group of organisms serve as substrates for others. The interacting association thus formed therefore consists of microbes which are highly dependent on each other's activities (Laanbroek and Veldkamp, 1982; Slater and Bull, 1982).

Enrichment cultures, however, particularly continuous culture enrichments, frequently result in the isolation of microbial associations in which a variety of interactions are apparent.

Many compounds including cyclohexane (Stirling, Watkinson and Higgins, 1976), Dalapon (Senior, Bull and

Slater, 1976), ligno-aromatic compounds (Balba and Evans, 1980b; Healy and Young, 1978, 1979; Kaiser and Hanselmann, 1982a; Grbic-Galic and Young, 1985), dodecyclohexane (Feinberg, Ramage and Trudgill, 1980), Lontrel (Slater and Lovatt, 1982), veratryl-glycerol--guaiacyl ether and guaiacoxyacetic acid (Chen, Supanwong, Ohmiya, Shimizu and Kawakami, 1985), and whey (Chartrain and Zeikus, 1986a, b) have been shown to be degraded by microbial associations.

Isolation of the component species of anaerobic interacting microbial associations has focussed essentially on the methane-producing bacteria (Sleat and Robinson, 1984) although for many years little was known about these species due to the inadequate isolation and culturing techniques for strict anaerobes. Hungate (1950), however, developed a novel technique to isolate and quantify strictly anaerobic bacteria from the rumen. This technique has since been used as a standard procedure for the isolation and cultivation of methanogens and other fastidious anaerobes although several modifications or alternatives of the original procedure have since been described (Bryant, 1972; Daniels and Zeikus, 1975; Balch, Fox, Magrum, Woese and Wolfe, 1979; Bhatnagar, Henriquet and Longin, 1983). With the development of anaerobic culture chambers, however, these procedures are now essentially obsolete (Edwards and McBride, 1975).

Severe problems may be encountered in attempting to characterise microbial associations, particularly where the component species are very similar in terms of their morphology, physiology and biochemistry. The medium used in the monoculture isolation technique must ensure that the component species with specific nutritional requirements, which are normally met by other members of the association, are able to grow. Besides the medium composition parameters such as pH, Eh, temperature and light must be carefully considered.

Despite these problems isolation of the component species of anaerobic interacting associations has been accomplished by a number of workers. For example, Ferry and Wolfe (1976) isolated Methanobacterium formicicum and Methanospirillum hungatei together with a bacterium similar in appearance to Pseudomonas PH-1 (Taylor, Campbell and Chinoy 1970) which grew aerobically but not anaerobically on benzoate, from a methanogenic enrichment culture. Balba and Evans (1977) isolated a facultative, non-sporulating, gram-negative bacterium which grew aerobically on p-hydroxybenzoate but not on benzoate.

McInerney et al (1981a) isolated and described bacterial species which were capable of oxidizing aliphatic acids to hydrogen, CO_2 and acetate in anaerobic syntrophic association with either sulphate-

reducing or methanogenic bacteria. An association of sulphate-reducing and methanogenic bacteria was also isolated by Abram and Nedwell (1978) from saltmarsh sediment. Ward (1978) isolated a methanogenic bacterium Methanosarcina, together with several obligately anaerobic, non-methanogenic bacteria, from an association isolated from a hot spring algal bacterial mat. Kaiser and Hanselmann (1982a) isolated an anaerobic microbial association, which contained 4 to 5 different species, capable of degrading syringic acid to methane and CO_2, although they did not attempt to characterise the component species. Chartrain and Zeikus (1986b) characterised the bacterial trophic populations and prevalent species which degraded the lactose component of whey to methane and CO_2 under open culture conditions and found that three dominant bacterial trophic group populations were present. The three prevalent species which utilised lactose were identified as Leuconostoc mesenteroides, Klebsiella oxytoca and Clostridium butyricum, whilst Clostridium propionicum and Desulfovibrio vulgaris were the dominant lactate-consuming, H_2-producing acetogenic bacteria. Methanosarcina barkeri and Methanothrix soehngenii were identified as the dominant acetate-utilising methanogens and Methanobacterium formicicum was the prevalent H_2-utilising methanogen.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Source of Inoculum

Anoxic freshwater sediment was sampled from Cumbernauld Burn, Scotland and then rapidly transported to the laboratory and stored in an anaerobic chamber (Forma Scientific Anaerobic Chamber, Model 1024). The sediment was used in closed culture enrichments to isolate interacting microbial associations capable of catabolising lignin-related aromatic compounds.

2.2 Growth Media

2.2.1 Basic mineral salts media

Two basic mineral salts media, A and B were used.

Medium A contained the following (g l^{-1} glass-distilled water): NH_4Cl, 0.9; K_2HPO_4, 1.5; $NaH_2PO_4.2H_2O$, 0.85; $MgCl_2.6H_2O$, 0.2; $NaHCO_3$, 0.5; vitamins solution, 5 ml; trace element solution, 9 ml; Na_2CO_3 (8% w/v), 2.5 ml; $FeCl_3$ (10% w/v), 0.03 ml and $Na_2S.9H_2O$ (100 g l^{-1}), 0.15 ml.

The vitamins solution contained (mg l^{-1} glass-distilled water): biotin, 5.0; folic acid, 2.0; pyridoxin HCl, 10.0; riboflavin, 5.0; thiamin, 5.0; nicotinic acid, 5.0; pantothenic acid, 5; cobalamin,

0.1; p-aminobenzoate, 5.0; and thioctic acid, 5.0.

The trace element solution contained the following (g l^{-1} glass-distilled water): EDTA disodium salt, 1.91; $MgSO_4$, 3.0; NaCl, 1.0; $MnSO_4$, 0.5; $FeSO_4$, 0.1; $CoCl_2$, 0.1; $ZnSO_4$, 0.1; $AlK(SO_4)_2$, 0.1; H_3BO_3, 0.1; and Na_2MoO_4, 0.1.

The medium was supplemented with aromatic compounds to a final concentration of 2 mM and the pH adjusted to 7.0 ± 0.1 with concentrated HCl or NaOH solution prior to filter sterilisation through a Sartorius membrane filter (0.2 µm, SM11307) under an atmosphere of oxygen-free nitrogen (OFN, British Oxygen Company). The medium was aseptically distributed into sterile bottles and overgassed with OFN, after trace oxygen removal with an oxygen trap (Phase Separation Ltd.), to give an Eh of less than -40 mV. The bottles were prewarmed to 30°C prior to inoculation.

Medium B was adapted from Kaiser and Hanselmann (1982a) and contained the following (g l^{-1} glass-distilled water): NH_4Cl, 0.25; NaCl, 1.0; KH_2PO_4, 0.2; $MgCl_2.6H_2O$, 0.4; KCl, 0.5; $CaCl_2.2H_2O$, 0.15; and HCl(1.0M), 5.0 ml. The following additions were made to the autoclaved (15 lbs psi, 121°C, 20 minutes), cooled medium from filter sterilised stock solutions (litre^{-1}); acidic trace elements, 1.0 ml; $NiCl_2.6H_2O$ (1mM), 1.0 ml; $NaHCO_3$ (1M), 20 ml; basic trace elements, 1.0 ml;

vitamins, 1.0 ml; resazurin (5 mM), 1.0 ml.

The acidic trace elements contained the following (mg l^{-1}): $FeCl_2.4H_2O$, 1491; H_3BO_3, 61.8; $ZnCl_2$, 68.1; $AlCl_3$, 50; $MnCl_2.4H_2O$, 90; $CuCl_2.2H_2O$, 17; $CoCl_2.6H_2O$, 238; and $NiCl_2.6H_2O$, 23.8 and were dissolved in 50 mM HCl.

The basic trace elements were dissolved in 100 mM NaOH and contained the following (mg l^{-1}): $Na_2MoO_4.2H_2O$, 48.4; $NaSeO_3.H_2O$ (31% Se), 2.55; and $Na_2WO_4 . 2H_2O$, 3.3

The vitamins solution contained (mg l^{-1}): biotin, 10; pyridoxin.HCl, 20; thiamin-HCl, 20; riboflavin, 30; and cyanocobalamin, 20; which were dissolved in $NaHCO_3$ (16.8 mg l^{-1}) which contained $(NH_4)_2\ SO_4$ (4 mg l^{-1}) to give a final pH of 6.6.

The medium was overgassed with OFN and 2.5 ml of $Na_2S.9H_2O$ (100 mM) aseptically added together with the appropriate carbon source and sodium dithionite (52.5 mg l^{-1}) under a continuous stream of OFN.

The C-sources (aromatic acids) were prepared in deaerated 0.5 M NaOH, sterilised by filtration and stored under an oxygen-free gas phase in the dark at 4°C.

The final pH of the medium was 7.0 ± 0.1.

Solidified medium was prepared by using double-strength basic mineral salts medium and

double-strength agar (2.4% w/v, Agar No. 3, Oxoid). The agar solution was autoclaved (15 lbs psi, 121°C, 20 minutes) after steaming and cooled to 50°C before aseptically mixing with the prewarmed (45°C) medium. The plates were poured inside the anaerobic chamber and maintained under an oxygen-free atmosphere (CO_2, 5%; H_2, 10%; N_2, 85%) overnight prior to use.

2.2.2 Media for cultivation of methanogenic bacteria

Three types of media were used to culture methanogenic species: acetate, H_2/CO_2 and complex medium.

Acetate and H_2/CO_2. The medium used was the same as that described in 2.2.1 (A) with the exception that the carbon source was either acetate (10 mM) or an atmosphere of H_2/CO_2 (80:20). The medium was supplemented with either pre-sterilised mefoxin (Merck, Sharp and Dohme), 100 $\mu g\ ml^{-1}$, or phenol, 2mM.

Complex medium. This medium contained the same basic mineral salts as those described in 2.2.1 (A) supplemented with($g\ l^{-1}$)yeast extract, 2; peptone, 4; meat extract, 1; acetate, 4; and $NiCl_2.6H_2O$ (1 mM), 1 ml. The medium was autoclaved at 15 lbs psi (121°C) for 20 minutes.

2.2.3 Media for cultivation of sulphate-reducing bacteria

Two media, A and B, were used for the isolation and cultivation of sulphate-reducing bacteria.

Medium A was the same as that described by Widdel (1980) and contained the following (g l^{-1} glass-distilled water): Na_2SO_4, 3.0; KH_2PO_4, 0.2; NH_4Cl, 0.25; NaCl, 1.0; $MgCl_2.6H_2O$, 0.4; KCl, 0.5; and $CaCl_2.2H_2O$, 0.15. The medium was autoclaved for 20 minutes at 15 lbs psi (121°C), cooled to room temperature and placed inside the anaerobic chamber prior to aseptic addition of the following components (ml l^{-1}) from sterile stock solutions: trace elements, 1; vitamins, 1; selenite and tungstate, 30; and sulphide, 3. The medium pH was poised at 7.2 ± 0.1 with either dilute sulphuric acid or sodium carbonate solution.

The trace element solution used contained the following (mg l^{-1} glass-distilled water): $FeCl_2.4H_2$, 1500; $CoCl_2.6H_2O$, 190; $MnCl_2.4H_2O$, 100; $ZnCl_2$, 70; H_3BO_3, 62; $Na_2MoO_4.2H_2O$, 36; $NiCl_2.6H_2O$, 24; $CuCl_2.2H_2O$, 17; and HCl (25% v/v), 10 ml. Care was taken to ensure that the ferrous chloride was first dissolved in the hydrochloric acid to ensure that the insoluble iron (III) hydroxides, which are normally present in Fe(II) salts as oxidation products, were

solubilised. The medium was autoclaved at 15 lbs psi (121°C) for 20 minutes.

The selenite and tungstate solution used contained the following (mg l^{-1} glass-distilled water): NaOH, 500; $Na_2SeO_3.5H_2O$, 3; and $Na_2WO_4.2H_2O$, 4 and was sterilised by autoclaving at 15 lbs psi (121°C) for 20 minutes.

The bicarbonate solution was prepared by dissolving 84 g of $NaHCO_3$ in 1 litre of sterilised glass-distilled water saturated with CO_2.

The sulphide solution was prepared by dissolving 120 g of $Na_2S.9H_2O$ in 1 litre of glass-distilled water. The resulting solution was then autoclaved, in a closed bottle, under a head space atmosphere of OFN, for 20 minutes at 15 lbs psi (121°C).

Medium B: The second medium used was the same as that described by Postgate (1979) Medium B, and contained the following (g l^{-1} tap water): KH_2PO_4, 0.2; NH_4Cl, 1.0; $CaSO_4$, 1.0; $MgSO_4.7H_2O$; sodium lactate, 3.5; yeast extract, 1.0; ascorbic acid, 0.1; thioglycollic acid, 0.1; $FeSO_4.7H_2O$, 0.5.

The pH was adjusted to between 7.0 and 7.5 and autoclaved for 20 minutes at 15 lbs psi (121°C).

This medium was essentially used for isolation and enumeration procedures.

2.3 Cultivation of Microorganisms

2.3.1 *Closed culture*

Closed cultures were made in 150 ml serum bottles (MacFarlane Robson Ltd., U.K.) in which 10 ml of inoculum were added to pre-reduced 90 ml mineral salts medium supplemented with 2 mM of the appropriate aromatic substrate. The bottles were closed with Suba-seals prior to overgassing for 15 minutes with OFM, after trace oxygen removal with an oxygen trap. The bottles were incubated without shaking at 30°C in the dark.

2.3.2 *Open culture*

Continuous cultures were established by conversion of closed culture vessels into chemostats by the method described by Al-Sarraj (1983) with the actual chemostat design adapted from Veldkamp and Kuenen (1973).

2.3.3 *Culture maintenance*

Cultures of interacting microbial associations were maintained by removal of 200 ml aliquots of culture fluid at weekly intervals and replacement of this with the same volume of fresh medium which had been supplemented with 4 mM of the appropriate carbon source.

2.4 Analytical Methods

2.4.1 Substrates

Quantitative and qualitative analyses of aromatic substrates were made with a UV scanning spectrophotometer (Pye Unicam PU8800). Before analysis, culture supernatant samples were centrifuged at 10,000 x g for 10 minutes, in a MSE centrifuge, and diluted with the appropriate volume of basic mineral salts medium. The characteristic UV absorption spectra of the different aromatic compounds were recorded between 320 and 200 nm.

2.4.2 Extraction and HPLC/TLC identification of aromatic compounds

500 ml volumes of culture supernatant were first centrifuged at 10,000 x g for 10 minutes and then extracted three times with 300 ml aliquots of anhydrous diethyl ether. The combined extracts were dried over anhydrous Na_2SO_4 and then evaporated to dryness under vacuum with a rotary evaporator (Gallenkamp) at 35° to 40°C.

For acidic compounds, culture supernatants were adjusted to pH2 with concentrated sulphuric acid and extracted as above.

Dried extracts were dissolved in 1 ml of methanol for High Performance Liquid Chromatography

(HPLC) analysis or in 1 ml of anhydrous diethyl ether for Thin Layer Chromatography (TLC) studies.

High performance liquid chromatography (HPLC)

1 µl aliquots of extract were injected via a valco valve (10 µl loop) and separated on a reversed phase column (Lichrosorb RP-8, 10 µm Merck). The mobile phase contained acetonitrile and methanol in degassed aqueous formic acid (10:30:70 v/v) in which the final concentration of formic acid was 5 mM. The flow rate (1 ml min^{-1}) was maintained by means of a piston pump (Varian LC5010) and the column effluent was monitored with an UV detector (Varian UV-50) at a fixed wavelength of 260 nm. Calibrations were made with 1 mM solutions of Analar grade standards which were re-crystallised for higher purity.

Thin layer chromatography (TLC)

Samples were chromatographed on precoated TLC selica gel plates (Kieselgel 60.F254; E. Merck AG, Darmstadt, FDR), together with purified aromatic standards (1 mM), with a two solvent system in which the first one contained benzene, dioxan and acetic acid at volume ratios of 90:25:10, while the second contained benzene, ethyl acetate and formic acid at volume ratios of 85:15:1.

Spot visualisation: acidic fractions (yellow spots against a blue background) were visualised by

spraying the plates, after drying, with a solution of bromocresol green, 0.2% (w/v), in ethanol. Other organic compounds (brown spots against a white background) were visualised by exposing the plates to iodine vapour in a closed chromatography tank for 5 minutes. Finally, unsaturated compounds were detected by their capacity to absorb ultra violet light.

Visualised spots were identified by comparison of RF values with known standards.

2.4.3 Short-chain fatty acids

Short-chain fatty acids were analysed by gas liquid chromatography (GLC) with a Perkin Elmer Sigma 115 GLC, fitted with a flame ionisation detector (FID). A stainless steel column (length 2m, internal diameter 2mm) was filled with 5% neopentyl glycol sebacate + 1% H_3PO_4 on Anakrompolyester (80-100 mesh). The flow rate of the carrier gas (nitrogen) was set at 40 ml min^{-1} and the injector and detector temperatures were maintained at 180°C. The column was maintained initially at 100°C for 5 minutes after which the temperature was increased to 130°C at a rate of 40°C min^{-1}. Samples from the cultures were either injected directly on the column or after acidification of 0.9 ml aliquots with 0.1 ml formic acid (Aristar, B.D.H.) prior to injection (1 µl). Quantification and peak identification were made with 10

mM standards of the C_2 to C_6 volatile fatty acids.

2.4.4 Methane

The methane concentrations of culture gas phases were determined by GLC analysis under the same conditions as those described for the short-chain fatty acids with the exceptions that the detector and oven temperatures were 150° and 80°C respectively. 50 µl of gas samples were injected and pure methane (BOC) was used as standard. The temperature and atmospheric pressure were recorded and the methane concentrations converted to molarity at standard temperature and pressure (STP). Total methane concentrations were calculated as the sum of the gas present in the head space and the gas dissolved in the liquid medium, by reference to the solubility of methane at 1 bar (10^5 Pa) and 35°C of 0.00173 g 100 ml^{-1}.

2.4.5 Sulphate assay

Culture samples were acidified then gassed with OFN to remove any soluble sulphides after which 10 ml aliquots were dispensed into 150 ml conical flasks followed by 0.5 ml of the conditioning reagent which contained a mixture of 30 ml concentrated HCl, 30 ml distilled water, 100 ml 95% ethyl- or isopropyl alcohol, 75g NaCl and 50 ml of glycerol. 1.0g of barium chloride

crystals (20-30 mesh) was then added to each after which the flasks were stirred gently for exactly 1 minute and the optical density determined at 420 nm after exactly 4 minutes.

The sulphate concentration was determined by reference to a standard curve (1 to 10 mg 100 ml^{-1}).

2.4.6 Sulphide assay

Acid-soluble sulphide was determined by acidification of 10 ml aliquots of culture supernatant in closed flasks with concentrated HCl. The evolved sulphide was trapped in zinc acetate (1% w/v) as zinc sulphide over a period of ≥30 minutes. 10 ml of 0.1 N iodine solution were then added to the zinc sulphide precipitate followed by 1 ml of concentrated HCl. Finally, the solution was back titrated with standard sodium thiosulphate (0.1 N) with starch (1g 100 ml^{-1}) as the indicator. The total sulphide was then calculated as follows:

Since 1.0 ml of 0.1 N Iodine = 1.6 mg sulphide

$$[HS^-] = \frac{(\text{ml iodine} - \text{ml } Na_2S_2O_3)\ 160(\text{mg } l^{-1})}{\text{ml sample}}$$

Gaseous H_2S (unionized H_2S) from the chemostat cultures was also trapped as zinc sulphide by bubbling

the gas exhaust through zinc acetate (1% w/v) and the concentration of sulphide determined as before.

2.4.7 pH determinations

Culture pH values were routinely determined by use of a Pye Unicam pH meter (Pye Unicam PW9418) fitted with a Pye Unicam electrode.

2.4.8 Biomass determinations

Optical densities

Growth was followed by measuring culture optical densities (OD) at 620 nm, with a Pye Unicam 8800 spectrophotometer, against basic mineral salts medium blanks.

Dry weights

Dry weights were determined on 50 ml aliquots of culture. The samples were acidified to between pH 2.5 to 3.0 with HCl (6M) to remove any sulphide precipitate and filtered through acetate filters (Millipore, 0.22 µm) which had been previously dried and weighed. After twice washing with 25 ml volumes of distilled water the filters were again dried to constant weight at 80°C.

2.4.9 *Enumerations of methanogenic and sulphate-reducing bacteria by the Most Probable Number technique*

Enumerations of methanogenic and sulphate-reducing bacterial populations utilising acetate and/or H_2/CO_2 were made on 1 ml aliquots of either the veratric or syringic acid-catabolising cultures after first serially diluting (10^{-1}-10^{-10}) with sterile saline solution (0.8% w/v NaCl). Subsequently, 1 ml samples of each dilution were individually added to 9 ml of basic mineral salts medium (2.2.2) for the enumeration of methanogens and Postgate's medium (B) (Postgate, 1979) for the sulphate-reducing bacteria. 5 serum bottles (25 ml) were used for each dilution and each medium and were closed with Suba-seals prior to overgassing for 5 minutes with H_2/CO_2 (80:20). The bottles were incubated at 30°C for 2 weeks in the case of the sulphate-reducing bacteria and 3 to 4 weeks for the methanogens.

The results for the methanogens were interpreted on the basis of methane production in the head space of the bottles, and for the sulphate-reducing bacteria on the appearance of a black precipitate. 95% confidence limits were used in the estimation of the Most Probable Number.

CHAPTER THREE

EVALUATION OF ACID, ALKALI AND TEMPERATURE TREATMENTS AND METHANOGENIC FERMENTATION OF CHEMICAL HYDROLYSATES

Research into the development of alternative fuels and chemical feedstocks has led to investigation of a variety of raw materials including lignocellulose. As discussed earlier (1.3.1) thermochemical pretreatment of lignocellulose has been reported to increase its biodegradation significantly both at high and low pH. Of these, methods involving the use of alkali and acids such as NaOH and H_2SO_4, have been most widely used.

The objectives of this study were to evaluate the potential for utilising NaOH and H_2SO_4 pretreatments under conditions of ambient temperature, steam and 121°C (15 lbs psi) to increase biodegradability and subsequent methane production from lignocellulosic material.

3.1 Acid, Alkali and Temperature Treatment

3.1.1 *Substrate*

The sawdust used in this study was from Scots Pine (Pinus sylvestris) which had been ground with a pin mill (Kek Ltd., Cheshire) to a particle size of <40 mesh prior to drying to constant weight at 100°C.

3.1.2 Analysis

Both the untreated and treated sawdust were assayed for lignin and ash content as follows:

Lignin was determined by use of the USSR standard method described by Pearl (1967). 1 g of dried sample was treated with 10 ml of hot demineralised water and allowed to stand for 15 minutes with occasional shaking. The hydrolysis was then made with 25 ml of 86% (v/v) H_2SO_4 for 3 h at ambient temperature, after which the preparation was diluted with 250 ml of demineralised water and boiled for 5 minutes. The resulting, cooled, mixture was then filtered through a Gooch crucible and the granular precipitate of lignin washed with demineralised water before drying to constant weight at 100°C. Finally, the weight of the extracted dried lignin was determined.

The ash content was determined according to the method of The Association of Official Analytical Chemists (1975).

The remaining fraction was assumed to be mainly cellulose, hemicellulose and related compounds and was quantified by subtracting the sum of the weight of the lignin and ash components from the weight of the dried sawdust.

3.1.3 *Physico-chemical hydrolysis*

Sodium hydroxide and H_2SO_4 were selected for the treatment of sawdust. In all, seven different concentrations of NaOH (0.1, 0.5, 1.0, 5.0, 10.0, 25.0 and 50.0% w/v) were used. Similarly, the same concentrations of H_2SO_4 were used with the addition of a final concentration of 72% (v/v). Three different temperatures: ambient (25 ± 2), 100 and 121°C were used. It must be noted, however, that the final temperature was reached by the use of autoclaves. Thus an additional variable of pressure (15 lbs psi) was also introduced. Control treatments (water) were also used at each of the temperatures.

1 g samples of ground, dried sawdust were placed in Erlenmeyer flasks (150 ml) and 10 mls of each concentration of NaOH and H_2SO_4 were individually added to triplicate flasks. The flasks were then sealed with Suba-seals (Gallenkamp) prior to incubation at the three different temperatures. Triplicate flasks were then sampled at discrete time intervals and treated as follows: the preparations were diluted with distilled water, neutralised to pH 7.0 with either 6 N NaOH or 6 N H_2SO_4, filtered through filter paper (Whatman No. 1) in Buchner funnels under vacuum, and washed with 200 ml distilled water. The residues were then dried to constant weight at 100°C. Finally, solubilisation was

determined by measuring the disappearance of insoluble dry matter compared with the distilled water controls.

3.1.4 Results and discussion

The sawdust used in this study was found to contain (% by weight): lignin, 27.8 and ash, 2.8, with the remainder (69.4%) composed of cellulose and hemicellulose together with components such as resins, phenolics, flavenoids, pectin and tannins.

The disappearance of insoluble dry matter when samples of sawdust were individually treated with NaOH and H_2SO_4 at various concentrations between 0.1 and 50% w/v and v/v respectively at ambient temperature for various time intervals is shown in Figures 5 and 6 with the results of heat treatments in Tables 2 and 3.

From Figures 5 and 6 it can be seen that after one hour for all the treatments, including the distilled water controls, significant solubilisation was apparent. This was possibly due to the disappearance of the readily-soluble materials present in the sawdust. Subsequently, solubilisation continued although the rates for all treatments were much less dramatic.

From Figure 5 it can be seen that even after 120 h incubation at ambient temperature, solubilisation continued in the presence of 50% and, to a lesser extent, 0.1, 0.5 and 1% (w/v) NaOH. Although, in general terms,

Figure 5. Effects of Increasing Concentrations of NaOH on Solubilisation of Pine Sawdust, after Grinding, at Ambient Temperature, H_2O (o——o), 0.1 (●——●), 0.5 (□——□), 1.0 (■——■), 5.0 (△——△), 10.0 (▲——▲), 25.0 (◇——◇) and 50.0% (◆——◆). Values Represent Means of Triplicate Determinations

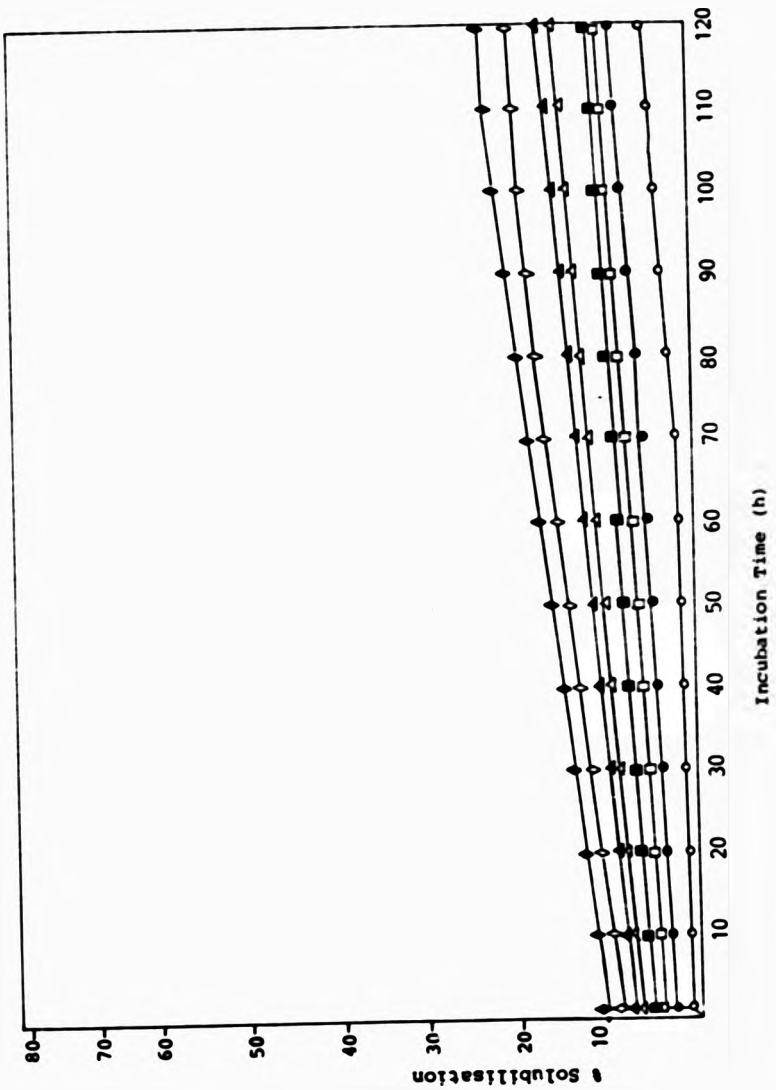

% solubilisation
10
20
30
40
50
60
70
80
10
20
30
40
50
60
70
80
90
100
110
120
Incubation Time (h)

Figure 6. Effects of Increasing Concentrations of H_2SO_4 on Solubilisation of Pine Sawdust after Grinding at Ambient Temperature, H_2O (o——o) 0.1 (●——●), 0.5 (□——□), 1.0 (■——■), 5.0 (Δ——Δ), 10.0 (▲——▲), 25.0 (◊——◊), 50.0 (◆——◆) and 72.0% (●——●). Values Represent Means of Triplicate Determinations

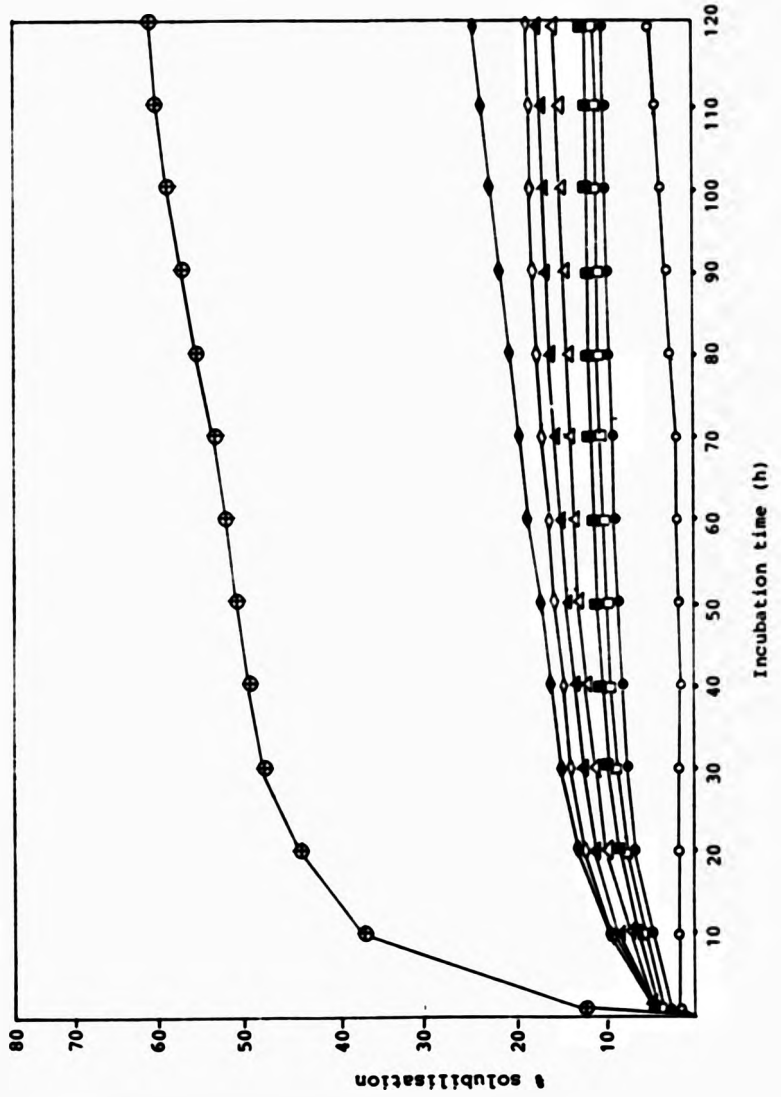

% solubilisation
10
20
30
40
50
60
70
80
Incubation time (h)
10
20
30
40
50
60
70
80
90
100
110
120

Table 2. Effects of Increasing Concentrations of NaOH and Heat Treatments on the Solubilisation of Pine Sawdust After Grinding. Values Shown Represent Means of Triplicate Determinations.

NaOH Concentrations	Percentage Solubilisation					
	Steam			121°C (15 lbs psi)		
% (w/v)	15 min	60 min	change (%)	15 min	60 min	change (%)
0.0 (H_2O)	1.7	4.2	2.5	6.0	9.2	3.2
0.1	9.6	7.6	-(2.0)	7.4	12.0	4.6
0.5	12.1	13.5	1.4	13.9	14.4	0.5
1.0	14.2	14.8	0.6	20.0	16.2	-(3.8)
5.0	17.0	19.8	2.8	19.7	24.1	4.4
10.0	18.2	22.1	3.9	19.6	26.3	6.7
25.0	19.4	24.1	4.7	20.2	28.5	8.3
50.0	23.1	29.7	6.6	31.6	17.6	-(14.0)

Table 3. Effects of Increasing Concentrations of H_2SO_4 and Heat Treatments on the Solubilisation of Pine Sawdust After Grinding. Values Shown Represent Means of Triplicate Determinations.

H_2SO_4 Concentrations	Percentage Solubilisation					
	Steam			121°C (15 lbs psi)		
% (v/v)	15 min	60 min	change (%)	15 min	60 min	change (%)
0.0 (H_2O)	1.7	4.2	2.5	6.0	9.2	3.2
0.1	3.7	4.6	0.9	11.0	15.4	4.4
0.5	3.9	10.0	6.1	17.2	24.3	7.1
1.0	3.9	19.9	16.0	19.9	25.6	5.7
5.0	9.8	26.0	16.2	27.4	34.2	6.8
10.0	14.6	26.1	11.5	30.3	38.8	8.5
25.0	13.9	32.1	18.2	35.2	44.7	9.5
50.0	23.6	34.0	10.4	40.0	51.9	11.9
72.0	39.0	44.0	5.0	52.2	52.1	-(0.1)

the greater the concentration of NaOH the higher the percentage solubilisation there did not appear to be any direct correlation between these two. A possible explanation for this, however, is not readily obvious.

In the presence of H_2SO_4 at concentrations of up to 25% (v/v) a similar picture was obtained (Figure 6) although solubilisation continued in all the treatments even after 120 h incubation. Although no direct correlation between H_2SO_4 concentration and percentage solubilisation was again apparent, a dramatic increase in solubilisation (both in terms of rate and percentage) resulted when the concentration was increased from 50 to 72% (v/v). This was possibly due to the fact that cellulose is not completely hydrolysed at concentrations below 65% (Sarkanen and Ludwig, 1971).

No direct comparisons between the effects of the two treatment types could be made for two reasons. Firstly, the same concentrations of acid and alkali were not used, and, secondly, the two chemicals have differing solubilisation effects. For example, acid treatment results in the hydrolysis of carbohydrates to leave the insoluble lignin fraction, while alkaline treatment results in fibre swelling, with some solubilisation of lignin and carbohydrates (Knappert et al, 1980; Pavlostathis and Gossett, 1985).

The results of the addition of steam in the

presence of NaOH are presented in Table 2 from which it can be seen that significant solubilisation was apparent, with the exception of the water controls, after 15 minutes, after which the rates slowed particularly at the lower concentrations. Once more as the concentration of NaOH was increased solubilisation also increased although no correlation was apparent. With the exception of the lowest concentration (0.1%) of NaOH, extended treatment from 15 to 60 minutes resulted in increased solubilisation although there was no distinct trend in the increase. Together with the process of solubilisation NaOH may be utilised by saponification of uronic and acetyl ester by reaction with free carboxyl groups, and for neutralisation of acidic products formed from alkaline degradation of cellulose, hemicellulose and lignin (Pavlostathis and Gossett, 1985). The same workers also reported that the effect of alkali treatment, as observed here, was not uniform and postulated that residual alkali could be used to predict treatment effect provided that the concentration used was in excess.

Table 2 shows the results of the treatment of sawdust at 121°C (15 lbs psi) for 15 and 60 minutes in the presence of NaOH. High percentage solubilisation with all the treatments, including the water control, was apparent after 15 minutes and, with the exception

of the 0.1% NaOH concentration, increased solubilisation resulted compared with the steam-treated preparations. The increases observed could possibly be attributed to the removal of extractive materials such as protein, tannin, pectin and possibly some of the hemicellulose. Extended treatment to 60 minutes resulted in rather variable results since concentrations of 0.1, 0.5, 5.0, 10.0 and 25.0% (v/v) were characterised by increased solubilisations whereas decreases were observed at the concentrations of 1 and 50% (v/v). With the exception of the final concentration, however, treatment by autoclaving resulted in elevated solubilisation compared with steam treatment. The one discrepancy could possibly be attributed to repolymerisation of lignin monomers in the presence of 50% (v/v) under the conditions of 121°C and 15 lbs psi.

The energy requirements for steaming and autoclaving were not calculated but, according to Pavlostathis and Gossett (1985), if no treatment is applied the inputs required for the digester heating, mixing and grinding are higher than the outputs, although they reported that this economic analysis of the overall process could be misleading.

As discussed earlier (1.3.1), the alkali treatment of sawdust results in the removal of lignin and also increases the surface area by swelling and

altering the component crystalline and amorphous structures (Han and Callihan, 1974; Carr and Doon, 1984).

The results of the addition of steam in the presence of H_2SO_4 are presented in Table 3, from which it can be seen that the lower concentrations (0.1, 0.5 and 1.0%) showed very little difference in their effects on solubilisation after steaming for 15 minutes, while with the higher concentrations solubilisation increased as the concentration increased. After 60 minutes, however, significant solubilisation was apparent with all the concentrations used. The solubilisation increase recorded at low concentrations of H_2SO_4 may possibly be attributed to the hydrolysis of the hemicellulose which has been reported by many investigators to be easily removed (Cowling, 1975; Knappert et al, 1980; Pavlostathis and Gossett, 1985; Gonzalez, Lopez-Santin, Caminal and Sola, 1986).

Table 3 summarises the results of sawdust treatment when subjected to 121°C (15 lbs psi) for 15 and 60 minutes in the presence of H_2SO_4. High percentage solubilisations were recorded with all the treatments, including the water control. Unlike the steam treated preparations, however, increases in solubilisation were recorded at all the concentrations although there was no direct correlation between the

concentration and resultant solubilisation. By extending the treatment to 60 minutes increased solubilisation was apparent at every concentration with the exception of 72% (v/v) although the percentage increases were not as dramatic as those observed in the presence of steam treatment. The discrepancy observed in the presence of 72% (v/v) H_2SO_4 could possibly be attributed to the recrystallisation of cellulose.

Acid treatment of lignocellulosic materials has been used as a preliminary treatment stage to degrade the polymeric structure. For example, for subsequent analytical purposes, enzymatic hydrolyses and for producing sugar liquors, particularly for fermentation to ethanol (Wilke et al, 1981; Knappert et al, 1980).

Han and Callihan (1974) used different concentrations of H_2SO_4 to hydrolyse rice straw and sugar cane bagasse to increase the subsequent microbial digestibility of the cellulose content and found that sugar production was maximal when the bagasse was treated with 5% (v/v) H_2SO_4 for 15 minutes at 121°C. Prolonged heating up to 2 h did not in fact increase sugar yield further. Bienkowski, Ladisch, Voloch and Tsao (1984) examined the effects of steeping corn residue with concentrations of H_2SO_4 between 15 and 25% (v/v), subjected to discrete temperatures between 95 and 103°C, and showed that a residence time of 5 h, together

with a temperature between 95 and 103°C and an acid concentration of 16% (v/v), were required for optimum depolymerisation. Wright and D'Agincourt (1984) in a similar study found that the hydrolysis of crystalline cellulose in the presence of concentrated and dilute acids differed. In the case of the former, 85% (v/v) H_2SO_4 resulted in a breakage of the bonds between adjacent cellulose chains thus destroying the crystallinity. Conversely, dilute H_2SO_4 mediated hydrolysis of the amorphous regions and as a consequence a crystalline residue remained.

3.2 Methanogenic Fermentation of Sawdust Hydrolysis Products

The effects of pretreatments of lignocellulosic materials on subsequent anaerobic digestion have previously been investigated (Wilson and Pidgen, 1964; Zeikus, 1980b; Chandler et al, 1980; Young and McCarty, 1981; Chynoweth and Jerger, 1985; Pavlostathis and Gossett, 1985). For example, Chynoweth and Jerger (1985) suggested that although biogasification of wood and wood residue as an energy source may be technically feasible, since high bioconversion efficiencies were achieved with some selected hardwood species after physical treatment to reduce the particle size to 0.8 mm, methane could in fact be obtained at

much more rapid rates following pretreatment with sodium hydroxide. Pavlostathis and Gossett (1985) examined methane production from wheat straw, after alkaline treatments, and found that concentrations of 16 and 50 g NaOH 100 g^{-1} total solids resulted in increased biodegradability and subsequent methane production in excess of 100%.

3.2.1 Preparation of hydrolysates

Although maximum solubilisation was obtained in the presence of H_2SO_4, at a concentration of 72% (v/v), after 1 hour of treatment at 121°C (15 lbs psi) this result was comparable with the lower concentration of 50% under the same conditions and as a consequence the latter concentration was chosen for all subsequent treatments and the temperature of treatment was standardised at steaming.

The hydrolysates from both NaOH and H_2SO_4 treatments were obtained as follows:

100 g samples of dried sawdust were individually suspended in 1 litre of NaOH or H_2SO_4, 50% (w/v and v/v respectively). After steaming for 1 h the pH of each preparation was adjusted to 7.0 with either NaOH (6N) or H_2SO_4 (6N) before filtration through a Whatman No. 1 filter paper. The filtrates were then freeze-dried by means of a vacuum freeze drying unit (Model

30.P.2.T.S. Edward High Vacuum Ltd., Sussex) after which the dried materials were extracted with diethyl ether to leave any Na_2SO_4 from the neutralisation step, and dried by rotary evaporation at 40°C (Gallenkamp Rotary Evaporator).

3.2.2 *Analysis of hydrolysis products*

The insoluble materials which resulted from the two sawdust treatments were dried at 100°C to constant weight, after which lignin and ash components were determined. Finally, the volatile matter in the freeze-dried extracts was determined by ignition at 550°C for one hour.

3.2.3 *Results and discussion*

The results of the hydrolysis products analysis are shown in Table 4 from which it can be seen that 52.9% of the lignin present in the sawdust was removed after treatment with NaOH, 50% (w/v), at 100°C for 1 h. Conversely, treatment with H_2SO_4, 50% (v/v), at 100°C for 1 h, increased the amount of residual lignin by a factor of approximately 2.5 compared with the untreated controls. These results emphasised the fact that alkali treatment removed lignin and hemicellulosic materials to leave mainly the crystalline α-cellulose (Brown, 1983; Pavlostathis and Gossett, 1985). Conversely, treatment

with H_2SO_4 showed that cellulose and hemicellulose were effectively removed and subsequently converted to sugar materials.

Table 4. Analysis of Untreated and Treated Pine Sawdust. Values Shown Represent Means of Triplicate Determinations and are Expressed as % Dry Weight.

Treatment	Lignin %	Ash %	Residual Material %
Untreated	27.8	2.8	69.4
NaOH (50% w/v) 100°C, 1h	14.7	2.6	82.7
H_2SO_4 (50% v/v) 100°C, 1h	69.9	5.2	25.9
NaOH extract	-	0.22	-
H_2SO_4	-	0.17	-

The ash content of the alkali-treated sawdust decreased slightly as the lignin content decreased, which was in direct contrast with the H_2SO_4-treated sawdust in which a considerable increase was observed. The latter was attributed to the increase in lignin con-

tent, since lignin has been reported to have an ash content of approximately 30% for softwoods (Brown, 1983).

Characterisations of the freeze-dried volatile extracts were not made. The relatively low concentrations of ash present in these possibly resulted from sodium sulphate after the neutralisation step.

3.2.4 *Methanogenic fermentation*

Closed culture methanogenic fermentations of acid and alkali hydrolysates, residual solids and untreated sawdust, together with acetate were investigated as follows:

A serum-bottle technique was used in which prereduced triplicate 90 ml aliquots of basic mineral salts medium (2.2.1 B), individually supplemented with 1 g l^{-1} of the extracted materials, acetate, residual solids or untreated sawdust as the sole carbon and energy sources, were maintained in an anaerobic culture chamber overnight to facilitate anaerobiosis prior to inoculation with 10% (v/v) inoculum from the veratric acid enrichment culture (2.3.1). The bottles were closed with Suba-seals and incubated at 30°C. Samples for methane gas and fatty acid analyses were taken at regular intervals over a period of 30 days (Figures 7 and 8) with the exception of the untreated sawdust and residual solids supplemented cultures (18 months).

Figure 7. Methane Production by the Interacting Microbial Association from Different Physico-Chemical Treatment Fractions and Acetate During Closed Culture Cultivation at 30°C. NaOH Hydrolysate (o——o), H_2SO_4 Hydrolysate (●——●), NaOH Residual Solids (□——□), H_2SO_4 Residual Solids (■——■), Acetate (Δ——Δ) and Untreated Pine Sawdust (▲——▲)

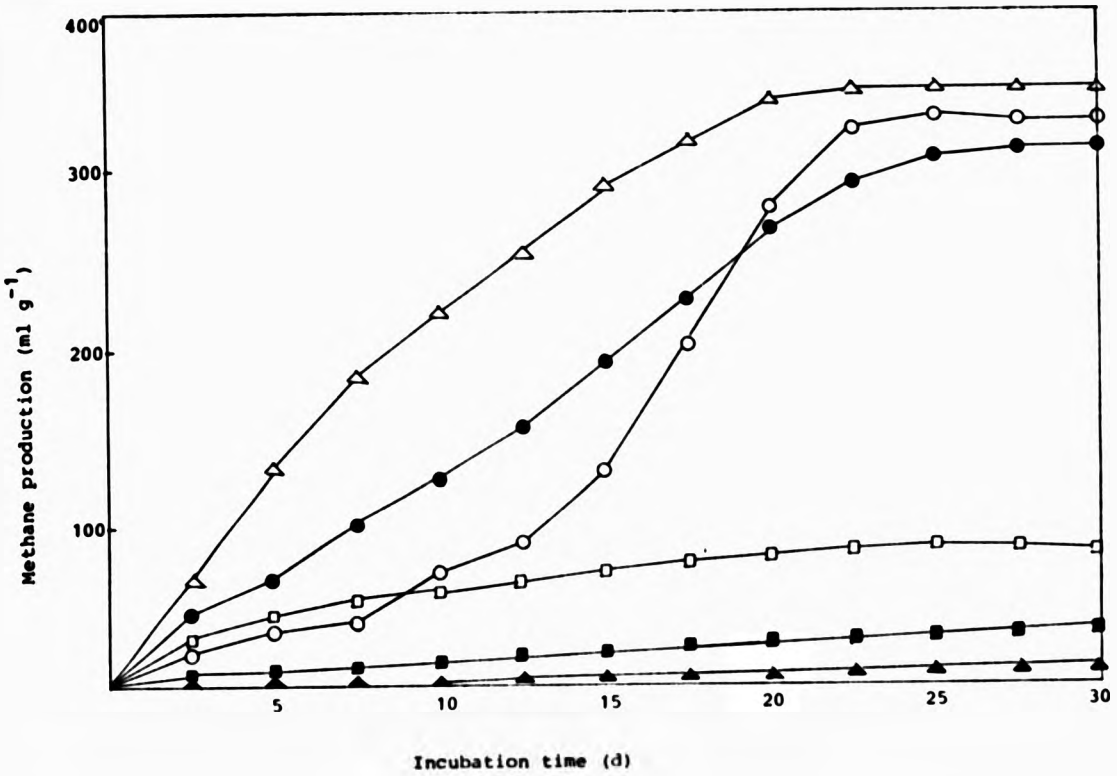
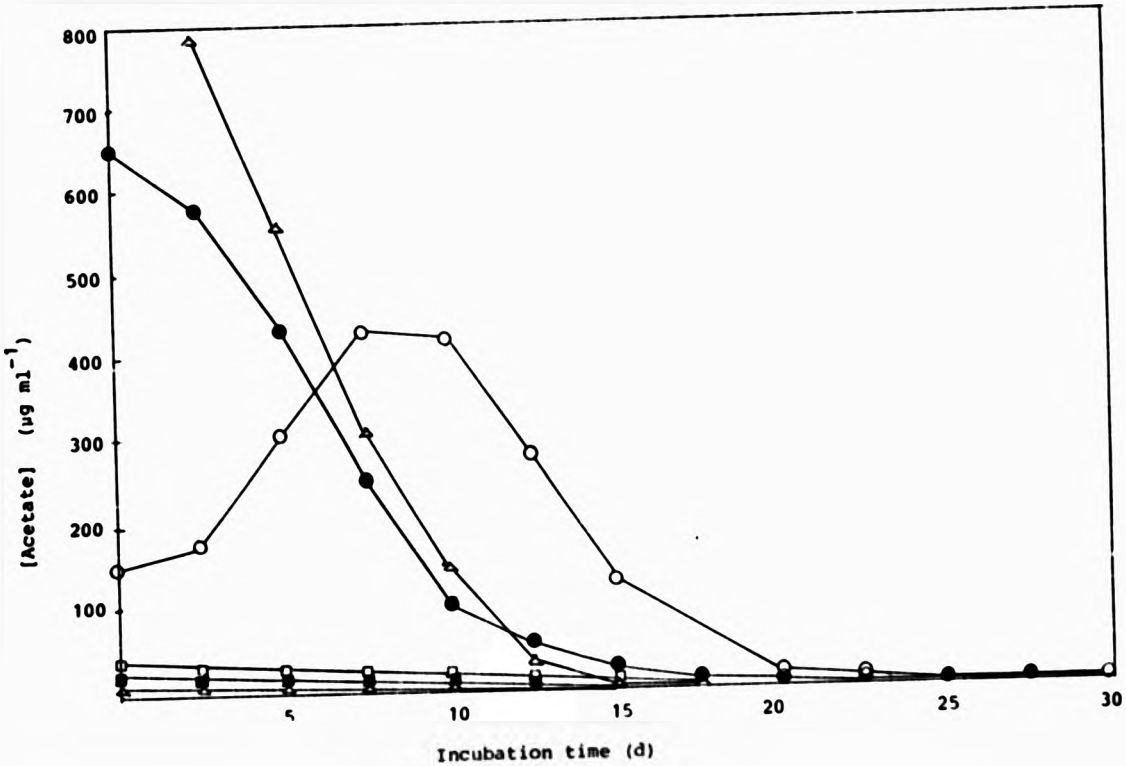

400
300
200
100
Methane production (ml g^{-1})
5
10
15
20
25
30
Incubation time (d)

Figure 8. Acetate Production and Utilisation by the Interacting Microbial Association from Different Physico-Chemical Treatment Fractions During Closed Culture Cultivation at 30°C. NaoH Hydrolysate (○—○), H_2SO_4 Hydrolysate (●—●), NaoH Residual Solids (□—□), H_2SO_4 Residual Solids (■—■), Acetate (△—△) and Untreated Pine Sawdust (▲—▲).


800
700
600
500
400
300
200
100
[Acetate] (µg ml^{-1})
5
10
15
20
25
30
Incubation time (d)

Terminal methane concentrations were determined after 30 days incubation and corrected to standard temperature and pressure. Corrections for the solubilities of methane and CO_2 were also made by the method described by Shelton and Tiedje (1984).

In addition to these closed cultures, total gas production was determined by use of three-neck round-bottom flasks (1 litre) fitted with graduated gas collection systems similar to the one described by Balba,(1978).

3.2.5 *Results and discussion*

From Figure 7 it can be seen that the concentration of methane produced from the untreated sawdust during the first 30 days incubation was very low and was equivalent to 16.7 ml g^{-1} which represented a digestibility of 4.9% only (Table 5). This low digestibility did not in fact change significantly even after protracted incubation over a period of 18 months. The percentage digestibility was, however, comparable with the results obtained by Chynoweth and Jerger (1985) who reported that anaerobic bioconversion efficiency for loblolly pine was 3% only, while the equivalent values for hybrid poplar and sycamore were 53.8 and 56.7% respectively. Holt and Jones (1983) also reported that Scots pine was more resistant to bacterial degradation

Table 5. Percentage Volatile Solids Digested, Total Gas Produced, Percentage Methane and Total Methane Produced by the Interacting Microbial Association from Different Physico-Chemical Treatment Fractions During Closed Culture Cultivation at 30°C.
Values Shown Represent Means of Triplicate Determinations.

Treatment Fraction	% Volatile Solids Digested	Total Gas Produced ($ml\ g^{-1}$)	Total Methane Produced ($ml\ g^{-1}$)	% of Methane in the Total Gas Produced
Untreated sawdust (control)	4.9	30.9	16.7	54.0
NaOH residue	23.7	143.5	81.8	57.0
H_2SO_4 residue	11.8	69.7	40.4	58.0

in anaerobic aquatic habitats than beech wood. Thus it would appear that not all woody materials are susceptible to bioconversion to the same extent although it is still not clear whether it is the lignin content, the type of structural association between the lignin and other components, or the presence of other constituents in some woody materials or a combination of these which is responsible for the recalcitrance.

Although it has been suggested that the association between lignin and other cell wall polymers is resistant to microbial attack (Cowling, 1975; Chandler et al, 1980; Zeikus, 1980a) this was later refuted by Chynoweth and Jerger (1985) who speculated that biogasification of wood was technically feasible.

From Figure 7 it can also be seen that methane production from the residual solids of acid and alkali treatment, whilst although still comparatively low, did increase quite significantly compared with the untreated sawdust with increases of 23.7 and 65.1 ml g^{-1} respectively observed. Similarly, increases in digestibility of 6.9% and 18.8% respectively were also apparent. These increases in both methane production and digestibility of the residual solids after acid and alkali treatment, compared with the untreated control, may be attributed to the effects of the two chemicals on the lignocellulosics which resulted in a higher lignin

content, and hence greater recalcitrance of the acid-treated than the alkali-treated sawdust (Han and Callihan, 1974). As discussed earlier (1.3.1), acid hydrolysis results in the removal of cellulose and hemicellulose to leave lignin which has been shown to be resistant to anaerobic degradation (Chandler et al, 1980; Zeikus, 1980b). Conversely, alkali treatment results in the removal of lignin, and this, together with swelling and physical alteration of the component crystalline and amorphous structures renders the residual materials more susceptible to microbial catabolism (Detroy et al, 1981; Carr and Doon, 1984).

Although cellulose and hemicellulose have been demonstrated to be biodegradable under anaerobic conditions (Stafford, Hawkes and Horton, 1980), the degree and rate of degradation are affected by factors such as the degree of crystallinity and polymerisation of the molecule and the capillary structure (Cowling, 1975). However, when cellulose and hemicellulose are associated with lignin in a lignocellulosic matrix both groups become increasingly resistant to microbial attack. Fan, Gharpuray and Lee (1981) concluded that the content of lignin governs the rate of hydrolysis of lignocellulosic materials although reduction in crystallinity and particle size were shown to have little effect.

Despite the contention that lignin is resistant

to microbial catabolism, Benner and Hodson (1985) did report dissimilation under anaerobic conditions. However, microbial conversion of most woody species is generally not considered technically feasible without some type of pretreatment to dissociate the biodegradable components from lignin. Pavlostathis and Gossett (1985) examined methane production from wheat straw, after subjection to alkaline treatments of 2, 16 and 50 g NaOH 100 g^{-1} total solids, and found that each of the treatments resulted in higher methane production compared with untreated controls. An increase in excess of 100% was in fact observed when concentrations of 50 g NaOH 100 g^{-1} total solids were used. In a similar investigation Chynoweth and Jerger (1985) showed that when cottonwood, hybrid poplar, and sycamore were individually treated with 5% and 50% by weight of NaOH per gram of feed volatile solids at 100°C for 2.5 h, the terminal methane yields achieved were obtained after 12 to 14 days compared with 60 days for untreated controls. It is now well documented, however, that low-molecular weight, polyaromatic lignin derivatives and known lignin degradation products, such as vanillic acid, cinnamic acid, catechol, protocatechuic acid, phenol, benzoic and, p-hydroxybenzoic acid and syringic acid can be catabolised under anaerobic conditions (Balba and Evans, 1979; Healy and Young, 1979; Taylor, 1982; Sleat and

Robinson, 1983; Colberg and Young, 1985).

From Figure 7 it can be seen that the methane production from both acetate and the acid extracts started immediately with no apparent lag period and followed the same pattern. The pattern of methane production from the NaOH extract, however, differed somewhat and was characterised by a long lag phase although both the rate of subsequent methane production and final concentrations were in fact comparable to the production from the acetate-supplemented culture. The protracted lag phase observed exemplified the different products obtained with the two types of hydrolysis and could possibly have been attributed to the synthesis of essential enzymes. Thus it may be speculated that a specific complement of constitutive enzymes existed within the microbial association which was able to catabolise the H_2SO_4 hydrolysis products, such as volatile acids and sugars, whereas adaptive enzymes were required to catabolise the sugars, phenolic compounds, lignin monomers and oligomers which resulted from the NaOH treatment. The results shown in Table 6 tend to support this possibility, and also show that the terminal concentration of gas produced from acetate was 566.3 ml of which 362.4 ml was methane, which represented 64.0% of the total gas. The remaining 36.0% was assumed to be essentially CO_2 together with trace con-

Table 6. Percentage Volatile Solids Digested, Total Gas Produced, Percentage Methane and Total Methane Produced by the Interacting Microbial Association from Different Physico-Chemical Treatment Fractions and Acetate During Closed Culture Cultivation at 30°C. Values Shown Represent Means of Triplicate Determinations.

Treatment Fraction	% Volatile Solids Digested	Total Gas Produced ($ml\ g^{-1}$)	Total Methane Produced ($ml\ g^{-1}$)	% of Methane in the Total Gas Produced
NaOH hydrolysate	94.3	545.7	332.9	61.0
H_2SO_4 hydrolysate	96.7	614.9	344.3	56.0
Acetate	98.7	566.3	362.4	64.0

centrations of other gases which were not determined. This value represented a total of 98.7% of the theoretical value. The terminal concentrations of methane produced from the acid and alkali hydrolysates were 344.3 and 332.9 ml g^{-1} respectively (Table 6) which represented 56.0 and 61.0% of the total gas produced by the respective culture. It is interesting to note that the methane productions were similar for the two hydrolysates even though the chemical compositions differed markedly.

3.3 Summary

Ground pine (Pinus sylvestris) sawdust was treated with various concentrations (from 0.1 to 50% w/v) of NaOH at ambient temperature. The results obtained showed that solubilisation increased as both the treatment time and concentration were increased. The highest value of 23.7% was obtained at a concentration of 50% (w/v) after 120h. This compared with a percentage solubilisation of 11% which was obtained with the lowest concentration (0.1%) after the same treatment period.

When the mixtures were steamed for 15 and 60 minutes the highest solubilisation (29.7%) was obtained with 50% (w/v) NaOH after 60 minutes. This result compared with a value of 7.6% when the 0.1% concentration

was used. At the higher temperature of 121°C (15 lbs psi), however, solubilisation in the presence of 50% (w/v) NaOH decreased as the treatment time was increased since 31.6% resulted after 15 minutes but only 17.6% after 60 minutes. Since the result of the 15 minutes treatment at 121°C was not significantly different to the 60 minutes steam treatment the latter was used in all subsequent studies.

When NaOH was replaced with H_2SO_4 similar results were obtained since solubilisation at ambient temperature again increased with concentration and treatment time with the highest value (64.2%) obtained with 72% (v/v) and a treatment time of 120h.

In the presence of elevated temperature (steam) and a shorter incubation period (1h) then a percentage solubilisation of 44% resulted in the presence of the same concentration of acid although decreases to 34 and finally 4.6% were apparent with the lower concentrations (50 and 0.1%) under the same conditions.

By increasing the temperature further to 121°C percentage solubilisations of 51.9 and 52.1% resulted from the treatments with 50 and 72% (w/v) H_2SO_4 after 60 minutes.

As a consequence of the relatively limited solubilisation increase at 121°C all subsequent treatments were made in the presence of steam with 50% (v/v)

H_2SO_4 and an incubation period of 1 h.

Biomethanation of the two resulting fractions, residual solids and the freeze-dried neutralised hydrolysate, of the two treatment types resulted in the production of higher concentrations (81.8 ml g^{-1}) of methane from the NaOH residual fraction than from the corresponding H_2SO_4 fraction (40.4 ml g^{-1}). Conversely, a lower concentration (332.9 ml g^{-1}) of methane was obtained from the NaOH hydrolysate than from the acid hydrolysate (344.3 ml g^{-1}). Thus, in total, 1 g of sawdust subjected to 1 h steam treatment in the presence of NaOH at 50% (w/v) concentration resulted in the production of 142.1 ml of methane compared with 143.8 ml from the corresponding H_2SO_4 pretreated material. Both these results were significantly higher than the methane concentration of 16.7 ml g^{-1} obtained with the sawdust control. The pretreatments, therefore, resulted in approximately 8.5 fold overall increases in methane production.

The total costs of energy and chemicals for the two treatments and subsequent neutralisations were not calculated since they were equivalent. Pavlostathis and Gossett (1985), however, reported that at the then price of natural gas, methane production from lignocellulosic materials, after such treatments, was not economically attractive. Thus, at the present time, with oil prices

considerably below the 1985 figures, it would appear that treatments such as these are even less financially attractive although this situation could well change in the future.

The use of other pretreatment methods, however, could prove more economically feasible in the short term. For example, the use of the enzyme "aryl etherase" discovered by Srinivasan, Chong, Cary and Narva (1984), which was shown to digest lignin to methanol and catechol in as little as 2 h.

CHAPTER FOUR

ENRICHMENT, ISOLATION AND ADAPTATION OF INTERACTING MICROBIAL ASSOCIATIONS TO SELECTED METHANOGENIC SUBSTRATES

4.1 Enrichment and Isolation

As discussed earlier (1.5.1) enrichment techniques are designed essentially to change the microbial environment in such a way that the organism or organisms under study successfully compete against all other species and hence become the dominant population(s) (Parkes, 1982). Once isolated, anaerobic microbial associations can then be adapted for the methanogenic fermentation of many naturally-occurring aromatic substrates (Balba and Evans, 1980b).

With respect to the present study preliminary investigations were made with anoxic freshwater sediment and fresh bovine rumen fluid (obtained from Glasgow City Abattoir) as follows:

Thirty three lignin-related aromatic compounds were selected for the enrichments in which between 4 and 5 g anoxic sediment or bovine rumen fluid were added to 15 ml of medium, supplemented with 2 mM of the specific aromatic compound. 25 ml serum bottles (MacFarlane Robson Ltd., U.K.) were used and these were incubated inverted and stationary at 30°C in the dark.

Closed cultures

For veratric and syringic acid closed cultures, three-neck, round-bottom pyrex flasks (1 litre), fitted with graduated gas collection systems, similar to the one described by Balba (1978), were used. The flasks were batched with 600 ml of medium, supplemented with either veratric or syringic acid to a final concentration of 2 mM, inoculated with 60 g of anoxic sediment and overgassed for 20 min. with OFM to establish anaerobiosis. The cultures were maintained at 30°C and sampled at regular intervals for methane and residual substrate concentrations for 3 months. Fermentation gases were collected by displacement of a solution of sodium chloride (20% w/v) and citric acid (0.5% w/v).

When rumen fluid was used as the source of inoculum methane production was high in all the treatments, including the controls, due to the presence of residual carbon and as a consequence it was not possible to use methane as a criterion for the determination of aromatic compound catabolism. Unfortunately, the UV absorption spectra were initially impossible to measure even when the cultures were first centrifuged for 15 min. at 10,000 x g. After 14 d of incubation at 30°C, however, all the treatments, including the non-amended controls, produced typical absorption bands at 275 ± 0.2 nm. Following this analysis each enrichment culture was

subcultured and reincubated for up to 8 weeks at which point some of the aromatic compounds were completely catabolised, as indicated by the volumes of methane formed and the UV absorption spectra, whilst others were either partially catabolised or recalcitrant (Table 7).

When the same compounds were incubated in the presence of freshwater sediment as inoculum, the UV absorption spectra were more distinct and, in addition, methane was only generated after initiation of catabolism of the respective aromatic molecule. For example, the catabolisms of syringic acid, gallic acid, sinapic acid and 3,5,dimethoxy - 4 hydroxycinnamic acid were first detected after 18 to 20 days incubation, as indicated by their corresponding absorption bands. Methane, however, was not generated until after between 25 and 30 days of incubation. Other compounds (mainly di-substituted aromatic monomers) such as veratric acid, vanillic acid, and vanilline were catabolised only after extended incubation periods of between 4 and 10 weeks, whilst the remainder were either partially catabolised, as evidenced by the accumulation of metabolic intermediates, or were recalcitrant.

Recalcitrance in this instance may be attributed to either recalcitrance *per se* or to the presence of toxic intermediate metabolites resulting from demethoxylation or decarboxylation. Bache and Pfennig (1981)

Table 7. Catabolism of Aromatic Compounds in Bovine Rumen Liquor and Anoxic Freshwater Sediment Enrichment Cultures Under Anoxic Conditions.

++++ Denotes full catabolism of compound

+++) Denotes degree of catabolism
++) relative to full catabolism
+)

\- Denotes no catabolism after 3 months incubation at 30°C.

Recorded intermediates, phenol and catechol, accumulated in these enrichments.

Compound	Bovine Rumen Liquor	Anoxic Fresh-Water Sediment	Lag Time (Weeks)
Veratric Acid	+	++++	4-5
Syringic acid	+++	++++	2-3
Ferulic acid	++	++++	4-5
Gallic acid	++	++++	2-3
Catechol	-	-	-
Vanillic acid	+++	++++	4-5
Vanilline	+++	+++	4-5
Guaicol	-	catechol	2
Pyrogallol	+	++++	2-3
p-cresol	-	-	-
m-cresol	-	-	-
o-cresol	-	-	-
Protocatechuic acid	catechol	++++	4-5
p-anisic acid	phenol	phenol	2
m-anisic acid	phenol	phenol	2
p-hydroxybenzoic acid	++	++++	4
Sinapic acid	++	++++	2-3
p-coumaric acid	+	phenol	2
Cinnamic acid	+	++	4-5
Phenol	-	-	-
Cinnamyl alcohol	-	+	4-5
Benzoic acid	++	++++	3-4
p-methoxy phenol	-	-	-
3,4-dimethoxycinnamic acid	+	++++	4-5
Piperonylic acid	-	-	-
3,4-dimethoxybenzoic acid	+	++	4-5
2,4-dimethoxybenzoic acid	++	++++	4-5
2,6-dimethoxybenzoic acid	+	+	4-5
Phloroglucinol	++	++++	2-3
Resorcinol	+	+	4-5
Hydroxy-hydroquinone	-	-	-
Quinol	-	-	-
Lignin extract	+	+	2-3
Sediment control	-	-	-
Rumen liquor control	-	-	-

reported that when Acetobacterium strains were grown on benzoic acid derivatives, which contained 1, 2 or 3 methoxy groups on the aromatic ring, the resulting corresponding hydroxy aromatic compounds after demethoxylation were not degraded. They reported that for some compounds, such as anisol, the residual product of the demethoxylation was phenol which was toxic to all Acetobacterium strains examined. Kaiser and Hanselmann (1982b) similarly reported that whilst syringic acid analogues with substituents in the 3, 4 and 5 position of the ring were completely catabolised, 3,4 substituted substrates were converted to catechol only.

In the present study ring cleavage of phenol and catechol under anoxic conditions did not result. The refractory nature of both these compounds has in fact been documented for both aerobic (Kuwahara, 1980) and anaerobic (Clark and Fina, 1952; Taylor, Campbell and Chinoy, 1970) conditions. Conversely, Healy and Young (1978) and Balba and Evans (1980c) reported complete catabolism of both phenol and catechol under anaerobic conditions.

These discrepancies may possibly be explained by the fact that catabolic rates of organic compounds are influenced by factors such as the source and the organic exposure history of the microbial species in a given environment (Anon, 1967). Thus, with the exception of highly polluted environments, the organic

exposure history of most aquatic microbial communities is dominated by naturally-occurring substrates (Kaiser and Hanselmann, 1982a, b; Shimp and Pfaender, 1985).

Healy and Young (1979) reported that the response of enrichments to the different compounds tested was little affected by the different sources of inocula and concluded that the substrate was the strongest selection pressure.

In the study of Healy and Young (1979), acclimation to syringic acid and catechol resulted after 2 and 21 days respectively. In the present study, however, acclimation to syringic acid took considerably longer (18 days) in both enrichments whereas even after a protracted incubation period of 3 years catabolism of catechol was still not detected which suggested that the source of inoculum was an important determinant. Healy and Young (1979) in fact used an inoculum from a laboratory digester fed with heat-treated refuse in which the two molecules were normally present and as a consequence the microbial species were continously exposed to these selection pressures.

Slater and Bull (1982) reported that in natural environments growth on a single carbon and energy source may occur although microbial populations usually encounter a diversity of these. The availability of two or more carbon sources frequently leads to sequential

use of the compounds, in closed culture, and characteristic lag phases between the exhaustion of one substrate and the metabolism of a second, a phenomenon known as diauxie.

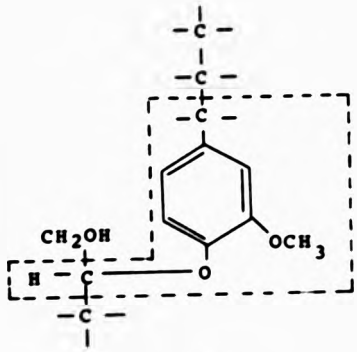
The results obtained in this preliminary investigation clearly demonstrated that whilst the organisms isolated from freshwater sediment had slightly greater metabolic versatilities compared with the rumen fluid enrichment their metabolic activities, as evidenced by complete substrate dissimilation, were significantly higher and as a consequence these cultures were selected for further study.

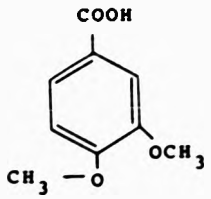
4.2 Methoxylated Aromatic Acids as Lignin Model Compounds

It is an exceedingly difficult task to study the biochemical mechanisms whereby microorganisms degrade lignin due to the pronounced structural complexity of the molecule. As has already been discussed (1.1.3), lignin, unlike other biopolymers, does not contain readily hydrolysable bonds which occur at periodic intervals along a linear backbone. Thus, it is particularly difficult to design experiments to elucidate specific enzymic transformations which occur during microbial catabolism of lignin (Muranaka, Kinoshita, Yamada and Okada, 1976; Cain, 1980; Crawford, 1981).

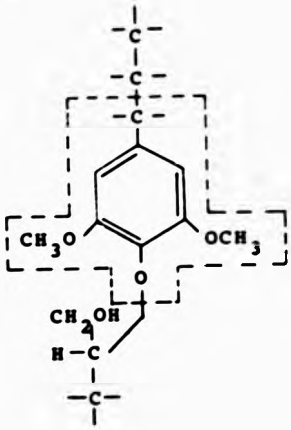
Theoretically, one way to circumvent this problem of chemical complexity is to study the microbial degradation of simple model compounds of known chemical structure (Umezawa, Nakatsubo and Higuchi, 1982). In this experimental approach low molecular weight compounds, which contain chemical structures known to occur in lignin, may be used (Hatakka, 1985). The assumption is then made that the biochemistry of degradation of these lignin models is analogous to the catabolic mechanisms of lignin biodegradation (Milstein, Shragina, Gressel, Flowers and Huttermann, 1983).

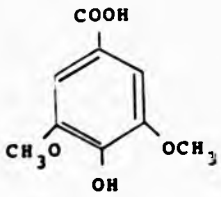
Molecules selected as representative monomers in this study were the simple methoxylated aromatic acids syringic and veratric acids. Since the initiation of this study, however, several reports have appeared in the literature with similar use of these compounds as model substrates for the enrichment and isolation of microorganisms for studies of lignin biodegradation mechanisms, both under aerobic and anaerobic conditions (Kaiser and Hanselemann, 1982a, b; Gold, Mayfield, Cheng, Krisnangkura, Sihimada, Enoki and Glenn, 1982; Ander, Eriksson and Yu, 1983; Leonowicz, Edgehill and Bollag, 1984; Hatakka, 1985; Chen, Ohmiya, Shimizu and Kawakami, 1985). Figure 9 shows the relationship between the structural elements of lignin and the selected monomers.





VERATRIC ACID





SYRINGIC ACID

Figure 9. The Relationship between Structural Elements of Lignin and the Selected Methoxylated Aromatic Monomers Veratric and Syringic Acids (Crawford, 1981)

4.2.1 Catabolism of syringic and veratric acids: acclimation times

Serum bottles of 250 ml capacity were amended with 150 ml aliquots of prereduced filter sterilised basic mineral salts medium (2.2.1A). Samples of medium were individually supplemented with syringic and veratric acids to final concentrations of 2 mM. The media were then prewarmed to 30°C, prior to inoculation with 4 to 5 g aliquots of freshwater sediment, after which the bottles were sealed with Suba-seals, over-gassed with OFW and incubated in the dark. Gas headspace samples were removed at regular intervals, replaced with OFW and analysed by GLC for methane. Liquid samples were similarly removed and assayed for the dissimilation of the aromatic compounds by examination of UV absorption spectra (320 to 200 nm).

The results for both syringic and veratric acid catabolisms are shown in Figures 10 and 11 from which it can be seen that catabolism of syringic acid started after 18 days incubation at 30°C and continued for a further 13 days at which point dissimilation was complete. The presence of methane, however, was not apparent until after 24 days although methanogenesis continued for a protracted period. It must be noted, however, that the concentrations of methane detected were particularly low.

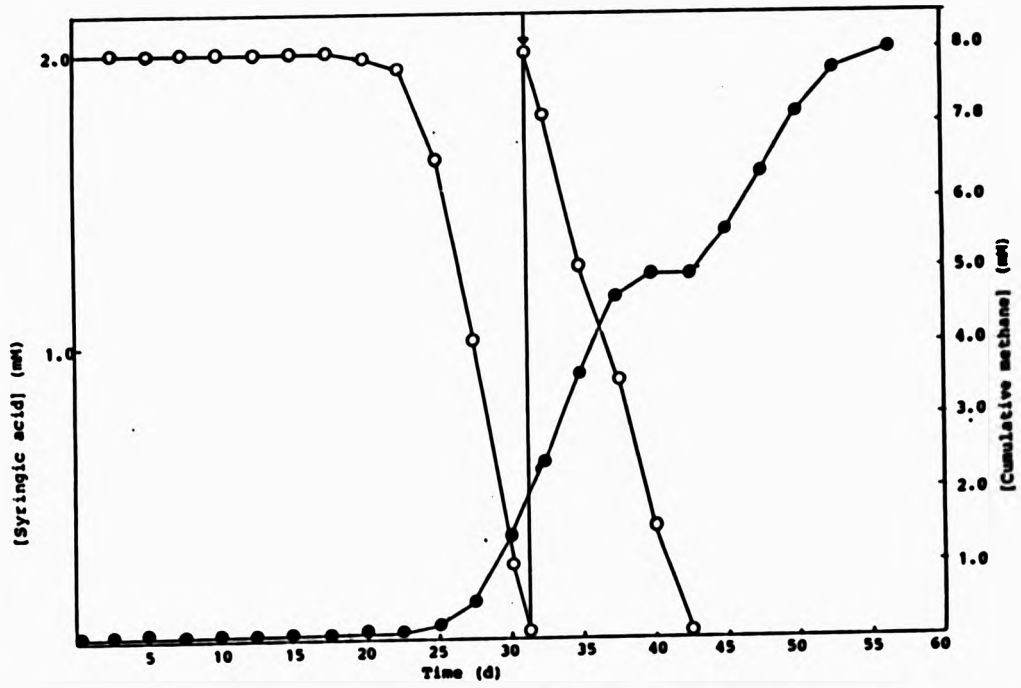


Figure 10. Changes in the Concentrations of Residual Syringic Acid (○—○) and Methane (●—●) During the Closed Culture Enrichment of the Syringic Acid - Catabolising Microbial Association
Arrow denotes the initiation of second batch culture

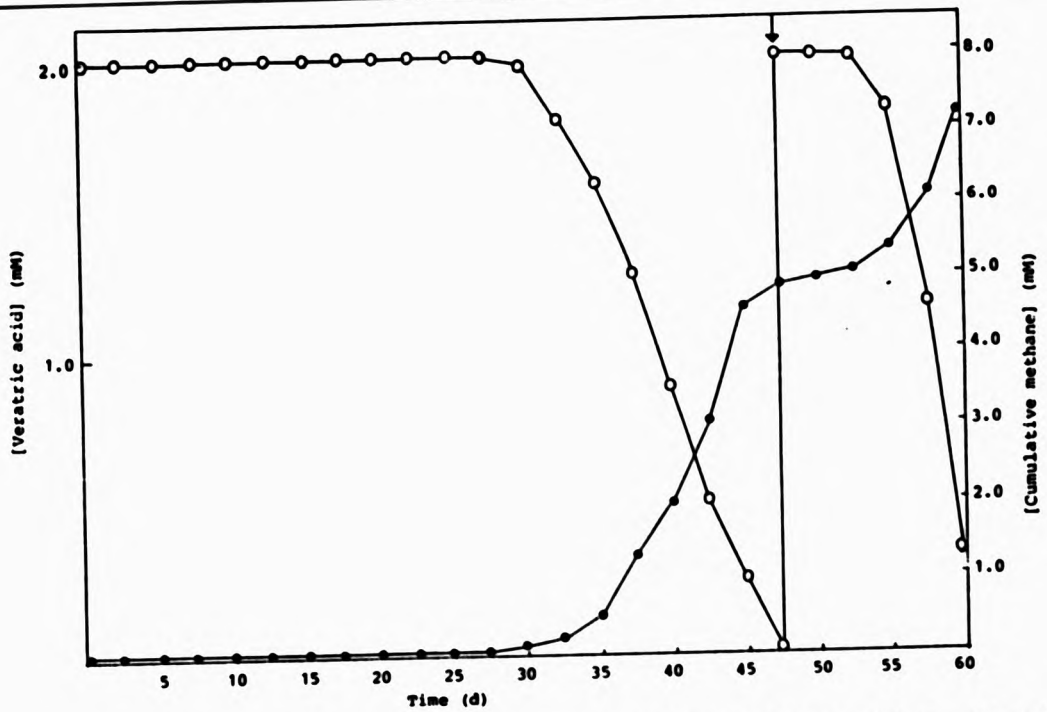


Figure 11. Changes in the Concentrations of Residual Veratric Acid (○—○) and Methane (●—●) During the Closed Culture Enrichment of the Veratric Acid - Catabolising Microbial Association
Arrow denotes the initiation of second batch culture

Following substrate dissimilation a second batch culture was initiated and this resulted in both the elimination of a lag period and also an increase in the rate of dissimilation. Throughout this period methanogenesis continued at an exponential rate.

With each repeated subcultivation the rate of substrate dissimilation increased until after ten such manipulations total catabolism was complete between 2 and 3 days with methanogenesis termination apparent after between 5 and 7 days incubation.

Veratric acid catabolism (Figure 11) was both much slower than that of syringic acid and also did not begin until after 30 days of incubation.

Once again there was a lag between initial substrate dissimilation and the initiation of methanogenesis, although this period was comparable with that obtained for syringic acid, and similarly the terminal methane concentration was also comparable.

Following initiation of a second batch culture a lag period was again apparent although this was much reduced to between 4 and 6 days. Between 4 and 5 subcultivations were in fact required before this lag period was eliminated. In all cases, however, substrate dissimilation proceeded at a much slower rate than the comparable closed cultures amended with syringic acid. Thus it would appear that it was easier for the cultures

to acclimatise to, and subsequently dissimilate, 3,4,5-trisubstituents than those with 3,4-disubstituents.

Similar observations have also been reported by other investigators (Healy and Young, 1979; Kaiser and Hanselmann, 1982a and b) but without possible explanations.

4.2.2 Carbon balance

After 10 subcultivations each microbial association was individually transferred to a 1 litre round bottom, 3-necked flask fitted with a graduated gas collection system. Closed cultures were again initiated and gas samples assayed at regular intervals until the stationary phases were reached at which point the terminal methane volumes were recorded and compared with the theoretical values according to the equation of Buswell and Muller (1952):

$$C_nH_aO_b + [n-\frac{a}{4}-\frac{b}{2}]H_2O \rightarrow [\frac{n}{2}-\frac{a}{8}+\frac{b}{4}]CO_2 + [\frac{n}{2}+\frac{a}{8}-\frac{b}{4}]CH_4 \quad (1)$$

from which concentrations of 4.75 and 4.5 mM methane, for veratric and syringic acids respectively, were obtained.

According to Kaiser and Hanselmann (1982a) veratric and syringic acids catabolism to acetate may be described by the following equations:

$$C_9H_9O_4 + 0.5\ HCO_3 + 4H_2O \longrightarrow 4.75CH_3COO^- + 3.25H^+ \quad (2)$$

veratric acid

$$C_9H_9O_5 + 4H_2O \longrightarrow 4.5\ CH_3COO^- + 3.5H^+ \quad (3)$$

syringic acid

with acetate catabolism then proceeding as follows:

$$CH_3COO^- + H_2O \longrightarrow CH_4 + HCO_3 \quad (4)$$

acetate

Table 8 shows the carbon balances of syringic and veratric acid catabolisms from which it can be seen that the volume of total gas generated from the former was 84.3%, of the theoretical maximum, of which 56% was methane. The remaining 44% was then assumed to be mainly CO_2 and, to a lesser extent, gases such as hydrogen and H_2S. Veratric acid dissimilation resulted in a slightly decreased gas volume although the methane concentration obtained 54% was comparable with that obtained for syringic acid.

Microbial biomass for the syringic acid culture accounted for 9.7% of the carbon, whilst the comparable percentage for the veratric acid was 6.3%. The biomass was calculated according to the method described by Kaiser and Hanselmann (1982a) in which it was calculated

Table 8. Carbon Balances for the Anaerobic Catabolism of Veratric and Syringic Acids

Substrate	Acclimation Lag Period (days)	Gas Production Period (days)	% Theoretical Total Gas Recovered	% Methane from the Total Gas	% Accounted for by Biomass	% Total Methane Equivalent Recovered %	Total Methane Equivalent Recovered mM	Theoretical Methane mM
*VA 2mM	30 ± 2.0	50	82.7	54	6.3	89	8.24	9.5
†SA 2mM	18 ± 1.2	31	84.3	56	9.7	93	8.14	9.0

*VA - Veratric Acid

†SA - Syringic Acid

that each mg of cell dry weight corresponded to 9 μ Mol of $C_4H_7O_2$ with the assumptions made that C, H and O accounted for 78.5% (w/w) of the biomass dry weight.

For both cultures no residual substrate or volatile fatty acids were detected at the end of the catabolisms when the pH values were 6.8 and 6.9 for the syringic and veratric acid cultures respectively.

4.2.3 Cross-acclimation

The ability of the syringic and veratric acid-catabolising cultures to dissimilate other aromatic compounds was examined in a series of cross-acclimation studies. A range of aromatic compounds (Table 9) were examined as follows:

800 ml aliquots of prereduced medium (2.2.1A), pH 7.0 ± 0.1, were individually inoculated with 200 ml of veratric or syringic acid-acclimated culture. The closed cultures were then incubated for 24 h at 30°C to dissimilate any traces of carbon source which were transferred with the inocula. 15 ml aliquots of the cultures were then individually transferred into sets of 5 replicate serum bottles and amended to 2 mM with the different aromatic compounds from concentrated, filter sterilised stock solutions. The cultures were incubated at 30°C for 3 months and sampled at regular intervals for methane. Terminal substrate concentrations and pH

Table 9. Aromatic Compounds to Which the Veratric and Syringic Acid-Isolated Interacting Microbial Associations Were Acclimated.

++++ Denotes full catabolism of compound

+++) Denotes degree of catabolism
++) relative to full catabolism
+

\- Denotes no catabolism after 3 months incubation at 30°C

Recorded intermediates, phenol and catechol, accumulated in these enrichments.

Compound	Veratric Acid Culture	Syringic Acid Culture
Sinapic acid	++	++++
2,6-dimethoxybenzoic acid	-	-
Protocatechuic acid	++++	-
Ferulic acid	++++	+ catechol
Vanillic acid	++++	+ catechol
p-coumaric acid	-	++
Phloroglucinol	+	+++
2,3-dimethoxybenzoic acid	-	-
2,3-dimethoxybenzoic acid	++++	++
p-dimethoxybenzene	-	-
Pipronylic acid	-	-
p-hydroxybenzoic acid	++++	+ phenol
Gallic acid	++++	++++
1,5-dihydroxy anthraquinone	-	-
3,5-dihydroxybenzoic acid	-	-
m-dimethoxy benzene	-	-
p-methoxy benzene	-	-
Resorcinol	-	-
Orcinol	-	-
Guaiacol	-	-
Trans-cinnamic acid	-	+
Salicylic acid	-	-
Pyrogallol	++	++++
Catechol	-	-
Phenol	-	-
Quinol	-	-
Benzoic acid	++++	++
o-cresol	-	-
p-anisic acid	-	+ phenol
m-anisic acid	-	-
Cinnamyl alcohol	-	-
Piperanol	-	-
Veratric acid	++++	+ catechol
Syringic acid	++++	++++

values only were recorded. From Table 9 it can be seen that, in general, aromatic compounds with similar structural configurations and substitutions were dissimilated mostly without lag periods or with very short lag periods compared with the original enrichments. Molecules such as sinapic acid and pyrogallol, in the case of veratric acid inoculum, and p-coumaric acid and benzoic acid for the syringic acid inoculum, however, required acclimation periods of up to 8 weeks while compounds such as catechol, phenol and guaicol showed no evidence of dissimilation even after protracted incubation periods in excess of 2 years.

The veratric acid-catabolising microbial association was able to catabolise 3,4,5-trisubstituted aromatic acids such as syringic, gallic and sinapic together with pyrogallol, and 3,4-disubstituted aromatic acids such as vanillic, ferulic and protocatechuic while molecules which did not contain substituents on the 4 position such as 2,6 and 2,3-dimethoxy benzoic acid were not completely dissimilated.

The syringic acid-catabolising microbial association also dissimilated 3,4,5-trisubstituted aromatic compounds although the 3,4 and disubstituted aromatics were converted mainly to catechol or phenol. Kaiser and Hanselmann (1982b) reported similar observations for a syringic acid-catabolising microbial

association although they offered no possible explanation for this. Healy and Young (1979), however, isolated a vanillic acid-catabolising interacting microbial association which dissimilated 11 aromatic model compounds. Included in these were vanillic acid, catechol, syringic acid and protocatechuic acid. The authors also reported that compounds with structural similarities were readily catabolised without any acclimation period.

The results obtained in this study showed that only compounds which represented possible catabolic intermediates were dissimilated without lag periods. Examples of these included the syringic acid catabolic pathway intermediates, gallic acid and pyrogallol and the veratric acid catabolic pathway intermediates, vanillic and protocatechuic acids. From these results it was apparent that the isolated microbial association which was acclimated to 3,4-disubstituents also degraded compounds with 3,4,5-trisubstituents although the trisubstituent-catabolising microbial association did not dissimilate the disubstituted compounds.

It is well documented that no single microorganism has been found to catabolise aromatic compounds completely to terminal products under anaerobic conditions. Only interacting microbial associations, each component of which has limited but specific metabolic

activities, can achieve complete catabolism under these conditions. The component species thus determine both the kinds and the amounts of catabolic end products, and also conversion rates. Since natural environments are characterised by the presence of diversities of substrates and populations dynamic states prevail in which species dominance reflects substrate availability. Thus, although the source of inoculum is important it is the substrate which is the strongest determinant.

4.3 The Demonstration of and Explanation for Loss of Metabolic Versatility in the Veratric Acid-Catabolising Microbial Association

The veratric acid-catabolising microbial association was subjected to open culture cultivation at a constant dilution rate of 0.005 h^{-1} for a period of 12 weeks at which point the substrate dissimilation potential was lost. The same loss was also observed after 20 closed culture subcultivations. The apparent effect in both culture types was that demethoxylation activity displaced ring cleavage and as a consequence catechol was formed with no further dissimilation. The characteristic absorption spectra, at 251 and 283 nm, were then replaced by an absorption spectrum at 275 nm. Extraction of this compound with diethyl ether followed by TLC identification confirmed the formation of

catechol. Although the presence of methane and acetate continued in both the open and closed cultures the concentrations were particularly low and were possibly indicative of methoxycarbon origin. However, this can only be confirmed with the use of ^{14}C-labelled veratric acids.

This phenomenon of loss of metabolic potential has been reported by Kaiser and Hanselmann (1982a, b) in studies on veratric and vanillic acid catabolisms although a possible explanation from thermodynamic kinetic considerations failed to explain the phenomenon. The possibility does exist, however, that autoinhibition by an intermediary metabolite could have been the mediating factor.

In the present study one possible explanation could be that ring cleavage occurred before demethoxylation or decarboxylation and thus a different veratric acid ctabolic pathway was operative compared with the one described by Balba, Clark and Evans (1979) where the aromatic substrate was demethoxylated before ring cleavage.

The veratric acid-catabolising microbial association was maintained in liquid culture at 4°C for 4 months. Subsequent inocula for both closed and open

cultures were then found to have retained their veratric acid-catabolising activity, a situation which remained unchanged even after repeated subcultivations and open culture cultivation for a period of more than 3 years.

A possible explanation for this retention is that the storage period might have reflected the need for a protracted adaptation time by the microbial association or alternatively might have resulted in some detrimental effect on the demethoxylating species.

To examine the two possibilities of promotion of the ring cleaving species or inhibition of the demethoxylating species a series of experiments were designed to evaluate the possible restoration of the loss of metabolic potential in the original veratric acid-catabolising culture.

4.3.1 Examination of the role of growth factors

Two types of growth factors, sterilised anoxic sediment and yeast extract, were examined in this study.

Sterilised mud extract: 500 g of anoxic freshwater sediment were autoclaved (15 lbs psi, 121°C, 20 min) with 500 ml of glass-distilled water. After cooling and filtration, through a Whatman No. 1 filter paper, 1 ml aliquots were added to 100 ml samples of mineral salts medium (2.2.1A) supplemented with veratric acid (2 mM). Closed cultures were initiated with

inoculum (50% v/v) from the microbial association which converted veratric acid to catechol. Subsequent closed cultures were then initiated at weekly intervals by reducing the inoculum size by increments of 10% until a final inoculum volume of 10% was reached. For each closed culture, concentrations of methane, residual substrate and catechol were monitored at regular intervals.

Although growth factors have been recommended for the establishment and maintenance of microbial associations (Balba, 1978) it was found that for the veratric acid-catabolising microbial association demethoxylation activity continued without ring cleavage even when the closed cultures were supplemented with mud extract over a period of 6 months. One possible explanation for this phenomenon is that the demethoxylating species present in the microbial association had a physiological advantage and as such competitively displaced the ring cleavage species. Alternatively, the resultant intermediates of demethoxylation could have inhibited the activities of other component species of the microbial association.

Yeast extract: In the second experiment sediment extract was replaced with yeast extract at a concentration of 0.1% (w/v) and the subcultivation strategy and analyses repeated.

Although Bache and Pfennig (1981) reported that the presence of 0.05% (w/v) yeast extract was necessary to ensure reproducible initiation of growth of an isolated microbial association which catabolised methoxylated aromatic substrates, the use of yeast extract (0.1%, w/v) in the present study did not result in a similar effect even after 6 months of closed culture cultivation with subcultivations on a weekly basis.

4.3.2 Examination of the role of acetate

Acetate accounts for approximately 70% of the methane produced from sewage sludge digestion (Smith and Mah, 1966) and freshwater lake sediments (Cappenberg, 1974). Acetate has also been shown to account for 68 to 90% of the methane produced in cattle-waste digesters (Mountfort and Asher, 1978; Mackie and Bryant, 1981; Boone, 1982).

Healy and Young (1979) reported that catechol and phenol can be fermented only if active methanogenic populations are present. Since it has been demonstrated that acetate is a major methanogenic precursor in the anaerobic habitats, the addition of this to the microbial association which converted veratric acid to catechol could possibly have stimulated the methanogenic population. Therefore, the experiment described in

(4.3.1) was repeated with the yeast extract replaced by acetate (2 mM). Once again the results showed that acetate had no effect on the activity of the association although the labile molecule was quickly dissimilated.

4.3.3 Examination of the effects of demethoxyxlation inhibitors

Demethoxylation of aromatic compounds under anaerobic conditions was reported by Bache and Pfennig (1981) to occur in the presence of Acetobacterium woodii which they selectively enriched on a number of methoxylated aromatic compounds as sole carbon and energy sources. The workers also reported that the residual products of demethoxylation of some aromatic compounds were toxic to the same species. For example, in case of anisol the residual product of demethoxylation was phenol which was apparently toxic for all Acetobacterium strains examined.

To examine the effect of demethoxylation inhibitors an experiment was made in which duplicate 2 mM veratric acid-supplemented closed cultures were initiated in the presence of both 2 and 4 mM phenol. The concentrations of residual substrate, phenol, catechol and methane, together with the optical densities, were monitored at regular intervals throughout the experiment.

In the presence of both 2 and 4 mM phenol, catabolism of veratric acid completely ceased which suggested that the demethoxylating species were sensitive to phenol.

4.3.4 Examination of the effect of the addition of Acetobacterium woodii strain NZ Va 16

Since Acetobacterium species have been shown to demethoxylate aromatic compounds (Sache and Pfennig, 1981), the addition of this species may be used to confirm the loss of ring cleavage activity. Thus an experiment was made in which mineral salts medium, supplemented with veratric acid (2 mM), was inoculated with 10% (v/v) of the veratric acid-catabolising microbial association together with 10% (v/v) of mid-exponential phase Acetobacterium woodii strain NZ Va 16. The concentrations of residual veratric acid, catechol and methane were then monitored at regular intervals.

As a result of the addition of A. woodii complete dissimilation of veratric acid to methane and CO_2 ceased and the intermediate catechol accumulated, thus it was apparent that the demethoxylation species, A. woodii, had a kinetic advantage to ring cleavage species although the possibility that the product catechol was also toxic to the latter can not be discounted at this stage.

4.4 Summary

The results of this study showed that of the 33 lignin related monomers examined 19 were catabolised by the microbial association isolated from bovine rumen liquor and 20 by the association isolated from anoxic freshwater sediment. From the results it was clear that catabolic response varied according to the number and position of the ring substituents since adaptation to 3,4,5-trisubstituted monomers necessitated shorter time intervals than corresponding periods for 3,4-disubstituted monomers.

It was also apparent that the interacting microbial associations adapted to 3,4-disubstituted monomers also dissimilated the 4-substituted and 3,4,5-trisubstituted monomers completely to methane and CO_2 whilst the interacting assoications adapted to 3,4,5-trisubstituted monomers only partially dissimilated the 4-substituted and 3,4-disubstituted monomers; thus agreeing with the results of Kaiser and Hanselmann (1982b).

Examination of the catabolisms of the selected monomers, veratric and syringic acids showed approximate stoichiometric conversion to methane and CO_2.

From the results of this study the conclusion may be made that the recalcitrance of lignin to microbial degradation is not necessarily related to the

structure of the component monomers but more likely to factors such as the high molecular weight and the three dimensional structure. Release of the component monomers is therefore an essential prerequisite for the utilisation of lignin for the production of energy and/or chemical feedstocks.

CHAPTER FIVE

OPTIMISATION OF CULTURE CONDITIONS FOR THE ISOLATED MICROBIAL ASSOCIATIONS

5.1 Introduction

Microbial growth and product formation occur in response to the environment. Therefore, to understand growth and product formation, it is essential to understand the relationships between regulation of microbial metabolism and the physical and chemical environment. Microorganisms each respond uniquely to the environment and their reaction provides them with a selective and competitive advantage in their usual ecological niche. Microbial response to the environment is often interactive since while growing in or adapting to an environment, the species will alter the environment, as a consequence of their growth activities, and, in some cases, as a means to improve their competitive advantage against other organisms. Microorganisms respond to both physical and chemical mechanisms which provide them with a selective advantage.

The objective of this study was to characterise growth in response to changing environmental variables as a prerequisite for the optimisation of growth and product formation.

5.2 Examination of the Effects of pH on Substrate Catabolism

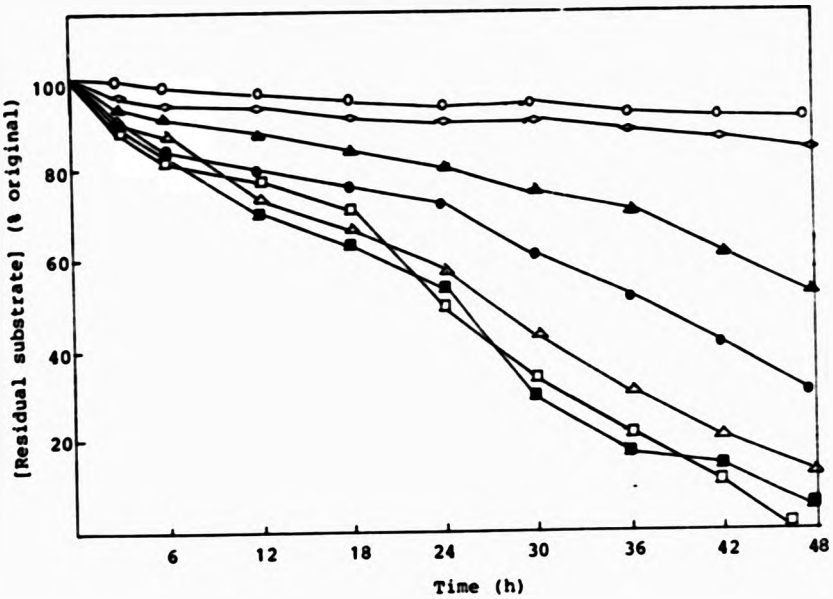
Examination of the effects of pH on substrate catabolism in closed cultures was made in 150 ml serum bottles, in which the pH values of prereduced 90 ml aliquots of basic mineral salts medium (2.2.1B) were adjusted to 6.0, 6.5, 6.8, 7.2, 7.6 and 8.0 with solutions of Na_2CO_3 10% (w/v) and $NaHCO_3$ 10% (w/v). For each discrete pH value five replicates were used. The bottles were individually supplemented with either veratric or syringic acid, to a concentration of 2 mM, as the sole carbon and energy sources and maintained in an anaerobic culture chamber overnight to facilitate anaerobiosis prior to inoculation with 10% (v/v) inoculum from the appropriate veratric - or syringic acid - catabolising stock culture (2.3.1). The bottles were closed with Suba-seals and incubated at 30°C. Samples for the determination of the residual substrate concentration were taken at regular intervals over a period of 48h, for the syringic acid, and 72h, for the veratric acid culture.

Figures 12a and b show that for the syringic acid-catabolising microbial association the optimum pH approximated to 7.2, although growth occurred throughout the pH range tested. Substrate catabolisms at both pH 6.0 and 8.0, however, were very slow and were equivalent

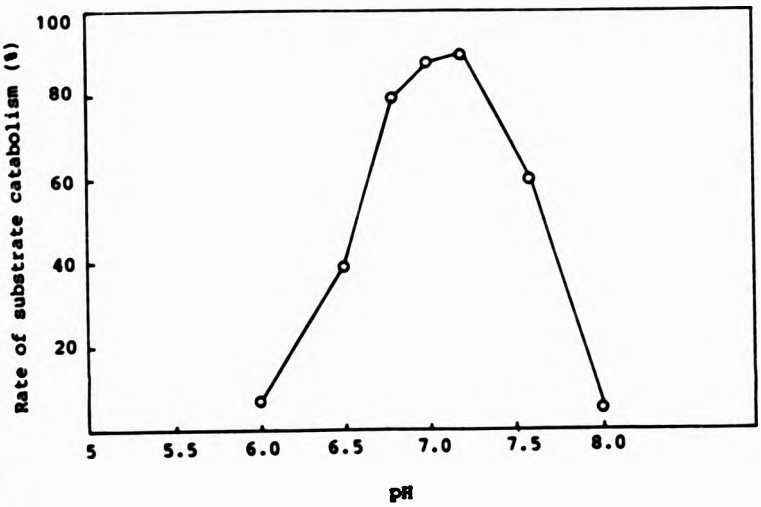
Figure 12

a) Changes in Residual Substrate Concentration During the Closed Culture Cultivation of the Syringic Acid-Catabolising Microbial Association Maintained at pH Values of 8.0 (o—o), 7.6 (●—●), 7.2 (□—□), 7.0 (■—■), 6.8 (△—△), 6.5 (▲—▲) and 6.0 (◇—◇),
b) Mean Syringic Acid Catabolic Rates of Closed Cultures Maintained under Different pH Regimes

(a)


[Residual substrate] (% original)
100
80
60
40
20
6
12
18
24
30
36
42
48
Time (h)

(b)


Rate of substrate catabolism (%)
100
80
60
40
20
5
5.5
6.0
6.5
7.0
7.5
8.0
pH

to 6.0 and 5.0% dissimilation after 42h incubation at 30°C.

From Figures 13a and b it can be seen that although the rates of catabolism of veratric acid under different pH regimes were much slower than those obtained with syringic acid the optimum pH was again of the order 7.0 to 7.2. Conversely, substrate catabolisms at pH 6.0 and 8.0 were somewhat higher than those obtained with syringic acid.

pH can exert both direct and indirect effects on bacterial cells and in addition can change the ionic form of the molecule under study (Al-Sarraj, 1983). Direct effects include the alteration of the intracellular pH which in turn can influence the constituent enzymes and transport mechanisms whereas indirect effects encompass the extracellular enzymes such as hydrolases.

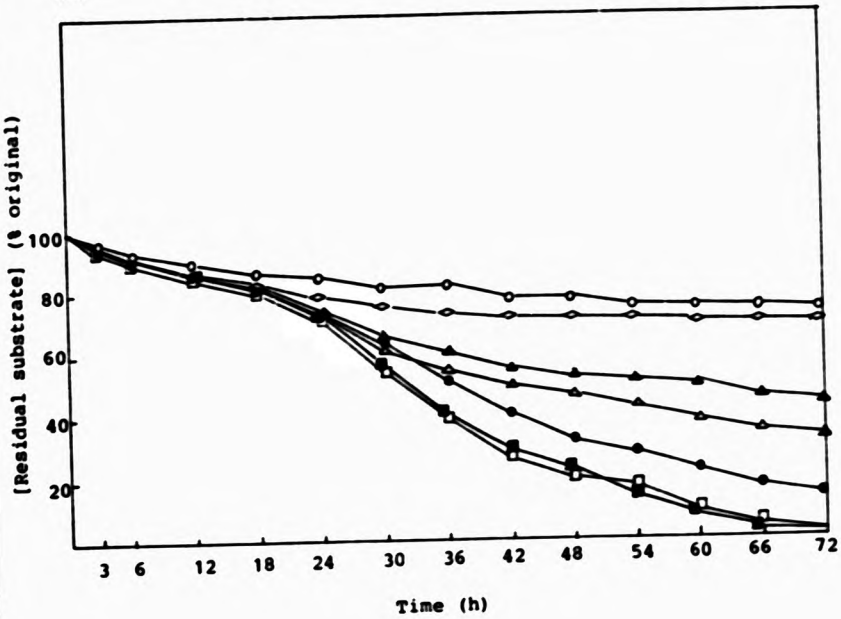
Leonowicz et al (1984) reported that laccases of the fungi Rhizoctonia praticola and Trametes versicolor formed different products from syringic acid and vanillic acid at different pH values. For example, four products were formed from syringic acid when the cultures were incubated at pH 6.9: 2, 6-dimethoxy-1, 4-benzoquinone, 6-methoxy-4-(2, 6-dimethoxy-4-carboxyphenoxy-)-benzoquinone, 2, 6-dimethoxy-4-(2, 6-dimethoxy-4-carboxyphenoxy) phenol and

Figure 13

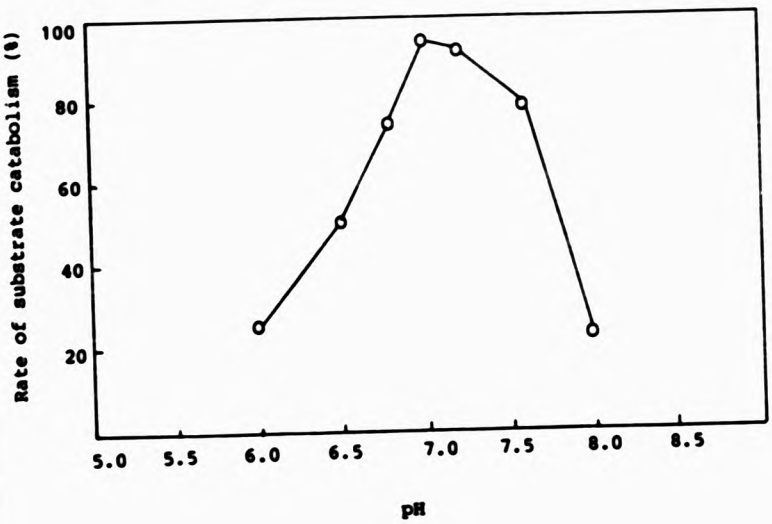
a) Changes in Residual Substrate Concentration During the Closed Culture Cultivation of the Veratric Acid-Isolated Microbial Association Maintained at pH Values of 8.0 (○—○), 7.6 (●—●), 7.2 (□—□), 7.0 (■—■), 6.8 (△—△), 6.5 (▲—▲) and 6.0 (◇—◇).

b) Mean Veratric Acid Catabolic Rates of the Isolated Microbial Association During Closed Culture Cultivation under Different pH Regimes.

(a)


[Residual substrate] (% original)
100
80
60
40
20
3
6
12
18
24
30
36
42
48
54
60
66
72
Time (h)

(b)


Rate of substrate catabolism (%)
100
80
60
40
20
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
pH

6-methoxy-4-[2, 6-dimethoxy-4-(2, 6-dimethoxy-4-carboxy-phenoxy) phenoxy]-1, 2-benzoquinone, although the last two products did not appear in an enzyme assay at pH 3.5. From these results they concluded that the catabolic pathways of syringic and vanillic acids were largely dependent on the pH of the culture during incubation. Unfortunately, no similar studies were made with anaerobic isolates.

5.3 Examination of the Effects of Temperature on Substrate Catabolism

Four discrete temperatures were used in this experiment: 25, 30, 35 and 40°C. The experimental design was the same as that described earlier (5.2) although in this case the pH was poised at 7.0 for all treatments. Concentrations of residual substrates were determined at regular intervals over periods of 36 and 60h for the syringic and veratric acid-catabolising cultures respectively.

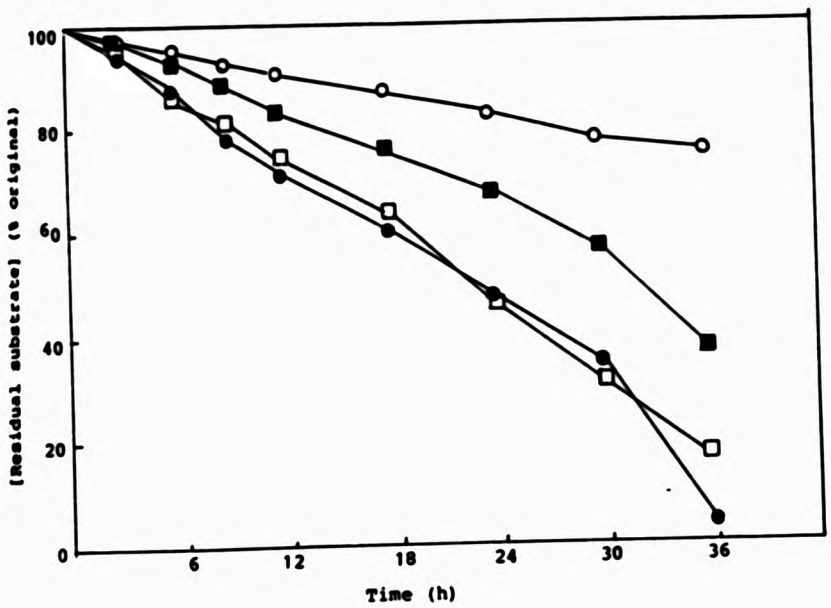
For both interacting microbial associations optimum temperatures of approximately 35°C were apparent (Figures 14a and b and 15a and b). From these figures it can also be seen that the catabolic rates slowed considerably at 40°C although the lowest temperature used (25°C) was not characterised by similar decreases. These results were possibly as expected since the

Figure 14

a) Changes in Residual Substrate Concentrations During the Closed Culture Cultivation of the Syringic Acid-Catabolising Microbial Association Maintained at Temperatures of 40°C (o——o), 35°C (●——●), 30°C (□——□) and 25°C (■——■)

b) Mean Syringic Acid Catabolic Rates of the Isolated Microbial Association During Closed Culture Cultivation at Different Temperatures

(a)


100
80
60
40
20
0
6
12
18
24
30
36
Time (h)
(Residual substrate) (% original)

(b)

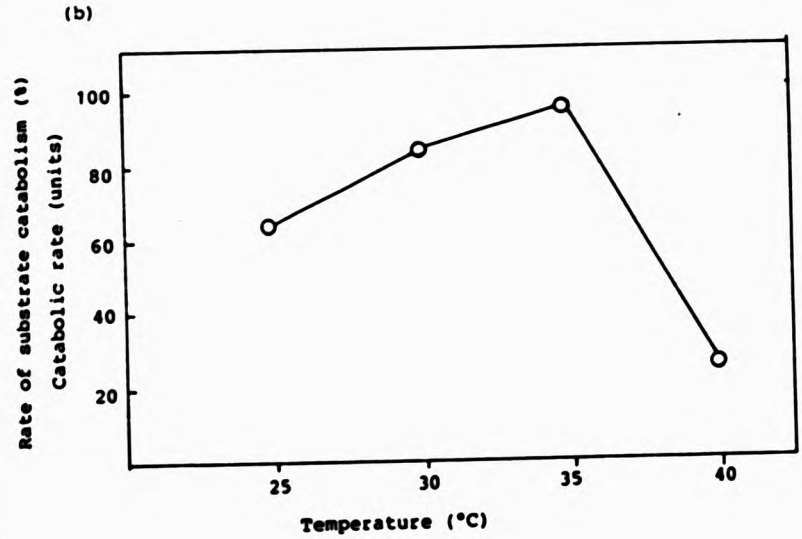
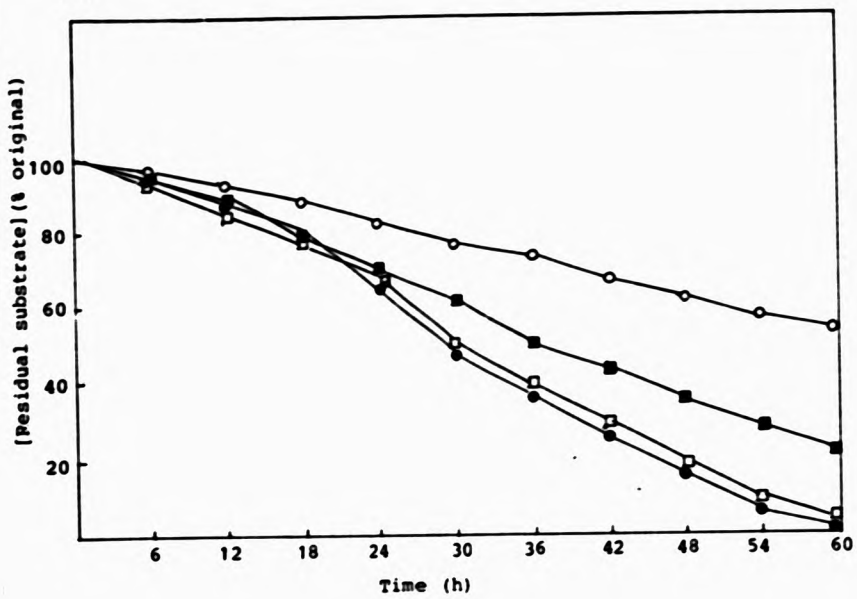

100
80
60
40
20
25
30
35
40
Temperature (°C)
Rate of substrate catabolism (%)
Catabolic rate (units)

Figure 15

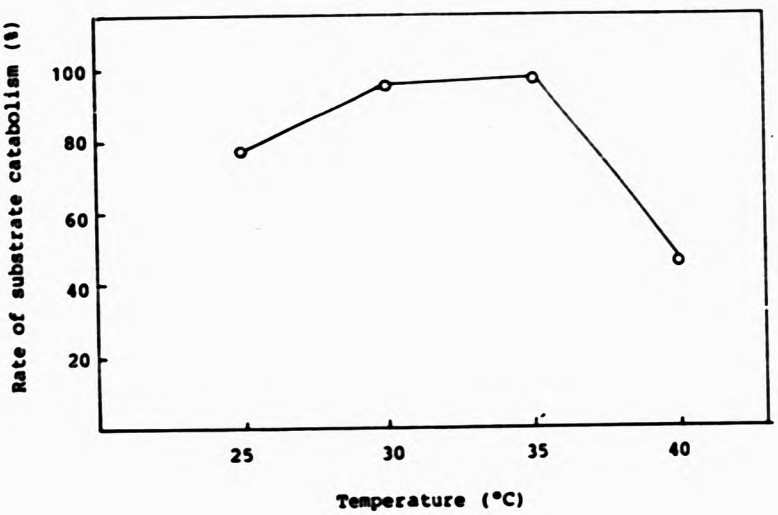
a) Changes in Residual Substrate Concentration During the Closed Culture Cultivation of the Veratric Acid-Catabolising Microbial Association Maintained at Temperatures of 40°C (o——o), 35°C (●——●), 30°C (□——□) and 25°C (■——■)

b) Mean Veratric Acid Catabolic Rates of the Isolated Microbial Association During Closed Culture Cultivation at Different Temperatures

(a)


[Residual substrate](% original)
100
80
60
40
20
6
12
18
24
30
36
42
48
54
60
Time (h)

(b)


Rate of substrate catabolism (%)
100
80
60
40
20
25
30
35
40
Temperature (°C)

interacting microbial associations had been subjected to the selection pressure of low in situ temperatures prior to sampling.

Since the isolated microbial associations contained more than one species it is possible that the temperature optima obtained (35°C) might not have been optimum for all components of the associations. It may be speculated, therefore, that the overall rate-limiting steps may be attributed to microbial catabolism at suboptimal temperatures.

5.4 Determination of the Effects of Sulphate Concentration on Methane Production

Although sulphate has been used in many synthetic media for isolation and cultivation of methanogens (Bryant, Tzeng, Robinson and Joyner, Jr., 1971; Zeikus and Henning, 1975; Patel, Roth, van den Berg and Clark, 1976), several reports have indicated that methane formation is generally absent in marine sediments in the presence of high concentrations of sulphate (Martens and Berner, 1974; Oremland and Taylor, 1978). Winfrey and Zeikus (1977) demonstrated that methanogenesis was inhibited by the addition of as little as 0.2 mM sulphate to fresh water sediments, and, similarly, the same inhibition was apparent when sulphate was added to a mixed culture of sulphate-reduc-

ing and methanogenic bacteria (Abram and Nedwell, 1978). These apparent inhibitions of methane formation have been attributed to a variety of factors including hydrogen sulphide toxicity (Cappenberg, 1974), kinetic competition for electron donors (Winfrey and Zeikus, 1977; Mountfort et al, 1980; Robinson and Tiedje, 1984) and thermodynamic considerations (Zehnder, 1978).

Against this background an experiment was made to examine the effects of increasing concentrations of sulphate on the catabolisms of syringic and veratric acids.

The mineral salts medium used in this study was the same as that described earlier (2.2.1B) with the exception that four sulphate concentrations, 2, 5, 10 and 20 mM were used, together with a control which contained no sulphate. Closed cultures were made in 150 ml serum bottles similar to those described earlier (5.2) and samples for methane analysis were taken at regular intervals over a period of 8 days, at which point terminal concentrations of residual substrate and sulphate were also determined.

From Figure 16 and Table 10 it can be seen that in the absence of sulphate addition to the veratric acid-catabolising microbial association methane formation showed a lag period of between 24 and 48h, after which exponential production was apparent over a period

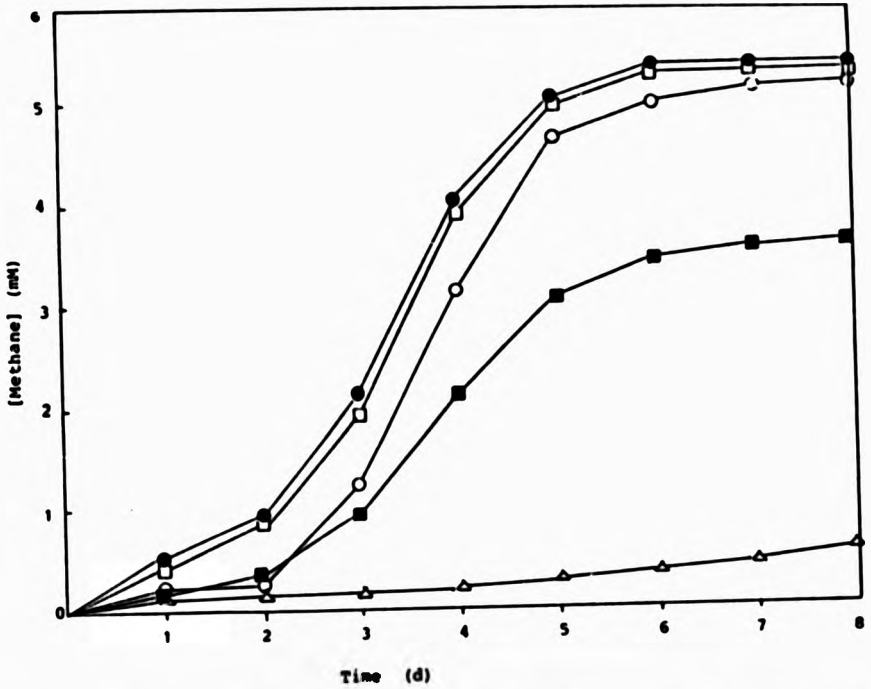


Figure 16. Methane Production from Veratric Acid by the Interacting Microbial Association During Closed Culture Cultivation in the Presence of 0.0 (O—O), 2.0 (●—●), 5.0 (□—□), 10 (■—■) and 20mM (△—△) Sulphate

Table 10. Changes in the Concentrations of Residual Sulphate and Residual Substrate During Closed Culture Cultivation of the Veratric and Syringic Acid-Catabolising Microbial Associations in the Presence of 0.0, 2.0, 5.0, 10.0 and 20.0 mM Sulphate.

SO_4^{2-} Concentrations (mM)	Veratric Acid		Syringic Acid	
	Residual SO_4^{2-} (%)	Residual Substrate (%)	Residual SO_4^{2-} (%)	Residual Substrate (%)
0.0	0.0	0.0	0.0	0.0
2.0	26.0	0.0	54.0	0.0
5.0	49.0	8.0	64.0	0.0
10.0	64.0	33.0	79.0	18.0
20.0	85.0	95.0	91.0	92.0

of approximately 3 days before a terminal concentration of 5.3 mM was recorded after 8 days incubation when no residual substrate remained. Addition of sulphate, at concentrations of 2 and 5 mM, did not affect either the rate of methane formation or the terminal concentration although the substrate catabolism was slightly affected. When 10 mM sulphate was added, however, the total substrate catabolism was reduced by 33% and methane formation by 28%. Similarly, the addition of 20 mM sulphate resulted in 94% inhibition of methane production with substrate catabolism similarly affected.

Figure 17 and Table 10 show the results of sulphate additions to the syringic acid-catabolising microbial association from which it can be seen that the residual sulphate concentrations were higher in all the treatments, and, as a consequence, the effects of sulphate were somewhat less dramatic with respect to substrate degradation. With one exception (10 mM) the methane concentrations, however, were comparable with those obtained with the veratric acid-catabolising microbial association. In the treatments in which the sulphate concentrations added did not exceed 5 mM no significant effects on the rates of methane generation and the residual substrate concentrations were apparent. The higher residual sulphate concentrations recorded (Table 10) were, however, accompanied by lower most

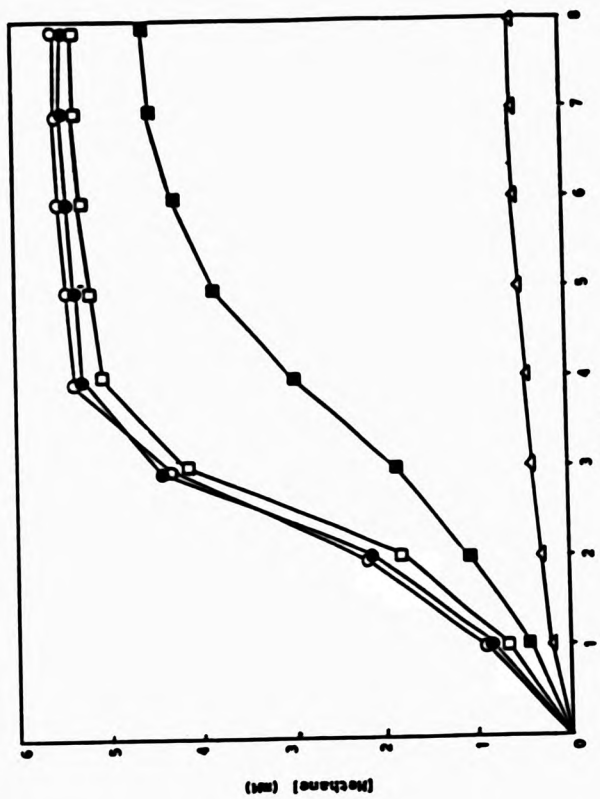


Figure 17. Methane Production from Syringic Acid by the Interacting Microbial Association During Closed Culture Cultivation in the Presence of 0.0 (○—○), 2.0 (●—●), 5.0 (□—□) 10 (■—■) and 20mM (△—△) Sulphate

probable numbers of sulphate-reducing bacteria, <100 ml^{-1} compared with 4 x 10^3 ml^{-1}, while the newly adapted veratric and syringic acid-catabolising associations contained approximately the same number of sulphate-reducing bacteria, 6.6 x 10^4 ml^{-1} for the veratric acid and 5.4 x 10^4 ml^{-1} for the syringic acid-catabolising associations. These observations have recently been supported by Isa, Grusenmeyer and Verstraete (1986a) who reported that sulphate reduction decreased from 71.5 to 49% 7 weeks after inoculation of a high-rate digester even though the digester was initially predominated by sulphate-reducing bacteria.

When 10 mM sulphate was added 18% of the substrate was not degraded during the incubation period and a decrease in the rate of methane formation was apparent, which may possibly be attributed to a proportional change in the CO_2:CH_4 ratio since Sleat and Robinson (1983) reported that in unaugmented sediment approximately equal volumes of methane and CO_2 were produced during benzoate fermentation whilst in the presence of sulphate, although the total volume of gas released was not affected, methane production was reduced. Inhibition of sulphate-reducing bacteria by molybdate (20 mM) then resulted in much higher proportions of methane to CO_2.

When the concentration of sulphate was further

increased to 20 mM, results directly comparable with the veratric acid data were obtained with a terminal concentration of methane equivalent to only 7% of the value of the unamended control recorded.

The results obtained with both microbial associations suggested that concentrations of sulphate below 5 mM did not effect either substrate degradation or methane production. These observations are consistent with the results obtained by Patel, Khan and Roth (1978) who found that the addition of sulphate up to a concentration of 4.6 mM did not affect methane production even though the optimum sulphate concentration was found to be in the range 0.16 to 0.52 mM. Sleat and Robinson (1983) similarly showed that the addition of 10 mM sulphate to stable isolated microbial associations which degraded benzoate did not affect either the stoichiometry or the rate of methane production. These results were somewhat surprising since it is well documented that sulphate-reducing bacteria have higher affinities for both acetate and hydrogen. Kristjansson *et al* (1982), for example, reported that *Methanobrevibacter arbophilus* had a K_s for H_2 of 6 mM while *Desulfovibrio vulgaris* had a K_s of 1 μM. Similarly, *Methanosarcina barkerii* was shown to have a K_s for acetate of 3 mM while *Desulfovibrio postgatei* had a K_s of 0.2 mM (Schönheit *et al*, 1982). Although there is

considerable evidence to support the theory that competition for acetate and H_2 is responsible for the inhibition of methanogenesis in sulphate-rich marine sediments, it has been reported that slow but detectable rates of methanogenesis were apparent even in the presence of sulphate (Oremland, 1975; Mountfort et al, 1980; Senior, Lindström, Banat and Nedwell, 1982; Winfrey and Ward, 1983). Since sulphate-reducing bacteria outcompete methanogenic species for common precursors one would predict that sulphate reduction would dominate methanogenesis in the presence of sulphate although the results obtained in this study did not support this theory. Similar results have also been reported by Isa, Grusenmeyer and Verstraete (1986a) who showed that sulphate-reducing bacteria were outcompeted by methanogenic bacteria in a high-rate anaerobic digester, with the former only competitive at hydrogen concentrations below 50 μM.

A lag phase was apparent when no sulphate was added to the veratric acid-catabolising culture but not with the syringic acid. Although a possible explanation was not readily apparent, a similar observation was recorded by Coutts (1986) who reported that methane production from butyrate was not detected for the first 45h of closed culture cultivation although subsequently butyrate was completely dissimilated.

Since the addition of 20 mM sulphate inhibited both veratric and syringic acid catabolisms it may be concluded that high concentrations of sulphate had some detrimental effect(s), either direct or indirect, on other members of the respective associations. For example, increases in pH, as a result of the formation of sodium sulphide from the reduction of sodium sulphate with subsequent hydrolysis to H_2S and NaOH (Postgate, 1979) may have been a contributory factor particularly with respect to the aromatic monomer-catabolising species since only 6 to 7% of the aromatic rings were actually dissimilated in each case. The increase in pH could also have resulted in increased hydrogen sulphide solubility which could have exerted inhibitory effects on the methanogens and other members of the associations. Kroiss and Wabnegg (1983) related inhibition of methanogenesis to the concentration of free H_2S in solution when they found 50% inhibition at a concentration of 50 mg l^{-1}. Additionally, sulphate-reducing bacteria have been reported by Isa et al (1986b) to be affected by high concentrations of free hydrogen sulphide (1,000 mg l^{-1}). The workers, however, did not provide evidence of whether the inhibition was due to toxicity by free H_2S or merely due to the unavailability of essential metallic elements as a result of insoluble sulphide precipitation.

5.5 Summary

The results presented showed that catabolism of veratric and syringic acids by the interacting microbial associations was detected at each discrete pH value used over the ranges 6.0 to 8.0 with optima between pH 7.0 and 7.2. Similarly, catabolisms of both molecules were detected at the discrete temperatures used between 25 and 40°C with optima at temperatures of approximately 35°C.

Catabolisms of these molecules were not affected by sulphate concentrations below 5 mM. Dissimilation of veratric acid in fact required an extended lag phase in the absence of sulphate thus possibly indicative of interaction between the veratric acid-catabolising and sulphate-reducing components of the association. For both microbial associations, however, the presence of sulphate at high concentrations (20 mM) exerted detrimental effects on substrate dissimilation and was possibly due to either the inhibition of methanogenic bacteria as a result of competition for the substrates H_2 and acetate or the unavailability of essential metallic elements as a result of insoluble sulphide precipitation.

CHAPTER SIX

METHANOGENIC FERMENTATIONS OF VERATRIC AND SYRINGIC ACIDS IN OPEN CULTURE

6.1 Introduction

The anaerobic catabolism of complex organic molecules to reduced products involves the formation of many metabolic intermediates mediated by the interactions of microbial species. Under ideal conditions the predominant end products are methane and CO_2. If, however, conditions are such that, for example, residence times are short, and principal intermediates such as acetate, propionate and butyrate are not rapidly removed, then these acids, rather than methane, become the dominant end products. In addition to the above other intermediates may also accumulate. For example, Kaiser and Hanselmann (1982b) showed that catechol was the major end product from veratric and vanillic acid dissimilations.

Accurate simulation of conditions to investigate interacting microbial associations can be best accomplished by the use of open cultures (Pfennig, 1984). Although, as discussed by Parkes (1982), open cultures have many advantages over closed cultures they have only been used to a limited extent in studies of anaerobic catabolism (Balba, 1978; Hansson and

Molin, 1981; Boone, 1982; Keith, Herbert and Hartfoot, 1982; Al-Sarraj, 1983; Traore, Fardeau, Hatchikian, Gall and Belaich, 1983; Chartrain and Zeikus, 1986a, b; Coutts, 1986).

In open cultures, fresh growth-limiting nutrients are continuously supplied and metabolic products, including potentially inhibitory products, continuously removed together with microbial cells. Conversely, closed cultures are characterised by the initial presence of excess substrate although no further input of growth substrates is made and metabolic end products, including any inhibitory end products, and cells, are not removed. Thus, the environmental conditions present in the system and hence selection conditions are constantly changing.

6.2 Open Culture Cultivations

The present study was made to examine the catabolisms of the selected aromatic monomers when subjected to a regime of increasing dilution rate. The continuous culture systems used were established by conversion of closed culture vessels into chemostats by the method described in 2.3.2. Two open cultures were established, with veratric and syringic acids (2 mM) as the limiting carbon and energy sources, and were subjected to step-wise dilution rate increases over the

range 0.005 to 0.025 h^{-1}. The results expressed at each particular discrete dilution rate were obtained after a minimum of 3 culture volume changes.

6.2.1 Veratric acid

The isolated veratric acid-catabolising microbial association was first grown in open culture for a period of 3 months, at a constant dilution rate of 0.005 h^{-1}, during which time quantitative analyses of residual substrate, residual sulphate, metabolic intermediates, methane, optical density, sulphide and pH were determined at regular intervals (Figure 18). Subsequently, a second culture was initated and was subjected to regular step-wise increases in dilution rate to 0.025 h^{-1} (Figure 19). The results are presented as the means of 4 to 5 replicate analyses.

From Figure 18 it can be seen that during the first 45 days of cultivation the influent veratric acid was completely catabolised to acetate and methane at approximate concentrations of 3.4 and 4.3 mM respectively, which, in total, accounted for 81.05% of the influent carbon. Subsequently, the concentration of residual substrate progressively increased and was accompanied by the appearance of an aromatic intermediate which was characterised by a spectrophotometric peak at 275 nm and thus identified as the demethoxylation product catechol. This molecule had a concentration of

Figure 18. Changes in the Concentrations of Residual Veratric Acid (o—o), Methane (e—e), Acetate (□—□), Residual Sulphate (■—■) and Catechol (△—△) During the Open Culture Cultivation of the Isolated Microbial Association at a Constant Dilution Rate of 0.005 h^{-1}

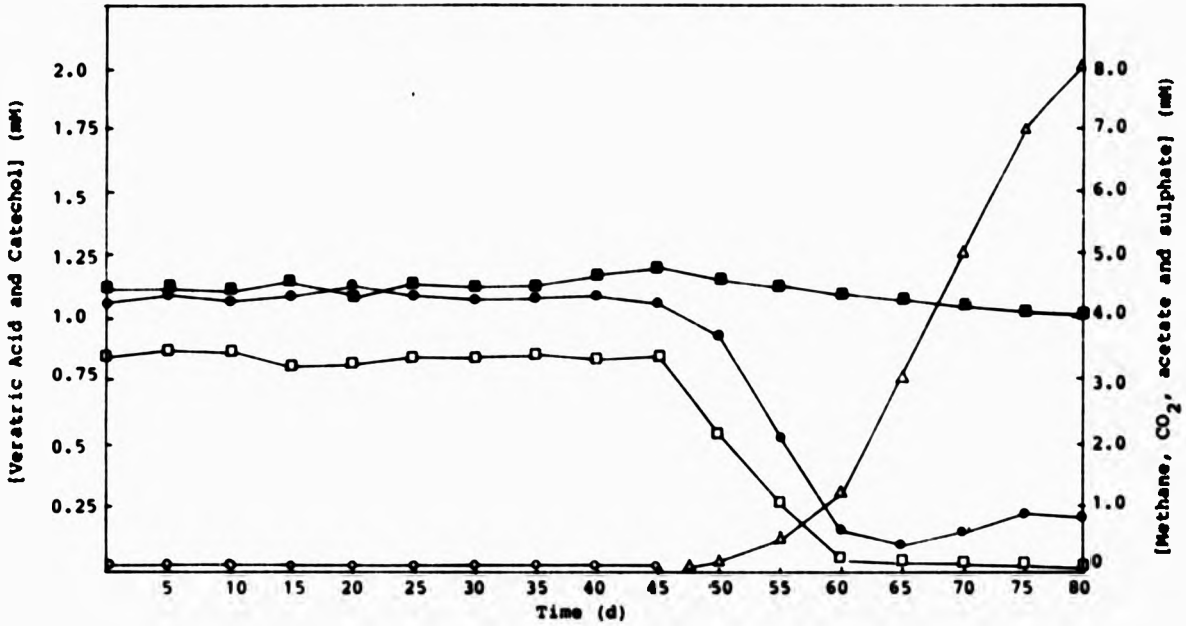
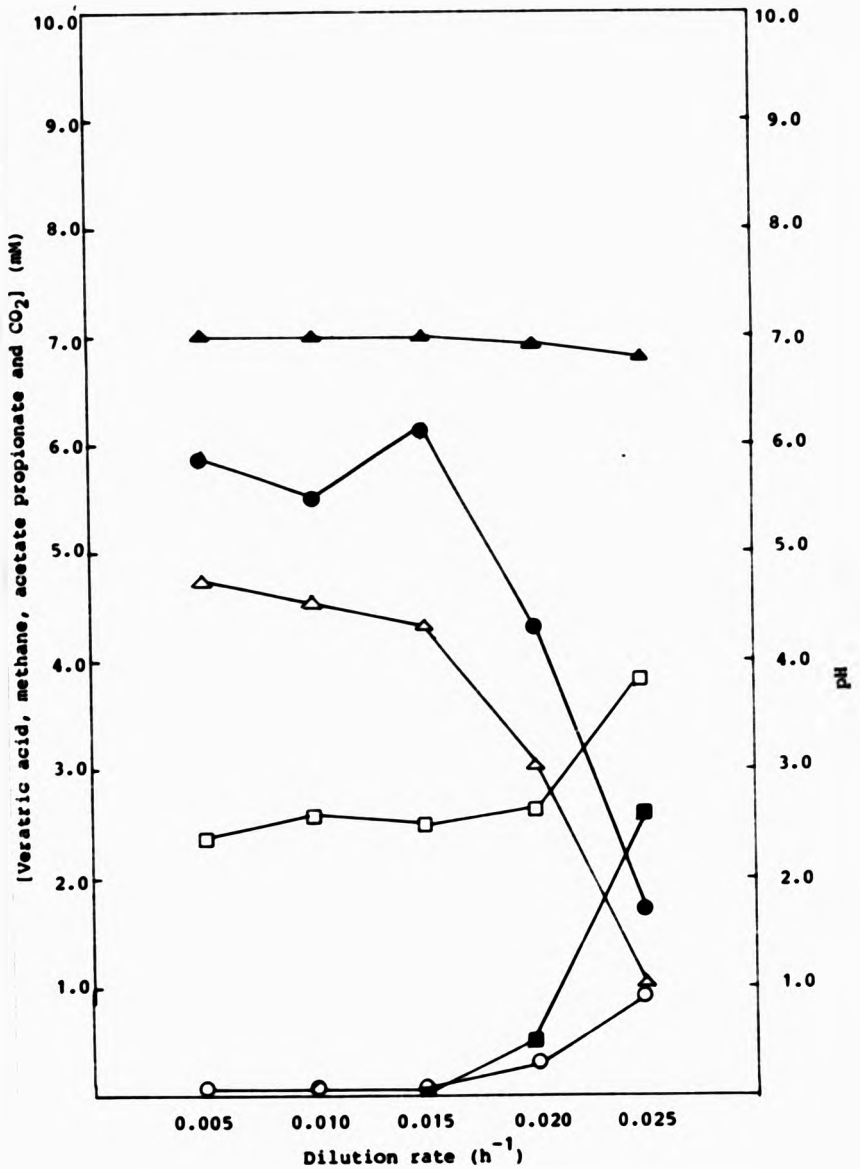

[Veratric Acid and Catechol] (mM)
[Methane, CO_2, acetate and sulphate] (mM)
Time (d)
2.0
1.75
1.5
1.25
1.0
0.75
0.5
0.25
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80

Figure 19. Changes in the Concentrations of Residual Veratric Acid (○—○), Methane (●—●), Acetate (□—□), Propionate (■—■), CO_2 (△—△) and pH (▲—▲) During the Open Culture Cultivation of the Isolated Microbial Association over the Dilution Rate Range 0.005 to 0.025h^{-1}


10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
[Veratric acid, methane, acetate propionate and CO_2] (mM)
pH
0.005
0.010
0.015
0.020
0.025
Dilution rate (h^{-1})

2 mM which was equivalent to a conversion of 100% of the original methoxylated substrate (veratric acid). The culture continued to produce this intermediate with no other change in the fermentation balance apparent even after a protracted cultivation period of two years. Concomitantly the concentrations of both acetate and methane progressively decreased to 0.33 and 0.65 mM respectively. The possibility existed, therefore, that a species with a similar metabolic potential to Acetobacterium woodii (Bache and Pfennig, 1981) or Clostridium pfennigii (Krumholz and Bryant, 1985), both of which have been shown to demethoxylate aromatic molecules, or the bacterium isolated by Mountfort and Asher (1986), which cleaved the phenyl-ether bonds of methoxylated aromatic substrates to give the corresponding hydroxy aromatic derivatives and mixed volatile fatty acids of chain length C_1, C_2 and C_4, was present in the microbial association. Since no spore forming bacterium was detected in the association and since no volatile fatty acids, other than acetate, were detected it was assumed that species similar to the one described by Krumholz and Bryant (1985) and by Mountfort and Asher (1986) were not present.

According to Bache and Pfennig (1981) the molar ratios of acetate formed: methanol consumed during the demethoxylation of aromatic monomers by monocultures of

Acetobacterium woodii were of the order of 0.75 (equation 4):

$$4CH_3OH + CO_2 \longrightarrow 3CH_3COOH + H_2O \qquad (4)$$

Similarly, with vanillic, syringic and veratric acids the ratios were again 0.75 according to the following equations:

$$4 \text{ vanillic acid} + 2CO_2 + 2H_2O \longrightarrow 4 \text{ protocatechuic acid} + 3CH_3COOH \qquad (5)$$

$$2 \text{ syringic acid} + 2CO_2 + 2H_2O \longrightarrow 2 \text{ gallic acid} + 3CH_3COOH \qquad (6)$$

$$2 \text{ veratric acid} + 2CO_2 + 2H_2O \longrightarrow 2 \text{ protocatechuic acid} + 3CH_3COOH \qquad (7)$$

Thus, each four methoxyl groups produced three acetates, so, from theoretical considerations, when the latter are converted to methane 3 mM should be produced. The results obtained showed that the mean concentration of methane produced during the first 45 days of cultivation was equivalent to 30% of the theoretical value. The decrease in methane production from the theoretical value was possibly due to the utilisation of the

precursors by sulphate-reducing bacteria since it has been reported that the affinity of sulphate-reducing bacteria to H_2 and acetate at low concentrations of substrate was 10 to 15 fold that of methanogens (Robinson and Tiedje, 1984). In addition, at low substrate concentrations the ratio of the growth rates of sulphate-reducing bacteria: methanogenic bacteria has been shown to be 18:8 (Isa et al, 1986b).

The results of the residual sulphate concentrations did not show the same dramatic changes as those observed for veratric acid, acetate and methane and, in fact, only a slight increase in sulphate reduction was observed during the 90 days cultivation period. This increase could possibly be attributed to limited changes in metabolic potentials similar to the ones reported by Kristjansson, Schönheit and Thauer (1982) who demonstrated that sulphate-reducing bacteria can competitively displace methanogenic bacteria due to their increased affinity for acetate and H_2.

In order to examine the shift in fermentation balance which was apparent after 45 days cultivation a second open culture was initiated under the same conditions. In this instance, however, complete substrate dissimilation continued and catechol did not appear. After two years of open culture cultivation the culture was subjected to step-wise increases in dilution rate

from 0.005 to 0.025 h^{-1} and the concentrations of residual sulphate, gaseous sulphide, soluble sulphide, pH and biomass was assayed after first allowing a minimum of three culture volume changes at each discrete dilution rate.

The results presented in Figure 19 show that residual veratric acid was not detected until the dilution rate was increased above 0.015 h^{-1} which suggested that the veratric acid-catabolising species had a D_{crit} (the dilution rate when the specific growth rate is maximum), within the constraints of the microbial association, of >0.015 and <0.020 h^{-1}. Even at a dilution rate of 0.025 h^{-1}, however, partial catabolism was still detected which suggested that either the constraints of the microbial association on the veratric acid-catabolising species had changed possibly as a result of the displacement of a component species or that catabolism was confined to species attached to the chemostat wall. For the dilution rate range of 0.005 to 0.015 h^{-1} the concentration of acetate was maintained at approximately 2.4 mM although subsequent increases to 2.6 and 3.8 mM were observed at the higher dilution rates of 0.020 and 0.025 h^{-1} respectively. At dilution rates below 0.020 h^{-1} propionate and other volatile fatty acids were not detected. However, when the dilution rate was raised to 0.020 h^{-1} propionate was

detected at a concentration of 0.5 mM and then further increased to 2.65 mM at a dilution rate of 0.025 h^{-1}. The appearance of propionate was accompanied by a reduction in methane concentration to 4.7 and then 1.5 mM at the higher dilution rates of 0.020 and 0.025 h^{-1} respectively. Similarly, CO_2 formation was also reduced to 3.1 and 1.0 mM at the same dilution rates. Thus it was apparent that at a dilution rate of 0.020 h^{-1} there was a major shift in the fermentation balance possibly as a result of the displacement of methanogenic species other than those attached to the chemostat wall. Although, in this instance, the concentration of propionate was not inhibitory, Hobson, Bousfield and Summers (1974) found that when propionate accumulated in digesters, methanogenesis was impaired and later Hobson and Shaw (1976) found that propionate was inhibitory to hydrogen-utilising methanogenic bacteria such as Methanobacterium formicicum.

The initially low concentrations of metabolites observed for the first three dilution rates showed that the influent veratric acid was effectively degraded to methane and the carbon balance of the biomethanation was equivalent to 74.29% of the theoretical maximum. The major products were methane (5.83 mM), CO_2 (4.72 mM) and acetate (2.4 mM). These values, however, deviated from theoretical values (Tarvin and Buswell, 1934) which

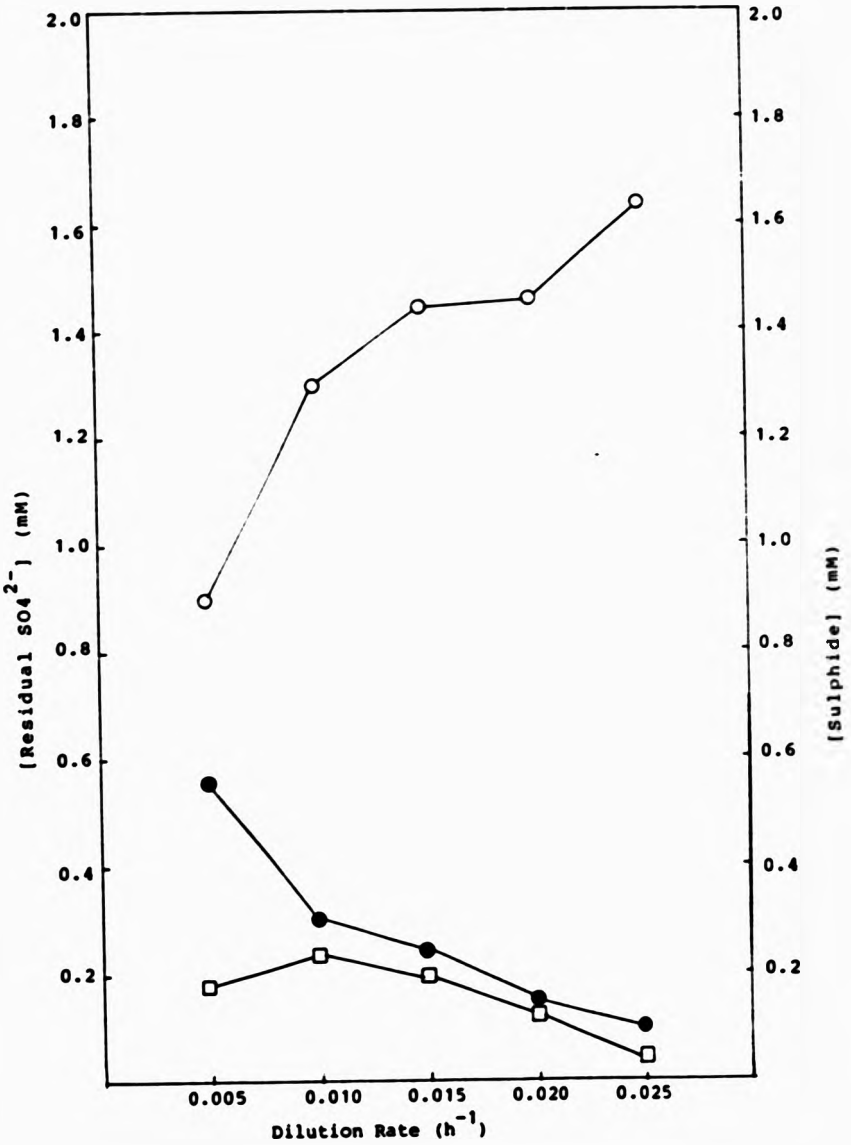
predicted that 9.5 mM of methane and 9.5 mM of CO_2 should have been produced from 2 mM veratric acid.

The residual sulphate, gaseous and soluble sulphide concentration data are presented in Table 11 and Figure 20, from which it can be seen that from the original 2 mM influent sulphate concentration the sulphate utilised decreased from 1.1 mM (55%) at a dilution rate of 0.005 h^{-1} to 0.36 mM (18%) at a dilution rate of 0.025 h^{-1}. Similarly, the gaseous and soluble sulphide concentrations decreased as the dilution rate increased. The gaseous sulphide concentration decreased from 0.56 mM (28%) at a dilution rate of 0.005 h^{-1} to 0.1 mM (5%) at a dilution rate of 0.025 h^{-1} whilst the soluble sulphide concentrations also decreased from 0.18 mM (9%) to 0.04 mM (2%) at the dilution rates of 0.005 and 0.025 h^{-1} respectively. These results could have been partially attributed to washout of free sulphate-reducing bacteria when the dilution rate was raised above 0.005 h^{-1} although this possibility does not explain the stepped changes observed. The presence of sulphate-reducing bacteria appeared to have little or no effect on the methanogenic fermentation of veratric acid despite the fact that a limited concentration of hydrogen sulphide was needed to satisfy the methanogenic population requirement for cell synthesis and maintenance of adequate redox conditions (Hungate, 1969).

Table 11. Sulphur Balances for the Open Culture Catabolism of Veratric Acid by the Isolated Microbial Association. Determinations Expressed as % of Influent Sulphate Concentration Presented in Brackets.

Dilution Rate (h^{-1})	Residual SO_4^{2-} (mM)	H_2S (mM)	HS^- (mM)	SO_4^{2-} (mM)
0.005	0.9 (45)	0.56 (28)	0.18 (9)	0.36 (18)
0.010	1.35 (65)	0.30 (30)	0.24 (12)	0.16 (8)
0.015	1.44 (72)	0.24 (12)	0.20 (10)	0.12 (6)
0.020	1.46 (73)	0.16 (8)	0.14 (7)	0.24 (12)
0.025	1.64 (82)	0.10 (5)	0.04 (2)	0.22 (11)

Figure 20. Changes in the Concentrations of Residual Sulphate (○—○), gaseous Sulphide (●—●) and Soluble Sulphide (□—□) During the Open Culture Cultivation of the Isolated Microbial Association, over the Dilution Rate Range 0.005 to 0.025h^{-1}, in the presence of 2mM Influent Veratric Acid


2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
[Residual SO_4^{2-}] (mM)
[Sulphide] (mM)
0.005
0.010
0.015
0.020
0.025
Dilution Rate (h^{-1})

Since the concentrations of residual sulphate, gaseous and soluble sulphides all changed with dilution rate, it would appear that at least two populations of sulphate-reducing bacteria were present, one of which was dilution rate independent and the other dilution rate dependent. At no dilution rate was all the sulphate reduced or assimilated thus it would appear that the sulphate-reducing bacteria were either carbon or electron limited.

The pH of the culture remained relatively constant at each discrete dilution rate although a decrease of 0.2 units was observed at the dilution rate of 0.02 h^{-1} (Figure 19).

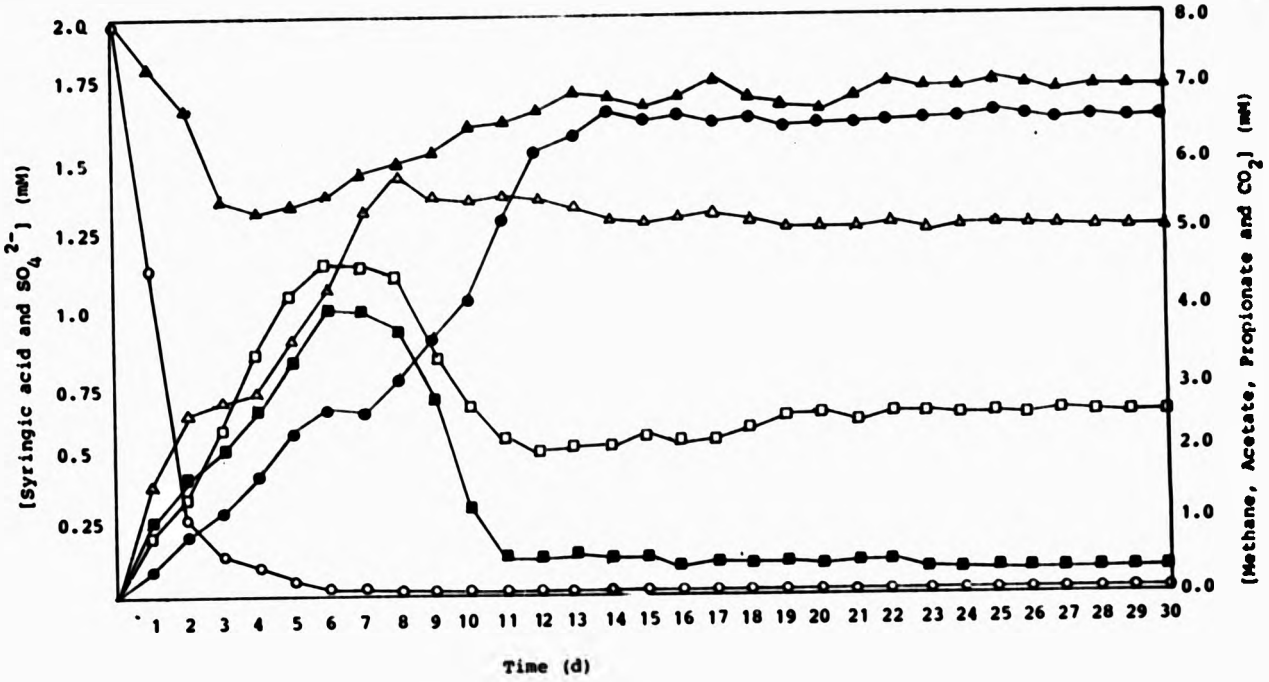
The results of biomass analyses fluctuated not only from dilution rate to dilution rate but also at the same dilution rate although at each discrete dilution rate the optical densities were particularly low. Similarly, when the population size was determined by dry weight estimation then fluctuations were again apparent. Parkes (1982) reported that great care must be taken if changes in culture absorbancy are used as a measure of biomass of a mixed community, both in batch and chemostat culture. The use of absorbance as a measure of biomass is quite acceptable for monocultures although the biomasses of individual species of an interacting microbial association may change quite markedly whilst the overall culture absorbance remains constant.

6.2.2 Syringic acid

The results for the catabolism of syringic acid by the isolated microbial association grown in open culture for a period of one month at a constant dilution rate are shown in Figure 21 from which it can be seen that at a dilution rate of 0.005 h^{-1} syringic acid was catabolised and methane, acetate, propionate and CO_2 were produced. After one week the concentrations of both acetate and propionate decreased from 4.5 to 2.3 mM (acetate) and from 4.0 to 0.5 mM (propionate) whilst the concentrations of methane and CO_2 increased from 2.5 to 6.5 mM and from 2.8 to 5.2 mM respectively. The increases in both methane and CO_2 concentrations could possibly have been due to the reduction in number of sulphate-reducing bacteria, as estimated by the MPN method, as a result of washout, which was accompanied by an increase in the concentration of residual sulphate. Thus it would appear that the net result was an increase in the availability of acetate and H_2 for methanogenesis. However, the presence of both acetate and propionate was possibly indicative of more efficient ring cleavage activity. These results were somewhat different to those obtained with the veratric acid-catabolising microbial association where propionic acid was not detected as an intermediate.

For the cultivation period 8 to 30 days the

Figure 21. Changes in the Concentrations of Residual Syringic Acid (○—○), Methane (●—●), Acetate (□—□), Propionate (■—■), CO_2 (△—△) and Residual Sulphate (▲—▲) During the Open Culture Cultivation of the Isolated Microbial Association at a Constant Dilution Rate of $0.005h^{-1}$


[Syringic acid and SO_4^{2-}] (mM)
[Methane, Acetate, Propionate and CO_2] (mM)
Time (d)
2.0
1.75
1.5
1.25
1.0
0.75
0.5
0.25
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

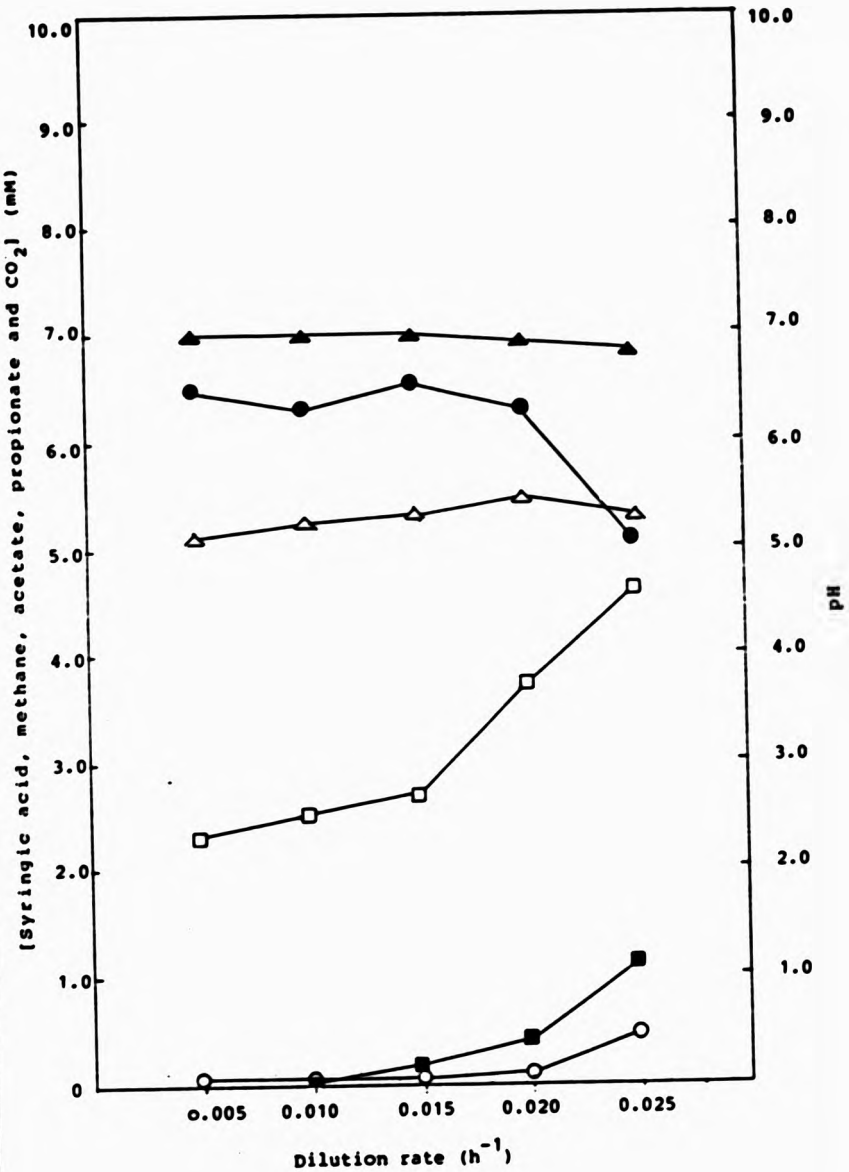
concentrations of methane, CO_2, acetate and propionate remained relatively constant and were 6.5, 5.2, 2.5 and 0.5 mM respectively.

Concomitant with syringic acid catabolism, sulphate utilisation after four days cultivation was equivalent to 1.25 mM (65% of the influent) and subsequently decreased to approximately 0.5 mM, which was equivalent to 25% utilisation, after 3 weeks cultivation from which point the residual concentration remained relatively constant.

The decrease in sulphate utilisation could again have been indicative of washout of free sulphate-reducing bacteria although at the applied dilution rate ($0.005\ h^{-1}$) one would not have expected this to have occurred. A similar observation was, however, reported by Al-Sarraj (1983) who examined catabolism of benzyl alcohol, anthranilic acid and cinnamyl alcohol by interacting microbial associations isolated from salt-marsh sediment.

The results presented in Figure 22 show the changes in the concentrations of residual substrate, acetate, CO_2 and methane after the chemostat was subjected to progressive increases in dilution rate over the range 0.005 to $0.025\ h^{-1}$ from which it can be seen that residual syringic acid was not detected at dilution rates of $0.020\ h^{-1}$ and below. Thus it was apparent that

Figure 22. Changes in the Concentrations of Residual Syringic Acid (○—○), Methane (●—●), Acetate (□—□), Propionate (■—■), CO_2 (△—△) and pH (▲—▲) During the Open Culture Cultivation of the Isolated Microbial Association over the Dilution Rate Range 0.005 to 0.025h^{-1}


10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
[Syringic acid, methane, acetate, propionate and CO_2] (mM)
pH
0.005
0.010
0.015
0.020
0.025
Dilution rate (h^{-1})

the culture was syringic acid limited. Corresponding total carbon balance data showed that 97.8% of the syringic acid was converted to methane, CO_2 and acetate. Since the syringic acid was not detected at each of the first three dilution rates examined it appeared that the syringic acid-catabolising species had a D_{crit}, within the constraints of the microbial association, of >0.020 h^{-1} and <0.025 h^{-1}.

The other results obtained were typical of open culture data since the concentrations of methane, CO_2 and pH did not change significantly over the range of dilution rates examined. Conversely, the concentration of propionate increased from 0.4 mM at a dilution rate of 0.015 h^{-1} to 1.2 mM at a dilution rate of 0.025 h^{-1}, at which point the acetate concentration also increased. The increases in acetate and propionate were accompanied by reductions in the concentrations of both methane and CO_2. Thus it was apparent that at a dilution rate of 0.025 h^{-1} the methanogenic species, other than those attached to the chemostat walls, had been displaced and, as a consequence, a major shift in the fermentation balance had resulted.

Once again the pH remained relatively constant at the first four dilution rates examined although there was a slight decrease when the dilution rate was increased to 0.025 h^{-1}. A possible explanation for this

was that the increases in the concentrations of volatile fatty acids were responsible.

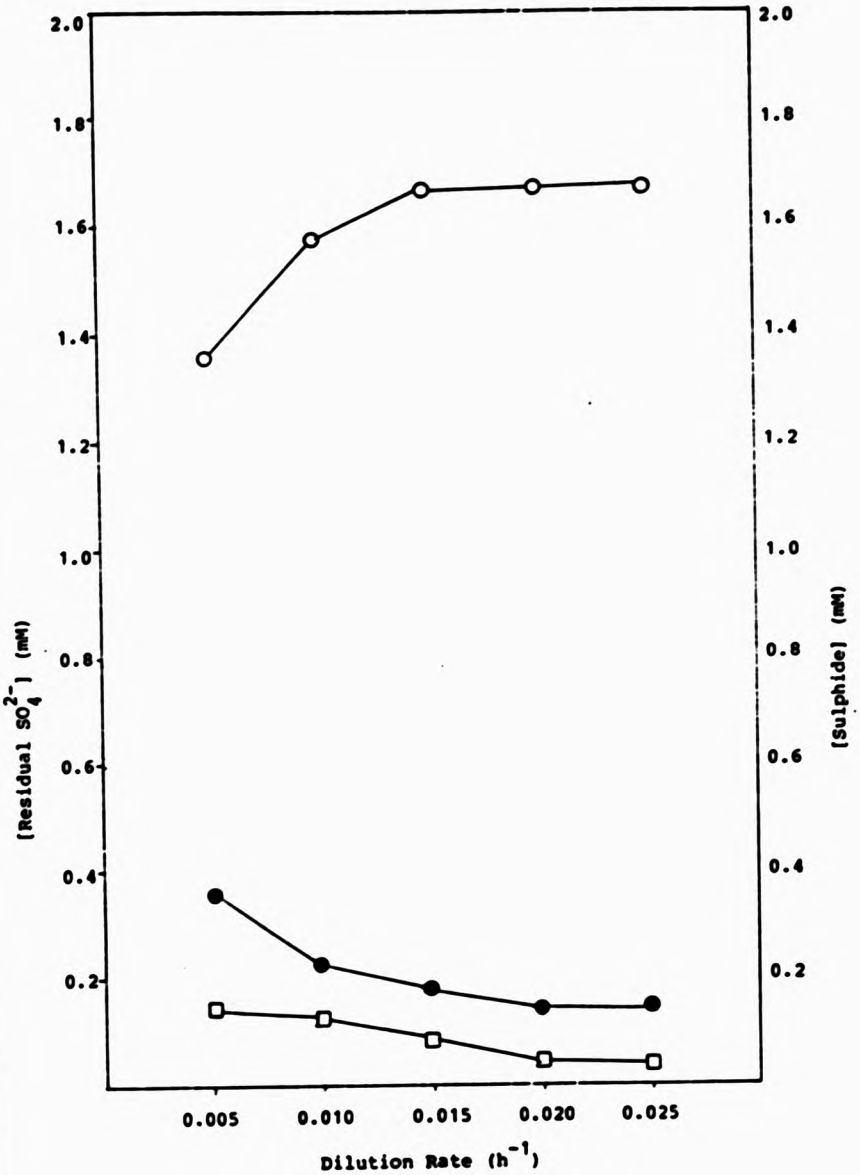
It is clear from the initially low concentrations of metabolites recorded for the first four dilution rates that the syringic acid was effectively degraded to methane and CO_2. The carbon balance showed that the total carbon recovered was equivalent to 89.39% of the theoretical maximum. Thus the observed discrepancies could possibly be attributed to biomass synthesis. The major products were methane (6.47 mM), CO_2 (5.33 mM) and acetate (2.3 mM).

Table 12 and Figure 28 show the sulphur balances for the dilution rate range examined from which it can be seen that the residual sulphate concentrations increased at each of the dilution rates examined from 1.36 mM (68.0%), at a dilution rate of 0.005 h^{-1}, to 1.7 mM (85.0%), at a dilution rate of 0.025 h^{-1}. Of the 2 mM sulphate used at the dilution rate of 0.005 h^{-1}, the gaseous and soluble sulphide concentrations accounted for 0.36 and 0.14 mM respectively. Subsequently, concentrations of both gaseous and soluble sulphide decreased to 0.14 mM and 0.04 mM at a dilution rate of 0.025 h^{-1}. These results were somewhat atypical of open culture data especially when one considers that the syringic acid was completely catabolised at each of the first three dilution rates examined when the methane

Table 12. Sulphur Balances for the Open Culture Catabolism of Syringic Acid by the Isolated Microbial Association. Determinations Expressed as % of Influent Sulphate Concentration Presented in Brackets.

Dilution Rate (h^{-1})	Residual SO_4^{2-} (mM)	H_2S (mM)	HS^- (mM)	SO_4^{2-} (mM)
0.005	1.36 (68)	0.36 (18)	0.14 (7)	0.14 (7)
0.010	1.58 (79)	0.22 (11)	0.12 (6)	0.08 (4)
0.015	1.66 (83)	0.18 (9)	0.08 (4)	0.08 (4)
0.020	1.70 (85)	0.14 (7)	0.04 (2)	0.12 (6)
0.025	1.70 (85)	0.14 (7)	0.04 (2)	0.12 (6)

Figure 23. Changes in the Concentrations of Residual Sulphate (O—O), Gaseous Sulphide (●—●) and Soluble Sulphide (□—□) During the Open Culture Cultivation of the Isolated Microbial Association, over the Dilution Rate Range 0.005 to 0.025h^{-1}, in the Presence of 2mM Influent Syringic Acid


2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
[Residual SO_4^{2-}] (mM)
[Sulphide] (mM)
0.005
0.010
0.015
0.020
0.025
Dilution Rate (h^{-1})

and CO_2 concentrations did not change considerably. These results once again could have been indicative of washout of free sulphate-reducing bacteria with subsequent activity confined to surface-attached species and thus growth rate independent. Again, since at no dilution rate was all the sulphate reduced, it would appear that the sulphate-reducing bacteria were either carbon and/or electron limited.

6.2.3 *Determination and evaluation of kinetic parameters*

Open culture:

The saturation constant (K_s) is a measure of the affinity a single species or microbial association has for the growth-limiting substrate. Thus, the lower the K_s then the higher the affinity and the greater the capacity to grow rapidly in an environment with low growth-limiting substrate concentrations.

K_s values for the veratric and syringic acid-catabolising microbial associations were determined from open culture data by plotting experimental values of $\frac{1}{D}$ against $\frac{1}{S}$ in the Lineweaver and Burk (1934) mode, where D is the dilution rate and S is the substrate concentration. The lines were fitted by regression analysis and the abscissa intercepts gave the $-\frac{1}{K_s}$ values.

The data presented in Table 13 shows that the K_s value for the veratric acid-catabolising microbial association was 0.11 mM and the corresponding value for the syringic acid-catabolising association was 0.18 mM.

The maximum specific growth rates (μ_{max}) were then estimated from the same plots since the intercepts on the y axes are equivalent to $\frac{1}{\mu}$. Thus, for the two associations (veratric and syringic acid-catabolising) values of 0.03 and 0.04 h^{-1} were obtained respectively.

From the results it can be seen that the veratric acid-catabolising association had a higher affinity for its growth-limiting substrate than did the syringic acid-catabolising association. However, it is interesting to note that the μ_{max} of the veratric acid-catabolising association (0.03 h^{-1}) was lower than the equivalent value (0.04 h^{-1}) for the syringic acid-catabolising association.

Closed culture:

A series of closed cultures were made in serum bottles with seven different substrate concentrations (0.03125, 0.0625, 0.125, 0.25, 0.5, 1.0 and 2.0 mM) of veratric and syringic acids and mid-exponential growth rates (μ) were calculated according to the equation:

$$\mu = \frac{\log_e X_t - \log_e X_o}{t_t - t_o} \, h^{-1} \qquad (8)$$

Table 13. Saturation Constant (K_s) and Maximum Specific Growth Rate (μ_{max}) Values of the Veratric and Syringic Acid-Catabolising Microbial Associations Calculated from Open and Closed Culture Data.

Culture	Open Culture		Closed Culture	
	K_s (mM)	μ_{max} (h^{-1})	K_s (mM)	μ_{max} (h^{-1})
Veratric acid-catabolising	0.11	0.03	0.28	0.03
Syringic acid-catabolising	0.18	0.04	0.32	0.04

where μ is the specific growth rate

X_t is the culture absorbance at time t

X_o is the culture absorbance at time o

Discrete values of μ were then plotted against S and μ_{max} calculated from the resultant plot; with K_s equivalent to $\frac{1}{2}\ \mu_{max}$.

The K_s values were also estimated by plotting values of $\frac{1}{\mu}$ against $\frac{1}{s}$ in Lineweaver and Burk (1934) mode. The lines were fitted by regression analysis and the abscissa intercepts gave the $-\frac{1}{K_s}$ values.

The results presented in Table 13 show that the K_s values were 0.28 and 0.32 mM for the veratric and syringic acid-catabolising microbial associations respectively thus, once again, the veratric acid-catabolising association had the higher affinity for its substrate. As with the open culture data calculations, the veratric acid-catabolising association had a lower (0.03 h^{-1}) μ_{max} than the syringic acid-catabolising association (0.04 h^{-1}). It is interesting to note that of the two cultivation system types (open and closed) for both associations higher substrate affinity and higher maximum specific growth rates were obtained with the former. The differences were possibly due to the different constraints of the two cultivation systems. For example, the results obtained showed that at the higher dilution rates there were shifts in

metabolic activities with both open cultures, which were possibly indicative of either species washout or changes in metabolic potentials. It is also possible that the removal of the metabolic products, including potentially inhibitory products, in the case of the open cultures, facilitated these differences. As a consequence of species displacement from the open cultures and thus possible differences in the final species compositions of the microbial associations cultivated in open and closed cultures then direct comparisons of kinetic data of the two are probably invalid.

6.3. Summary

Veratric and syringic acids were effectively degraded by the interacting microbial associations in open culture to methane and the carbon balances of the biomethanations were equivalent to 74.29 and 97.8% respectively. In both cases, at dilution rates below 0.015 h^{-1} complete catabolism was apparent. The veratric acid-catabolising species had a D_{crit}, within the constraints of the microbial association, of >0.015 and <0.020 h^{-1}. At a dilution rate of 0.020 h^{-1} increases in the concentrations of volatile fatty acids were apparent and were accompanied by reductions in the concentrations of both methane and CO_2 thus indicative of a major shift in the fermentation balance possibly as

a result of the displacement of growth-dependent methanogenic species. The syringic acid-catabolising species, however, had a D_{crit}, within the constraints of the microbial association of >0.02 and <0.025 h^{-1}. Increases in the concentrations of volatile fatty acids were again apparent but this time at a dilution rate of 0.015 h^{-1} when reductions in the concentrations of methane and CO_2 were also observed. Again the displacement of growth dependent methanogenic species appeared to have been responsible for the shift in fermentation balance.

The results for the sulphate metabolism patterns were different from those of the substrate catabolisms since the concentrations of residual sulphate, gaseous and soluble sulphides all changed with dilution rate. At the lower dilution rate of 0.005 h^{-1}, 1.1 mM (55.0%) and 0.64 mM (32.0%), of the influent sulphate was metabolised by the veratric and syringic acid-catabolising associations respectively, while at the higher dilution rate of 0.025 h^{-1} the corresponding values were 0.35 mM (18.0%) and 0.30 mM (15.0%). Since at no dilution rate was all the sulphate reduced or assimilated it would appear that the sulphate-reducing bacteria were either carbon or electron limited.

The kinetic determinations showed that the K_s and μ_{max} values for the veratric and syringic acid-

catabolising microbial associations in open culture were 0.11 mM and 0.03 h^{-1}, and 0.18 mM and 0.04 h^{-1} respectively. Thus it was apparent that of the two, the veratric acid-catabolising association had the higher affinity for its growth-limiting substrate but the lower μ_{max}. Corresponding determinations with closed cultures resulted in higher K_s and lower μ_{max} values for both associations thus exemplifying the different constraints operative within the two cultivation systems.

As a result of species displacement with increasing dilution rates and thus changing populations, the validity of determining values of K_s and μ_{max} from open culture data must be questioned. As a consequence of this direct comparisons of estimates made with open and closed culture data are not valid. Thus, for comparative purposes closed culture data only may be used.

CHAPTER SEVEN

BIOCHEMISTRY OF VERATRIC AND SYRINGIC ACID CATABOLISMS UNDER ANOXIC CONDITIONS

7.1 Introduction

In the presence of oxygen oxygenases are involved as key enzymes in the ortho-(intradiol) and meta-(extradiol) cleavages of the aromatic ring. Ortho fission involves the oxidative rupture of a bond between adjacent carbon atoms bearing the hydroxyl groups of an o-dihydroxyphenol, while meta fission involves the oxidative rupture of the bond between a carbon atom bearing a hydroxyl group and one bearing a hydrogen atom or side chain substituent (Cain, 1980). In the absence of light, nitrate or sulphate many simple aromatic compounds such as benzoate, cinnamate, and ferulic, syringic and vanillic acids are completely degraded to carbon dioxide and methane under anoxic conditions by naturally-occurring interacting microbial associations, although different enzymatic routes are necessitated. Evans (1977) postulated that the aromatic ring is converted to a cyclohexane during the initial reductive phase. In an oxidative step two electrons are then removed to give the corresponding enol whose carbonyl tautomer represents the ultimate substrate for ring cleavage. The aliphatic fission product is then further oxidised to acetate.

7.2 Detection and Identification of Metabolic Intermediates

Basic mineral salts medium (2 litres), supplemented with either veratric or syringic acid (2 mM), was inoculated with the appropriate isolated microbial association at a ratio of 1:10 (v/v). The closed cultures were then incubated at 30°C and monitored for substrate dissimilation, volatile fatty acids and methane evolution at regular intervals by the methods described in 2.4. After approximately 20 to 25% reduction in the concentrations of the residual substrates, 2-Bromoethanesulfonic acid (BESA) was added to give final concentrations of 2 mM in order to enhance the accumulation of intermediates to detectable levels. BESA was chosen as a specific inhibitor of methane production since it is a structural analogue of coenzyme M (2-mercaptoethanesulfonic acid) (Healy, Young and Reinhard, 1980; Zinder, Anguish and Cardwell, 1984). BESA is thought to competitively inhibit methyl transfer reactions at the terminal reductive step during methane formation from H_2/CO_2 (Taylor and Wolfe, 1974).

After addition of BESA the rate and magnitude of substrate utilisation remained approximately the same as that observed with the unamended control cultures, whereas the total gas productions were reduced to less than 20% for syringic acid and 25% for veratric acid

(Figures 24a, b and 25a, b). From these figures it can also be seen that acetate, propionate, butyrate and isobutyrate all accumulated in the BESA-treated cultures. In addition other volatile fatty acids such as isovalerate and valerate were detected by GLC analysis in both cultures but were not quantified.

Subsequently, samples from both the BESA-amended veratric and syringic acid-supplemented cultures were taken for extraction after 4.5 and 3 days of cultivation respectively at which point 81.25 and 78.0% of the substrates were dissimilated (Figures 24b and 25b). The extraction procedure was carried out by the method described earlier (2.4.2). Both neutral and acidic fractions were analysed by TLC, GLC, HPLC and spectrophotometry as described in 2.4.1 and 2.4.2.

7.2.1 Veratric acid

In addition to the volatile fatty acids listed above the following compounds were detected as intermediates of veratric acid catabolism: vanillic, protocatechuic, p-hydroxybenzoic, benzoic and cyclohexanecarboxylic acids and catechol.

A summary of the detected metabolites and the proposed pathways is illustrated in Figure 26. The first step is the demethylation of veratric acid to vanillic acid and further to protocatechuic acid. Bache

Figure 24. Changes in the Concentrations of Residual Veratric Acid (○—○), Methane (●—●), Acetate (□—□), Propionate (■—■) and Valerate (△—△) During the Closed Culture Cultivation of the Isolated Microbial Association in the Absence (a) and Presence (b) of BESA (2mM)

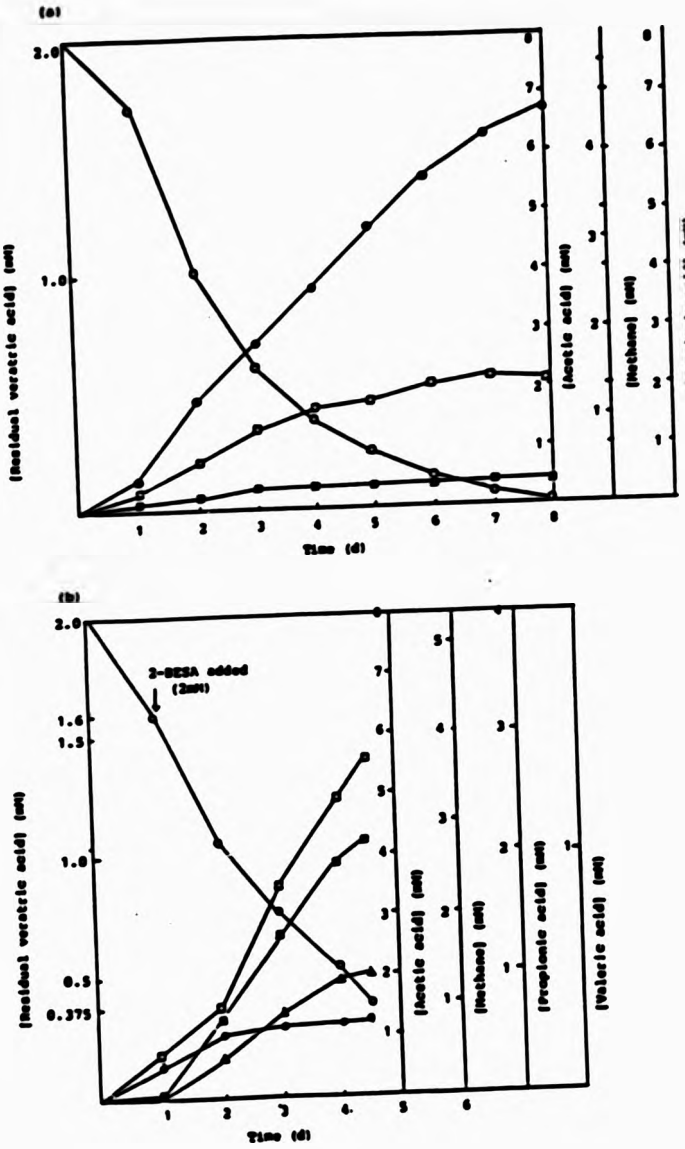

(a)
(Residual veratric acid) (mM)
(Acetic acid) (mM)
(Methane) (ml)
(Propionic acid) (mM)
Time (d)
(b)
2-BESA added (2mM)
(Residual veratric acid) (mM)
(Acetic acid) (mM)
(Methane) (ml)
(Propionic acid) (mM)
(Valeric acid) (mM)
Time (d)

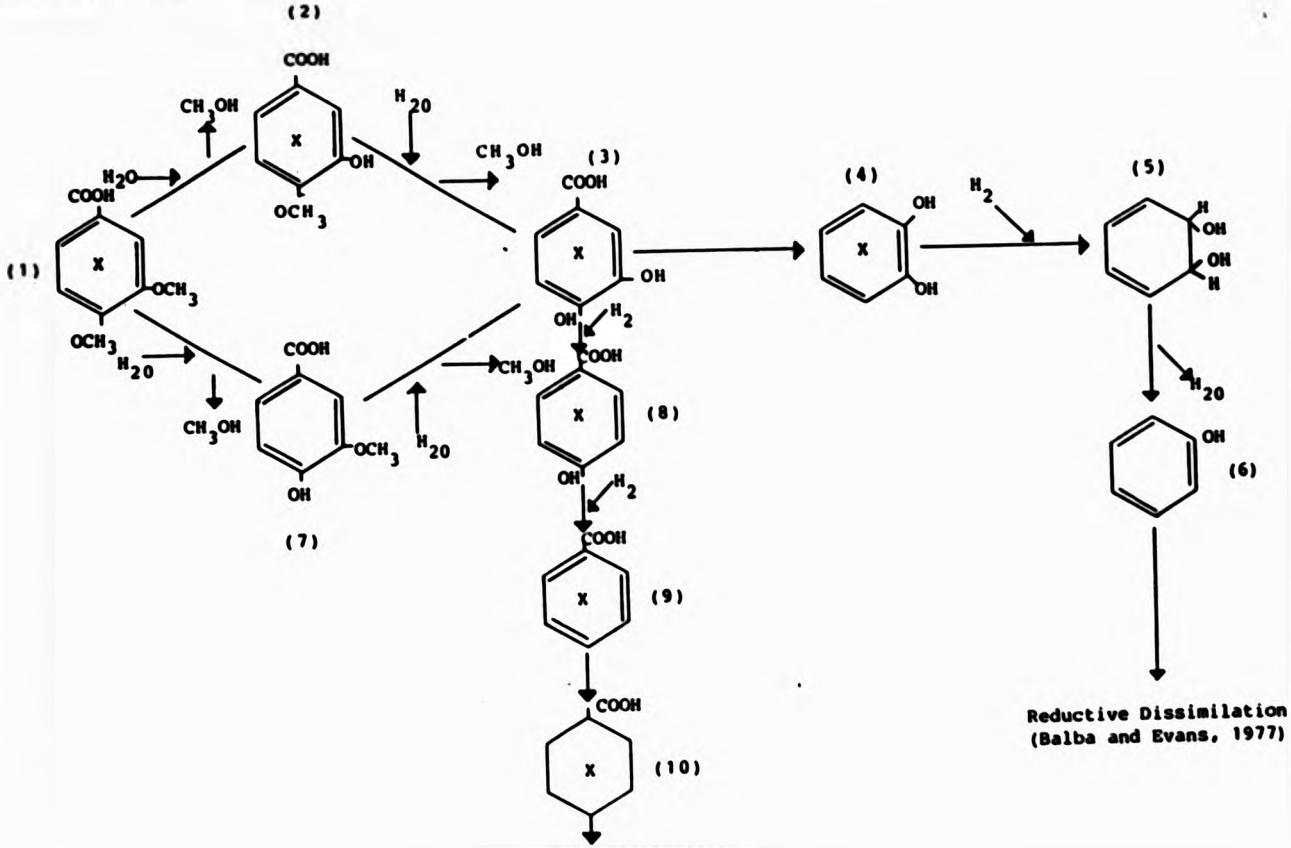
Figure 25. Changes in the Concentrations of Residual Syringic Acid (○——○), Methane (●——●), Acetate (□——□), Propionate (■——■) and Valerate (△——△) During the Closed Culture Cultivation of the Isolated Microbial Association in the Absence (a) and Presence (b) of BESA (2mM)

(a)
2.0
1.0
[Syringic acid] (mM)
Time (d)
[Methane] (ml)
[Acetic acid] (mM)
[Propionic acid] (mM)
(b)
2-BESA added
1.5
0.5
0.44
[Syringic acid] (mM)
Time (d)
[Methane] (ml)
[Acetic acid] (mM)
[Propionic acid] (mM)
[Valeric acid] (mM)

Figure 26. Possible Pathways for the Catabolism of Veratric Acid by the Isolated Interacting Microbial Association

(1) 3,4-Dimethoxybenzoic Acid (Veratric Acid) (2) 3-Hydroxy-4 methoxybenzoic Acid (Vanillic Acid) (3) 3,4-Dihydroxybenzoic Acid (Proto catechuic Acid) (4) 1,2 Dihydroxybenzene (Catechol) (5) cis-Benzendiol (6) Phenol (7) 3-Methoxy-4 Hydroxy Benzoic Acid (8) p-Hydroxybenzoic Acid (9) Benzoic Acid (10) Cyclohexanecarboxylic Acid

* Designate Intermediates Detected


(2)
COOH
CH3OH
X
OH
OCH3
H20
H2O
(1)
(3)
(4)
(5)
H2
(6)
(7)
(8)
(9)
(10)
Reductive Dissimilation
(Balba and Evans, 1977)
Reductive Dissimilation
(Evans, 1977)

and Pfennig (1981) first reported the anaerobic demethoxylation of aromatic compounds by an obligate anaerobe, Acetobacterium woodii, although other anaerobic bacteria have subsequently been found to demethoxylate aromatic monomers as reported by Krumholz and Bryant (1985) and Mountfort and Asher (1986).

After demethoxylation a reductive dehydroxylation appeared to have resulted to yield p-hydroxybenzoic acid, which is a precursor of benzoic acid. Although the sequence of appearance of the above aromatic compounds was not determined, since a time course experiment was not made, it seemed that benzoic acid was the last aromatic intermediate found and thus possibly the substrate for ring reduction since cyclohexanecarboxylic acid was also detected.

Catechol was also found in the culture supernatant and could have resulted from decarboxylation of protocatechuic acid, with no further catabolism as reported by Kaiser and Hanselmann (1982b). The workers showed that during conversion of veratric acid to catechol a new intermediate, 3-hydroxy-4-methoxy benzoic acid, was formed instead of vanillic acid thus indicating the preferential removal of methoxyl groups in the meta position. However, since vanillic acid was detected in the culture supernatant it was clear that a different catabolic route was operative. A second

possibility is the dissimilation pathway proposed by Balba and Evans (1980c) in which catechol was converted to phenol through cis-benzenediol. In the experiment made, however, phenol cyclohexanol or cyclohexanone were not detected. Grbic-Galic and Young (1985) detected several unusual reduced compounds, such as toluene, benzene, cyclohexane and methoxycyclohexane during the methanogenic fermentations of ferulate and benzoate in the presence of BESA by a stabilised microbial association and suggested that these intermediates might be electron sinks under conditions in which the methanogenic bacteria were suppressed.

In addition to the above two possibilities a third route could have been operative in which methyl cyclohexanone was formed from 2-oxocyclohexanecarboxylate. However, since no heptanoate or isocaproate were detected the assumption was made that 2-oxocyclohexanecarboxylate was converted to pimelate before subsequent dissimilation to non-volatile fatty acids and CO_2 and finally to methane.

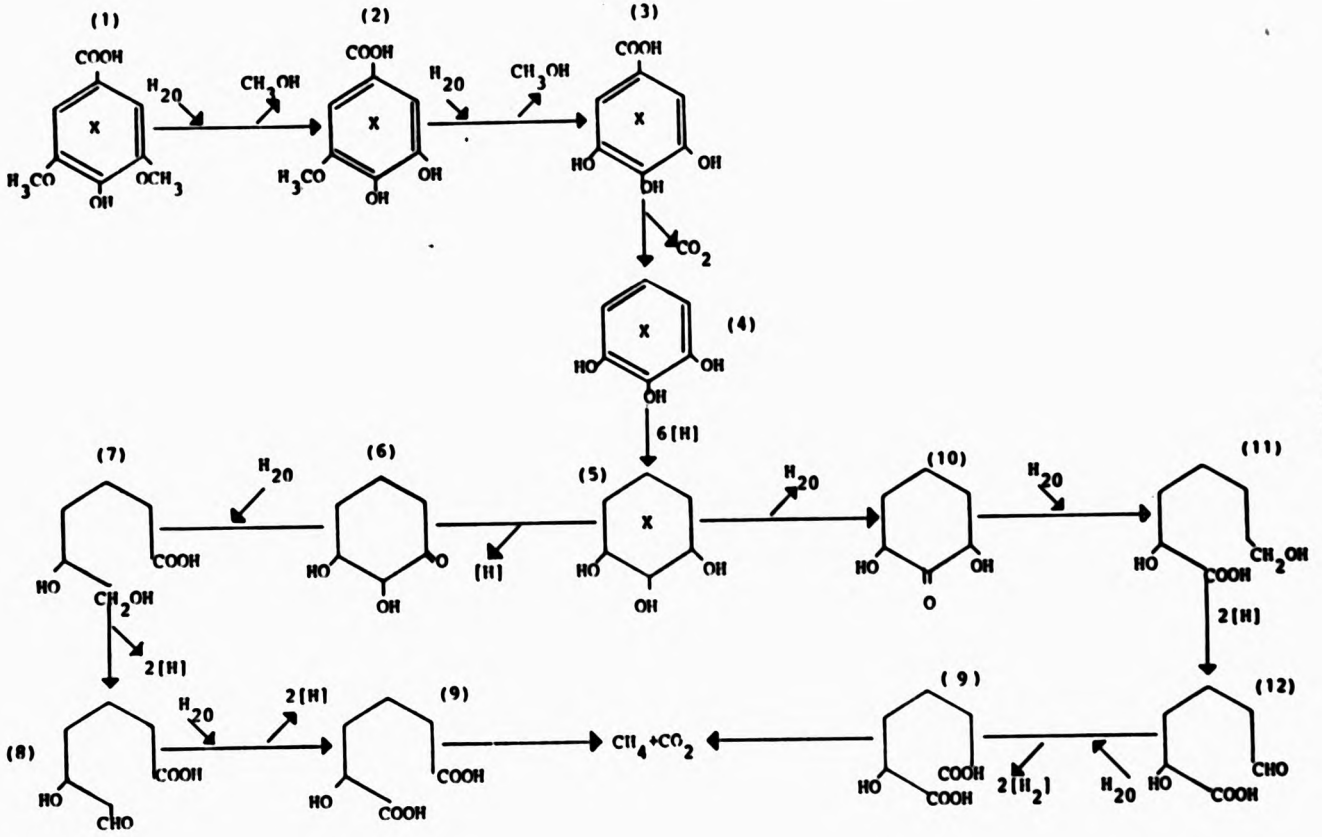
7.2.2 Syringic acid

Figure 27 illustrates the proposed pathways for syringic acid catabolism which was constructed from the detection of the following compounds: 3,4-dihydroxy-5-methoxybenzoic acid, gallic acid, pyrogallol and tri-

Figure 27. Possible Pathways for the Anaerobic Catabolism of Syringic Acid by the Isolated Interacting Microbial Association

(1) Syringic Acid (2) 5-Methoxy-3,4-dihydroxy benzoic acid (3) 3,4,5-Trihydroxybenzoic Acid (Gallic Acid) (4) 3,4,5-Trihydroxybenzene (Pyrogallol) (5) 1,2,3-Trihydroxycyclohexane (6) 2,3 dihydroxy-1-Oxocyclohexane (7) 5,6-Dihydroxyhexanoic Acid (8) 6-hydroxy-6-Oxo-hexanoic Acid (9) 5-hydroxyadipinic Acid, (10) 2,6 dihydroxy-1-Oxocyclohexane (11) 2,5 Dihydroxyhexanoic Acid (12) 5-Hydroxy 2-oxo-Hexanoic Acid

* Designate Intermediates Detected


(1)
(2)
(3)
(4)
(5)
(6)
(7)
(8)
(9)
(10)
(11)
(12)
COOH
X
H_2O
CH_3OH
H_3CO
OCH_3
OH
HO
CO_2
6[H]
[H]
2[H]
CH_2OH
CHO
CH_4+CO_2
$2[H_2]$
O

hydroxycyclohexane together with the volatile fatty acids acetate, propionate, butyrate, isobutyrate, valerate and isovalerate. The first step once again appeared to be the demethoxylation of syringic acid on the meta position to produce 3,4-dihydroxy-5-methoxy-benzoic acid while 4,5dihydroxy-3-methoxybenzoic acid was not detected. After the first demethoxylation the compound was again demethoxylated to gallic acid and then decarboxylated to pyrogallol. Pyrogallol was subsequently reduced to trihydroxycyclohexane. Since several possible intermediary ring fission products were not detected these have been postulated in the proposed pathway. For example, trihydroxycyclohexane can be converted to either 2, 3 dihydroxy-1-oxocyclohexane (Kaiser and Hanselmann, 1982a) or to 1,3-dihydroxy-2-oxocyclohexane (M.T.M. Balba, personal communication), both of which are in accordance with the pathways postulated by Evans (1977).

Unfortunately, since the analyses were carried out by several methods and some intermediary compounds were not detected it was not possible to determine the carbon balance. Although BESA has been reported by Healy et al (1980) and Kaiser and Hanselmann (1982a) to successfully block methane production and thus result in the accumulation of intermediates, Grbic-Galic and Young (1985) suggested that in BESA-amended cultures the

inhibitor might have changed some of the dominant catabolic reactions and, as a consequence, other hydrogen-utilising microorganisms could have assumed the role of methane producers. Bryant (1979) and Wolin (1982) both showed that in the absence of methanogens, more reduced products such as ethanol, propionate, butyrate, lactate, succinate and adipate were formed.

Since the aromatic compounds veratric and syringic acids were both catabolised by BESA-amended (2 mM) cultures, it may be postulated that their catabolisms to acetate by components of the microbial associations were independent of interactions with the methanogenic species. Subsequent attempts to isolate the ring-cleaving populations from the associations were, however, unsuccessful.

Comparison of the two pathways shows that the syringic acid pathway appeared to differ from that of veratric acid since in the case of the former no dehydroxylation occurred before ring reduction/cleavage of pyrogallol and benzoic acid was not detected as an intermediate, whereas in the case of the latter dehydroxylation occurred before ring reduction.

The production of catechol from veratric acid in the original culture via demethoxylation and subsequent decarboxylation was also reported by Kaiser and Hanselmann (1982b). In this report catabolism was shown

to take a different route in which the methoxyl group in the meta position was the first one to be demethoxylated although in the present study the methoxyl group in the para position was the first to be demethoxylated to produce vanillic acid.

Since the methanogenic fermentations under study were accomplished by interacting microbial associations, the exact components of which have not been fully characterised, these observed differences could possibly be attributed to the different associations used.

A detailed understanding of the microbiology of such fermentations would ultimately lead to the possibility of increased substrate utilisation and thus a greater end product formation.

7.3 Summary

The biochemical transformation of veratric acid by the microbial association was initiated with two demethoxylation steps, first to vanillic acid and then to protocatechuic acid. From this point two possible pathways could have been operative, the first one by the decarboxylation of protocatechuic acid to catechol with this intermediate then reductively dissimilated by the mechanism proposed by Balba and Evans (1977). However, since catechol and phenol were not catabolised

by the interacting microbial association it would appear more likely that a second pathway was operative. In this case the protocatechuic acid was dehydroxylated to benzoic acid before reductive dissimilation to methane and CO_2. Provisional confirmation of this was obtained from identification of benzoic acid, cyclohexane-carboxylic acid and pimilate as intermediary products, all of which were utilised as substrates by the microbial association.

Syringic acid transformation was also initiated with demethoxylation steps followed by decarboxylation to pyrogallol. The pyrogallol was then reduced to trihydroxycyclohexane. Two possibilities then existed for the subsequent conversion of this intermediate to methane and CO_2. Firstly, by conversion to 2,6dihydroxy-1-cyclohexane and then 5-hydroxyadipinic acid and secondly by conversion to 2,3-dihydroxy-1-oxocyclohexane prior to dissimilation to either 6-hydroxy-2-oxohexanoic acid or 1,2-dihydroxyhexanoic acid. However, since several of the possible intermediary ring fission products were not detected at this stage it was not possible to confirm if either or both of the pathways were operative. Considerable biochemical analyses would in fact have been required to identify all the intermediates involved and to establish the exact pathway(s) for the catabolism of syringic acid. Confirmat-

ion of the postulated pathway(s) could then have been made by use of radiotracer techniques.

The major apparent differences between the catabolisms of the two molecules examined were that the veratric acid was dehydroxylated to benzoic acid before ring reduction while the reduction of pyrogallol occurred with the hydroxyl groups still attached to the ring on the 3,4,5 positions.

CHAPTER EIGHT

ENUMERATION, ISOLATION AND PRELIMINARY CHARACTERISATION OF COMPONENT MONOCULTURES

Under anaerobic conditions complete dissimilation of aromatic compounds to methane and CO_2 requires the intervention of different groups of microorganisms. In order to gain better understanding of the activities of the component members of the interacting microbial associations used in this study attempts were made to enumerate and then isolate the different species involved. Particular emphasis was given to the sulphate-reducing bacteria and methanogens.

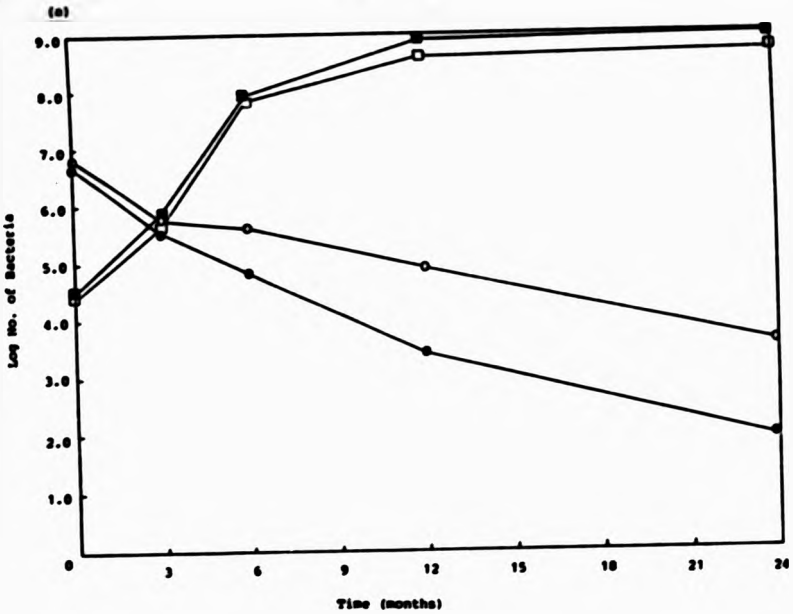
8.1 Enumeration of Sulphate-Reducing Bacteria and Methanogens

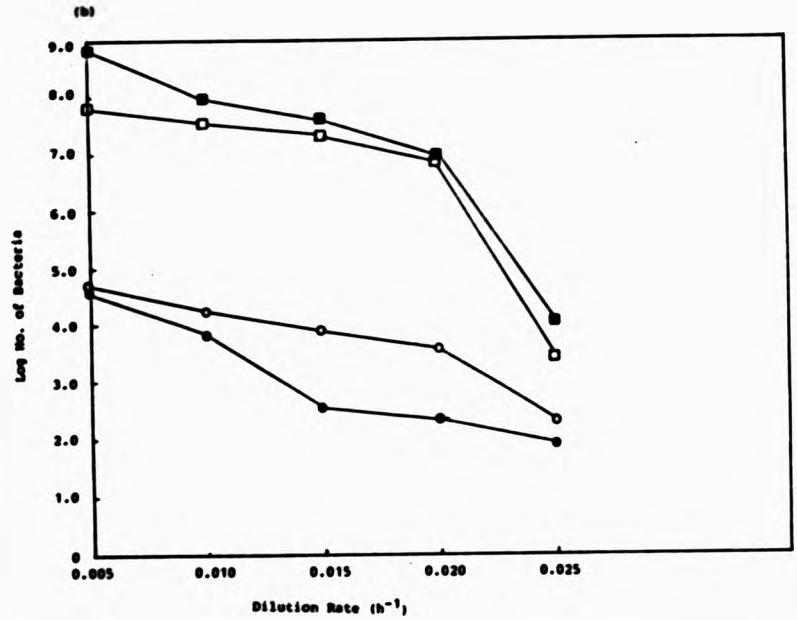
8.1.1 Sulphate-reducing bacteria

Enumerations were made on 1 ml aliquots of chemostat culture supernatants, taken at different dilution rates, and also on samples taken from the closed cultures, which were subjected to 1 week subcultivation regimes, at different time intervals. The method used was the same as that described earlier (2.4.9).

From Figure 28a it can be seen that, in general, the closed cultures were characterised by dramatic

Figure 28. Changes in the MPN's of the Component Sulphate-Reducing Bacteria (○—○, ●—●) and Methanogens (□—□, ■—■), During the Closed (a) and Open (b) Culture Cultivations of the Veratric and Syringic Acid-Catabolising Microbial Associations Respectively


(a)
Log No. of Bacteria
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
3
6
9
12
15
18
21
24
Time (months)


(b)
Log No. of Bacteria
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
0.005
0.010
0.015
0.020
0.025
Dilution Rate (h⁻¹)

reductions in numbers of sulphate-reducing bacteria from the time of establishment. For example, in the case of the veratric acid-catabolising association the number reduced from 6.8×10^6 ml^{-1} to 4.0×10^3 ml^{-1} over a period of two years. Similarly, the number of sulphate-reducing bacteria present in the syringic acid-catabolising culture reduced from 5.4×10^6 ml^{-1} to less than 100 ml^{-1} over the same period. Both these reductions in number were accompanied by decreases in sulphate-reduction.

A possible explanation for both the reductions in number and activity was competitive displacement by the methanogenic bacteria although similar observations by Sleat and Robinson (1984) were reported with no explanation offered.

From Figure 28b it can be seen that similar counts made on the open cultures showed decreases with increasing dilution rate. For example, at a dilution rate of 0.005 h^{-1} the veratric acid-catabolising association contained 5.2×10^4 free-living sulphate-reducing bacteria ml^{-1} and the syringic acid-catabolising association contained 4.8×10^4 ml^{-1}. After the dilution rates were increased to 0.025 h^{-1}, however, the corresponding numbers fell to 215 and 74 ml^{-1} respectively, and were again accompanied by decreases in sulphate reduction. Since reductions in species number

and activity were observed for each dilution rate increase simple competitive displacement would not offer a full explanation for this unless a number of different sulphate-reducing bacteria was present which seems unlikely. The possibility therefore existed that a combination of competitive displacement and changes in metabolic potential was operative.

8.1.2 Methanogenic bacteria

Methanogenic bacteria utilising acetate and/or H_2/CO_2 were enumerated by the method described in 2.4.9.

From the results presented in Figure 28a it can be seen that for the closed cultures the initial numbers of methanogenic bacteria were 2.6×10^3 ml^{-1} for the veratric acid-catabolising association and 3.6×10^3 ml^{-1} for the syringic acid-catabolising association. After two years of closed culture cultivation, with subcultivation at weekly intervals, the numbers of methanogenic bacteria had increased in both the veratric and syringic acid-catabolising associations to 5.7×10^8 and 8.9×10^8 ml^{-1} respectively. Thus, it would appear that these increases were due to the methanogenic species outcompeting the sulphate-reducing bacteria for the common precursors acetate and H_2.

The results presented in Figure 28b show that the number of constituent methanogenic bacteria present

in the two associations remained relatively constant at the first three dilution rates (0.005, 0.010 and 0.015 h^{-1}). At dilution rates above 0.015 h^{-1}, however, the numbers of methanogens decreased to 3.9 x 10^3 ml^{-1} in the veratric acid-catabolising association and to 1.2 x 10^4 ml^{-1} in the syringic acid-catabolising association thus indicating species washout. These results, therefore, suggested that each association contained more than one species of methanogenic bacteria.

8.2 Isolation of Component Monocultures

8.2.1 Sulphate-reducing bacteria

10 ml aliquots of the two microbial associations were individually inoculated into 90 ml of Postgate medium (2.2.3B). The bottles were sealed with Subaseals, overgassed with H_2/CO_2 (80:20) and incubated at 30°C for 2 weeks. At this point samples were subcultured onto the same medium, solidified with 1.2% (w/v) agar, amended with 20 mM sulphate. The plates were then incubated for 2 weeks in anaerobe jars after which discrete colonies were picked off and used to inoculate similar plates. After two weeks incubation cell morphology was determined by use of a light microscope. Two distinct colony types were isolated from each association. The first one was black coloured and consisted of gram-negative curved rods. This

species was subsequently shown to metabolise lactate, ethanol and H_2. Colonies of the second isolate were also black coloured and again consisted of gram-negative curved rods which metabolised lactate, ethanol and H_2, thus the possibility existed that the two isolates were in fact the same species.

Morphology, however, is not a good taxonomic guide to the identification of sulphate-reducing bacteria because, as reported by Postgate (1979), a single pure strain of Desulfovibrio may form vibrioid, spirilloid, semilunar, straight and sometimes half-empty coccoid forms in response to age and environment. Therefore, considerable experimentation is required in order to identify the above isolates.

8.2.2 Methanogenic bacteria

10 ml aliquots of the two microbial associations were individually inoculated in 90 ml of phenol(2 mM)-supplemented medium (2.2.2). The carbon sources used were acetate (10 mM) and an atmosphere of H_2/CO_2 (80:20). After 3 weeks incubation at 30°C samples were subcultured onto the same medium, solidified with 1.2% (w/v) agar. The plates were incubated for 3 weeks at 30°C in anaerobe jars which contained filter papers soaked with a 2.5% (w/v) Na_2S. The acetate-supplemented cultures were incubated under an atmosphere of OFN

whereas the H_2/CO_2 supplemented cultures were incubated under this atmosphere (80:20).

Discrete colonies were then examined for visual purity by use of a light microscope. Subsequently, individual colonies were suspended in liquid mineral salts (2.2.2) amended with 2 mM phenol and supplemented with either acetate or H_2/CO_2. The cultures were overgassed with either OFM or H_2/CO_2 as appropriate and methanogenic identity confirmed by methane generation. Finally, positive isolates were subjected to Gram characterisation and phase contrast studies from which it was apparent that each association contained two distinct methanogenic populations. The two isolates from the veratric acid-catabolising association were designated VAM1 and VAM2 and had the following characteristics:

VAM1: nonmotile Gram-negative cocci which utilised acetate and H_2/CO_2; and

VAM2: nonmotile gram negative short rods which also utilised both acetate and H_2/CO_2.

The corresponding isolates from the syringic acid-catabolising association which were designated SAM1 and SAM2 had the following characteristics:

SAM1: nonmotile gram-negative cocci which utilised H_2/CO_2 only; and

SAM2: nonmotile gram-negative vibrio which utilised acetate and H_2/CO_2.

8.3 Facultative Anaerobic Bacteria

5 ml aliquots of closed cultures and chemostat supernatants were individually transferred to 150 ml of mineral salts medium (2.2.1) supplemented with veratric or syringic acids (2 mM). The resulting cultures were incubated at 30°C on an orbital shaker (Gallenkamp, U.K.) at 100 rpm. Since no significant growth was observed in any of the flasks after two weeks of incubation, similar cultures were prepared and incubated at 30°C unshaken. Here, growth was apparent in all the flasks within 3 to 4 days. Subsequently, samples of the cultures were inoculated onto the same media set with 1.2% (w/v) agar and incubated until discrete colonies developed.

Distinct morphological types of colonies were recognised on both substrates. For the veratric acid-amended plates three colony types were seen, one of which was blue (VA1) (Plate 1) whilst the other two (VA2 and VA3) were colourless but different in size. For the syringic acid-amended plates two colony types only were found, one of which was brown coloured (SA1) (Plate 2) whilst the other was colourless (SA2).

Substrate specificities of the five colony types were determined by use of replicate plates which contained mineral salts medium individually supplemented with benzoate, p-hydroxybenzoate, protocatechuic acid,

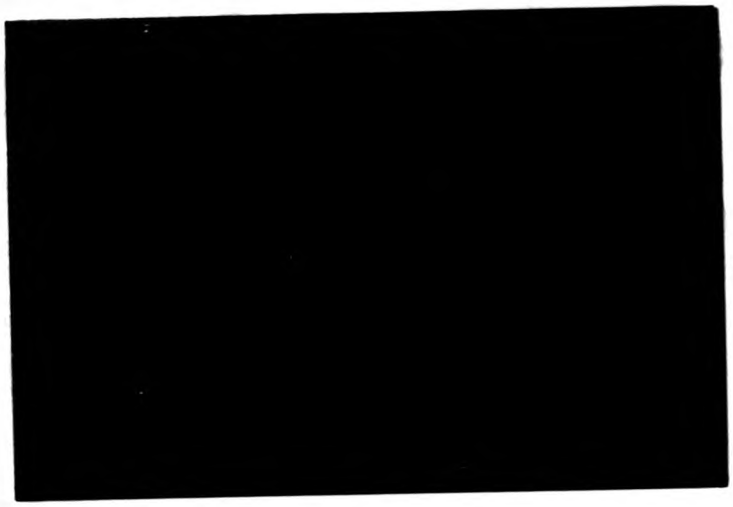


Plate 1. Facultative anaerobic bacterium isolated from the veratric acid-catabolising microbial association, designated as VA1

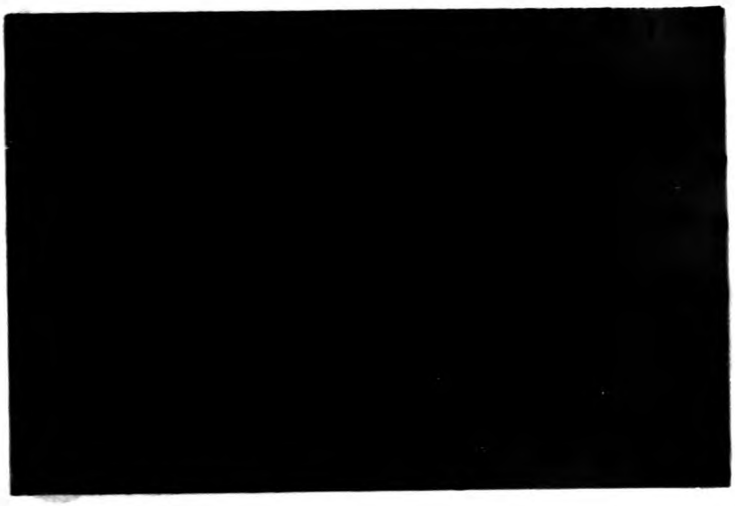


Plate 2. Facultative anaerobic bacterium isolated from the syringic acid-catabolising microbial association, disignated as SA1

pyrogallol, hexanoic acid, butyrate, succinate, pyruvate, propionate and acetate (2 mM) (Table 14).

From the results presented in Table 14 it can be seen that although the three isolates cultured on the veratric acid-amended plate had distinct morphological colony types, they all metabolised the same compounds. It is interesting to note that all the aromatic compounds metabolised by these isolates were detected as intermediates of veratric acid catabolism.

Similarly, although the two isolates cultured on the syringic acid-amended plates also had distinct morphological colony types, they metabolised the same compounds. Once again the aromatic compounds metabolised by the two isolates were also detected as intermediates of syringic acid catabolism.

None of the isolates from the veratric and syringic acid-catabolising associations utilised either catechol or phenol although all of them metabolised the fatty acids and simple sugars tested.

It can be seen from the results presented that considerable experimentation is still required in order to identify these isolates and to characterise the activities of each one within the interacting associations.

Table 14. Preliminary Carbon Nutrition Tests of Facultative Anaerobic Bacteria Isolated from the Veratric and Syringic Acid-Catabolising Microbial Associations.

+ and - denote growth and no growth respectively.

Isolates	Substrates																		
	Veratric Acid	Syringic Acid	Benzoic Acid	p-hydroxy Benzoic Acid	Protocate-chuic Acid	Catechol	Phenol	Gallic Acid	Pyrogallol	Hexanoic Acid	Succinic Acid	Pyruvate	Butyrate	Propionate	Acetate	Glucose	Sucrose	Mannose	Lactose	Maltose
VA1	+	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
VA2	+	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
VA3	+	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
SA1	-	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
SA2	-	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+

8.4 Summary

Most probable number estimates made on the microbial associations subjected to both closed and open culture cultivation showed that the sulphate-reducing bacteria were progressively displaced. For the syringic acid-catabolising association, reduction from 5.4×10^6 to 92 ml^{-1}, was apparent after 2 years and this compared with the reduction from 4.8×10^4 to 92 ml^{-1} obtained in open culture when the dilution rate was increased stepwise from 0.005 to 0.025 h^{-1}. It is interesting to note that although the initial population sizes were different the final population sizes were identical. Similar decreases from 6.5×10^6 to 4000 ml^{-1} and from 5.2×10^4 to 215 ml^{-1} were apparent for the veratric acid-catabolising association although here the final population sizes were somewhat higher than the corresponding ones obtained with the syringic acid-catabolising association. Progressive displacement of the sulphate-reducing bacteria from both open cultures suggested that either the cultures were not in steady state or that the associations contained a number of species.

Although in closed culture reductions in the numbers of sulphate-reducing bacteria were accompanied by increases in the numbers of methanogens from 3.6×10^3 to 8.9×10^8 and from 2.6×10^3 to 5.7×10^8 for the

syringic acid and veratric acid-catabolising associations respectively, thus possibly confirming competitive displacement of the former by the latter, this trend was not continued in open culture since reductions from 7.8×10^{8} to 1.2×10^{4} ml^{-1} and from 6.7×10^{7} to 3.9×10^{3} were apparent for the syringic and veratric acid-catabolising associations respectively. From these results it was clear that the constraints of the two culture types were different.

For both associations monoculture isolations showed that a minimum of one sulphate-reducing and two methanogenic bacteria were present. The veratric acid-catabolising association contained possibly three species of facultative anaerobic bacteria while the syringic acid-catabolising association contained two species. However, before full characterisation of these species could be accomplished and the actual bases of the interspecies interactions determined, then, considerable experimentation would be necessitated.

CHAPTER NINE

GENERAL SUMMARY

Solubilisation of ground pine (Pinus sylvestris) sawdust pretreated with various concentrations of NaOH and H_2SO_4 at temperatures of ambient, steam and 121°C (autoclaved) were examined. Steaming for 1 h in the presence of 5OZ (w/v and v/v) NaOH or H_2SO_4 respectively, was selected as the optimum pretreatment method and subsequently used for this study. Total methane generation of the two pretreatment products, hydrolysates and residual solids, in closed cultures by isolated microbial associations, resulted in 8.5-fold increases compared with untreated controls. Thus it was apparent that pine wood sawdust was not susceptible to biomethanation without such physico-chemical pretreatment. However, it was still not clear whether it was the lignin content and/or the type of structural association between the lignin and other components and/or the presence of other constituents in the pine wood material which was responsible for the recalcitrance.

Although the total costs of energy and chemicals for the pretreatments in this instance were not calculated, the use of other pretreatment methods such as the use of the enzyme "aryl etherase" could prove more economically feasible.

Although the catabolic response of the lignin monomers examined varied according to the number and position of the ring substituents, many of the monomers were catabolised by the interacting microbial associations which were isolated from bovine rumen liquor and from anoxic freshwater sediment. Thus, it appeared that recalcitrance of lignin to microbial degradation under anoxic conditions was not necessarily related to the structure of the component monomers but more likely to factors such as the high molecular weight, the three dimensional structure or to factor(s) as yet unidentified.

Examination of the catabolisms of the selected monomers, veratric and syringic acids, showed approximate stoichiometric conversion to methane and CO_2. The veratric and syringic acid-catabolising microbial associations catabolised both molecules at each discrete pH value examined over the range 6.0 to 8.0 with optima between 7.0 and 7.2 and temperature optima at approximately 35°C. Catabolisms of these molecules were not affected by sulphate concentrations below 5 mM, although the presence of sulphate at high concentrations (20 mM) exerted detrimental effects. The exact nature of this inhibition, however, was not established and thus necessitates further investigation.

The veratric and syringic acids were also

catabolised at near stoichiometric values by the interacting microbial associations in open culture although the concentrations of residual sulphate, gaseous and soluble sulphides all changed with dilution rate. Since at no dilution rate was all the sulphate reduced or assimilated, it would appear that the sulphate-reducing bacteria were either carbon or electron limited.

Kinetic determinations showed that of the two associations, the veratric acid-catabolising association had the higher affinity for its growth-limiting substrate but the lower maximum specific growth rate. Higher K_s and lower μ_{max} values were, however, apparent for both associations when subjected to closed culture cultivation, thus exemplifying the different constraints operative within the two cultivation systems. The displacement of component species in the open cultures resulted in different associations at different dilution rates and possibly accounted for the kinetic discrepancies obtained. As a consequence of these the validity of the kinetic estimates derived from open culture data must be questioned and as a consequence comparisons of the kinetic parameters of open and closed cultures can not be made with confidence.

Although the biochemical transformations of both veratric and syringic acids were initiated with demethoxylations to the corresponding hydroxy derivat-

ives, dehydroxylation of the veratric acid occurred before ring reduction and cleavage whereas the corresponding pathways for syringic acid differed since no dehydroxylation occurred before ring reduction and cleavage. Since many possible intermediates were not detected by the methods used in this study, the use of radiotracers and/or other analytical techniques would be required to confirm the, as yet, tenuous pathways for the catabolism of these compounds.

Most probable number estimates made on the microbial associations subjected to both closed and open culture conditions showed that the sulphate-reducing bacteria were progressively displaced by the methanogens. Considerable experimentation, however, would still be required before full characterisation of the actual bases of the interspecies interactions could be achieved.

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