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Chemical Principles of the Biotechnology of the Preparation of Fuel

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The chemical aspects of the biotechnology of the preparation of fuels together with the potential resources and possibilities of the biofuel technology are described. The present state of research into the preparation of biogas (mixture of methane and carbon dioxide), ethanol, acetone, butyl alcohol, and hydrogen from the biomass is examined. The technological features and economic aspects of the processes listed above are analysed.
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The last decade has been characterised by an increasing interest in the problem of the application of renewable sources of energy. One of the main aspects of this research is associated with the use of solar energy. In recent years, biotechnological methods for the conversion of solar energy into fuel have developed significantly. The world interest in this problem is high, numerous symposia, conferences, and seminars being held annually.

A new scientific and technical field concerned with "fuel from the biomass" has been developed fairly vigorously in our country.[1-6] A number of projects have reached the level of technical solutions, whereas other are passing through the stage of laboratory research and experimental tests.

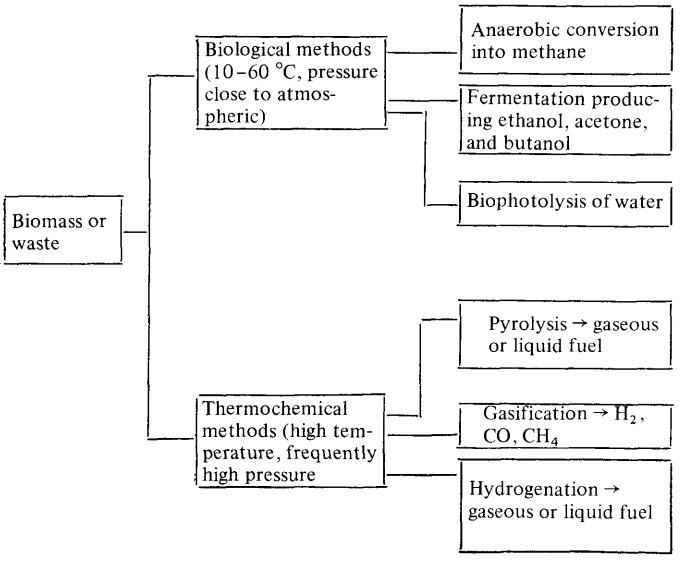
I. POTENTIAL RESOURCES AND POSSIBILITIES OF THE BIOFUEL TECHNOLOGY

The biomass is the cheapest form of renewable energy which can be accumulated on a large scale. By the term biomass, we understand any materials of biological origin, metabolic products, and the organic waste formed in the course of their processing. The daily increment in the biomass on Earth is 200 billion tonnes, which have an energy equivalent of 3×10^{21} J.[7,8]

The systems for the conversion of energy by preparing biofuels are fairly varied and include processes involving the preparation of methane, hydrogen, ethanol, methanol, butyl alcohol, and acetone as well as a number of other components of interest as fuel materials. The overall possibilities of the biofuel technology are fairly considerable and have been discussed in a review.[9]

The products derived from natural vegetation, agricultural waste, a wide variety of industrial waste and waste produced by the municpal economy and finally the crops specially grown in the so called "energy plantations" (sugar cane, manioc, potatoes, plankton, etc.) are potential raw materials for the production of biofuel.

The energy content of certain types of fuel, waste, and biomass are presented in Table 1. The simplest method of extracting energy from many materials is their combustion. Other methods of processing the initial raw material are presented in the Scheme below:



Estimates of the potential contribution of biofuel to the overall energy provision to the community are of fundamental importance. Evidently, biofuel technology may serve as a basis of new power engineering in the developing countries which have relatively poor energy resources but are located in zones with a high bioproductivity. Estimates of the percentage contribution of biofuels in highly developed countries are the subject of lively discussion in the literature.

The utilisation of the biomass in the USA amounts nowadays to 2% of the total energy consumption (1.5 quad g^{-1} or 1.6×10^9 GI g^{-1}).† Various estimates for the year 2000 range from 4 quad g^{-1} (4.2×10^9 GI g^{-1}) to 17 quad g^{-1} (1.8×10^{10} GI g^{-1}). The latter quantity amounts to 15-20% of the total energy production in the USA.[11]

† 1 quad corresponds to 10^{15} British Thermal Units, each of which is equal to 1.06×10^9 GI. This quantity corresponds to approximately 50 million tonnes of coal [Ed.of Translation].

Considerable attention has been devoted to prospects for the utilisation of renewable energy sources in europe. The problem of the preparation of biogas which has been subjected to a detailed and thorough analysis for various European countries.[10-12] The principal conclusion reached in these investigations is that biofuels will play a significant role in the power generation in these countries. By the year 2000, the contribution of the biotechnology of the preparation of fuel to the overall energy requirements of the countries in the Common Market will be 5–10%. For comparison, we may point out that the production of hydroelectric energy in the overall energy balance is only 2%.

Table 1. The energy contents of certain types of fuels, waste, and biomass.[10]

Fuel	Energy content, MJ kg^{-1}	Biomass and waste	Energy content, MJ kg^{-1}
Coal	29 3	Typical biomass	14.0
Coke	28.4	Bacteria	22.7
Dry ash-free peat	21,0	Algae	20.0
Air-dry peat (25% H_2O)	16.3	Straw (15% H_2O)	14.2—15.5
		Dry straw	17.0
Brown coal	14.7	Grassy materials	17.5
Lignite, commercial fuel	26.8	Natural plants	18.3
		Wet wood	16.0
Unrefined petroleum	42.3	Dry ash-free wood	18.7
Diesel fuel	41.0		
Petrol	43.5	Unpurified waste	9.0—11.0
Kerosine	45.6—47.8	Fuel waste	14.7—15.6
Purified gas	41.0	Cattle manure	21.4
Natural gas[a]	31.7—39.8		
Coking gas[a]	16.1	Pig manure	20.0
Hydrogen[a]	12.1	Monosaccharides	15.5
Methane[a]	37.7	Polysaccharides	17.6
Biogas (60% CH_4)[a]	22.3	Lignin	31.1
Methanol	22.4	Protein	23.9
Ethanol (95%)	28.2	Fat	31.4
Ethanol (100%)	29.7	Carbohydrates	16.7—17.6
Isopropyl alcohol	33.0	Unpurified fibre	18.8—19.7
n-Propyl alcohol	33.6		
n-Butyl alcohol	36,2		

[a]Energy content in MJ m^{-3}.

The role of the biomass in the energy supply in the countries of Latin America has been analysed and it has been shown that 10–20% of the overall energy requirement in these countries will be met by employing the biomass and the biotechnology for its conversion into fuel.[13]

The energy programme in China has been discussed[14,15] and it has been shown that up to 30% of the energy provision in that country will be achieved by employing biofuels.

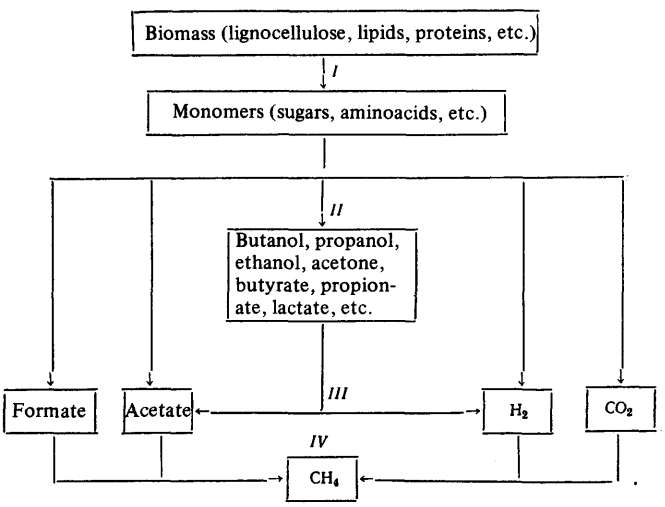
The ecological problems of the utilisation of the biomass and the advantages associated with it have been discussed.[16]

II. BIOGAS

The technology of the preparation of biogas (a mixture of 50–80% of methane and 20–50% of carbon dioxide) by the anaerboic conversion of various types of plant raw materials and organic waste has been developed to the greatest extent.[17-20] At the present time, studies in this field are being prosecuted both within the framework of fundamental research into the formation of methane by complex microbial populations and within the framework of the creation of an improved technology for the intensification of the process.

1. Principles of the Preparation of Methane

The anaerobic conversion of the biomass into methane (methane fermenation) is a complex multistage decomposition of a wide variety of organic materials under anaerobic conditions under the influence of bacterial flora, whose final result is the formation of methane and carbon dioxide. According to modern ideas, the anaerobic conversion of virtually any biomass into methane proceeds via four successive stages: the stage involving the hydrolysis (cleavage) of complex biopolymer molecules (proteins, cellulose, lipids, etc.) into simpler molecules, for example, monomers: aminoacids carbohydrates, etc.; the stage involving the fermentation of the resulting monomer to still simpler substances—lower acids and alcohols; the acetogenic stage (the formation of H_2, CO_2, formate and acetate), and then directly the methanogenic phase, which leads to the final cleavage product—methane:[19,21]



The study of the kinetics of methanogenesis from various substrates (cellulose, hemicelluloses, monosaccharides, ethanol, methanol, butyrate, and acetate) has been described in a number of communications,[21-29] kinetic schemes have been devised, and computer models describing processes involving the formation of methane in such systems, in the presence of natural methanogenic microbial populations, the *Methanobacillus kuzneceovii* system, and the pure *Methanosarcina vacuolata* have been constructed. As an example, Fig.1 illustrates the dynamics of the anaerobic conversion of glucose into methane in the presence of the methanogenic *Methanobacillus kuzneceovii* system. The overall experimental data accumulated as a result of the study of this process are summarised by the following kinetic scheme:

$$C_6H_{12}O_6 + H_2O \rightarrow 2H_2 + 2CO_2 + C_2H_5OH + CH_3COOH,$$
$$C_2H_5OH + H_2O \rightarrow CH_3COOH + 2H_2,$$
$$CH_3COOH \rightarrow CH_4 + CO_2,$$
$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O.$$

The first two reactions are formed by a non-methanogenic microorganism via the Embden–Meyerhof pathway and the third and fourth reactions are performed by acetate- and water-utilising methanologens.

A mathematical model of the process, consisting of a system of nine non-linear differential equations, has been devised on the basis of the proposed kinetic scheme.[27-29] The adequacy of the proposed model has been tested by solving the so called direct problem. On the other hand, the solution of the reverse problem made it possible to determine from the available experimental data the kinetic parameters and constants for the conversion of glucose into methane. Mathematical calculations have confirmed the satisfactory quality of the description of the experiment.

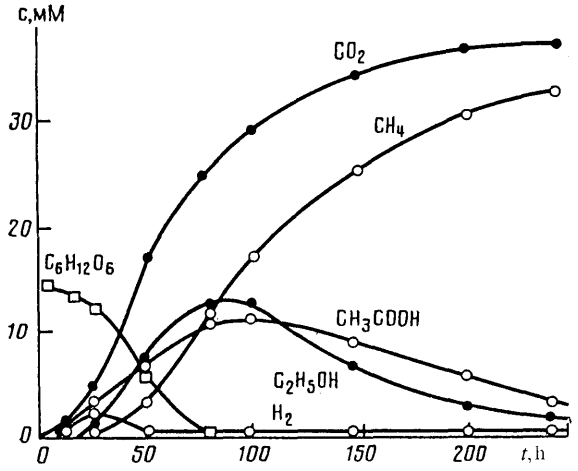


Figure 1. Dynamics of the anaerboic conversion of glucose in the presence of the *Methanobacillus kuzneceovii* system of microorganisms.[24]

Methane fermentation can occur in the temperature range from 10° to 65 °C. It has been shown that thermophilic methane fermentation (45–65 °C) is 2–3 times faster than mesophilic fermentation (25–30 °C), the change in temperature influencing only the rate of the process but not the qualitative composition of the product formed.

The acid generating stage, determined by the activity of the primary anaerobes, occurs at pH from 5 to 6.5–7 and is characterised by an intense growth of bacteria which ferment free carbohydrates. This period is accompanied by a decrease in the content of carbohydrates in the medium, a decrease in the pH of the medium, and an increase in the overall content of volatile aliphatic acids represented by formic, acetic, propionic, butyric, and other acids. Propionic and butyric acids predominate during this period.[17,21,30] The microbiological and biochemical processes in this stage have so far been studied inadequately.[17,20,34]

Among the large group of acid generating bacteria, acetogenic, lactic acid, and butyric acid bacteria as well as producers of caproic and succinic acids, ethanol, butanol, and other lower alcohols and ketones are of special importance (in the literature, this entire group is frequently referred to as solvent synthesising bacteria).

A definite interest has arisen in recent years in a comprehensive investigation of anaerobic bacteria active in the acid generating stage, which are important not only from the standpoint of methane generation but also because they are direct producers of biofuel substances.

2. Methane Forming Bacteria

Methanogens are obligatory anaerobes sensitive to oxygen. The oxidation–reduction potential of the medium used for their growth is -300 mV and less.[31] More than 45 species of methane forming bacteria are known at the present time.

Depending on the species, methanobacteria, can use a limited number of substrates as sources of energy (Table 2). Among them acetate is the most important, occupying the first place among precursors (more than 75%).[17,23-29,32-35] Acetate is followed by carbon dioxide,[26-29,36-38] formate, methanol,[22,23,41] methylamines,[42] methylcobalamin,[17] and carbon monoxide.[17] According to the available data, no other carbon compounds support the growth of methane forming bacteria.

Table 2. Chemical reactions effected by methanogens.[54]

Chemical reaction	ΔG_0° relative to CH_4 kJ mol^{-1}
$CO_2+4H_2\rightarrow CH_4+2H_2O$	−139
$4HCOO^-+4H^+\rightarrow CH_4+2H_2O+3CO_2$	−119
$4CO+2H_2O\rightarrow CH_4+3CO_2$	−186
$CH_3COO^-+H^+\rightarrow CH_4+CO_2$	−31
$4CH_3OH\rightarrow 3CH_4+CO_2+2H_2O$	−103
$4CH_3NH_2+2H_2O\rightarrow 3CH_4+CO_2+2NH_3$	−75
$2(CH_3)_2NH+2H_2O\rightarrow 3CH_4+CO_2+2NH_3$	−74
$4(CH_3)_3N+6H_2O\rightarrow 9CH_4+3CO_2+4NH_3$	−75

Methanogenic bacteria convert 90–95% of the carbon used into methane in order to accumulate energy for constructive metabolism via the coupled generation of a transmembrane potential and only 5–10% of the carbon is converted into the biomass. By virtue of the above feature, up to 80–90% of the organic matter decomposed in the course of the development of the methanogenic consortium is converted into gas.[17]

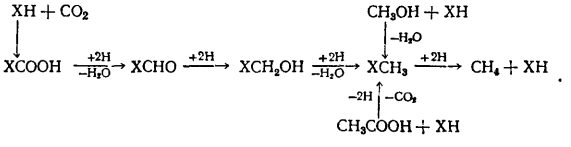
In mixed cultures, methane-forming organisms can be readily determined with the aid of fluorescence microscopy:[43-44] they give rise to either a green fluorescence by virtue of the F_{420} factor or a blue fluoresence by virtue of 7-methylpterin[45] (see below).

Fairly important data have been obtained by investigating rRNA. It was found that methane-forming bacteria differ from all other organisms in terms of their 16S RNA nucleotide sequence.[46,47] In addition, methane-forming bacteria differ sharply from other organisms as regards the composition of the electron transferring agent as well as a series of coenzymes. The processes involved in their metabolisms also have characteristic features.[41,47]

On this basis and primarily as a result of the studies of the 16S rRNA, methane-forming bacteria are regarded as a special branch in the evolution of organisms and are classified as archebacteria.[46]

3. The Mechanisms of the Formation of Methane

The biosynthesis of methane is the main reaction leading to the generation of the energy necessary for the development of the cell. The universal scheme for the formation of methane by bacteria was first proposed in 1976 by Barker, who combined processes involving the biosynthesis of methane from H_2/CO_2, formate, acetate, and methanol:[48]



Since it had been shown even earlier that formate, formaldehyde, and methanol are not intermediates released into the culture medium in the course of the formation of methane from CO_2, the authors suggested that these C_1-compounds, reduced to different extents during their transformation into methane, should be linked to one or several compounds "X". Vigorous biochemical and physicochemical studies during the last 10–15 years made it possible to elucidate to an appreciable extent the nature of the compounds "X". In the first place, at least six unique coenzymes were isolated from methanogens and identified. These coenzymes are hardly encountered in other species of bacteria.

The compound

$$HS{-}CH_2{-}CH_2{-}SO_3^- \ ,$$

which was called coenzyme M (CoM), was the first to be discovered and its involvement in the formation of methane was demonstrated. Coenzyme M can combine with a methyl group, being thereby converted into methyl coenzyme M ($CH_3SCH_2CH_2SO_3^-$). The latter plays a central role in the terminal stage of the formation of methane, which is associated with the demethylation of $CH_3-S-CoM$:[47]

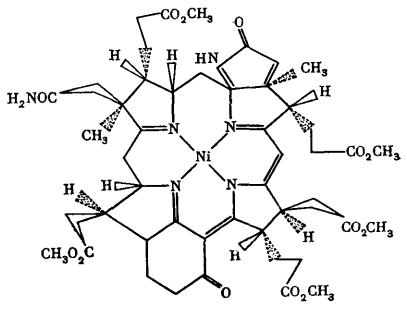
$$CH_3{-}S{-}CoM + 2H^+ + 2\bar{e} \xrightarrow[\text{methylcoenzyme M reductase}]{\text{comp. A, comp. B, } Mg^{2+}\text{, ATP}} CH_4 + HS{-}CoM \ .$$

This formally simple reaction is catalysed by an involved complex of enzymes and cofactors.[51] Component A is a protein complex exhibiting a hydrogenase activity. Four components were isolated from this complex: three proteins (A_1, A_2, and A_3) and the coenzyme FAD.[52] A_1 is an oxygen-resistant hydrogenase, which uses coenzyme F_{420} as the substrate. The hydrogenase A_1 may be identical with the F_{420}-reducing hydrogenase whose isolation has been described by Jacobson et al.[53] Compounds A_2 and A_3 are oxygen-labile proteins. It has been suggested that A_2 may participate in the reduction both at a formal level and as the activator methyl coenzyme M of the reductase system.[54] The function of A_3 is still not understood.

Component B is an oxygen-sensitive, thermally stable cofactor,[51] which has a molecular weight of about 1000 D and which contains an adenosine residue.[54] Catalytic amounts of ATP and Mg^{2+} are required for the functioning of the methyl coenzyme M reductase system. ATP probably activates enzymes or the coenzymes linked to the enzymes via acylation and phosphorylation reactions.[55]

Methyl coenzyme M reductase is an oxygen-stable protein consisting of three subunits and having a maximum absorption near 425 nm.[56] Two molecules of the recently discovered factor F_{430} function as prosthetic groups.[57]

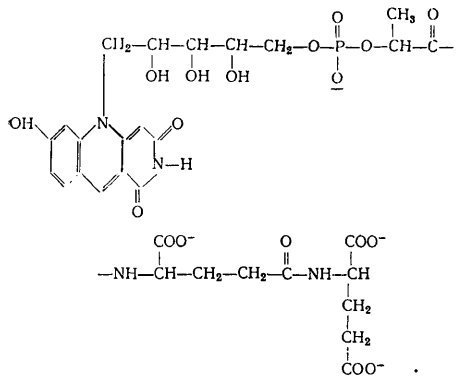
The factor F_{430}, given this name after the observation of its characteristic absorption maximum at 430 nm,[58] is a nickel-containing tetrapyrrole.[59–65] The structure of this chromophore, modified by methylating the carboxymethyl and carboxyethyl groups, has been established by Pfaltz et al.:[66]



The factor F_{430} usually forms ~1 : 1 complexes with coenzyme M and the complex is usually designated by $CoMF_{430}$.[67]

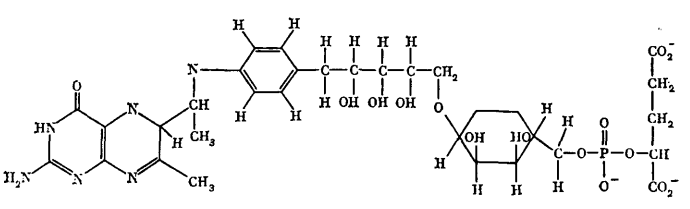
The function of $CoMF_{430}$ has not been ultimately established. From the chemical standpoint, this compound most probably functions as an electron transferring agent.[54]

The formation of methane from carbon dioxide represents the transfer of eight electrons from hydrogen to CO_2. Data obtained in the isotope fractionation of *M. thermoautotrophicum* have shown that H^+ from water is converted into methane, that H_2 serves solely as an electron donor, and that carbon dioxide is used in the gaseous form.[70,71] The coenzyme F_{420}, which has been given this designation on the basis of its fluorescence maximum in the oxidised form, first described in 1972 and having a low oxidation-reduction potential [E_0 = –0.34 V with respect to the normal hydrogen electrode (n.h.e.)], functions in this process as the electron transferring agent:[72–73]



This compound is the primary electron acceptor of hydrogenase,[39,53,53] formate dehydrogenase,[75–77] and F_{420}-dependent NADP-reductase[78] and is an electron donor in the terminal strage of the reduction of CO_2. Furthermore, F_{420} is an electron donor for two critical reactions in cell synthesis effected by pyruvate dehydrogenase and α-ketoglutarate dehydrogenase.

In 1978 experiments with labelled $^{14}CO_2$ showed that, over a period from 2 to 20 s, 25–50% of the label is bound into a compound giving rise to a bright yellow fluorescence.[36] These results suggested that the compound, later identified as 5,10-methenyl-5,6,7,8-tetrahydromethanopterin (5,10-methenyl-THF),[80] is an intermediate in the reduction of CO_2 to CH_4. After incubation under non-reducing conditions, 5,10-methenyl-THMP is converted into a compound which now exhibits a blue fluorescence and is a metabolic precursor of 5,10-methenyl-THMP (it does not contain the C_1 unit derived from CO_2). This compound has been called methanopterin.[81] Methanopterin has been isolated from the *Methanobacillus thermoautotrophicum* cells in 1983.[82] Its structure was determined in 1984 by high-resolution NMR spectroscopy (500 MHz)—by two dimensional ^1H and ^{13}C NMR:[83]

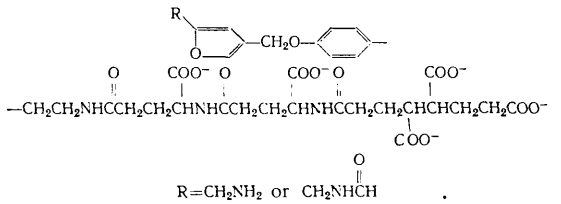


Methanosarcina barkeri contains another methanopterin derivative, which was called *sarcinapterin*. Its difference from methanopterin consists in the presence of glutamic acid joined to α-hydroxyglutaric acid.[81]

Brief incubation of the *M. thermoautotrophicum* cell suspension with $H^{13}CO_3^-$ (in a hydrogen atmosphere) and subsequent isolation of 5,10-methenyl-THMP showed that the label is present as the methenyl (=CH−) group.[54]

Thus methanopterin behaves as a C_1-transferring agent in two consecutive stages of the reduction of CO_2, which is manifested by the formation of methylene-THMP and methyl -THMP respectively.[54] These processes have been confirmed experimentally.[79,84]

Direct demonstration of the role of methanopterin in the reduction of CO_2 to methane has been achieved in a study[15] where it was shown that the absorption of CO_2 stimulated by coenzyme M and the so called RPG effect (see below) require the concerted action of two compounds stable with respect to oxygen and heating, one of which is methanopterin. The second compound, called the CDR-factor (the carbon dioxide reducing factor), was discovered in 1982 [86] and is yet another coenzyme in methanogens; the CDR-factor is encountered in two forms, namely as the free amine and as a formamide:



It has been suggested that in the latter case the formyl group originates in CO_2, since this form is in fact accumulated in the reaction mixture on incubation in an atmosphere of H_2 and CO_2 in the absence of methanopterin. This shows that the CDR-factor acts as a C_1-transferring agent before the THMP-derivative. Another name for the CDR-factor can also be encountered in the literature, namely methanofuran.[83] CO_2 is activated by the energy derived from ATP.[89] Reductive carboxylation is the energetically preferred type of CO_2 fixation.

The explanation of the socalled RPG effect, the sharp increase in the rate of reduction of CO_2 after the addition of $CH_2-S-CoM$ to the reaction mixture, is important.[90] This effect is produced also by a series of what are at first sight heterogeneous compounds: serine,[54] formaldehyde, hydroxymethylcoenzyme M,[91] pyruvate, L-ketoglutarate, L-malate, and isocitrate.[92] A common property of these compounds is that they can all function as donors of C_1-intermediates at different stages of the reduction process: of the formyl group (pyruvate, L-ketoglutarate, malate, and isocitrate), of the =CH− group (serine, formaldehyde, and hydroxymethylcoenzyme M), and of the methyl group ($CH_3-S-CoM$). Furthermore, the RPG effect clearly indicates a direct relation between the terminal (reduction of $CH_3-S-CoM$) and initial (activation of CO_2) stages of methanogenesis.[47,91]

Despite the fundamental correctness of the scheme proposed for methanogenesis by Barker, it is nevertheless too general in character. During the last 30 years, several attempts have been made to make this scheme more concrete,[46,48] but inadequate knowledge of the biochemistry of methanogens made it impossible to go outside the framework of general considerations and postulates. It was not until 1984 that Keltjens, having summarised the latest data, proposed a molecular model of the formation of methane from CO_2 and hydrogen.[54]

The basis of the Keltjens model is that CO_2 is activated by the methylreductase system (Fig.2). The one-electron reduction of the complex CH_3SCoMF_{430} leads to the formation of methane and a highly active thiyl radical, which activates and captures CO_2 with formation of a thiocarboxy-radical. The possibility of the existence of a thiyl radical in molecular methane-forming systems has been demonstrated experimentally.[69] The thiocarboxy-radical is subjected to attack by the CDR-radical coupled with one-electron reduction, which results in the formation of a complex of thiocarbamate, the CDR-factor, and $CoMF_{430}$ (Fig.2). Yet another one-electron reduction stage leads to the formation of the formamide form of the CDR-factor and the liberated thiyl radical. The subsequent stages involve the transfer of the formyl group of THMP and the formation of methyl-THMP. They take place with consumption of four electrons and the intermediate formation of methenyl- and methylene-THMP. Methyl-THMP is a relatively weak methylcarbonium (CH_3^+) donor. In order to be transferred to the thiyl radical, the CH_3^+ ion must be initially reduced to the methyl radical. This may be the function of component B of the methylcoenzyme M reductase system (Fig.2).

After the addition of the RPG-effectors, the increase in the degree of reduction of CO_2 is caused, as already mentioned, by the donation of C_1 units, which ultimately leads to an increase in the concentration of the thiyl radicals necessary for the binding of CO_2. However, the RPG-effect is transient apparently for two reasons: reaction of radical trapping agents and the exhaustion of the C_1 intermediates in the reduction of CO_2 for the synthesis of cell carbon.

Above we examined in detail the metabolism and biochemical characteristics of the methanogenic organisms specialising mainly in the conversion of H_2/CO_2 and/or formate. The metabolism and biochemical features of the organisms whose main substrates are acetate, methanol, and methylamines have been investigated to a much lesser extent, which can be explained both by the smaller number of organisms of this group and by certain methodological difficulties in their cultivation and investigation.[42,43] Hitherto it has been firmly established by the tracer atom method and certain other procedures that a common terminal methyl group transferring agent is coenzyme M for all methanogens.[36,50,93–96] It has been suggested that the primary recipients of the methyl group may be methanopterins or corrinoids, while the further transfer of the methyl group takes place as in the reduction of CO_2 with hydrogen.[93,97]

There exist two basic mechanisms of the transformation of acetate into methane and CO_2. The mechanism I, also referred to as the acetoclastic mechanism,[98,99] was described above and involves the cleavage of acetate in such a way that the methyl group is converted into methane and the carboxy-group is converted into CO_2. The mechanism II was proposed in 1936 by Barker and Van Neel and is a two-stage process.[100] Acetate initially decomposes to hydrogen and CO_2, which is then followed by the reduction of CO_2 with hydrogen. According to Van Neel's hypothesis, all the potential methanogenic substrates are initially converted into oxidised products and CO_2 and methane are formed as a result of the reduction of CO_2.[100] These two mechanisms can be differentiated using ^{14}C-labelled substrates.

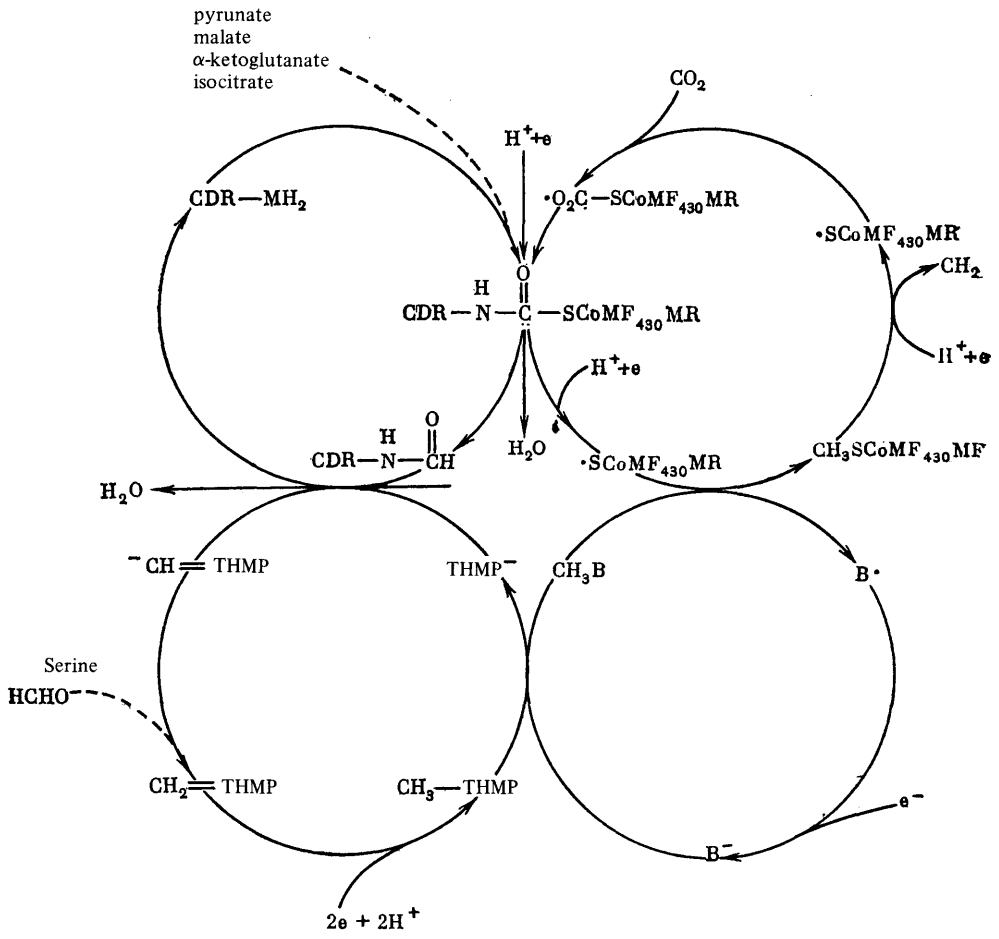


Figure 2. Keltjens molecular model for the reduction of CO_2 to methane.[54]

Numerous experiments have demonstrated that pure cultures belonging to the *Methanosarcina* family and also *Methanotrix soengenii* convert acetate via the mechanism I.[98,101,102] There is also no doubt that formate, methanol, and methylamines converted into methane by pure cultures of methanogens via the mechanism I.[46] Van Neel's hypothesis has been confirmed for other substrates, such as alcohols (C_2 and higher) and aliphatic acids (C_3 and higher), but subject to its significant modification which is that the conversion of these substrates takes place with participation of not one but several micro-organisms. The interaction of several micro-organisms takes place as a result of a process which has come to be called the interspecies hydrogen transfer.[103] This phenomenon was first demonstrated for an ethanol oxidising culture, the micro-organism called *Methanobacillus omelianskii*.[103] A similar interaction of cultures has been demonstrated in the methanogenic degradation of cellulose and hemicellulose,[21,104] glucose,[24–26,105] butyrate,[21,106,107] propionate,[108] acetate,[109] and also methanol, ethanol, and acetate by the methanogenic association of micro-organisms *Methanobacillus kuzneceovii*.[23–26,108,110] Interspecies hydrogen transfer is especially important in the anaerobic degradation of aliphatic acids, since their oxidation can proceed, for thermodynamic reasons, only at very low partial pressures of oxygen in the system, which is in fact ensured by the activity of hydrogen consuming organisms.[111–113]

Methanogens have played an enormous role in the genesis of natural gas on Earth. It has been demonstrated by geochemical analytical methods that more than 80% of gas deposits of industrial importance in the USSR consist of biological methane which has accumulated in such colossal amounts during billions of years of the activity of methanogens.[17] The advances in modern biotechnology permit the hope that in the near future it will be possible to replace a considerable proportion of the extracted natural gas by the industrial synthesis of methane.

4. Technological Principles of the Preparation of Biogas

The phenomenon of the evolution of combustible gas on decomposition of organic material under anaerobic conditions has been known for a long time. The first practical application of biogas dates back to the end of the XIXth century when in 1885 in the English city of Exeter methane obtained from a septic tank was used to illuminate streets.[114]

The first-scale factory for the manufacture of biogas was constructed in 1911 in Birmingham for the sterilisation of the solids from the sewage in that city. The methane formed was converted

into electrical energy. At the present time there exist a number of examples of the use of methane tanks in various countries of the World, this field of biotechnology passing through a period of intense development at the present time.[1,5,10,11,14]

The industrial preparation of biogas from organic waste has a number of advantages:[5]

(1) The heat generating capacity of biogas amounts to 20–28 MJ m^{-3} (0.7–0.8 kg of conventional fuel); the processing of 1 tonne of organic matter (dry weight) yields up to 800 m^3 of biogas.

(2) The process takes place periodically, semiperiodically, and continuously in large-scale reactors whose volume ranges from several cubic metres to thousands of cubic metres. The reactors are as a rule simple to construct.

(3) Methane fermentation achieves the effective purification of sewage and the processing of waste from animal husbandry and the cultivation of plants and of active sludge leads to the preparation of sterile high-quality fertilisers and to the complete mineralisation of nitrogen and phosphorus.

(4) The high coefficient of the conversion of the energy of organic substances into biogas, reaching 90%.

(5) Biogas can be used very effectively also directly as a fuel or can be converted with 83% efficiency into electrical and thermal energy by means of gas generators.

(6) In the anaerobic processing of waste, it is possible to obtain valuable biologically active compounds, for example, vitamin B_{12}, together with biogas.

A critical analysis of the economic problems associated with the introduction of biogas technology has been carried out.[115] It has been shown that the manufacture of biogas may be economically justified only when a series of conditions are observed: there must be a supply of raw materials with minimum and negligible transport and storage costs; there must exist a system for the utilisation of the gas without its accumulation and storage; there must exist a cheap source of low-potential heat for the heating and thermostatting of the methane tank.

Studies directed to the conversion into biogas of the waste from animal farms, which is regarded as an integral component part of modern animal farming industry, are of greatest practical interest.[116] The large-scale application of biogas technology makes it possible to solve in one process three inter-related problems—the preparation of a fuel, the conversion and sterilisation of waste, and the preparation of concentrated fertilisers.

An improved technology for the conversion of waste from the animal farming industry has been described.[117,120]

The rate-limiting stage in the preparation of biogas is the degradation of the biomass to oligomeric and monomeric components. Studies designed to intensify the depolymerisation processes by various methods are being prosecuted on these lines. One of the latest aspects of this field is associated with the use of ionising radiation. It has been shown that preliminary irradiation of biomass with γ-rays can intensify severalfold the methane formation process.[29,121]

Studies are being carried out on the automation and control of the methane generating process. For example, a correlation has been discovered between the productivity of the methane generator and the content of the coenzyme F_{420}. Methods have been developed for the determination of F_{420} in mixtures and procedures have been proposed for the estimation of the "methane generating force" of a mixed culture in terms of the content of coenzyme F_{420}.[123]

Fundamentally new possibilities in the biotechnology for the preparation of fuel are associated with the development of methods for the biogasification of peat.[124] Large deposits of peat fuel are known to exist in many countries, the development of their use being held back by the lack of an ecologically acceptable technology.

The first attempts to convert peat into biogas date back to the 1920's.[125] At the present time several projects designed to obtain biogas from peat are being developed. The project being developed by the Institute of Gas Technology (Chicago, USA), which has been called the "Biotherm Gas-process", provides for the combination of the conversion of peat into methane with participation of stages involving biological and thermochemical gasification.[126,127]

A second project, work on which is being carried out by the Dynatech Company (USA), provides for the chemical processing of peat with alkali coupled with an oxidative process and subsequent anaerobic conversion into biogas.[128,129]

The technical-economical parameters of various processes, including traditional processes have been compared and it has been shown that both projects may reach the stage of an economically significant technology in the immediate future. The main possibilities for the improvement of the technology are associated with the development of methods for the preliminary treatment of peat involving the improvement of its capacity for biodegradation.[124]

The rates of methane formation have been compared[130] for cellulose and hemicellulose in mesophilic and thermophilic decomposition processes. In the mesophilic process (35 °C), the degree of conversion of hemicellulose was higher than that of cellulose, but hemicellulose was converted significantly less effectively compared with cellulose under the conditions of thermophilic decomposition (55 °C). The rates of formation of gas from hemicellulose-containing materials are 0.3–0.6 of a volume of biogas per volume of the reactor per day for a methane content of 52–63% as a function of the process conditions.

The attempts to combine in a single system the generation of atomic energy and biogas are of great interest. There have been reports according to which a system for the preparation of biogas, based on the use of the heat of the cooling water, has been created within the framework of an atomic electric power station in Belgium. Algae converting biogas in the subsequent methanogenic stage have been cultivated using the low-potential discarded heat from the electric power station in water reservoirs.[131,132]

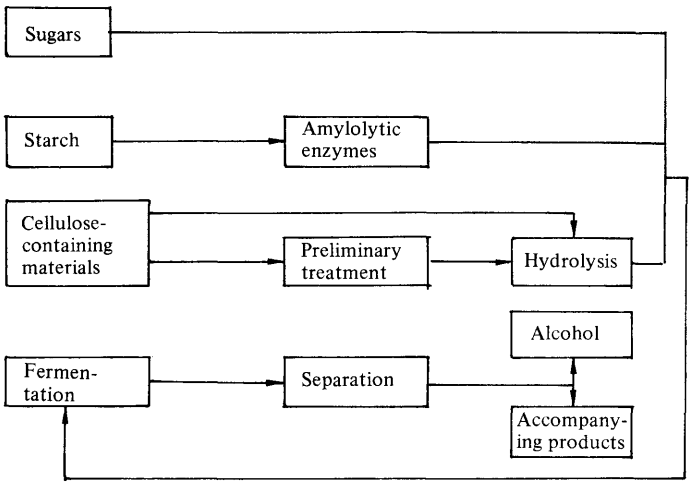
The present state of the biogas industry in the world has been surveyed in a number of reviews.[5,10,11]

III. ETHANOL

1. Chemical Principles of the Synthesis

With increasing deficiency of liquid mineral fuels, it has become necessary in many countries to replace petroleum by ethanol as a fuel, in the first place for the needs of automobile transport. The sources of raw material for the manufacture of fuel ethanol are plants with a high sugar content (sugar cane, sugar beet, etc.), starch (potatoes, maize, etc.), grain crops, as well as a wide variety of cheap plant materials of the lignocellulose type.

The biotechnological preparation of ethanol can be represented schematically as follows:



Only part of this scheme, based on the use of starch or sugars (molasses) as the initial raw material, has so far been realised on a wide scale. Low grade maize (corn) starch is thinned with α-amylase at a high temperature (80 °C, 20–30 min), hydrolysed with gluco-amylase to soluble sugars (mainly glucose), and this is then followed by the yeast fermentation of glucose to ethanol, which is subsequently fractionally distilled.[133]

The rate-limiting stage in the conversion of the cheap cellulose-containing raw material is the hydrolysis stage (cellulose is approximately 100 times more difficult to hydrolyse than starch). At the present time, three principal methods of hydrolysis of cellulose are in the process of development and compete with one another: (1) with dilute inorganic (sulphuric) acids at a high temperature; (2) with concentrated inorganic acids (sulphuric, hydrochloric, or hydrofluoric acid) at a low temperature; (3) enzyme hydrolysis at a low temperature. Each method has definite advantages and disadvantages.

When concentrated acids are used, high rates of hydrolysis of cellulose are achieved, but problems associated with the recovery of the acids and the corrosion of the equipment arise.

Processes using dilute acids require high temperatures in order to attain technological rates of conversion of cellulose, but the rate of decomposition of glucose increases at the same time and equipment is again corroded.[143,144]

Enzyme hydrolysis does not suffer from the above disadvantages, but at the present time the cost of cellulases (enzymes hydrolysing cellulose) is fairly high. Furthermore, the attainment of high degrees of conversion of cellulose frequently requires an intense preliminary treatment to decompose the lignin shell and to convert cellulose from the crystalline to an amorphous state. Other problems in enzyme hydrolysis consist in the inhibition of cellulases by the hydrolysis products and in the inactivation of enzymes.

In general, hydrolysis with dilute acids makes it possible to achieve only 50–60% yields of glucose, while hydrolysis with concentrated acids or a multistage process involving the decrystallisation of cellulose (the Tsao–Purdue process) is potentially capable of reaching yields of 90%. The yield of glucose in enzyme hydrolysis depends greatly on the type of preliminary treatment (mechanical milling, delignification with solvents, treatment with live steam, preliminary hydrolysis of hemicelluloses with dilute acid) and can vary from less than 50% to more than 90%.[134–140]

Jones and Semrau[134] carried out an economic analysis of the cost of the final product (ethanol) for different methods of hydrolysis of cellulose. None of them has economic advantages, compared with the preparation of ethanol from grain, which has been traditional in the USA. However, the economic situation can change significantly if furfural, the principal side product of the acid hydrolysis of wood, becomes commercially significant as a chemical agent.[134]

The use of various component of the biomass not susceptible to alcohol fermentation is being studied in this connection. An interesting aspect of such research is associated with the development of methods of preparation of monomers from the biomass.[141,142] Thus a procedure has been described for the preparation of 2-vinylfuran—the component capable of serving as a basis for new polyvinyl polymers.

The use of a hybrid process, in which a dilute acid is employed for the preliminary treatment and separation of the corresponding fraction of wood (mainly the hemicellulose fraction), while enzymic hydrolysis is used to convert the residual cellulose into glucose, also appears very promising.[154]

Partial degradation of cellulose can likewise be achieved with the aid of a number of physical methods, for example, by treating cellulose with live steam or with γ-radiation. The effect on the enzyme and acid hydrolysis of the preliminary treatment [with radiation] of the biomass has been investigated and it has been shown that, for doses greater than 10 Mrad, bagasse (the residue from sugar cane after the juice has been pressed out) is degraded; bagasse irrigated with a dose of 100 Mrad affords 2–4 times more glucose than the non-irradiated specimen. Irradiation influences the acid hydrolysis to a greater extent than the enzyme hydrolysis.[145–147]

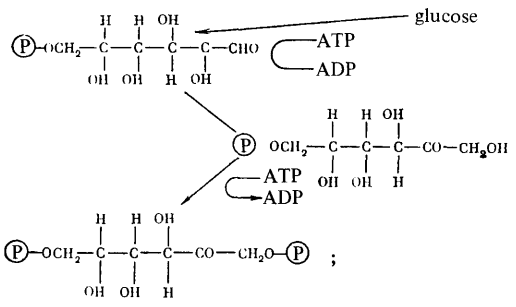
The second stage in the processing of cellulose—the conversion of carbohydrates into ethanol—is one of the oldest microbiological processes used by man:

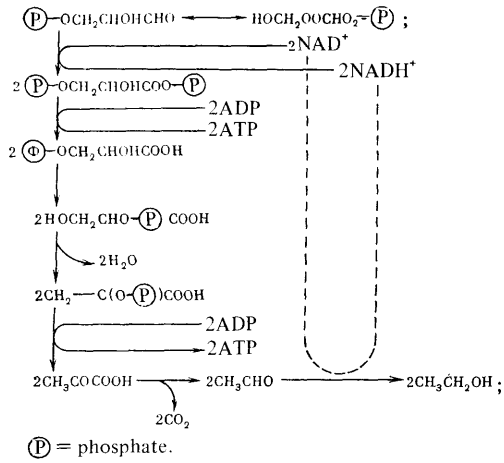
$$C_6H_{12}O_6 \rightleftharpoons 2C_2H_5OH + 2CO_2 .$$

The coefficient of the conversion of energy in this reaction is 98%.

The mechanism of the microbiological preparation of ethanol is fairly complex, but it has been investigated in great detail at the microbiological and biochemical levels[1] (see Scheme 1).

Scheme 1





(P) = phosphate.

On a technological scale, sugars are fermented to ethanol as a rule by yeast (*Candida brassicae, Candida utilis, Saccharomyces cerevisiae, etc.*). The theoretical yield of ethanol is 51 wt.% of the initial concentration of glucose, while the practical yield is 47 wt.% as a result of the growth of the yeast and the formation of side products. The inactivation of yeast by high concentrations of ethanol (in excess of 10–12%) creates many problems and for this reason methods for the extraction of ethanol from the culture medium are being vigorously developed.[148] In this sense, much hope is associated with the bacteria *Zymomonas mobilis* which ferments sugars twice as fast as yeast.[149] Thus the *Z. mobilis* cells immobilised in calcium alginate pellets afforded at the outlet from the reactor 102–116 g litre h^{-1} of ethanol for an incoming concentration of glucose of 200 g $litre^{-1}$.[150]

The idea of direct effectively single-stage conversion of polysaccharides into ethanol has gained wide currency in recent years. Thermophilic anaerobic bacteria are very promising in this connection: they do not require aeration, grow at high (60–65 °C) temperatures, grow rapidly on cellulose and exhibit a high metabolic activity, contain the complete set of enzymes of the cellulase complex, and ferment cellulose and sugars with formation of a limited spectrum of metabolites: ethanol, lactate, H_2, and CO_2.[137] These bacteria also have a number of advantages characteristic of thermophilic organisms in general. Thus at high temperatures there is, firstly, a decrease in the solubility of oxygen and other gases which lowers their toxic effect and increases the time during which anaerobic conditions are maintained, secondly, the viscosity of the solutions diminishes and the solubility of the substrates increases, which improves their accessibility to enzymes, and finally and thirdly, the volatility of ethanol increases, which permits its continuous distillation using a low vacuum, reducing thereby its inhibiting effect.

The technological application of thermophilic anaerobic bacteria is limited by a number of disadvantages: the lack of balance in the cellulase enzyme complex; the relatively low yield of ethanol to the fermented sugar molecule; the increased sensitivity to ethanol (as a rule their metabolism is almost completely suppressed already in the presence of 0.5% of ethanol in the medium); the low capacity to ferment the products of the hydrolysis of hemicelluloses.[137]

The natural strains of micro-organisms suitable for the direct conversion of plant raw material into ethanol require at the present time an improvement in their biological characteristics. This can be achieved by methods involving the selection of the appropriate mutants by the creation of recombinant strains with specified properties by genetic engineering and also by searching for new natural strains and by the selection of micro-organisms suitable for joint cultivation. The development of improved methods of preparation of ethanol, the creation of new strains of micro-organisms tolerant to an increased ethanol content and a low pH have been described.[152,153]

2. Technological Features and Economics

Brazil has the greatest experience in the use of ethanol as a fuel. The National Alcohol Programme (PROALCOOL), adopted by the Brazilian government in 1975, was the outcome of almost half a century's research in this field. The principal aim of this programme was to reduce the import of petroleum by partly replacing petrol by ethanol and made provision for the manufacture by 1985 of 107 billion litres of ethanol annually.[156] The source of alcohol was in this instance the fermentation of sugar obtained from sugar cane.[155] It is noteworthy that Brazil as a whole has successfully fulfilled these plans. The campaign conducted in that country in favour of replacing petrol by alcohol has enabled Brazil to save 2 billion dollars per year. The results of the Brazilian experiment for the development of systems for the preparation of fuel ethanol from sugar cane are described in a report by Khan and Fox.[157]

As already mentioned, the manufacture of ethanol from grain is the traditional procedure in the USA, the cost of such alcohol being 42 cents per litre.[134] The use of grain as the substrate is explained by the low prices and certain surpluses in the production of mainly maize grain.[157,158] These factors as well as the energy crisis led to the adoption in the USA of a national alcohol programme designed mainly to produce gasohol (90% of petrol and 10% of ethanol).[159] The popularity of this new fuel was due to its satisfactory anti-knocking properties (10–12% of ethanol is equivalent to 1–2 cm^3 of tetraethyl-lead per gallon)[159] and its economy—the consumption of gasohol is smaller by 25.3 g km^{-1} than that of non-ethylated petrol.[160]

Among the developments in the acid hydrolysis of lignocellulose materials, attention must be concentrated on the process developed at the New York University.[134] In order to obtain technologically acceptable rates of hydrolysis of wood by dilute sulphuric acid and in order to reduce the losses resulting from the decomposition of sugars at a high temperature, the process is carried out under continuous conditions with a short (10 s) residence time in the high-temperature zone in a specially designed reactor for a temperature of 232 °C and a pressure of 34.02 atm. Insoluble substances lignin, unreacted cellulose, ash components, and gypsum) are separated from the resulting suspension and the solution obtained, containing soluble sugars at a concentration of 10–11 wt.%, is supplied periodically into the reactor, where it is fermented to ethanol by yeast. The recycling time is 48 h. The concentration of ethanol at the outlet is 5.2 wt.% or 6.5 vol.%. The last stage is the distillation of ethanol.[134]

Economic calculations carried out for a factory with a productivity of 914.6 million litres of ethanol annually have shown that for an overall capital investment of about 132 million dollars the calculated cost of the ethanol would be 72 cents per litre. Thus it is seen from the calculation that the cost of the ethanol obtained by acid hydrolysis is fairly high and such technology cannot at present develop without government subsidies.

A group of scientists at the Michigan University have recently proposed a new process for the hydrolysis of wood with the aid of gaseous hydrogen fluoride. Dry wood is treated at a reduced pressure for 1 h. This results in the formation of glucosyl fluoride (up to

99% yield) from cellulose. Next, glucosyl fluoride is hydrolysed with water (1 h, 140 °C) with formation of glucose and hydrogen fluoride. The latter is recycled by evacuation or with the aid of slight heating and its residues in the solution are neutralised with calcium carbonate. The CaF_2 deposit is treated with sulphuric acid in order to achieve a more complete recyclisation of HF. The principal products of the process are glucose and lignin. The glucose is subsequently fermented to ethanol. The estimated cost of glucose is 12 cents per kilogram of which 7.5 cents represents the cost of the initial wood.[161] Unfortunately, there have been no communications so far about the scale of this process.

The entire wide variety of scientific and technological solutions of the problems concerning the enzymic conversion of cellulose into ethanol in the USA can be reduced to three basic processes.

(1) The use of separate reactors for the accumulation of cellulases, hydrolysis of cellulose, and the fermentation of sugars to ethanol (the Army Laboratory at Natick).

(2) The use of a separate reactor for the accumulation of cellulases and the combined process involving the conversion of cellulose into sugars and the fermentation of the sugars formed to alcohol (Gulf Oil Chemicals Co.—Arkansas State University).

(3) The use of a single reactor for the direct conversion of cellulose into ethanol with the aid of one or several species of microorganisms (Massachusets Institute of Technology).

The technology of the Gulf Oil Chemical Co. has been most thoroughly developed from the standpoint of technique and economics. Cellulose is hydrolysed by the cellulose enzymes of the fungus *Trichoderma reesei* and the sugars formed are fermented to ethanol by the cells of *Candida brassicae*.[135,162,163] It was proposed to scale up this technological process in three stages and a factory for the combined processing of 3000 tonnes of dry waste per day is to be built in the last stage, which is planned for the end of the 1980's. Apart from the production of 200 million litres of absolute alcohol annually, the factory will generate also 52.5 MW of electrical energy annually as a result of the combustion of the unused cellulose. The total cost of the planned factory is 230 million dollars. The commercial cost of the alcohol produced will be 58 cents per litre.[135]

The laboratory in Natick in collaboration with the New Brunswick Company constructed a pilot plant where wood is treated with steam and then hydrolysed by cellulases obtained by the deep culture of a mutant strain of *T. reesei* in a separate 400 litre fermenter. The sugars formed are fermented in a 250 litre reactor to give alcohol, which is distilled. The lignin and the unreacted cellulose are burnt. The apparatus has been automated to the highest possible extent for analysis and control.[134,164]

The process economics has been analysed for a factory with a productive capacity of 100 million litres of 95% ethanol annually, processing 495 000 of town waste containing 375 000 tonnes of cellulose. The capital investment in the construction of this factory is expected to be 83.5 million dollars and the intrinsic cost of ethanol is to be 42 cents.[135,164] According to other data, the intrinsic cost of ethanol from this process is 55 cents per litre.[134]

The process developed by the Massachusets Institute of Technology (MIT) is based on the employment of mutant strains of the thermophilic bacteria *C. thermocellum* and *C. thermosaccharolyticum* for the direct preparation of ethanol from agricultural cellulose-containing waste. In the laboratory experiments, the yield of alcohol reached 85%.[165] A disadvantage of this development is the relatively low rates of conversion of cellulose into ethanol. The economic analysis carried out was based on plans for a factory using the MIT technology processing 1500 tonnes of maize stems per day and capable of producing ~100 million litres of ethanol annually. The intrinsic cost of the ethanol is 28 cents per litre, while the cost of the raw material is 30 dollars per tonne. The capital outlay on the construction and operation of the factory is 34.4 million dollars.[135]

The Tsao–Purdue process is based on the preliminary treatment of cellulose-containing materials consisting in the dissolution of the cellulose fraction in cyclohexane, ethylenediamine, and concentrated phosphoric, sulphuric, and hydrochloric acids with subsequent precipitation of cellulose already in an amorphous form with methanol or water.[166] Recently the Tsao–Purdue process was criticised because of the considerable technical complexities in scaling up and is believed to be economically unfavourable.[135]

The Aietick Canadian Company developed an effective method for the preliminary treatment of lignocellulose materials for subsequent hydrolysis. Here Mason's method (or the co called steam explosion) is used, where live steam under pressure permeates into the biomass, whereupon the ordered structure of lignocellulose is broken down as a result of instantaneous decompression and then converted into a loose amorphous mass. The lignin then melts and can be readily separated from the cellulose fraction. Furthermore, such treatment results in the extraction of the hemicellulose fraction, which passes to water after the condensation of steam. Cellulose is hydrolysed by the enzyme preparations from *T. reesei* C-30 and *A. niger*. Under these conditions, 90–95% of cellulose and 80–85% of hemicellulose are converted into sugars in 100 h at 45–50 °C. The fermentation of the resulting glucose to ethanol takes place in 95% yield. The yield of ethanol is 270 litres from 1 tonne of wood. Xylose is not fermented by yeast and its concentrated solution serves after evaporation as a commercial product under the name "Feed molasses". If an effective fermentation of xylose to alcohol is also achieved, then the yield of ethanol may be 360 litres per tonne of wood. The lignin remaining after hydrolysis is used for the manufacture of phenol-formaldehyde plastics and adhesives.

Technical-economical calculations for the factory planned by the Aietick Company, designed to process 250 tonnes of lignocellulose daily with a productivity of 30 million litres of ethanol per year, have shown that the overall capital investment will be in the region of 10 million dollars and the cost of the ethanol will be 45 cents per litre.[133,135] One of the Canadian companies is planning to expand the factory in Manitoba which is already producing ethanol from barley by the Aietick process.

An analogous project has been developed by another Canadian firm—Stake Technology. Its difference from the Aietick process consists in the application of a continuous preliminary treatment of the lignocellulose raw material.[133,135]

The combined approach in Canada to the utilisation of biomass has also been manifested in the development of integrated agricultural projects including, for example, the growing of maize and the production of beef and fuel ethanol.[167]

The French government set aside in 1981 the sum of 26 million dollars for a programme designed to develop the manufacture of gasohol. It is proposed that the realisation of this programme by 1990 will enable France to satisfy the national requirements for transport fuel to the extent of 25–50%. It is planned to produce ethanol from wood, straw, and artichokes; the fermentation of the glucose syrups by immobilised cells will be combined with the removal of ethanol by extraction to prevent the inhibition of the process.[168]

In Japan interest in the preparation of ethanol from the biomass arose in the 1950's. Japanese technologists developed three processes for the hydrolysis of cellulose-containing raw material by concentrated acids at atmospheric pressure and a temperature not exceeding 100 °C.[169] Unfortunately the Japanese investigators were unable to eliminate fully the disadvantages characteristic of the hydrolysis by concentrated acids and more attention has been devoted to enzymic hydrolysis in the national project for the preparation of fuel ethanol, which has been executed by the Ministry of International Trade and Industry between 1980 and 1986. Japanese investigators have reported that they devised a very simple method for the enzymic conversion of cellulose-containing materials into sugars and the preparation of low cost sugars and ethanol with a considerable saving of energy.[135,155]

Scientific and technological activity in the development of processes for the preparation of fuel ethanol from biomass has also been manifested in India, Finland, Italy, Sweden, Austria, Australia, New Zealand, South Africa, Mauritius, and other countries.[114,170,171]

IV. THE ACETONE–BUTYL FERMENTATION

Interest in the preparation of organic solvents by a microbiological procedure has arisen anew recently. Examination of past history shows that interest in the acetone–butanol–ethanol fermentation waned and waxed anew, such interest determining the possibility of utilising the final fermentation products and also stimulating the research in this field.

The first major improvements in the process for the conversion of carbohydrates into butanol, acetone, and ethanol on an industrial scale were achieved during and after the First World War. The need for acetone was very high, which led to the rapid development of its manufacture. At the same time, butanol was not of much value and its storage and utilisation presented difficulties. However, after a time it was discovered that butanol is an extremely valuable chemical product and some of its esters came to be used in the manufacture of automobile varnishes and in other branches of industry.

It is essential to note that the USSR has been a pioneer in the development of the microbiological acetone-butyl industry, the scientific principles of which were developed by Academicians Shaposhnikov and Ierusalimskii.

The principal processes achieved on an industrial scale before the Second World War were the fermentation of the waste from the industrial manufacture of starch and molasses and the industrial processing of maize. Whether or not the industry was economical depended mainly on the cost of the raw material and after the Second World War the microbiological synthesis of solvents was abandoned in most countries as economically unfavourable in connection with the development of the production of synthetic solvents from cheap petroleum.[172,173]

The growing cost of the petroleum processing products during the 1970's stimulated the creation of more sophisticated technologies for the preparation of butanol. A commercially viable manufacturing process was developed on the basis of the fermentation of sugar cane molasses by a *C. acetobutylicum* culture.[174]

Butanol has effective characteristics as a fuel. By mixing up to 50% of butanol with petrol, a satisfactory fuel is produced.

The cultures used for the acetone–butanol fermentation consist of the *C. acetobutylicum* species and are distinguished, apart from morphogolical differences, also by the carbohydrate medium which they convert, by the rate of fermentation, by the type of nitrogen-containing nutrient substances required, and by the ratio of the solvents obtained.

The search for new strains is associated with the need to employ raw materials which are cheaper and more readily available than the expensive carbohydrates. The possibility of obtaining butanol from hydrolysed pine wood was investigated with the aid of *C.acetobutylicum* NC113-2951.[176] The fermentation was carried out at 30 °C for 5–7 days. The amount of hydrolysate obtained was 30 g litre^{-1} (4% of xylose, 6% of mannose, 2% of galactose, and 88% of glucose). The main hydrolysate was decolorised and steam-treated only in a combined process, which led to the removal of inhibitors and made it possible to obtain up to 1.6 g litre^{-1} of butanol which is equivalent to 90% of the yield of the product in relation to the carbohydrates adsorbed. However, during decolorisation the hydrolysates lost up to 30% of the sugars present. The use of a combination of cation- and anion-exchange resins for the purification of the hydrolysates made it possible to increase the yield of butanol as a result of subsequent fermentation (5.7 g litre^{-1} or 17% of the carbohydrates employed).

A *C. acetobutylicum* ATCC 324 culture was used to determine the optimum conditions for the preparation of butanol and acetone from acid buttermilk.[177] The culture of the above producer is capable of utilising several carbohydrates, including lactose, with formation of butanol, acetone, and ethanol in proportions of 6:3:1. The maximum synthesis of solvents was observed when sterile buttermilk was used in the fermenter without stirring. After 120 h, the pH fell to 5.2 and the concentration of solvents reached 9.2 g litre^{-1} and that of the residual lactic acid in the medium was 2.5 g litre^{-1}. Treatment in an autoclave and the absence of stirring increased the yield of solvents. Periodic fermentation of whey ultrafiltrate, glucose, lactose and galactose as carbon sources, by *C. beijerinckii* made it possible to compare the conditions for the preparation of butanol from the whey ultrafiltrate with those in its preparation from other substrates and to determine the optimum process parameters.[178]

The main aspects of the research on the improvement and reduction of the cost of the manufacture of butanol in our country involve the replacement of the expensive alimentary raw material (flour) by a non-alimentary raw material (for example sugar beet, molasses). Use of the *C. acetobuylicum*, strain S, culture, which made it possible to alter the solvent ratios and to obtain a fairly high yield of butanol (69–72%) for a content of residual sugar from 0.6 to 0.3%, is promising. The process terminates after 42 h, the content of the solvents at the end of the process amounting to 15.07 g litre^{-1} and including 11.03 g litre^{-1} of butanol, 3.38 g litre^{-1} of acetone, and 0.6 g litre^{-1} of ethanol.[179]

The utilisations of various sugars from hemicellulose hydrolysates by *C. acetobutylicum* have been compared.[177] A 90% yield of butanol can be observed in the fermentation of glucose. Five other sugars ensured the formation of butanol in decreasing amounts: cellobiose > mannose > arabinose > xylose > galactose.

An important problem involves the search for strains ultilising cellulose. It was found that only two out of 21 of the solvent producing *Clostridium* species strains tested exhibit a cellulolytic activity.[180] Furthermore, subsequent investigations revealed that the same strains exhibit endoglucanase and cellobiase activities, but their overall activity is insufficient for the hydrolysis of crystalline cellulose.

The biochemical principles of the anaerobic metabolism have been elucidated in fair detail and described in a number of studies.[181–183] *C. acetobutylicum* metabolises glucose to pyruvic acid via the

Embden–Meyerhof pathway with formation of two molecules of ATP and two molecules of NADP. Acetyl-CoA, CO_2, and H_2 are formed as a result of the phosphoroclastic pyruvate cleavage reaction. The amount of hydrogen in *C. acetobutylicum* varies as a function of the presence of NADH-ferredoxin oxidoreductase.[184] The acetyl-CoA formed is converted into acetate (ATP is synthesised), ethanol (two molecules of NADH are required), and acetoacetyl-CoA, which is either transformed into acetone or is reduced (two molecules of NADH are needed) to butyryl-CoA. *C. beijerinckii* reduces acetone to isopropyl alcohol.[185] It had been suggested earlier that the formation of butyrate is associated with transferase activity, but the latest studies have demonstrated that the main metabolic pathway proceeds via butyryl phosphate and the formation of ATP.[186,187] Butyryl-CoA is subsequently reduced to butanol with participation of two NADH molecules. The hydrogen, evolved in the first stages of the conversion of glucose, is consumed in the formation of NADH used to reduce the butanol.

It is known that in the course of the growth phase in the periodic cultivation of *C. acetobutylicum*, the main products are acetic and butyric acids, hydrogen, and CO_2. The acids are reassimilated when the cultivation pH falls and are converted into solvents with simultaneous consumption of the sugar. There exists the view that the accumulation of butyrate in the medium activates a new fermentation system, which leads to the formation of acetone and butanol and disturbs the usual mechanism of the cycle. The transition from the acid to the synthesis of the solvent can be partly accounted for by the latest enzymological studies at the level of the enzymes of these two phases. It has been shown that the activity of phosphate acetyltransferase and acetylkinase was greatly reduced and that of phosphate butyryltransferase vanished during the stage involving the synthesis of the solvent by *C. acetobutylicum* ATCC824.[187] A similar situation has been observed for *C. acetobutylicum* DS 14: the butyraldehyde and butanol dehydrogenase activities were determined only for cells synthesising the acetone–butanol mixture.[186] The same phenomenon has been observed for *C. beijerinckii*.[185] *C. acetobutylicum* possesses a transferase system which transfers the acetoacetyl-CoA from the cycle to the synthesis of acetoacetate and the latter can be decarboxylated to acteone. Acetate(butyrate):CoA transferase is an enzyme with a broad substrate specificity, which also exhibits a higher activity in the butanol synthesising cells than in the cells synthesising acid.[174,186] The decarboxylation of acetoacetate to acetone stimulates the transferase reaction via the well known acetoacetate decarboxylase, which is again induced only in the butanol synthesising cells.[174,186,187]

The synthesis of the reduced final products depends on the reducing potential of the cell. During the acid phase in the periodic culture portions of them are lost in the form of H_2. In the synthesis of the solvent, two moles of NADH are formed per mole of glucose and a certain amount of the former is also produced from the reduced ferredoxin. Consequently, in order to increase the amount of butanol, it is essential to increase the total amount of NADH. This can be achieved, for example, by inhibiting the hydrogenase at the acid formation stage[145] or via continuous cultivation in the solvent formation stage. The maximum theoretical yields from 1 g of glucose are 0.41 g of butanol, 0.32 g of acetone, and 0.5 g of ethanol. Thus, in the formation of a mixture of these products, the maximum theoretical yield depends on the product ratio. If this ratio is 6:3:1 (butanol–acetone–ethanol), which is typical, the maximum theoretical yield of solvents is 0.39 g g^{-1}.

It has been established that at a high pH mainly acids are synthesised whereas at a low pH an acetone–butanol mixture is formed. The optimum pH for the synthesis of solvents varies as a function of the strain and the cultivation conditions, lying as a rule in the range 4.0–6.0.[180,189] This is apparently associated with the fact that acetoacetate decarboxylase has a pH optimum in the region of 5.0.[190] Furthermore, the pH influences the ratio of the dissociated and undissociated forms of acetic and butyric acids and it is known that only the undissociated form permeates through the cell membrane.[191]

However, these conclusions cannot be applied to all the butanol synthesising *Clostridium* species. Significant synthesis of butanol by *C. beijerinckii* PI 13436 is attained at pH 6.8 and neither an acid concentration nor a pH which might be correlated with the onset of the formation of butanol were observed.[185] The synthesis of butanol by *C. acetobutylicum* NCIB 8052 (ATCC 824) can be induced at pH 7.0 by adding high concentrations of acetate and butyrate (100 mM each)[192] and in certain *C. acetobutylicum* cultures, isolated in Egypt, the final pH did not prevent the synthesis of butanol (20 g $litre^{-1}$) down to 6.4.[193] Whatever the process mechanism, the addition of acetate and butyrate to the periodic culture influences the time of the onset of synthesis,[185,192,194] the final product ratios, and the concentration and yield to an extent which cannot depend exclusively on the simple conversion of the acids into the acetone–butanol–ethanol mixture.[194]

An important factor in the formation of the acetone–butanol mixture is the concentration of the carbon source. Unduly low glucose concentrations[186,196,197] afforded as a result only acids. In a periodic culture, the organisms did not enter into the butanol formation phase under these conditions and in continuous systems the substrate concentration must be not less than 5 g $litre^{-1}$.[198]

Detailed studies of the kinetics of the formation of products were carried out by chemostatic cultivation with limiting glucose, nitrogen, and phosphate.[199] The specific rate of consumption of glucose increased with increase in the rate of dilution. An increase in the rate of dilution increased the rate of formation of acid, which did not appear at low rates of growth. A completely different picture obtained as regards the rate of formation of acetone, butanol, and ethanol. It has been shown that their amounts increase with increasing rate of dilution up to a certain point (0.1–0.2 h^{-1}) and then again decrease rapidly. Extrapolation indicates the formation of an acetone–butanol mixture at a zero rate of growth. Thus the synthesis of butanol is only partly associated with growth. If the butanol-synthesising cells are again placed in a buffer solution, they continue to synthesise the solvents. Thus non-growing cells can be used for the prolonged preparation of butanol.[198,200]

A weak point in acetone–butanol fermentation is the low concentration of the target products (about 2%). Solvents, especially butanol, are toxic to bacterial cells. In test experiments, the rate of growth of *C. acetobutylicum* ATCC 824 was 50% inhibited at a butanol concentration of 7–13 g $litre^{-1}$, an acetone concentration of 40 g $litre^{-1}$, and an ethanol concentration of 50–60 g $litre^{-1}$.[201] Complete growth inhibition was observed in the presence of 14–16 g $litre^{-1}$ of butanol and 70 g $litre^{-1}$ of acetone and ethanol. The lower concentration limit at which no decrease in the rate of growth takes place is 4.4–8 g $litre^{-1}$ of butanol. Other strains responded analogously to organic solvents and in certain cases they are even more sensitive than the strain 824. For a culture of *C. acetobutylicum* P 262, the addition of 10 g $litre^{-1}$ of butanol virtually stopped growth.

The difference between the inhibiting concentrations of the solvents can be accounted for by the differences in the hydrophobic properties of the alcohols.[202] The lipid layers in the cell membranes are acted upon by the solvents. It has been shown that butanol (0.5–1.5 vol.%), added to the lipid fraction obtained from cells in

the middle of the exponential phase of the growth of the *C. acetobutylicum* ATCC 824 strain, appreciably increases the mobility of the spin-labelled hydrocarbons in lipid dispersions or, in other words, butanol leads to the "thinning" of the lipid double layer.[203] It has been established that the composition of the lipid acyl chains changes, on passing from the exponential to the stationary growth phase to one with a higher percentage content of saturated chains. The addition of butanol (0.5–1 vol.%) to the cultures influences the composition of the chains in the same way. It has been concluded that the ability to increase the percentage content of saturated aliphatic acids in the membrane in response to the increase of the butanol level acts against the "thinning" effect of the latter.[203]

C. acetobutylicum is sensitive not only to butanol but also to acetic and butyric acids. The inhibiting effect by acids is correlated with the intercellular concentration of undissociated structures and with the pH.[204] A twofold decrease in the rate of growth occurs in the presence of 8–15 g litre^{-1} of acetic acid and 6–13 g litre^{-1} of butyric acid.[201]

At the present time, industrial fermentation with periodic cultivation has approached the limit of the biological possibilities of the cultures employed. For example, the Weisman strain of *C. acetobutylicum*, cultured in a 90 m^3 periodic fermenter on sugar cane molasses, affords an overall yield of solvent of 19.5 g litre^{-1} in the proprotions 6:3:1 (butanol–acetone–ethanol) after 30–34 h in 0.3 g g^{-1} yield and with a productivity of 0.6 g litre^{-1} h^{-1}. For comparison, we may note that the best result obtained on a laboratory apparatus with glucose as the source of carbon is as follows: overall yield of the acetone–butanol mixture 18.4–21.3 g litre^{-1} and a productivity of 0.3–0.9 g litre^{-1} h^{-1}.[205]

The change from periodic continuous cultivation regimes made it possible to increase the productivity in acetone–butanol fermentation. A two-stage chemostatic cultivation of *C. acetobutylicum* DS 1731, optimised with respect to pH and temperature, has been described.[199] The first stage was carried out at a rate of dilution D = 1.25 h^{-1} (37 °C, pH 4.3) and the second stage at D = 0.03 h^{-1} (33 °C, pH 4.3). The stream of synthetic medium contained 0.1 g litre^{-1} of KH_2PO_4 and 54 g litre^{-1} of glucose. These conditions permitted the complete utilisation of the carbon source, affording a yield of the final product of 18.2 g litre^{-1} [4.87:12.78:0.78 (g litre^{-1}) acetone–butanol–ethanol mixture].

Chemostatic cultivation of *C. acetobutylicum* ATCC 824 with glucose limitation (50 g litre^{-1}) and the use of a complex medium at D = 0.1 h^{-1} gave an overall solvent yield of 15.9 g litre^{-1} [5.4:9.5:1 (g litre^{-1} acetone–butanol–ethanol mixture]; a butanol yield of 0.32 g g^{-1} and a productivity of 2.56 g litre^{-1} h^{-1} were achieved at D = 0.22 h^{-1}. The high productivity of this system is associated with the high concentration of the biomass as a result of the employment of a complex medium. Evidently the productivity can be increased still further by recirculating the biomass.

The continuous formation of acetone and butanol can also be achieved by employing immobilised cells. In order to reduce the losses of activity, a technique designed to meter the neutrient substances has been developed.[198] Using this technique, the continuous production of an acetone–butanol mixture in the presence of *C. acetobutylicum* ATCC 824 cells immobilised on the calcium alginate gel or adsorbed on beechwood chippings has been achieved.[198] In the system with adsorbed cells, the best results were attained for D = 0.2 h^{-1}: the solvent yield was 6.3 g litre^{-1}, the butanol yield 0.33 g g^{-1}, and the productivity 1.2 g litre^{-1} h^{-1}. These are highly promising figures bearing in mind that the rate of growth of the micro-organisms was not raised to the maximum and the medium was not optimised.

The growing cells of *C.beijerinckii* MD 27.6 were immobilised in a calcium alginate gel for the continuous preparation of butanol and isopropyl alcohol. The medium contained glucose and a yeast extract. The best result was obtained for D = 0.3 h^{-1}: an overall solvent yield of 9–10 g litre^{-1} and a productivity 1.5 g litre^{-1} h^{-1}.

Thus, despite the fact that the acetone–butanol fermentation is one of the first purely microbiological processes developed at the industrial level, the renewal of interest in this manufacturing process can be justified only if a number of problems, capable of resulting in economically viable results, are solved. The use of an improved technology, such as continuous cultivation and immobilised cells, can increase the productivity of the fermentation. The attempts to solve the problem of the toxicity of butanol and acetone and to increase the yield of the products synthesised have been concentrated on the adaptation and selection of tolerant strains and also on the creation of various mutants in the study of the genetic control of the production of butanol. At the present time, studies have been published on the development of methods for genetic analysis which make it possible to manipulate the corresponding genes for the creation of strains capable of more productive synthesis of solvents and of altering their ratio .

It is noteworthy that the acetone–butyl fermentation is a waste-free industrial process. The carbon dioxide of hydrogen formed during fermentation can be regarded as secondary products. Carbon dioxide, liquid and solid, is used in the food industry and hydrogen is employed as a fuel or in methane formation processes. The liquid waste from industry containing riboflavin, vitamins, and protein is used as cattle feed.

V. HYDROGEN

An important natural phenomenon, which is of great interest from the standpoint of the conversion of energy, was discovered in 1942.[206] After adaptation to the dark, under anaerobic conditions, the cells of microscopic algae begin to evolve hydrogen vigorously in the course of several hours. Since the microscopic algae are capable of producing molecular oxygen in the light, there exists the possibility, in principle, of employing the mechanism of photosynthesis for the biophotolysis of water with conversion of light energy into a form of fuel.[1]

In recent years, the phenomenon involving the formation of hydrogen by microscopic algae has been investigated in a series of laboratories. The present state of this problem has been discussed in review articles.[1,2,206,207] There exist several examples of laboratory processes leading to the formation of hydrogen. The use of thermophilic algae *Mastigoladus laminosus* has been described.[208] The system for the formation of hydrogen acted in the course of several days with an efficiency in the utilisation of the energy of sunlight of 2.7%. The algal culture was projected against the action of oxygen with the aid of a mixture of argon, nitrogen, and CO_2.

The blue-green algae, exhibiting both hydrogenase and nitrogenase activities, are of interest in this sense. The limitation in the supply of nitrogen promotes the formation of hydrogen by a factor of 2.5–3.5 compared with the normal conditions.[133] The experiments performed with the blue-green algae *Anabaena cylindrica* showed that the yield of hydrogen increases by ~70% on periodic illumination (10 s period). An apparatus for the manufacture of

hydrogen has been created under laboratory conditions in which a suspension of algae circulated between dark and illuminated zones.[209]

The practical use of the phenomenon of the photoevolution of hydrogen by microscopic algae has not as yet been achieved in view of the low efficiency of the evolution of hydrogen under stationary conditions. It may be that further studies and the optimisation and increase in the process efficiency will create conditions for the technological exploitation of this phenomenon.

Experiments performed on photosynthesising bacteria have shown that the best candidates for the preparation of hydrogen are the purple bacteria and cyanobacteria. It has been reported that the use of immobilised cells of photosynthesising bacteria made it possible to achieve a productivity in the evolution of hydrogen of 40 ml H_2 per gramme of the dry cells per hour.[210] Fairly high productivities—180 ml H_2 from a litre of the culture medium per hour—have also been achieved by the continuous cultivation of the purple bacteria *Rhodospirillum rubrum* on a medium with lactate.[211]

At the end of the 1970's and the beginning of the 1980's, studies on artificial photosynthesis were also prosecuted vigorously.[1,68,76,213] Best results were attained on artificial systems containing illuminated chloroplasts and enzyme preparations of bacterial hydrogenases. Such systems were capable of evolving hydrogen at rates up to 94 μmol H_2 per milligramme of chlorophyll per hour in the course of several hours.[212]

The genetic modification of the photosynthesising organisms, the study of their biochemical pathways, the increase of the hydrogenase and nitrogenase activities, and the optimisation of the nutrition, illumination conditions, and immobilisation of micro-organisms are all ways leading to an increase in the efficiency of the photosynthesis and its industrial application.[133]

The ability of certain chemotropic organisms to evolve molecular hydrogen was also noted a fairly long time ago. Subsequently, this phenomenon was investigated in detail in our country and abroad. The present state of the problem has been analysed in detail.[79]

Hydrogen-forming micro-organisms are common in nature and hydrogen can be formed from carbohydrate compounds, including starch and cellulose and from aminoacids and ketoacids.

On complete decomposition of glucose solely to CO_2 and H_2, the limiting energy conversion efficiency of 99% can be achieved theoretically:

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 12H_2, \quad \Delta G_0' = -260 \text{ kJ mol}^{-1}.$$

In practice, under the conditions of clostridial fermentation the process proceeds with formation of acetate or acetate and butyrate apart from CO_2 and H_2:

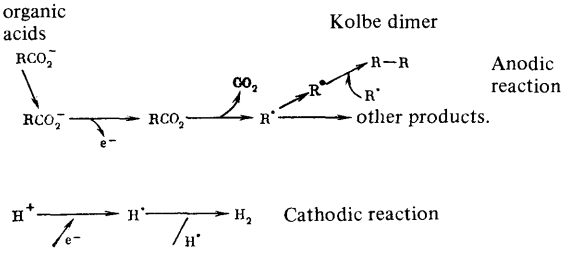
$$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2, \quad \Delta G_0' = -206{,}6 \text{ kJ mol}^{-1};$$
$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2CH_2COO^- + 2HCO_3^- + 3H^+ + 2H_2, \quad \Delta G_0' = -255{,}2 \text{ kJ mol}^{-1}.$$

In the first case, 33% of the energy stored in glucose is converted into hydrogen form and in the second 16.7% is converted. This is determined by the fact that a considerable proportion of the energy remains in the aliphatic acids.[1] In order to increase the efficiency of the energy transformation, the aliphatic acids formed can be converted into fuel by the Kolbe electrolysis.[118] The process has been vigorously investigated by the Dynatech Company (USA).[133]

Organic acids are selectively extracted from the culture liquid by kerosine and are re-extracted with an aqueous solution. Organic acids are converted into liquid and gaseous fuel via the reaction

$$2CH_3CH_2CH_2COOH \rightarrow C_6H_{14} + 2CO_2 + H_2.$$

The fundamental anodic and cathodic reactions in the electrolysis are presented below:



The alkanes formed can serve as an effective fuel for internal combustion engines and the mixture of hydrogen and Co_2 produced is used for the generation of elecricity.

Anaerobic bacteria, which are capable of forming ethanol together with hydrogen, are also of definite interest. A new thermophilic hydrogen-forming culture, isolated in hydrothermal sources in Kamchatka, which decomposes glucose in accordance with the equation

$$C_6H_{12}O_6 + H_2O \rightarrow 2H_2 + 2CO_2 + C_2H_5OH + CH_3COOH, \quad \Delta G_0' = -201{,}44 \text{ kJ mol}^{-1},$$

has been described.[119] In this system the efficiency of the transformation of energy (of the overall energy into the hydrogen and ethanol forms) is 79%.

One of the latest advances in the preparation of hydrogen from water involves the creation of microbiological systems for the biophotolysis of water on the basis of cultrues of micro-organisms coupled via their metabolites.[122,175] Laboratory tests of a system for the photodecomposition of water into hydrogen and oxygen by sunlight with the separate formation of the gases have been carried out and completed. Productivities up to 0.5 litre H_2 per litre of the medium per hour (correspondingly 0.25 litre O_2 per lite of the medium per hour) have been obtained.[175]

In conclusion of this Section, one may note definite advances in laboratory studies of photobiological processes leading to the formation of hydrogen and also the creation of several demonstration plants. The scaling up of these processes still remains a complex biotechnological task, but the colossal advantages of the hydrogen power engineering inspire the hope that studies on these lines will continue and will bring about the expected practical results.

VI. CONCLUSION

The recent years have been characterised by an increasing interest in the development of renewable energy and raw material sources. Biotechnological processes occupy an important place in these projects. National programmes in fuel and biotechnology are being actively developed in the USA, Great Britain, France, Brazil, Japan, Canada, China, and other countries.

During the last ten years, studies on the utilisation of biosystems for the synthesis of fuel-type compounds with a high energy content have been carried out in a number of countries, including the USSR. These investigations have been directed towards the creation of a fundamentally new energy technology using renewable energy sources, in the first place solar energy.

At the present time two categories of systems capable of contributing significantly to the energy supply of the country are considered as likely prospects.

The first generation bioenergy technology presupposes the wide-scale industrial introduction of methanogenesis, which has now reached the level of technical solutions. The process represents anaerobic microbiological conversion of cellulose-containing materials into a mixture of methane and carbon dioxide. The quantitative characteristics of this process have not been investigated, the pathways leading to the transformation of the substances in a complex sequence of reactions have been studied, the key intermediates have been discovered, and a mathematical model and methods for the regulation of methanogenesis have been created for the first time.

The large-scale application of the biogas technology makes it possible to solve three inter-related problems—the preparation of the fuel, the concentration of organomineral fertilisers, and the problem of the protection of the environment against biological contamination (agricultural waste). By utilising the waste in the USSR alone, it should be possible to obtain up to 150 million tonnes of conventional fuel, which fully covers the future energy needs of agriculture.

At the present time agriculture consumes 60–62 million tonnes of petroleum products. By generating biogas, the agricultural industry can attain self-sufficiency in terms of energy.

The preparation of fuel ethanol and the acetone–butanol mixture from cellulose may make a significant contribution.

The second generation bioenergy technology is associated with the creation of a large-scale process for the biophotolysis of water—the reaction leading to the decomposition of water into hydrogen and oxygen by sunlight. At the present time, a reaction of this kind is achievable on the basis of microbiological cultures coupled via their intermediate compounds.

The complexities in the creation of systems for the transformation of solar energy involve the necessity to occupy large territories by technical constructions. Because of this, the constructions must be as simple as possible, should be convenient to use, and should not require expensive materials.

It has been suggested that biopower engineering plants will develop in the period 1990–2000, which will create a new power enginnering technology using high-energy and ecologically pure fuel. After sufficient development, processes of this category will meet a significant proportion of the energy needs of the community.

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