PRODUCTION OF ACIDS BY ANAEROBIC FERMENTATION

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Summary

Some 400 isolates of cellulolytic and 150 isolates of amylolytic anaerobic bacteria were made from mesophilic animal-waste digesters. They were classified and tested for their ability to produce acids and alcohols of commercial interest, and manipulations of fermentation systems to maximise production of butyric acid from starch by one isolate were investigated.

1. INTRODUCTION

Hobson (1) described, on the basis of laboratory tests, the possible use of anaerobic bacteria in industrial fermentations. The work was not followed up at that time as SCP was the only microbial product commercially required and its production was then uneconomic. The background to the present work and some industrial uses of the fermentation products have been described in previous papers (2,3,4). Briefly, it was to survey the fibre-digesting bacteria from anaerobic digesters for their potential to produce chemicals from cellulosic materials and to see how far the production of one acid (butyric) in a mixed fermentation could be enhanced by manipulation of the fermentation system.

2. BACTERIA AND INITIAL TESTS

Isolations were made in media based on those used for rumen bacteria, but containing digester fluid as a source of growth factors, under strictly anaerobic conditions. The cellulolytic isolates were from a mesophilic cattle waste digester sampled over a period of 15 days. The amylolytic bacteria were from a number of mesophilic digesters sampled over 3 years. Isolations of thermophilic amyolytic bacteria were made from a piggery-waste digester running at 50, 53 or 59°C, but these bacteria proved unstable and died off after some weeks of subculture.

The cellulolytic bacteria were tested for ability to degrade filter paper and 128 isolates of high activity and two of low activity were tested in detail. These were grouped as *Clostridium*, species *butyricum*, *beijerinckii*, *acetobutylicum*, *bifermentans*, *sporogenes* and strains not of known species; *Sporolactobacillus* sp.; two strains of *Sarcina*; a sporing *Streptococcus*; other isolates of uncertain classification. Cellulolytic activity (tested on cotton) varied both in and between species. Most isolates had hemicellulolytic activity, but ability to degrade untreated barley straw varied from effectively zero to a maximum in batch culture of 45-50%. Some isolates had an almost homolactic fermentation; the majority produced mixtures of two or more of the lower VFA, succinic and lactic acids, ethanol and butanol with CO_2 and H_2. Further details of these isolates are given in references 4,5,6 and papers in press.

The amylolytic bacteria were grouped by morphology and fermentation products. Acetic and lactic acids were the main fermentation products of many of the strains; some produced propionic and succinic acids. Only a minority produced butyric acid (with other acids). Of these, four which produced the highest proportion of butyric acid in mixture with acetic and lactic acids were selected. These were tested in batch and continuous culture and all had similar properties, so the main experiments were carried out with one isolate, A2012. One criterion for selection was ability to grow on ammonia as sole N source to reduce the cost of a commercial medium. However, growth was slower in the ammonia medium than in a medium with ammonia, casitone and yeast extract (A2012, μ_m [maltose]: NH_3-N, 0.4 h^{-1}; complex-N, 1.63 to 1.35 h^{-1} tested at different times over the project period). The bacterium fermented a number of sugars and was classified as a species of Clostridium (3).

3. PRODUCTION OF BUTYRIC ACID

Ammonia was the principal nitrogen source for the bacteria and the 'simple, S' medium base contained only NH_4-N. The 'complex, C' medium contained Casitone and yeast extract, mainly for growth factors, and NH_4 as the main nitrogen source. The starch used was commercial 'soluble'. Maltose was used to obviate problems caused by the viscosity of starch media. Cultures were incubated under oxygen-free CO_2, with the medium reduced by cysteine, and at 39°C, unless otherwise stated. Determinations of residual substrate as maltose were inexact, as starch dextrins, bacterial polysaccharide and lytic products could be present. So substrate utilisation was calculated as the maltose or starch equivalent of the fermentation products.

3.1 Batch cultures

There was no growth in the S basal medium, and very slight growth (1-2mM acids formed in 2 days) on the C basal medium. There was no growth when acetic or butyric acids were added to these basal medium. A2012 was shown to require anaerobic conditions with a chemical reducing agent, but not necessarily CO_2 in the gas phase. The other three isolates were similar but varied in ability to grow without CO_2 or a reducing agent. When A2012 was grown in medium S with maltose, buffered to different pH values, growth was a maximum at pH 6.7 with no growth at pH 5.4 or 7.7. Batch cultures in medium C with pH electronically controlled showed that acids were produced from maltose between pH 4.9 and 7.7, with reasonable acid production beginning about pH 5.2. Over pH values from 5.9 to 7.6 more butyric than acetic was formed, the highest proportion of butyric being formed from about pH 5.9 to 6.4. Lactic acid was always very much less than the other acids, but tended to increase in proportion above about pH 6.4. In the following experiments pH-controlled cultures were run at pH's between 6 and 6.5.

Batch cultures with different proportions of ammonia in medium S showed that N and C appeared to be balanced at about 22mM NH_4-N to 28mM maltose. In media with excess maltose growth and acid production were proportional to N present, with the reverse in excess N media. When N was limiting there was some evidence for an uncoupled fermentation of maltose after growth and ceased and as N concentration was decreased growth became slower and the proportion of butyric acid in the fermentation products higher. The bacteria produced an α-amylase, induced by maltose or starch; typical activities in glucose, maltose and starch media C were 16, 260 and 243 units. In S medium with starch, activity was only 146 units. This might explain the generally lower fermentations of starch found in batch cultures with the S medium base than with the C medium. The C medium base was generally used with starch or

maltose. Since maltose was a hydrolysis product of starch, maltose fermentations gave essentially the same results as starch fermentations without the problems of viscosity and starch retrogradation association with starch media. The ammonia concentration in the media was varied in proportion to the carbohydrate concentration to approximately balance out as described above. In pH-controlled batch cultures, up to 10% maltose or starch was fermented. With 10% maltose at pH 6.5-6.6 in C medium, final acid concentrations were (mM): acetic 109, butyric 215, lactic 35 (with H_2 + CO_2). With 10% starch, concentrations were: acetic 166, butyric 249, lactic 30. Allowing for alkali added in pH control these represent C recoveries in fermentation products of 90% and 72% of the initial maltose and starch. In S base media final results were similar, but overall growth was slower. Various amounts of bacterial polysaccharide were formed in batch and other cultures and this could account for some substrates not recovered in fermentation products. These and other tests showed that high substrate concentrations could be used and that there was no substrate or product inhibition. Butyric:acetic ratios were usually about 1.5 or 2:1, but slow growth, in medium S for instance, increased this ratio. Continuous cultures run during these experiments had also shown an increased proportion of butyric acid at low growth rates. So it seemed that low growth rates would give maximum butyric acid and least acetic and lactic acids, but that a continuous system with higher flow rate would be desirable for economic production.

3.2 Continuous cultures

A number of stirred-tank continuous cultures were run with starch or maltose as substrates and pH controlled at 6.5. Results were similar and showed that butyric:acetic ratio decreased and lactic increased with increase in dilution rate beyond about $0.16h^{-1}$, and recovery of substrates in fermentation products tended to be lower with starch than with maltose because of starch retrogradation and low amylase activity at higher D values; amylase activity declined rapidly with increase in D. Some results were, (2% maltose, C base): D = $0.01h^{-1}$; amylase 2427 units; butyric:acetic 3.21; lactic 3.3mM; recovery 96%. D = $0.07h^{-1}$; amylase 353 units; butyric:acetic 2.50; lactic 16.5mM; recovery 68%. D = $0.20h^{-1}$; amylase 40 units; butyric:acetic 2.36; recovery 60%. Starch, 2%: D = $0.058h^{-1}$; butyric:acetic 1.20; lactic 9mM; recovery 80%. D = $0.129h^{-1}$; butyric:acetic 1.35; lactic 5mM; recovery 53%.

Continuous retained-biomass cultures of various designs were run each for some hundreds of hours, but were generally stopped because of clogging of the matrixes with bacterial extra-cellular polysaccharide. Some steady-state values show the general trend of results with these cultures. Fermenter (a) was a downflow anaerobic filter of length to diameter 18:1, packed with graded unglazed pottery chips and with a water jacket at 39°C: CO_2 was passed slowly over and through the filter and medium vessels to keep the system anaerobic. Dilution rates are liquid flow/void volume of filter. The bacteria were allowed to grow in the filter filled with medium and the medium was then drained and fresh medium trickled down the filter. Some results:- 2% starch, S base; D = $0.06h^{-1}$ butyric:acetic 1.87; lactic 1.4mM; recovery 30%. D = $0.28h^{-1}$; butyric:acetic 1.92; lactic 2.9mM; recovery 21%. D = $0.8h^{-1}$; butyric:acetic 2.52; lactic 2.1mM; recovery 11%. Fermenters (b) and (c) were squat tanks packed with pottery chips and with medium flowing up or down, but with the matrix always covered with medium and with a slow flow of CO_2 to give gentle mixing. Results, 2% starch, S base:- (b) D = $0.10h^{-1}$; butyric:acetic 3.59; lactic 1.2mM; recovery 17%. (c) D = $0.32h^{-1}$; butyric:acetic 2.18; lactic 2.3mM; recovery 17%. Butyric:acetic ratios were good and lactic acid was low, but recovery was low, probably because of fall in pH

between the inflowing and outflowing medium. pH is almost impossible to control in a filter system. Fermenters (d) and (e) were stirred-tanks with pH control but with biomass retained by nylon-mesh supports of two different designs, the second to try to prevent the build-up of bacteria and polysaccharide which occurred in the first. Fermenter (d). Results: 10% maltose, C base, pH 6.6: D_1 = 0.05h^{-1}; butyric:acetic 1.57; lactic 17.3mM; recovery 67%. D_1 = 0.10^{-1}; butyric:acetic 1.86; lactic 14.3mM; recovery 46%. D = 0.40h^{-1}; butyric:acetic 1.38; lactic 16.7mM; recovery 21%. These results showed, again, that low dilution rates were needed to get higher recoveries and butyric acid concentrations (275mM at D = 0.05h^{-1} above) and low lactate. The maltose not recovered as fermentation products at the lowest growth rate was converted to bacterial cells and polysaccharide. Fermenter (e). Results: 2% maltose, C base, pH 6.3: D_1 = 0.035h^{-1}; butyric:acetic 2.45; lactic 12.3mM; recovery 85%. D_1 = 0.207h^{-1}; butyric:acedtic 2.32; lactic 13.7mM; recovery 68%. D = 0.247h^{-1}; butyric:acetic 1.64; lactic 25.8; recovery 67%. The recovery at low D values was better here because of the lower maltose concentration, but butyric acid was formed in much lower concentration (73mM) than with 10% maltose. The modified nylon mesh support still eventually blocked up with bacterial polysaccharide.

Two-stage continuous culture using pH-controlled stirred-tanks were run. In the first stage 2% maltose or starch, C base medium was used at low D values, with sufficient ammonia to give utilisation of the carbohydrade and production of a good concentration of amylase and active bacteria. It was thus hoped that maltose or starch alone (4,6 or 8%) pumped into the second stage would be there converted to acids with a high proportion of butyric by non- or slowly-growing cells (cf. results of N-limitation above). The pH was controlled in both stages at 6.5. The results are too extensive to give here. In summary, the first stage behaved as a single-stage culture and at low D values converted about 70 to 80% of the maltose or starch to acids with the usual ratios of butyric to acetic. In the second stage, conversion of the added maltose or starch, and the proportion of butyric acid, increased as the flow rate of carbohydrate into the vessel decreased. The proportion of butyric acid could be increased over that in the first stage, but only at the expense of a low flow rate into stage 2. From a production point of view it would seem that the 2-stage system was little, if any, better than a 1-stage fermenter running at a low D and high concentration of starch in the feed. The 2-stage fermenter also had more pipes, etc., to become fouled with bacterial polysaccharide.

Fed-batch cultures were run in stirred tanks with and without pH control. The bacteria were grown in starch or maltose, C or S base, media as a batch culture to get good starch conversion. A small portion was then removed for testing and fresh medium added to the remaining culture, and this was repeated every 1 or more days. When the volume of culture had (say) doubled, medium was removed to bring the culture to its initial volume and the process continued, the culture attaining a steady-state. As in other cultures, high starch conversion and butyrate proportion depended on low turnover rates. As an example, with 8% starch in C medium, pH 6.3, and starting volume of culture 200ml, 50ml medium added. Added every 1 day; starch conversion 60%; butyric:acetic 1.6. Every 2 days; conversion 61%; butyric:acetic 1.55. Every 3 days; conversion 65%; butyric:acetic 2.05. Every 5 days; conversion 80%; butyric:acetic 2.3.

4. GENERAL CONCLUSIONS

The survey of cellulolytic bacteria showed that the digester population was complex and that it could provide bacteria capable of fermenting plant fibres to any of the acids and alcohols of commercial importance. Selection would have to be made to find isolates of particular reactions and of high-fibre digesting capacity: the straw used here is a residue and most resistant to degradation, other plant material could be more easily and more extensively degraded.

Digesters also contain many varieties of amylolytic bacteria and starch as usually prepared can, unlike fibres, be completely degraded. In the present experiments eight or 10% starch solutions could be converted to give high concentrations of acids in a fermenter effluent. Most of the amylolytic (and cellulolytic) bacteria gave mixed fermentation products and if a mixture of acids were required, or certain acids were to predominate, then growth, and so high medium turnover, rates could probably be higher than those used here. In the present case, the desired acid (butyric) was formed in high proportion, along with high amylase and so starch hydrolysis and conversion capacity, at low bacterial growth rates. Although lactic acid could be almost eliminated in the mixture, the limit to butyrate predominance over acetate would be determined by a commercially-viable production rate. For a continuous process a low turnover rate is not necessarily a disadvantage, and a single-stage stirred tank or a fed-batch might be best out of the fermenter types tested. The mechanical problems of handling starch media found in the laboratory could be eliminated on a large scale, and a relatively simple medium based on those used here could be devised

REFERENCES

1. HOBSON, P.N. (1969). Rumen microorganisms. (Their possible use in vitro). Process Biochem. 4. 11, 53-56
2. HOBSON, P.N. (1982). Production of alcohol and organic acids with anaerobic bacteria. In Fuels and Organic Chemicals from Biomass. ed. D.L. Wise, CRC Press, Boca Raton, Florida.
3. HOBSON, P.M. and REID, W.G. (1983). The production of acids from starch by anaerobic bacteria. Fourth specialized meeting (EEC), Anaerobic digestion, Broni, 17-19 May.
4. HOBSON, P.N., REID, W.G. and SHARMA, V.K. (1984). Anaerobic conversion of agricultural wastes to chemicals or gases. In Anaerobic Digestion and Carbohydrate Hydrolysis of Waste, ed. G.L. Ferrero et al., Elsevier Applied Science Publishers, London
5. SHARMA, V.K. (1983). Isolation and characterisation of cellulolytic bacteria from a cattle-waste digester. Ph.D. Thesis, Aberdeen.
6. SHARMA, V.K. and HOBSON, P.N. (1985). Isolation and cellulolytic activities of bacteria from a cattle waste anaerobic digester and the properties of some Clostridium species. Agric. Wastes 14, 173-196