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Fermentation of arabinose to ethanol by *Sarcina ventriculi*

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Summary. In the absence of oxygen, a strain of *Sarcina ventriculi,* isolated from soil, could rapidly and completely ferment up to 20 g/l of arabinose. The principal products were ethanol, acetate, CO_2 and H_2. The yield of alcohol, up to 30% by weight of the sugar fermented, was not appreciably influenced by the pH of fermentation in the range 4–7. Sugar concentrations up to 100 g/l did not affect initial growth, but fermentation was incomplete at high sugar levels. This was probably due to the accumulation of end products other than ethanol, because the cells could grow in the presence of up to 25 g/l of added ethanol. Glucose, galactose and arabinose were sequentially utilized, in that order, when initially present as a mixed substrate. These sugars are major components of the hemicellulose from some agricultural residues. Practical implications for the general problem of pentose conversion to alcohol are discussed briefly.

Introduction

The microbial conversion of agricultural or forestry residues into ethanol continues to be of worldwide interest. While most attention has been lavished on cellulose because of its abundance, there is increasing recognition that hemicelluloses may afford a more commercially attractive source of fermentable carbohydrate in the near future. A major advantage in using hemicelluloses is the relative ease with which they can be hydrolyzed to yield a mixture of simple sugars (Buchholz et al. 1981; Lee et al. 1978). In contrast to cellulose, which consists solely of glucose units, hemicelluloses vary in composition according to their source. They are characteristically rich in pentoses, however, and therein lies the problem of easy and complete conversion to ethanol. Neither the *Zymomonas* bacteria nor any of the commonly used fermentative yeasts can assimilate a mixture of aldopentoses to form ethanol.

There have been two approaches to solving the problem of aldopentose conversion, one using special semi-aerobic yeasts (Gong 1983; Gong et al. 1983), the other using bacteria such as the *Clostridia* (Zeikus 1980; Murray and Khan 1983). Simply stated the first approach, with yeasts, continues to be plagued by the slow and incomplete uptake of a variety of sugars, while bacterial processes suffer instead from the low yields of alcohol, i.e., coproduction of unwanted metabolites like acetate. A general review of pentose fermentation has been published by Rosenberg (1980).

Most investigators have studied the conversion of xylose to ethanol because xylose is a main constituent of hemicelluloses from hardwood, corn stover and wheat straw. Less common, but still abundant, are hemicelluloses rich in L-arabinose. These come from such sources as sugar beet residues (Schneider 1968), rice bran (Mod et al. 1979) and corn hulls (Wolf et al. 1953). We have chosen to study the fermentation of arabinose by bacteria.

Only a few bacterial species contain pyruvate decarboxylase, EC 4.1.1.1 (Scrutton 1971), a key enzyme which is mainly responsible for the high yields of ethanol by both yeast and *Zymomonas*. However, *Sarcina ventriculi* has long been known to perform a yeast-like fermentation of sugars (Smit 1930; Kluyver 1931). Indeed it does possess an active pyruvate decarboxylase, although more recent isolates have also shown considerable formation of acetate, H_2 and CO_2 by way of ferredoxin-linked thioclastic enzymes typical of the *Clostridia* (Stephenson and Dawes 1971). While *S. ventriculi* is unable to ferment xylose, some strains have been reported to

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ferment arabinose (Claus and Wilmanns 1974). The above facts prompted us to study this organism further, especially since no quantitative information on its growth has yet been published (Kuhn 1977).

S. ventriculi is a gram-positive, obligate anaerobe which forms large cell packets, 10–50 μm in size, held in a matrix of cellulose. Because of its almost unique ability to grow at pH levels as low as 1.5 it is easily isolated from soil. Sugars are required for growth, but there is also an absolute requirement for some 11 amino acids as well as biotin and nicotinic acid. The cells are notoriously short-lived when not actively growing. The difficulties of laboratory maintenance and the inability of vegetative cells to withstand freeze-drying probably account for the lack of study in many laboratories. Cultures can be maintained in liquid nitrogen, however. Other aspects of the physiology of both *S. ventriculi* and a related organism, *Sarcina maxima*, have been reviewed by Smit (1930) and Canale-Parola (1970).

Materials and methods

Isolation and cultivation. The strain of *S. ventriculi* was isolated from soil near the laboratory in Jülich using maltose enrichments at pH 2.2 followed by repeated plating (Gas-Pak System, Baltimore Biol. Labs.) to obtain a pure culture (Canale-Parola 1970). Plating efficiencies, it should be noted, were very poor even for fresh young cultures. When it was found that the isolate also fermented L-arabinose, cultures were carried routinely by the daily transfer of 1–2 ml of actively gassing inoculum with a syringe into 10 ml of liquid YEPA broth containing 0.5% yeast extract (Difco), 0.5% Bacto peptone (Difco) and 0.5% L-arabinose (Sigma) adjusted to pH 4.1 with HCl or H_2SO_4. All incubations and cultivations have been at 37° C unless otherwise stated. For carrying the culture over weekends, up to 4 days at room temperature, a medium of 2% glucose, 0.2% yeast extract at pH 6.5–7.0 was used. Tubes were made anaerobic before sterilization by heating them in a boiling water bath to remove dissolved air, and then closing them with rubber stoppers under a stream of oxygen-free nitrogen.

Identification of the isolate as *S. ventriculi* was based not only on its morphology and pH tolerance but also on a positive test for cellulose using the specific fluorescent dye Calcofluor (American Cyanamid Company, Bound Brook, NJ) and the absence of a butyric odor.

The studies on sugar utilization and alcohol tolerance were also done in stoppered anaerobic broth tubes to which ethanol and/or the appropriate carbon source, separately sterilized, had been added by syringe. These tubes contained small Teflon-coated magnetic stirring bars to permit continuous agitation during growth. The tubes were immersed in a water bath at 37° C and growth was followed by measuring the optical density at hourly intervals directly in the tubes.

Batch cultivation was done either in a 700 ml water-jacketed glass bottle with 400 ml of magnetically stirred medium or in a 7 l general purpose fermentor (Bioengineering A.G., Wald, Switzerland). In both there was provision for gassing with N_2 and for control of the pH to within ± 0.05 units by the automatic addition of 2 N NaOH. The YEPA medium, with various amounts of L-arabinose, was used.

To measure the total production of H_2 and CO_2, two successive runs were made. In one of these the total amount of gas evolved was measured directly in 500 ml gas burettes over acidfied water. In the other run, made under identical conditions, the CO_2 was first absorbed in a gas scrubbing bottle with 20% KOH. The volume of H_2 gas was thus measured directly, and CO_2 was accounted for by the difference in volumes between the two runs.

Analytical methods. After centrifuging to remove the cells, broth samples were analyzed for ethanol and acetate by gas chromatography (Hewlett-Packard Model 5840A, with automatic sampler and flame ionization detector). The $^1/_4$-inch glass column was packed with 3 feet of 80–100 mesh Poropak QS. Flow rate of the carrier gas, N_2, was 30 ml/min and temperatures of the injection port, column, and detector were 250°, 150°, and 230° C respectively. The presence of other acids at the end of fermentation was estimated by isotachophoresis (Mikkers and Everaertz 1981). Arabinose was determined with dinitrosalicylic acid reagent (Miller 1959). In the experiment with multiple substrates, individual sugars were assayed by low-pressure liquid chromatography (Biotronik, Model ZA 5100, Munich) with borate buffer elution and photometric detection as furfural/orcinol complexes. The resin used was Durrum DA-X4-70 (Capek and Stanek 1975).

Optical densities (OD) were measured at 546 or 600 nm with either an Eppendorf or Bausch and Lomb Spectronic 88 photometer. Dry weights were measured by filtering 50 ml samples of the culture through pre-washed and tared membrane filters (0.45 μm, Sartorius) which were then weighed after drying overnight at 105° C.

Results

The ability of our isolate to ferment various carbon sources at pH 4.1 is indicated primarily by the doubling times shown in Table 1. Growth was equally rapid on sucrose, maltose and all of the monosaccharides tested except mannose. Others have reported good growth on mannose (Canale-Parola 1970; Kuhn 1977). Lactose and cellobiose were

Table 1. Growth on various carbon sources at 37° C

Carbon source	ΔOD	End value of pH	t_D, h
Control	0.26	4.13	–
Glucose	3.20	3.85	1.5
Sucrose	3.31	3.83	1.8
Maltose	3.28	3.83	1.7
Fructose	3.04	3.82	1.6
Galactose	1.81	3.83	1.3
Arabinose	1.59	3.90	1.8
Lactose	0.97	4.10	3.8
Cellobiose	0.63	4.09	5.8
Mannose	0.45	4.07	> 10
Citrate	0.41	4.87	2.4
Galacturonate	0.36	4.15	5.3

Doubling times t_D, based on changes in OD at 600 nm

utilized more slowly than either glucose or galactose. Ability to grow on organic acids was tested by Kuhn (1977), who observed growth on malate but no growth on citrate. Her tests were made at an initial pH of 6.0, however, at which pH our isolate also failed to grow on citrate. Further evidence for citrate utilization at pH 4 is the rise in pH accompanying the growth, as noted in Table 1. Galacturonate was tested here because of its practical importance as a hydrolysis product of pectin. No significant growth on xylose was detected.

The changes in optical density shown in Table 1 do not accurately reflect the extent of growth in all cases. Previous workers have suggested that OD measurements with *Sarcina* are inaccurate because the cells settle out so rapidly. In our experience, that problem can be overcome by careful and rapid manipulation. A more serious bias, amounting to as much as a factor of two, is attributable to changes in the size of cell clusters. If large aggregates are present, then the transmission of light is increased as compared to a more finely dispersed suspension containing the same amount of biomass. Shown in Fig. 1 is a comparison between OD measurements and changes in dry weight during a typical batch fermentation. The two measures of biomass are parallel only during the initial period of exponential growth. Despite such drawbacks, the OD provided a convenient measure of growth, especially for preliminary screening experiments.

The maximum growth rates on arabinose at various controlled levels of pH are shown in Fig. 2. At pH 7.0 the doubling time was less than 1 h, and even at pH 3.0 growth was rapid. Ultimately of course μ must fall to zero at low pH and must reach some limiting value at high pH. The pH of cultivation did not seem to influence the yield of alcohol in any consistent manner (Table 2). As indicated by the values in parentheses, utilization of sugar was incomplete at the lower pH values. At pH 4.0 the amount of sugar consumed in 24 h was independent of the initial sugar concentration, a fact which suggests that the accumulation of acidic end products was limiting the extent of fermentation.

The time course of a typical batch fermentation, during the first 10 h is shown in Fig. 3 for a pH of 5.0 and an initial arabinose concentration of 10 g/l. Three phases can be distinguished. During the first 5 h there is an increasing rate of sugar utilization and parallel increases in the concentrations of both acetate and alcohol. At the end of this period there is an abrupt change in the OD curve, which signals slower growth and/or formation of larger cell aggregates. Some minor nutritional deficiency or accumulation of an end product may be responsible; more than 6 g/l of fermentable sugar are still present. At this time two other changes can also be noted. The rate of sugar usage starts to decline and the alcohol/acetate ratio increases somewhat. After 8–10 h all of the rates are much slower and most of the sugar has been utilized.

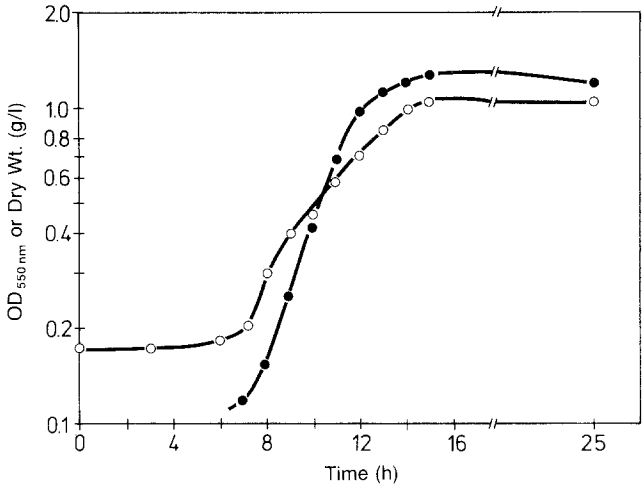

Fig. 1. Comparison of OD and dry weight as measures of growth. The initial concentration of arabinose was 10 g/l. ○, optical density (OD); ●, dry weigth

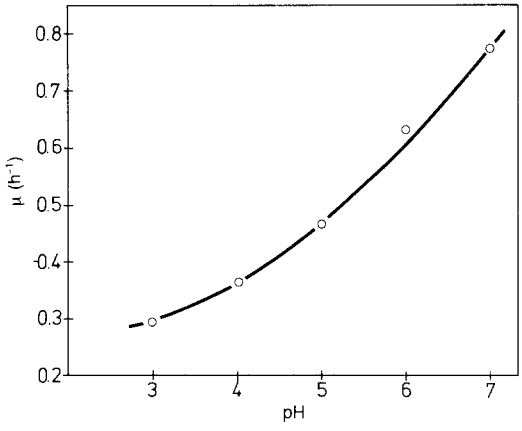

Fig. 2. Maximum growth rates on arabinose at 37° C: experiments with controlled pH based on changes in OD. The values at pH 4 and pH 5 are average values from several runs

Table 2. Yield of alcohol at various pH levels

pH, controlled	Arabinose concentration (g/l)		Alcohol yield, wt. %
	Initial	Consumed[a]	
3.0	11.3	4.3 (38)	18.8
4.0	19.4	6.7 (35)	29.1
4.0	10.3	6.6 (64)	24.5
5.0	20.4	18.4 (90)	28.3
6.0	10.1	9.4 (93)	21.6
7.0	19.4	15.3 (79)	30.5

[a] Consumed after 22–24 h. The values in parenthesis show per cent of initial sugar that was consumed

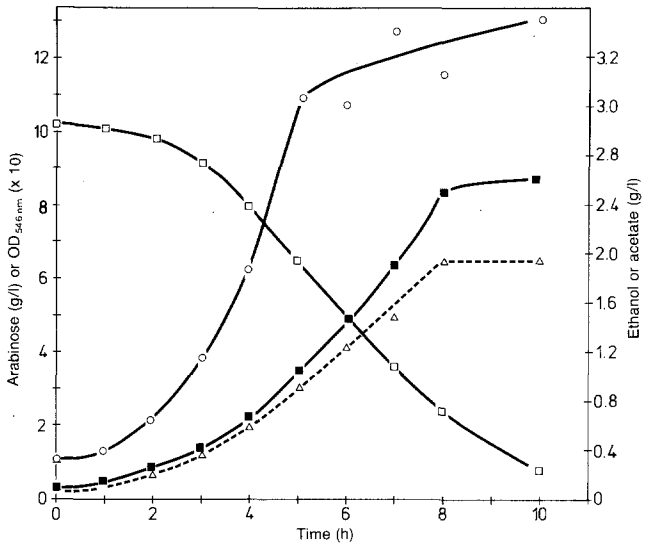

Fig. 3. Batch fermentation of arabinose at a controlled pH of 5.0, 37° C. □, arabinose; ○, OD; ■, ethanol; △, acetate

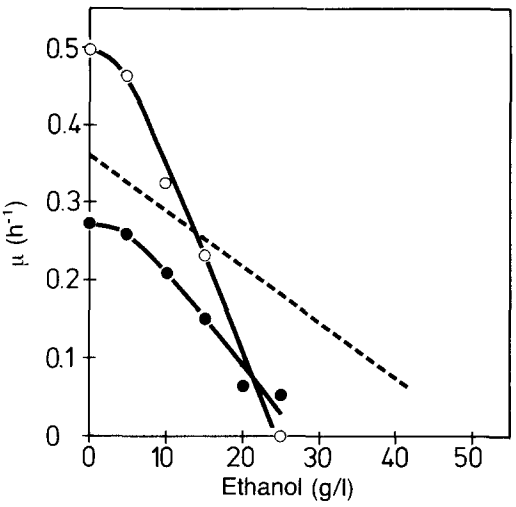

Fig. 4. Effect of added ethanol on growth rate as measured by changes in OD at pH 4.0. The graphs show results from repeated tests. The broken line represents data from a freshly isolated culture (see also text)

Table 3. End products formed from arabinose at pH 5.0 based on 100 mmol fermented

Ethanol	89.1 mmol
Acetate	58.9 mmol
CO_2	148.6 mmol
H_2	117.8 mmol

Carbon accounted for: 86.9%
O/R balance: 1.0

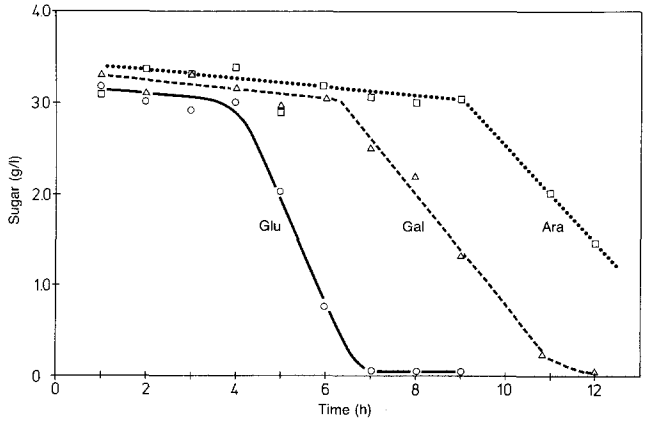

Fig. 5. Sequential utilization of mixed sugars at pH 5.0. The inoculum was grown on glucose. ○, glucose; △, galactose; □, arabinose

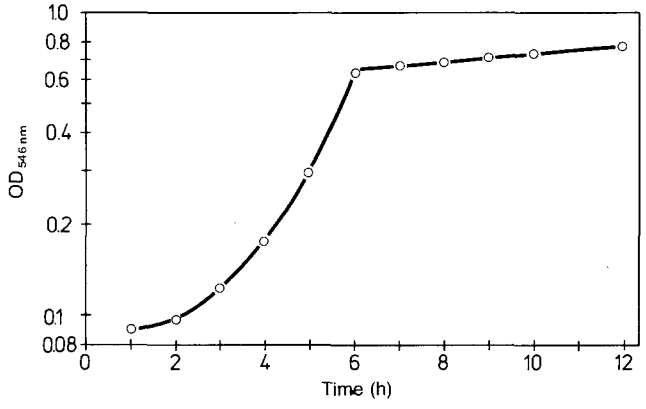

Fig. 6. Fermentation of a sugar mixture: changes in growth pattern upon exhaustion of the glucose. Data are from the same run as shown in Fig. 5

At the end of the experiment (22 h), the alcohol level was substantially unchanged, 2.65 g/l, but acetate had increased slightly, to 2.28 g/l. The final sugar concentration was 0.5 g/l. The yield of alcohol at 10 h was 33%, w/v, based on sugar utilized, a value 64% of that obtainable theoretically from a yeast or *Zymomonas* fermentation.

In separate experiments at pH 5.0 it was found that ethanol and acetate, along with CO_2 and H_2, accounted for most of the arabinose fermented (Table 3). A similar distribution of end products, with minor amounts of lactate, succinate, and formate has been reported for glucose (Canale-Parola 1970; Kuhn 1977).

Repeated tests of the influence of added ethanol on growth have failed to give consistent results, but the general pattern is evident from Fig. 4. Growth can continue up to a level of 25 g/l of added ethanol. Microscopically, and from the plots of optical density, one can detect an earlier formation of large cell aggregates as the ethanol concentration is increased. The data shown by the open circles represent an

average of duplicates run at the same time with the same inoculum, for which there was very close agreement in individual values of μ.

The growth of *S. ventriculi* is rather insensitive to the concentration of sugar. Neither lags nor changes in growth rates were observed in tests where the initial concentration of arabinose was increased up to 100 g/l.

Since a typical hydrolysate of hemicellulose would contain other sugars besides just arabinose, it was of interest to know how *S. ventriculi* would grow on a sugar mixture. Figure 5 shows a sequential utilization of glucose, galactose and finally arabinose. The total starting sugar concentration was 10 g/l. No time lags were noted and the less readily used sugars began to disappear when about 1 g/l of more readily used sugar was still present. The disappearance of glucose after about 6 h, as shown in Fig. 5, was accompanied by an abrupt break in the exponential increase in OD at this time (Fig. 6). The effect is similar to that observed in the batchwise growth on arabinose alone.

Discussion

Our data confirm the observations of Claus and Wilmanns (1974) that some strains of *S. ventriculi* can ferment both arabinose and cellobiose, sugars that were previously considered unsuitable for growth. Crowther (1971) has even reported weak growth of *S. ventriculi* on xylose. The closely related species, *S. maxima,* is known to ferment xylose, but it produces mainly butyric acid rather than ethanol (Canale-Parola 1970). It will be interesting to learn if some of these catabolic differences among strains, such as noted here also for mannose, are plasmid determined. Knowledge of the uptake mechanisms is completely lacking. The sequential utilization of multiple substrates, as shown in Fig. 5, is not uncommon however (Hsiao et al. 1982; Harder and Dikhuizen 1982). It is likely due to catabolite inhibition rather than repression (McGinnis and Paigen 1973; Russell and Baldwin 1978). The complicating effects from formation of large cell clusters when growing on certain sugars could probably be eliminated by the use of cellulolytic enzymes. As reported also by Kuhn (1977), cellulase does not seem to interfere with the growth of these bacteria.

Stephenson and Dawes (1971) have investigated the branched pathways for sugar catabolism by way of pyruvate. In cell extracts of *S. ventriculi* they found a pH optimum of 5.6 for pyruvate decarboxylase but an optimum of 6.8 for enzymes on the thioclastic route, which leads to H_2 formation and equimolar amounts of ethanol and acetate. At a pH of about 5 they expected little contribution of the latter pathway and therefore almost theoretical yields of CO_2 and ethanol. Their studies of batchwise fermentation of glucose at 30° C and controlled pH levels showed that while the thioclastic route was predominant at a pH near neutrality, about half of the glucose was still metabolized by this pathway in the range of pH 4–5. They surmised therefore, that the intracellular pH remains relatively high even when the cells are grown in acidic medium. From Table 3 it appears that our isolate growing on arabinose retained about 70% of the thioclastic pathway at pH 5 (59 mmoles of acetate were formed from 100 mmoles of pentose, whereas a yield of 83 mmoles of acetate would represent full operation of a thioclastic route). Stephenson and Dawes (1971) also found that at pH 4.7 the ratio of ethanol to acetate increased as growth proceeded, an effect similar to that noted in Fig. 3 for arabinose. Their explanation that the accumulation of acetate tends to suppress its further formation could also account for the somewhat higher yields of alcohol observed when the initial sugar concentration was 20 g/l instead of 10 g/l (Table 2). Overall, though, the effects of pH on yield of alcohol were less pronounced than those found by Stephenson and Dawes (1971).

The fact that the growth pattern changes after only 3–4 g/l of either glucose or arabinose has been fermented (Figs. 3, 6) is being further investigated using a simpler, better-defined growth medium. It seems likely that some nutritional deficiency is responsible, since end-product concentrations are still low at such an early stage in the fermentation. At later stages, product inhibition does seem to occur as suggested by the data at pH 4.0 in Table 2. Tilak (1970) has proposed that the accumulation of formic acid rather than acetic acid is responsible for the inhibition, but that possibility has not been tested here.

Because of the low concentration of sugar and relatively low yields of alcohol, it seems premature at this stage of the development work to report productivities or similar process parameters. One such parameter, the yield of cells, is noteworthy however. The yield was 12–13 g/l dry weight from 100 g/l of arabinose (Fig. 1), which is the quantity to be expected for an Embden-Meyerhof glycolysis (Stephenson and Dawes 1971).

No attempt has yet been made to select for alcohol tolerant strains of *S. ventriculi.* Judging from the success of others with *Clostridia* (Zeikus 1980), it should be possible to attain levels of 3–5% ethanol. Some of the variability in tolerance may be due to traces of oxygen, since *S. ventriculi* has neither catalase nor superoxide dismutase to provide protection for the cells (Bringer, unpublished). Little if any improvement in alcohol tolerance was noted in tests

at lower temperatures than 37° C or at pH values above 4 (data not shown).

On balance, further study of *S. ventriculi* as a potential producer of ethanol seems warranted. Although the requirement for peptone as a nitrogen source might be disadvantageous in some cases, many agricultural residues already contain sufficient amino acids, as protein, to support growth (Wolf et al. 1953). In any event, there are compensating advantages in using these fast-growing bacteria. In non-viscous media the cell clusters settle rapidly, so that cell recycle could be easily accomplished. Also, the low pH of the fermentation would favor co-fermentation with yeasts. Contamination problems might be less severe than with the *Clostridia,* not only on account of the pH but also because the cellulose coating of the cells would provide some protection against bacteriophage.

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