The Effect of Alkaline pH on Growth and Metabolic Products of a Motile, Yellow-pigmented *Streptococcus* sp.

By ANN F. GRAHAM AND BARBARA M. LUND*

ARC Food Research Institute, Colney Lane, Norwich NR4 7UA, U.K.

(Received 10 February 1983)

Streptococcus BL 78/7 was isolated from alkaline potato-processing effluent, and resembled *S. casseliflavus* in morphology, motility and pigment. In aerobic conditions, at controlled pH, growth occurred between pH 5 and 11 with a maximum growth rate between pH 8 and 9 and a maximum yield at pH 8. The major metabolic products formed from glucose were lactate and acetate. The molar proportion of lactate to acetate decreased with increasing pH, from 4:1 at pH 5 to approximately 1:1 above pH 9. Growth occurred under nitrogen over the pH range from below 6 to 11, with a maximum growth rate and yield at pH 7 to 8. Lactate was the major metabolic product, with formate, acetate and ethanol also present in the molar ratio of 2:1:1. The molar proportion of lactate to formate decreased from 5:1 at pH 6 to 1.3:1 at pH 11.

INTRODUCTION

The use of sodium hydroxide in the process of lye-peeling potatoes can result in the production of effluents with a pH of up to 12, which can be neutralized by bacterial fermentation (Gee *et al.*, 1974; Sistrunk *et al.*, 1979) but there is little information regarding the microbiology of the process. Several strains of bacteria that grow optimally at alkaline pH have been isolated by us from such wastes. These include five strains of a *Streptococcus* capable of relatively rapid growth at pH 10.3.

Although the ability to grow at pH 9.6 is characteristic of group D streptococci, there is little published information on the effect of controlled pH in the alkaline region on the rate of growth and the metabolism of streptococci. Gunsalus & Niven (1942) showed that when *Streptococcus faecalis* subsp. *liquefaciens* was grown anaerobically with the pH controlled at 5, 7 and 9, the proportion of glucose metabolized to lactate was 92% at pH 5 but decreased to 70% at pH 9, lactate being replaced by formate, acetate and ethanol. Studies of the effect of pH in the region of pH 4 to 8 on the metabolism of several species of *Streptococcus* and of *Lactobacillus bulgaricus* have been made by Platt & Foster (1958) and Rhee & Pack (1980).

The purpose of the present work was to determine the effect of alkaline pH on the rate of growth, the maximum cell density and the metabolic products of a *Streptococcus* isolated from alkaline potato-processing waste.

METHODS

Organism. *Streptococcus* strain BL 78/7 was isolated at 20 °C from a sample of potato waste, resulting from a commercial lye-peeling process, which had undergone a microbial fermentation and in which the pH had decreased to approximately pH 5. The isolate was obtained by enrichment in Medium 1 at pH 10 to 10.5, purified by plating on solidified Medium 1 and maintained on slopes of same medium.

Media. Medium 1, used for maintenance of strain BL 78/7, contained (g l⁻¹): peptone (Difco), 5; yeast extract (Difco), 1.5; glucose, 5; Na₂HPO₄ · 12H₂O, 1.5; NaCl, 1.5; Na₂CO₃, 5.3. A solution (100 ml) of Na₂CO₃ at 10 times the final concentration was autoclaved separately and added aseptically to 900 ml of the autoclaved medium. The solid form of Medium 1 was made by the addition of New Zealand Agar (15 g l⁻¹). The pH was adjusted to between 10.8 and 11.0 with sterile NaOH (0.5 M) after autoclaving.

Medium 2, used for studies of growth rates in fermenters, contained (g l^{-1}): tryptone (Difco), 10; yeast extract (Difco), 3.7; glucose, 5; NaCl, 2.5; Na_2CO_3 , 5.3. The Na_2CO_3 was prepared as for Medium 1, and the glucose was added in the form of a 20% (w/v) solution which had been sterilized by filtration through a cellulose acetate filter (0.22 μm pore size, Millipore). Appropriate volumes of these solutions were added aseptically to the autoclaved basal medium in the fermenter. The pH was adjusted with sterile NaOH (5 M) or HCl (1 M). When Medium 2 was used at a pH value of 9 or less, the Na_2CO_3 content was reduced to 1.1 g l^{-1} .

Characterization tests. The following tests suggested by Sherman (1937) and by Deibel & Seeley (1974) for the differentiation of enterococci from other streptococci were made. 1. Growth at 10 °C; 2. Growth at 45 °C; 3. Growth in media containing 6.5% (w/v) NaCl; 4. Growth in media containing 40% (v/v) bile. The tests were carried out at an initial pH of 7 to 7.5, on strain BL 78/7 and on reference strains of *S. faecalis*, *S. faecium* and *S. casseliflavus* using the media and methods of Barnes *et al.* (1956).

Lancefield precipitin test. Test organisms were grown in static culture for 48 h at 37 °C in Medium A of Barnes *et al.* (1956). Antigen extract was prepared by the method of Shattock (1949), layered on to group D antiserum (Wellcome) in a narrow glass tube and observed for the formation of a white precipitin ring.

Fermentation of sugars. Tests were made in the medium of Deibel *et al.* (1963), modified as follows: 1. 15 ml l^{-1} of a 0.2% (w/v) solution of bromothymol blue was added; 2. glucose was omitted, and raffinose or xylose was sterilized by filtration and added to a final concentration of 10 g l^{-1} . Control experiments used sterile distilled water in place of the sugar solution. Cultures grown for 18 h on slopes of Medium 1 were used to inoculate 10 ml test medium and acid production was observed as a colour change from blue to yellow.

Studies of the effect of pH on growth. Strain BL 78/7 was grown in Medium 2 at controlled pH in batch culture using a modular fermenter (LH 500, LH Engineering Co., Stoke Poges, Bucks.). A culture volume of 800 ml was used in a 1 litre vessel. For aerated cultures, air was pumped into the vessel at 800 ml min^{-1} with a stirrer speed of 1000 r.p.m., and for non-aerated cultures oxygen-free nitrogen was bubbled slowly through the medium with a stirrer speed of 500 r.p.m. All cultures were grown at 37 °C. Foaming was controlled by the automatic addition of a sterile 20% (v/v) aqueous suspension of Silicone DC Antifoam RD emulsion (Hopkin & Williams) and pH was controlled by the automatic addition of sterile NaOH (5 M) or HCl (1 M). The medium was inoculated with 40 ml of a culture grown for 18 h in Medium 2 at an initial pH of approximately 9.8. Samples (approximately 5 ml) were withdrawn from the fermenter at intervals of 30 min, the pH measured using a separate pH meter and the absorbance measured at 680 nm. The increase in $\log_{10} A_{680}$ was plotted against time, and the specific growth rate (μ) was determined from the slope of the graph. A calibration curve relating A_{680} to dry weight of cells was prepared using cells grown to late-exponential phase at pH 9.5.

Chemical analyses. Samples were removed from the fermenter at the beginning of stationary phase, centrifuged (10000 g for 10 min at 10 °C) and the supernatants stored at -20 °C prior to analysis for glucose, volatile fatty acids and ethanol. Glucose was determined using the Glucose Oxidase test combination (Boehringer). Volatile fatty acids and lactate were determined by titration of acidified samples against tetrabutylammonium hydroxide, followed by preparation of benzyl esters and separation by GLC (Jones & Kay, 1976). Chromatography conditions were as follows: column temperature was maintained at 80 °C for 20 min then increased to 150 °C at a rate of 5 °C min^{-1} ; argon (50 ml min^{-1}) was used as the carrier gas; hydrogen (40 ml min^{-1}) and air (500 ml min^{-1}) were supplied to the detector; attenuation was between 200 and 10⁹. Reference acids (10 $\mu\text{mol ml}^{-1}$) were treated in the same way as the samples, and crotonic acid was used as an internal standard.

Ethanol was determined by GLC using a PYE-104 instrument fitted with a flame-ionization detector. A 1 μl sample of the supernatant to be tested was injected on to a glass column (1.5 m $\times$ 6 mm o.d.) packed with Porapak Q (80–100 mesh, Waters Assoc. Inc., Mass., U.S.A.). Conditions were as follows: column temperature, 150 °C; detector oven temperature, 200 °C; argon carrier gas, 50 ml min^{-1} ; hydrogen, 40 ml min^{-1} ; air, 500 ml min^{-1} ; attenuation, 10⁹/100. Samples (1 μl) of a solution of ethanol (10 $\mu\text{mol ml}^{-1}$) were used as a reference.

RESULTS

Properties of strain BL 78/7

Strain BL 78/7 was an elongated coccus, 1–2 μm in length and 1 μm in diameter, occurring in pairs or short chains. Motility was observed during the exponential phase of growth when grown aerobically in Medium 2 at pH values between 6 and 8, but not when grown under nitrogen. After incubation for 48 h on Heart Infusion Agar (initial pH 7.4), cream/white colonies less than 1 mm in diameter were formed, whereas on Medium 1 (initial pH 10.8) colonies were yellow and greater than 1 mm in diameter. The properties shown in Table 1 indicated that strain BL 78/7 resembled *S. casseliflavus*.

Table 1. *Properties of isolate BL 78/7 and of reference strains of Streptococcus*

Test*	Property	<i>S. faecalis</i> NCTC 775	<i>S. faecium</i> NCTC 7171	<i>S. casseliflavus</i>		Strain BL 78/7
1	Growth at 10°C	+	+	—	+	+
2	Growth at 45°C	+	+	+	+	+
3	Growth in presence of 6.5% NaCl	+	+	+	+	+
4	Growth in presence of 40% bile	+	+	+	+	+
5	Lancefield group D	+	NT	—	+	+
6	Formation of acid from raffinose	—	—	+	+	+
7	Formation of acid from xylose	—	—	+	+	+
8	Motility	—	—	+	+	+†
9	Pigment‡	—	—	+	+	+

NT, Not tested.

* Tests 1–4 were made by the methods of Barnes *et al.* (1956) and test 5 by the method of Shattock (1949). Growth media for tests 1–8 had an initial pH of 7–8.

† Motility was not observed when strain BL 78/7 was grown under nitrogen.

‡ The strains recorded as + produced pigment on solidified Medium 1 (initial pH 10.8), but not on Heart Infusion Agar (initial pH 7.4).

Fig. 1. Effect of pH on the specific growth rate (μ) of *Streptococcus* BL 78/7. Cultures were grown in fermenters in Medium 2 in aerated conditions (□) with air provided at 800 ml min⁻¹ and stirring at 1000 r.p.m., or in non-aerated conditions (■) with N₂ bubbled slowly through the medium and stirring at 500 r.p.m. pH was controlled at the values indicated.

Effect of pH on growth

The effect of pH on growth at 37 °C was studied in batch cultures with continuous control of pH, and growth was observed, without an initial lag phase, at pH values up to 11. An exponential increase in A_{680} followed by a sharp cessation of growth occurred at pH 5 to 10.5, while above 10.5 the growth rate tended to decrease gradually. Organisms tended to occur mainly in pairs for the first 1–2 h of the exponential phase, after which chains were formed, the tendency to form long chains increasing with pH. At pH values above 10.3, after two to three cell divisions had occurred some chains were twisted or appeared refractile, and above pH 10.5 some cells also appeared swollen and misshapen from late-exponential phase onwards.

In aerated cultures the maximum specific growth rate (μ) was approximately 1.2 h⁻¹ at pH values of 8–9 (Fig. 1) corresponding to a doubling time of 34 min. Growth rates at pH 5 and pH 11 were 0.48 h⁻¹ and approximately 0.2 h⁻¹, respectively. In cultures sparged with nitrogen

Fig. 2. Effect of pH on maximum cell density of *Streptococcus* BL 78/7. Cultures were grown in fermenters in Medium 2 in aerated conditions (□) with air provided at 800 ml min^{-1} and stirring at 1000 r.p.m., or in non-aerated conditions (■) with N_2 bubbled slowly through the medium and stirring at 500 r.p.m. pH was controlled at the values indicated.

values for μ at pH 8 and pH 10 were similar to those determined for aerobic cultures, but at pH 6 a rate of 1.12 h^{-1} was found, which was significantly higher than the corresponding value for aerated cultures (0.85 h^{-1}). The rates determined at pH values close to 11 are approximate since a constant rate of growth was not maintained.

The highest cell densities were obtained at pH values 8 and 9 in aerated cultures ($1.5 \text{ mg dry wt ml}^{-1}$) and at pH 8 in cultures under nitrogen ($1.3 \text{ mg dry wt ml}^{-1}$) (Fig. 2). The growth rates were also highest at these pH values. A sharp decrease in maximum cell density occurred at pH 10.5. Except at pH 11, cultures grown under aerobic conditions gave slightly higher cell densities than cultures grown under nitrogen.

Effect of pH on metabolic products

In both aerated cultures and cultures under nitrogen at least 97% of the total glucose in the medium ($27.8 \mu\text{mol ml}^{-1}$) had been utilized by the beginning of stationary phase at pH values up to and including 10.5. At pH values above 10.5, however, growth was not terminated by exhaustion of glucose, only 75–85% of the available glucose being utilized at pH 11.

In cultures grown under nitrogen the major products were lactate, formate, acetate and ethanol, the last three occurring in the molar ratio of approximately 2:1:1 (Fig. 3a). The proportion of lactate formed was highest at low pH, accounting for 86% of the carbon recovered in the end-products at pH 6, but only 60–63% at pH 8, 10 and 11. At pH 6, 8 and 10, the carbon recovered was equivalent to more than 100% of the glucose present, indicating that medium constituents other than glucose contributed to the products. At pH 11 the carbon recovery was only equivalent to 73% of the glucose carbon.

In aerated cultures the major products were lactate and acetate (Fig. 3b). Lactate accounted for 86% of the carbon recovered at pH 5, and only 47–50% at pH values between 10.0 and 10.5. The carbon recoveries were less than 100% of the glucose carbon at all pH values, this was probably due to loss of carbon as CO_2 , which was not trapped.

DISCUSSION

The isolate BL 78/7 was a group D *Streptococcus*, apparently of the enterococcus group, but differed from *S. faecalis*, *S. faecium* and *S. avium* in that, under certain conditions, it was motile and pigmented. A similar bacterium was isolated from plant material by Mundt & Graham (1968) and described as *S. faecium* var. *casseliflavus*; fermentation of xylose and raffinose were

Fig. 3. Effect of pH on the metabolic products of *Streptococcus* BL 78/7. (a) Non-aerated culture; (b) aerated culture. Growth conditions as for Figs 1 and 2. Lactate (○); acetate (●); ethanol (△); formate (▲).

further properties in which it differed from the enterococci. Vaughan *et al.* (1979), from the results of DNA homology studies, proposed that this bacterium was in fact a distinct species. Although in the present work *S. casseliflavus* and strain BL 78/7 did not produce pigment on Heart Infusion Agar, *S. casseliflavus* was shown by Mundt & Graham (1968) to form pigment on a variety of non-alkaline media. Strain BL 78/7 resembled *S. casseliflavus* in morphology, pigment and fermentation of raffinose and xylose.

Although tolerance of pH values up to 9.6 is common in streptococci, the ability to grow at pH values as high as 11 has not previously been reported. Chesbro & Evans (1959) reported growth of *S. faecalis* and *S. faecium* at pH 10.5 but growth was preceded by a lag phase of over 12 h, during which the pH of the medium, which was not controlled, may have fallen. Moreover, the growth rates achieved were lower than those determined for strain BL 78/7. The pH value for optimum growth of strain BL 78/7, approximately 8.5, was similar to that of another bacterium also isolated from alkaline potato waste (Gee *et al.*, 1980) and subsequently described as *Exiguobacterium aurantiacum* (Collins *et al.*, 1983). Many alkalophilic bacteria have been reported to have a higher pH optimum for growth; cultures of *Bacillus alcalophilus* with malate as a carbon source grew most rapidly at pH 10.5 (Guffanti *et al.*, 1978), and a coryneform species (Souza & Deal, 1977), an *Ectothiorhodospira* species (Grant *et al.*, 1979) and a *Halobacterium* species (Tindall *et al.*, 1980), isolated from alkaline springs, grew optimally between pH 9 and 10. These species, however, did not grow at neutral pH, whereas strain BL 78/7 was capable of growth at pH values at least as low as 5. Such a wide range of pH tolerance is unusual. The only bacterium previously reported, to our knowledge, with a similar pH range was a strain of *B. sphaericus* (Bornside & Kallio, 1956).

The decrease in growth rate and maximum cell density at pH values higher than 9.5, despite the fact that at least 97% of the glucose was utilized at pH values up to pH 10.5, suggests that there is an increase in energy requirement to enable growth at pH higher than 9.5. The reason for the higher growth rate in non-aerated than in aerated cultures at pH 6 is not known.

Lactate was the major end-product of metabolism of strain BL 78/7, but the fermentation became increasingly heterolactic with increase in pH. A similar trend in the pH range 5 to 9 has been reported for *S. faecalis* subsp. *liquefaciens* (Gunsalus & Niven, 1942), in the acidic to neutral pH range for *S. thermophilus* (Platt & Foster, 1958) and in the pH range 4 to 8 for *Lactobacillus bulgaricus* (Rhee & Pack, 1980). The occurrence, in N₂-sparged cultures of strain BL 78/7, of formate, acetate and ethanol in the molar ratio 2:1:1 indicated the involvement of the phosphoroclastic split of pyruvate. In aerated cultures, it appeared that metabolism of pyruvate to acetate and CO₂ occurred. As 1 mol of ATP would be produced during the formation of 1 mol of acetate from pyruvate the shift from a homolactic to a heterolactic fermentation would be expected to result in an increase in ATP production per mol of glucose utilized. In fact, a similar shift in metabolism has also been reported to occur in *Lactobacillus* and *Streptococcus* spp. as a result of glucose limitation (de Vries *et al.*, 1970; Yamada & Carlsson, 1975; Thomas *et al.*, 1979).

The production in aerated cultures of acetate alone instead of formate, acetate and ethanol, could be due to oxygen sensitivity of the pyruvate formate-lyase enzyme system. In purified extracts from *S. faecalis* the formate-pyruvate exchange enzyme was shown to be highly sensitive to oxygen (Lindmark *et al.*, 1969), and in *Enterobacteriaceae* the enzyme system is present but inactive in aerobically grown cells (Thauer *et al.*, 1977). In aerated cultures higher cell densities were obtained than in cultures grown under nitrogen. This may be due to the greater quantity of acetate produced aerobically since the production of acetate from pyruvate would generate 1 mol of ATP per mol of pyruvate, whereas the formation of formate, acetate and ethanol would result in the formation of 1 mol of ATP per 2 mol of pyruvate.

The formation of acetate from pyruvate, in contrast to the formation of ethanol, does not regenerate NAD, and since slightly less lactate was produced in aerated than in non-aerated conditions, less NAD would be regenerated by lactate dehydrogenase. It is possible, therefore, that there is an alternative method for the oxidation of NADH in cells grown in aerobic conditions. There is evidence for the presence of a flavin-linked NADH oxidase in *S. faecium* (Ritchey & Seeley, 1976), and such an enzyme in strain BL 78/7 would remove the necessity for the reduction of pyruvate to oxidize NADH, allowing pyruvate to be used for ATP production by the formation of acetate.

A. F. G. was the recipient of an ARC research studentship.

REFERENCES

- BARNES, E. M., INGRAM, M. & INGRAM, G. C. (1956). The distribution and significance of different species of faecal streptococci in bacon factories. *Journal of Applied Bacteriology* **19**, 204–211.
- BORNSIDE, G. H. & KALLIO, R. E. (1956). Urea-hydrolysing bacilli. I. A physiological approach to identification. *Journal of Bacteriology* **71**, 627–634.
- CHESBRO, W. R. & EVANS, J. B. (1959). Factors affecting the growth of enterococci in highly alkaline media. *Journal of Bacteriology* **78**, 858–862.
- COLLINS, M. D., LUND, B. M., FARROW, J. A. E. & SCHLEIFER, K. H. (1983). Chemotaxonomic study of an alkalophilic bacterium, *Exiguobacterium aurantiacum* gen. nov., sp. nov. *Journal of General Microbiology* **129**, 2037–2042.
- DE VRIES, W., KAPTEIJN, W. M. C., VAN DER BEEK, E. G. & STOUTHAMER, A. H. (1970). Molar growth yields and fermentation balances of *Lactobacillus casei* L3 in batch cultures and in continuous cultures. *Journal of General Microbiology* **63**, 333–345.
- DEIBEL, R. H. & SEELEY, H. W. (1974). *Streptococcaceae*, In *Bergey's Manual of Determinative Bacteriology*, 8th edition, pp. 490–517 Edited by R. E. Buchanan & N. E. Gibbons. Baltimore: Williams and Wilkins.
- DEIBEL, R. H., LAKE, D. E. & NIVEN, C. F. (1963). Physiology of the enterococci as related to their taxonomy. *Journal of Bacteriology* **86**, 1275–1282.
- GEE, J. M., LUND, B. M., METCALF, G. & PEEL, J. L. (1980). Properties of a new group of alkalophilic bacteria. *Journal of General Microbiology* **117**, 9–17.
- GEE, M., HUXSOLL, C. C. & GRAHAM, R. P. (1974). Acidification of "dry" caustic peeling waste by lactic acid fermentation. *American Potato Journal* **51**, 126–131.
- GRANT, W. D., MILLS, A. A. & SCHOFIELD, A. K. (1979). An alkalophilic species of *Ectothiorhodospira* from a Kenyan soda lake. *Journal of General Microbiology* **110**, 137–142.
- GUFFANTI, A. A., SUSMAN, P., BLANCO, R. & KRULWICH, T. A. (1978). The protomotive force, and α -aminoisobutyric acid transport in an obligately alkalophilic bacterium. *Journal of Biological Chemistry* **253**, 708–715.
- GUNSALUS, I. C. & NIVEN, C. F. (1942). The effect of

- pH on the lactic acid fermentation. *Journal of Biological Chemistry* **145**, 131–136.
- JONES, D. W. & KAY, J. J. (1976). Determination of volatile fatty acids C₁–C₆, and lactic acid in silage juice. *Journal of the Science of Food and Agriculture* **27**, 1005–1014.
- LINDMARK, D. G., PAOLELLA, P. & WOOD, N. P. (1969). The pyruvate formate-lyase system of *Streptococcus faecalis*. I. Purification and properties of the formate-pyruvate exchange enzyme. *Journal of Biological Chemistry* **244**, 3605–3612.
- MUNDT, J. O. & GRAHAM, W. F. (1968). *Streptococcus faecium* var. *casseliflavus*, nov. var. *Journal of Bacteriology* **95**, 2005–2009.
- PLATT, T. B., & FOSTER, E. M. (1958). Products of glucose metabolism by homofermentative streptococci under anaerobic conditions. *Journal of Bacteriology* **75**, 453–459.
- RHEE, S. K. & PACK, M. Y. (1980). Effect of environmental pH on fermentation balance of *Lactobacillus bulgaricus*. *Journal of Bacteriology* **144**, 217–221.
- RITCHEY, T. W. & SEELEY, H. W. (1976). Distribution of cytochrome-like respiration in streptococci. *Journal of General Microbiology* **93**, 195–203.
- SHATTOCK, P. M. F. (1949). The streptococci of group D; the serological refractory group D strains. *Journal of General Microbiology* **3**, 80–92.
- SHERMAN, J. M. (1937). The streptococci. *Bacteriological Reviews* **1**, 3–97.
- SISTRUNK, W. A., ISMAIL, M. L. & COLLINS, J. A. (1979). Bacterial fermentation of high alkaline wastes from Irish potatoes. *Journal of Food Science* **44**, 439–442.
- SOUZA, K. A. & DEAL, P. H. (1977). Characterization of a novel extremely alkalophilic bacterium. *Journal of General Microbiology* **101**, 103–109.
- THAUER, R. K., JUNGEMAN, K. & DECKER, K. (1977). Energy conservation in chemotrophic anaerobic bacteria. *Bacteriological Reviews* **41**, 100–180.
- THOMAS, T. D., ELLWOOD, D. C. & LONGYEAR, V. M. C. (1979). Change from homo- to heterolactic fermentation by *Streptococcus lactis* resulting from glucose limitation in anaerobic chemostat cultures. *Journal of Bacteriology* **138**, 109–117.
- TINDALL, B. J., MILLS, A. A. & GRANT, W. D. (1980). An alkalophilic red halophilic bacterium with a low magnesium requirement from a Kenyan soda lake. *Journal of General Microbiology* **116**, 257–260.
- VAUGHAN, D. H., RIGGSBY, W. S. & MUNDT, J. O. (1979). Deoxyribonucleic acid relatedness of strains of yellow-pigmented, group D streptococci. *International Journal of Systematic Bacteriology* **29**, 204–212.
- YAMADA, T. & CARLSSON, J. (1975). Regulation of lactate dehydrogenase and change of fermentation products in streptococci. *Journal of Bacteriology* **124**, 55–61.