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EXPERIMENTS ON THE CULTURAL CONDITIONS OF CHEESE STARTERS IN WHEY AND ON THEIR ACID PRODUCTION IN MILK

Factors Affecting Growth and Acid-Producing Ability of Streptococcus Lactis

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Abstract. Factors affecting the growth of two isolates of *Streptococcus lactis* in a whey-based medium, and their acid-producing ability in milk were studied. Supplementation of diluted whey with peptone and extracts of yeast and beef allowed maximal growth of both the bacteria. Evidence has been presented which shows the presence of stimulatory factor(s) common to both peptone and yeast extract. An almost linear relationship occurred between the growth and increase in the peptone concentration up to 0.5%. A pH of 7.1 and stationery incubation in air provided optimum growth conditions for the bacteria. Associative growth of the two species resulted in symbiotic effect and both growth and acid production increased under the condition. Supplementation of milk with adenine and yeast extract brought about increased acid production in milk by AH2. Such potentiation of acid production was more pronounced by yeast extract alone and also in combination with adenine.

The species of streptococci are known to be fastidious and exact in growth requirements. Studies dealing with their nutritional requirement have established the growth stimulatory effects of many factors notably hydrolysed peptone,[1] amino acids, vitamins,[2–4] purines, pyrimidines [5–7] and biological extracts.[8,9] However, there is always a great variability in response of lactic streptococci to growth conditions and stimulants.[9] Difficulty is encountered in culturing a number of these species in defined medium. Some of the media[2,10] which satisfy their growth requirement cannot be employed for cultivation on commercial scale because of the cost and relative difficulty in the preparation. Milk medium, usually employed for culturing on large scale, is not always adequate to affect prompt and proper growth of some of the bacteria unless suitably supplemented.[1,3] Besides, utilization of milk for the purpose would not be economically feasible as our country is already short of milk. Best material, from the standpoint of both availability and economy for the propagation of the culture, would be whey, a waste product of cheese industry. The present investigation developed from attempts to develop a whey medium, and to find optimum cultural conditions for the growth of two locally isolated species of *S. lactis* in this medium. Results of the effect of certain factors on acid producing ability of the bacteria in milk are also presented.

Experimental

Organisms and Maintenance. Two strains of *S. lactis* designated as AH2 and C10 were isolated respectively from a sample of curd and imported starter culture. Their identification as strains belonging to *S. lactis* sps. was based on complete morphological and physiological tests. Both produced acid from glucose, lactose, maltose and sucrose. However, acid was not produced when mannitol, sorbitol, glycerol or arabinose was the source of carbon. Both the strains showed strong reducing power, grew at pH 9.2 and in the presence of 0.1% methylene blue. Stock cultures were maintained on agar slants composed of dextrose 0.1%, tryptone 0.5%, beef extract 0.3%, and $CaCO_3$ 0.1%, at 5°C (±0.2), and were subcultured fortnightly.

Media and Growth Conditions. Unless stated otherwise, the medium used in the growth experiments was composed of dextrose 0.1% (w/v), beef extract DIFCO 0.3% (w/v), yeast extract 0.1% (w/v), peptone 0.5% (w/v), and whey 25% (v/v), adjusted to pH 7.0. The medium used in preliminary growth experiments and for subculturing purpose did not contain whey and has been referred to in the text as basal medium. Phosphate buffer of appropriate pH was added to the medium to yield 1.7% phosphate salt by weight as described by Hargrove *et al.*[11] Both the media and the buffer were sterilized separately and mixed at the time of inoculation. 100 ml of the medium, dispensed in a 300-ml Erlenmeyer flask, was inoculated with 1 ml of inoculum and incubated at 30°C for 16–20 hr as described in the text.

Preparation of Whey. Whey was prepared from skim milk (total soluble solid 10%) essentially by the method of Mackie and McCartney.[12] After filtration through cotton wool, the filtrate was adjusted to pH 6.9 (±0.1), autoclaved at 10 lb for 10 min and filtered. The clear whey so obtained was used in the medium after necessary dilution.

Inoculum. Inoculum was prepared by inoculating 10 ml of the subculturing medium contained in a test tube with loopful of freshly growing cells. After about 16–18 hr of incubation at 30°C most of the spent medium from the tube was decanted off and 9 ml of sterile water was added to the cell deposit. The cell suspension so obtained was used as inoculum. Per cent inoculum as mentioned in the text means the amount of inoculum in ml used to inoculate 100 ml medium.

For the study of acid production in milk cell suspensions were prepared from 16 to 18 hr old broth culture. Cells were harvested by centrifuging at 3,000×g, and washed twice with sterile water before making suspension in sterile saline solution.

Measurement of Growth. Growth of cells was measured turbidimetrically in a Unikamp S.P. 600 spectrophotometer at 700 nm. At the end of incubation period, growth was stopped by immersing the flasks in to crushed ice. Aliquots from the flask were suitably diluted with appropriate medium before the measurement.

Acid Production. Titrable acidity was determined using 0.1N NaOH, with phenolphthalein as indicator.

Results

Effect of Whey on Growth. Results of the growth of AH2 and C10 in basal medium and in media containing different proportions of whey are presented in Table 1. Basal media containing lactose 1 and 2% were included in order to know whether whey acted merely as a source of lactose. Whey had a marked stimulatory effect on growth of both the strains. Maximum growth occurred in the presence of 25% of whey. With further dilution, the effect was diluted and the growth decreased. Less growth at concentration higher than 25% may well be attributed to the presence of lactose in excess of what would normally be assimilated. Under these conditions, the excess of carbohydrates is fermented with the result that more acid is formed and the pH of the culture becomes suboptimum for growth. The growth in the presence of lactose in either of the concentration was always less than in the presence of whey.

Effect of Omission of Different Ingredients from Complete Medium on Growth of AH2. Different media were prepared by omitting one or two components in order to study the effect of each of them on growth. Results of the growth of AH2 in complete and deficient media are summarized in Table 2. Omission of any of the ingredients except glucose caused marked reduction in growth of the bacteria. Poorest growth occurred when both peptone and yeast extract were omitted. Omission of neither peptone nor yeast extract alone produced such a pronounced effect.

Omission of yeast extract (0.1%) was almost as effective as beef extract (0.3%) thus demonstrating that yeast extract acts more as a growth stimulatory factor than as a source of nitrogen.

Effect of Peptone on Growth. The results of the effect of different concentrations of peptone in the medium on the growth of AH2 and C10 are shown in Fig. 1. Growth of both the strains increased with the increase in peptone concentration up to 0.5%, beyond which the effect levelled off.

Effect of pH on Growth of AH2 and C10. Media adjusted at different pH values with either HCl or NaOH, were buffered with phosphate buffer of appropriate pH. Growth of the two bacteria at different pH levels as recorded after 16-hr incubation is shown in Fig. 2. Optimum pH for both the strains seems to lie at about 7.1. Deviation in pH towards alkaline side retarded the growth to a lesser extent than deviation towards acid side.

Effect of Oxygen Tension on Growth of AH2 and C10. Results of the experiments designed to study the effect of varied level of oxygen-tension on growth of AH2 and C10 are presented in Table 3. Thunberg tubes were employed for incubation of cultures under anaerobiosis. The tubes containing respective cultures were completely evacuated prior to incubation. Incubation in air was carried out in conical flasks as usual. One set of each of the Thunberg tubes and flasks was incubated under stationary condition, and the other set with shaking on Gallenkamp shaking incubator at 60 oscillation/min.

The results (Table 3) revealed that the growth of both the bacteria was retarded most by increased oxygen-tension under aerobic incubation with shaking. Growth under anaerobiosis, under either of the incubation conditions, was always greater than under aerobic shaking. Maximum growth, however, occurred under stationary incubation in air.

Effect of Growing Cells of AH2 and C10 in Association on Total Growth. The study was undertaken to demonstrate whether the two species of *S. lactis* exhibit phenomenon of symbiosis when grown together. Medium for growing AH2 and C10 in combination was inoculated with 50% each of the two inocula, and incubated alongwith the standard containing cells of single strains under identical conditions. Results of two separate experiments performed with

TABLE 1. EFFECT OF WHEY ON THE GROWTH OF AH2 and C10.

(Incubation. Media inoculated separately with 1% inocula of AH2 and C10 were incubated for 20 hr).

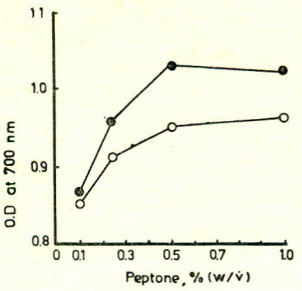
Media composition	Absorbance at 700 nm	
	AH2	C10
Basal med. (control)	1·15	0·90
,, +whey*	1·65	1·3
,, +diluted whey †(1:1)	1·70	1·35
,, +diluted whey (1:2)	1·75	1·40
,, +diluted whey (1:3)	1·75	1·45
,, +diluted whey (1:4)	1·70	1·40
,, +lactose (1%)	1·3	1·0
,, +lactose (2%)	1·28	0·95

*Ingredients of medium dissolved in undiluted whey; †whey diluted with different proportions of water, v/v as indicated.

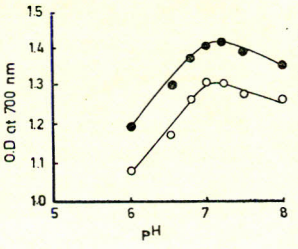
TABLE 2. EFFECT OF OMISSION OF DIFFERENT INGREDIENTS FROM COMPLETE MEDIA ON THE GROWTH OF AH2.

(Incubation. Complete and deficient media inoculated with 1% inoculum of AH2 were incubated for 18 hr).

Omission	Absorbance at 700 nm
Nil (complete med.)	1.45
Yeast extract	1.20
Beef extract	1.18
Peptone	0.85
Peptone+yeast extract	0.46
Dextrose	1.45



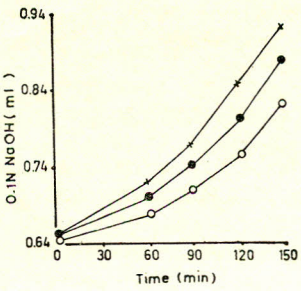
(1)



(2)

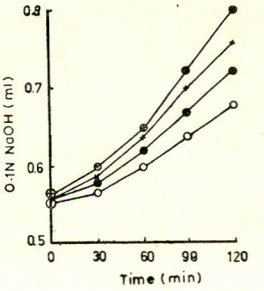
Fig. 1. Effect of peptone on growth of AH2, (●-●-●) and C10, (O-O-O)

Fig. 2. Effect of pH on growth of AH2, (●-●-●) and C10, (O-O-O)



(3)

Fig. 3. Acid production in milk separately by C10 (O-O-O), and AH2, (●-●-●) and by the two in combination (×-×-×).



(4)

Fig. 4. Effect of adenine and yeast extract on acid production in milk by AH2 (O-O-O), control (●-●-●), with adenine, 20 γ/ml (×-×-×), with yeast extract 20 γ/ml(⊗-⊗-⊗), with adenine and yeast extract 20 γ/ml each.

TABLE 3. EFFECT OF OXYGEN-TENSION ON THE GROWTH OF AH2 and C10.

(Incubation. Medium, 25 ml, cantained in Thunberg tubes (ca. 50 ml) and in conical flasks (ca. 100 ml) for incubation under vacuum and in air respectively, inoculated with 1% inoculm. Different vessels were incubated with and without shaking for 16 hr).

	Absorbance at 700 nm			
	In air		Under vacuum	
Bacteria	Stationary	With shaking	Stationary	With shaking
AH2	0.82	0.53	0.75	0.76
C10	0.65	0.5	0.58	0.6

TABLE 4. EFFECT OF GROWING CELLS OF AH2 and C10 IN ASSOCIATION ON GROWTH.

(Incubation. Media in experiments 1 and 2 respectively inoculated with 1 and 2% inocula were incubated under identical conditions for 20 hr).

Bacteria	Absorbance at 700 nm	
	Expt. I	Expt. II
AH2	0.78	1.45
C10	0.70	1.35
AH2+C10	0.85	1.55

different preparations are presented in Table 4. It is seen that in both the experiments more growth occurred when the bacteria were grown together.

Acid Production in Milk by Washed Cells of AH2 and C10. Washed cells from each of the two cultures grown separately for 20 hr were so suspended in saline solution that O.D. at 700 nm of these suspensions matched with each other. A third suspension was made by combining the two suspensions in equal proportions. 5 ml of each of the suspensions were added to 50 ml sterilized milk in flask. The contents of flasks were mixed thoroughly and titrable acidity determined at 0 hr. The flasks were then incubated at 30°C and acidity determined in aliquots drawn at regular intervals. Amounts of 0.1N NaOH in ml consumed per 5 ml of milk are plotted against time in min (Fig .3). AH2 produced more acid in milk than C10 in the same period up to 2.5 hr. Maximum acidity, however, was produced when the cells of the two bacteria were allowed to act in combination.

Effect of Adenine and Yeast Extract on Acid Production in Milk by Washed Cells of AH2. Since AH2 produced more acid in milk than C10, the former was selected for further studying the effect of adenine and yeast extract on acid production. Each of 50 ml milk with and without supplementation with adenine and yeast extract, was inoculated with 5 ml cell suspension. Results of the titration of 5 ml milk sample against the alkali at 0 hr and at different intervals are recorded in Fig. 4. It is seen that supplementation of milk with either yeast extract or adenine allowed more acid production in milk by AH2. Yeast extract was slightly more stimulatory than adenine. However, maximal acid production took place when milk was supplemented with both adenine and yeast extract.

Discussion

The stimulation in growth of the two bacteria by whey points to its utilization by lactic streptococci. The stimulatory effect may, at once, be ascribed to the water-soluble components of milk including vitamins, minerals and other factors of importance to lactic acid bacteria. Poor growth in the absence of whey indicates its dependence on the factors obviously missing in the components of other media. That the increase in growth is not due to the carbon source, lactose, in whey was clearly demonstrated when lactose was substituted for whey in the medium.

Whey alone, however, was not adequate for maximum growth unless supplemented with yeast extract, beef extract and peptone (Table 2). In the absence of either of the three the growth of AH2 was markedly reduced. The fact that the growth is reduced by the omission of either peptone or yeast extract but is not as pronounced as by the omission of both together, indicates the presence of certain growth factor(s) common to both peptone and yeast extract. The possibility of complementary role of either of them also may not be ruled out. Yeast, in fact, has been reported to contain accessory growth factors for lactic streptococci,[9] haemolytic streptococci[13] and other bacteria.[14] Observations

have also been made which show that the growth of certain strains of lactic streptococci could be increased by peptone and hydrolysed peptone.[9,15] Reportedly, these nitrogenous compounds are very well utilized by the bacteria. Growth is less in the absence of (Table 2) or in the presence of suboptimal concentrations of peptone (Fig. 1) showing that peptone provides easily assimilable nitrogen for protein synthesis.

Streptococci are deficient in cytochrome system and utilize anaerobic respiratory mechanism even when grown under aerobic conditions.[16] The results of the effect of oxygen-tension on growth presented in this paper clearly demonstrate that both AH2 and C10 are unable to utilize oxygen—the best of all hydrogen acceptors from the energy point of view. This is not due to the actual sensitivity to oxygen (cf. typical anaerobes) since both grew readily in air. However, growth in both the cases was retarded by increased oxygen tension. In this respect, the results are compatible with the observations made by Rahn *et al.*[17] In their experiment, continued agitation by current of air retarded the fermentation of *S. lactis* while nitrogen increased the rate. Maximum growth in our experiments, however, occurred under stationary incubation in air, thus pointing to the fact that minute quantity of oxygen favours the metabolic steps leading to the biosynthesis of protein in the bacteria.

The results of the associative growth of AH2 and C10 demonstrate a symbiotic relation between them. Higher growth (Table 4) and higher acid production in milk (Fig. 3) resulted from the combined growth. Several experiments to the same effect have been recorded in literature.[18–21] In all these cases, too, the lactic cultures were able to grow independently but their associative growth always resulted in higher acid production. The main component responsible for higher acid production due to combined growth has been identified as adenine.[21] Pure adenine possessed similar properties and was stimulatory to the cultures when added to milk. This is further confirmed by our results (Fig. 4) showing marked increase in the activity of AH2 due to adenine and yeast extract. Such potentiation of acid production in milk by yeast extract has been found to take place with Lactobacilli[22] and lactic streptococci.[9] Better stimulation by yeast extract than by adenine alone, suggests that there is at least one other potent factor in yeast extract which is acting as an acid potentiator. Speck *et al.*,[9] in fact, were able to identify such acid potentiators as peptides present in the extracts of pancreas, liver and yeast. Maximal acid production in milk supplemented with both adenine and yeast extract is obviously an indication of the deficiency of adenine in the system.

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References

1. A.W. Anderson and P.R. Elliker, J. Dairy Sci., **36,** 608 (1953).
2. C.F. Niven, Jr., J. Bacteriol., **47,** 2343 (1944).
3. A.W. Anderson and P.R. Elliker, J. Dairy Sci., **36,** 161 (1953).
4. W.E. Sandine, M.L. Speck and L.W. Aurand, J. Dairy Sci., **39,** 1532 (1956).
5. E.E. Snell and H.K. Mitchell, Proc. Nat. Acad. Sci., **27,** 1(1944).
6. J.A. Kaburger, M.L. Speck and L.W. Aurand, J. Bacteriol., **85,** 1051 (1963).
7. R.S. Dahiya and M.L. Speck, *ibid.*, **85,** 585 (1963).
8. E.E. Snell, F.M. Strong and W.H. Peterson, Biochem. J., **31,** 1789 (1937).
9. M.L. Speck, J.K. McAnelly and Jeanne D. Wilbur, J. Dairy Sci., **41,** 502 (1958).
10. E.B. Collins, F.E. Nelson and C.E. Parmella, J. Bacteriol., **59,** 69 (1950).
11. R.E. Hargrove, F.E. McDonough and R.P. Tittsler, J. Dairy Sci., **44,** 1799 (1961).
12. T.J. Mackie, and J.E. McCartney, *Hand Book of Practical Bacteriology* (Livingstone, London, 1953), ninth edition, 1964.
13. L. Freedman and C. Funk, J. Metabolic Res., **1,** 457, 469 (1922).
14. H.G. Wood, A.A. Anderson and C.H. Werkman, J. Bacteriol., **36,** 201 (1938).
15. E.I. Gravie and L.A. Mabbitt, J. Dairy Sci., **39,** 305 (1956).
16. T.E. Friedmann, J. Biol. Chem., **130,** 757 (1939).
17. Otto Rahn, C.P. Hegarty and R.E. Denel, J. Bacteriol., **35,** 547 (1938).
18. V.W. Green, J. Dairy Sci., **42,** 906 (1959).
19. H.C. Olsen, J. Dairy Sci., **43,** 439 (1960).
20. R.S. Dehiya and M.L. Speek, J. Dairy Sci., **45,** 607 (1962).
21. R.S. Dahiya and M.L. Speck, J. Bacteriol., **85,** 589 (1963).
22. C.N. Huhtanen and W.L. Williams, App. Microbiol., **11,** 20 (1963).