CI. CHEESE-RIPENING STUDIES¹.

THE NITROGEN REQUIREMENTS OF THE LACTIC ACID BACTERIA: THE EFFECT ON THE SUGAR-FERMENTING ABILITIES OF ENRICHING PEPTIC CASEINOGEN DIGEST BROTH WITH YEAST EXTRACT.

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IN studies on the quantitative determination of the sugar-fermenting abilities of lactic acid bacteria, Orla-Jensen [1919], Orla-Jensen, Orla-Jensen and Spur [1926], and Knudsen and Sørensen [1929] have shown that for certain strains—including in the genus *Betacoccus* or *Betabacterium* Orla-Jensen—the enrichment of the nitrogen source with autolysed yeast (yeast extract) assures a marked increase in the amount of acid produced from many of the carbohydrates submitted to fermentation. In view of these results, we have employed yeast extract in enriching various media used in our work on the bacteria isolated from ripening Kingston cheese [Sadler, Eagles and Pendray, unpublished results]. At the outset, milk and sugar broth respectively were enriched with yeast extract at the rate of 0.15 %—a concentration approximating to the amount used by Orla-Jensen [1919] in preparing some of his standard media.

As the study of the sugar-fermenting abilities of the bacteria progressed, we were led to enquire into the influence thereon of enriching the standard nitrogen source broth with higher concentrations of yeast extract.

EXPERIMENTAL.

Peptic caseinogen digest broth having a total nitrogen content of approximately 0.5 %—the standard broth employed by Orla-Jensen [1919]—was used as the nitrogen source. The method of preparation is given by Orla-Jensen [1919], and by Eagles and Sadler [1932]. The distribution of nitrogen in the source and also in Bacto yeast extract has been shown [Eagles and Sadler, 1932]. For the comparative studies we enriched the broth with Bacto yeast extract at the rate of 0.15, 1, 2 and 5 % respectively—an enrichment with yeast extract at the rate of 0.15 % being equivalent in terms of nitrogen to an enrichment of 0.01 % total nitrogen [Eagles and Sadler, unpublished results]. Having found that provided the “kind” of nitrogen is suitable the “amount” is equally important [Sadler, Eagles and Pendray, 1932, 1], peptic caseinogen digest broth containing approximately 1 % total nitrogen was also employed as a nitrogen source.

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The enriched broth is prepared as follows. The necessary weight of yeast extract is dissolved in a portion of the required volume of peptic caseinogen digest broth by heating at 80° for about 30 minutes—approximately 2 g. yeast extract in 100 cc. broth. The solution is then filtered, the filtrate is made up to volume with the digest broth and the whole is thoroughly mixed.

The method of preparing each sugar broth and the procedures followed throughout the fermentation study have been described already [Sadler, Eagles and Pendray, 1932, 1].

Determinations were made on 7 cultures isolated from Kingston cheese. Cultures EMB₁ 173 and 195 and cultures EMB₂ 168 and 173 are Gram positive, non-gelatin-liquefying coccus forms that have been already reported upon [Sadler *et al.*, 1932, 1]. Cultures EMB₁ 58, EMB₂ 189 and EMB 33 are Gram positive rod forms. Culture EMB₁ 58 was isolated from a Kingston cheese 13 days old, culture EMB₂ 189 from a cheese 2 months old, and culture EMB 33 from a cheese which, when 2 weeks old, had been placed in cold storage and held at about 37° F. for 4 months.

The results of the determinations of the total titratable acidity—recorded as g. lactic acid per litre—produced by the organisms from 7 carbohydrates, when employing the nitrogen sources defined, are given in Table I.

When employing nitrogen source 2—peptic caseinogen digest broth containing approximately 0.5 % total nitrogen [Eagles and Sadler, 1932]—the total titratable acidity produced from each of the carbohydrates by culture EMB₁ 173 is closely comparable, in every case, with the total amount of acid produced when the nitrogen is supplied as nitrogen source 2 enriched with yeast extract at the rate of 0.15 %. Enrichment of the broth with higher concentrations of yeast extract has a slight influence on the amount of acid produced from glucose, mannose and salicin; but the increase in the amount of acid produced from these carbohydrates and from fructose is not appreciable even when enrichment of the broth with yeast extract is at the rate of 5 %—equivalent to 0.33 % total nitrogen. Actually, the enrichment with 5 % yeast extract appears to have an inhibiting effect on the fermentation of galactose. The fermentation of lactose is not affected when peptic caseinogen digest broth (0.5 % T.N.) is supplemented by yeast extract. The influence of the nitrogen source on the fermentation of galactose and lactose confirms our previous observation that “in some measure there is a protein-carbohydrate-streptococcus linking up” [Sadler *et al.*, 1932, 2].

Apart from the fermentation of mannitol, culture EMB₁ 195 is strictly comparable with culture EMB₁ 173 in its sugar-fermenting abilities, whether the nitrogen is supplied as peptic caseinogen digest broth (0.5 % T.N.) or as the same broth enriched with yeast extract as defined. For the fermentation of mannitol it would seem that culture EMB₁ 195 requires nitrogen of the kind available in yeast extract—an observation again in harmony with our previous finding.

When peptic caseinogen digest broth (approx. 1 % T.N.) is employed as the nitrogen source, the total titratable acidity produced by cultures EMB₁ 173 and 195 from fructose, glucose, mannose, galactose, lactose and salicin respectively is much greater than the amount produced when the same broth diluted to contain approximately 0.5 % total nitrogen is used, and previous results, where comparison is possible, are confirmed.

Both cultures produce a much higher total titratable acidity from fructose, glucose, mannose, lactose and salicin when the nitrogen source is peptic caseinogen digest broth containing approximately 1 % total nitrogen than is produced when the diluted broth enriched with yeast extract as defined is supplied.

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Table I. *Titratable acidity in g. lactic acid per litre.*

Culture No.	Nitrogen source	Mannitol	Fructose	Glucose	Mannose	Galactose	Lactose	Salicin
<i>Coccus-forms.</i>								
EMB ₁ 173	2	2.5	5.0	4.7	4.5	3.6	4.5	3.4
		2.3	5.0	5.0	5.0	3.6	4.5	3.8
	2 a	2.7	5.4	5.2	5.0	3.6	4.7	4.3
	2 b	3.4	5.0	5.4	4.5	3.4	4.5	5.2
		3.4	5.6	5.6	5.4	4.3	4.7	5.2
	2 c	3.2	5.6	6.1	5.9	4.3	4.5	5.6
	2 d	3.6	6.5	6.1	6.3	2.0	4.7	5.2
	1	3.8	8.3	8.3	7.9	7.4	7.0	6.1
EMB ₁ 195	2	0.0	4.7	5.0	4.5	3.4	4.5	4.1
		0.2	5.2	4.7	4.5	3.2	4.3	3.2
	2 a	2.5	4.7	5.0	4.7	3.2	4.1	3.8
	2 b	1.6	5.0	5.4	5.2	3.4	4.3	4.3
		2.0	5.2	5.2	5.0	3.2	4.5	4.3
	2 c	1.1	5.9	5.4	5.9	4.1	4.5	3.8
	2 d	3.2	6.8	5.9	6.5	2.3	5.0	5.4
	1	0.7	7.4	7.7	7.9	7.2	6.5	5.9
EMB ₂ 168	2	0.2	4.5	5.0	5.9	5.4	5.6	0.7
	2 b	0.2	7.7	7.7	8.3	7.7	6.8	0.0
	2 d	0.5	9.2	7.7	9.7	6.3	8.6	2.0
	1	0.9	7.0	8.3	7.9	9.0	8.8	0.7
EMB ₂ 173	2	0.2	3.2	4.5	5.2	4.3	5.0	0.0
		0.0	4.1	5.2	5.0	4.5	5.9	1.4
	2 a	0.2	6.1	7.0	7.7	5.4	7.0	0.5
	2 b	0.5	8.8	8.3	8.6	7.2	7.9	5.9
		0.5	7.9	6.8	7.9	7.2	7.2	3.6
	2 c	0.2	7.2	7.2	8.3	7.2	7.4	4.3
	2 d	0.5	9.5	8.6	9.9	7.4	8.8	7.0
	1	0.2	7.4	8.1	8.6	8.3	9.9	3.2
<i>Rod-forms.</i>								
EMB ₁ 58	2	2.0	6.5	5.6	5.6	5.2	4.5	7.4
		2.5	7.2	5.4	7.2	5.4	5.9	6.1
	2 a	2.7	8.6	7.4	9.7	7.7	8.8	8.6
	2 b	3.4	9.9	8.8	11.3	9.2	9.7	9.7
		3.8	9.7	9.7	11.9	10.6	9.5	9.5
	2 c	3.8	10.6	9.9	12.4	—	10.1	9.5
	2 d	6.1	10.8	11.7	14.0	8.6	11.7	11.3
	1	2.5	11.9	10.4	11.9	10.4	8.8	11.0
EMB ₂ 189	2	2.5	7.0	7.9	9.2	7.4	9.0	8.8
		2.3	8.8	6.8	9.7	6.5	7.7	8.6
	2 a	2.7	9.9	9.2	10.4	6.5	9.5	8.3
	2 b	3.4	11.0	10.4	11.7	9.7	10.1	9.7
		3.6	11.5	10.8	11.7	10.8	10.4	9.9
	2 c	5.6	11.9	11.3	12.8	10.8	10.8	9.5
	2 d	6.8	12.8	12.6	14.2	9.0	12.8	12.2
	1	2.9	15.3	15.1	14.6	12.4	14.2	11.5
EMB 33	2	2.3	7.0	7.2	9.5	7.0	8.1	9.0
		2.5	7.9	6.8	8.8	6.1	7.2	8.3
	2 a	2.3	8.6	6.8	9.5	6.5	8.3	—
	2 b	3.4	10.6	9.5	11.7	10.1	8.8	9.0
		3.6	9.9	9.5	10.7	8.6	9.5	9.0
	2 c	3.8	11.3	9.7	11.7	9.0	10.4	9.5
	2 d	5.6	11.9	11.9	13.5	8.8	11.9	11.9
	1	3.2	14.4	15.1	14.9	12.6	14.2	13.1

Nitrogen sources:

- 2 Peptic caseinogen digest broth containing approx. 0.5 % total nitrogen [Eagles and Sadler, 1932].
- 2 a Nitrogen source 2 enriched with Bacto yeast extract at the rate of 0.15 %—equivalent to an enrichment of 0.01 % total nitrogen [Eagles and Sadler, unpublished results].
- 2 b Nitrogen source 2 enriched with Bacto yeast extract at the rate of 1 %—0.066 % T.N.
- 2 c Nitrogen source 2 enriched with Bacto yeast extract at the rate of 2 %—0.132 % T.N.
- 2 d Nitrogen source 2 enriched with Bacto yeast extract at the rate of 5 %—0.33 % T.N.
- 1 Peptic caseinogen digest broth containing approx. 1 % total nitrogen [Eagles and Sadler, 1932].

When employing nitrogen source 2 enriched with 0.15 % yeast extract for determining the sugar-fermenting abilities of culture EMB₂ 173, the total titratable acidity produced from fructose, glucose and mannose respectively is more than 50 % higher than is produced when the same nitrogen source without yeast extract enrichment is used; and, although less marked, there is an increase in the total titratable acidity produced from galactose and lactose when the enriched broth is employed. When the basic broth is enriched with higher concentrations of yeast extract the increase in the total titratable acidity produced by the culture from fructose, glucose, mannose and lactose respectively is marked, particularly when enrichment is at the rate of 5 %; even so, the critical differences in the total titratable acidity produced from fructose, glucose, mannose and lactose are to be seen when comparing the influence of nitrogen source 2 and nitrogen source 2 enriched with a trace only (0.15 %) of yeast extract. Throughout, the culture is unable to ferment mannitol. On the other hand, salicin, which is not fermented by the culture when the nitrogen is supplied as peptic caseinogen digest broth (0.5 % T.N.) or as the same broth enriched with 0.15 % yeast extract, is fermented to a marked degree when the broth is enriched with yeast extract at the rate of 5 %, an indication of the protein-carbohydrate-streptococcus linking up shown before [Sadler *et al.*, 1932, 2].

Culture EMB₂ 168 ferments salicin feebly to acid, only when the nitrogen source is peptic caseinogen digest broth (0.5 % T.N.) enriched with yeast extract at the rate of 5 %, and, in this respect, differs from culture EMB₂ 173. Otherwise, as far as comparison is possible, nitrogen sources 2a and 2b not having been used for culture EMB₂ 168, cultures EMB₂ 168 and 173 are closely similar in their ability to ferment the carbohydrates employed in the study.

The total titratable acidity produced by cultures EMB₂ 173 and 168 from fructose, glucose, mannose, galactose and lactose is about 100 % higher when the nitrogen source is peptic caseinogen digest broth containing approximately 1 % total nitrogen rather than the same broth diluted to contain approximately 0.5 % T.N.; and our previous results when glucose, mannose and lactose only were submitted to fermentation, are confirmed. It is also seen that, responding as they do to enrichment of the diluted broth with small amounts of yeast extract, these cultures, nevertheless, produce from fructose, glucose, galactose and lactose almost as much acidity when the nitrogen source is peptic caseinogen digest broth (approx. 1 % T.N.) as is produced when the same broth diluted (approx. 0.5 % T.N.) is enriched with yeast extract at the rate of 5 %. For the fermentation of salicin, on the other hand, peptic caseinogen digest broth, total nitrogen content 1 or 0.5 %, is not a suitable nitrogen source; but a greater or less amount of acid is produced when enrichment of the broth with yeast extract is provided—an indication of protein-carbohydrate-streptococcus linking up [Sadler *et al.*, 1932, 2].

When the peptic caseinogen digest broth containing approximately 0.5 % total nitrogen is enriched with yeast extract at the rate of 0.15 %, the total titratable acidity produced by culture EMB₁ 58 from glucose, mannose, galactose and lactose is much higher than is the case when the same broth without enrichment is employed as the nitrogen source—the increase in total acidity being particularly evident in the fermentation of mannose and lactose. The influence of the enrichment on the increase in the total acidity produced from glucose, mannose and lactose is more marked as higher concentrations of yeast extract are made available; and when the broth enriched with yeast extract at the rate of 5 % is used as the nitrogen source there is an increase in the total titratable acidity of over 200 %. In the fermentation of mannitol, the total titratable acidity

produced is comparatively low; but, comparing the influence of the basic broth and the same broth enriched with yeast extract at the rate of 5 %, there is seen to be an increase of 300 % in the amount of acid produced when the latter nitrogen source is employed. The influence of yeast extract enrichment on the fermentation of galactose is to be seen in the marked successive increases in the amount of acid produced when the nitrogen sources are 2a and 2b respectively; but enrichment with yeast extract at the rate of 5 % provides a less suitable nitrogen source than enrichment at the rate of 1 %. In the fermentation of salicin, enrichment of the basic nitrogen source with yeast extract provides for an increase in the total titratable acidity produced; yet the increase is not more than approximately 20 % until the concentration of the enrichment is 5 %.

When peptic caseinogen digest broth containing approximately 1 % total nitrogen is used as the nitrogen source, the total titratable acidity produced from fructose, glucose, mannose, galactose, lactose and salicin is from 50 % to 100 % higher than the amounts produced when the nitrogen is supplied as the same broth diluted to contain approximately 0.5 % total nitrogen; and our previous results [Sadler *et al.*, 1932, 1] in the study of lactic acid-producing coccus types are confirmed.

For the fermentation of mannitol, the peptic caseinogen digest broth containing approximately 1 % total nitrogen is no more suitable as a nitrogen source than is the same broth diluted to contain approximately 0.5 %; and, as a nitrogen source, it cannot be compared with the diluted broth enriched with yeast extract at the rate of 5 % (0.33 % T.N.), the suspicion thereby arising that, as has been shown already for the lactic acid streptococci [Sadler *et al.*, 1932, 2], the lactic acid-producing rods may require a certain moiety of a particular nitrogen fraction for the fermentation of a particular carbohydrate.

When the nitrogen is supplied as peptic caseinogen digest broth containing approximately 0.5 % total nitrogen, cultures EMB₂ 189 and EMB 33 produce a higher total titratable acidity from fructose, glucose, mannose, lactose and salicin than is produced by culture EMB₁ 58. There is no further increase in the amount of acid produced by culture EMB 33 when the nitrogen source broth is enriched with yeast extract at the rate of 0.15 %, and the influence of the same enrichment on the sugar-fermenting abilities of culture EMB₂ 189 is only slight. The increase in the total titratable acidity produced by both cultures from fructose, glucose, mannose and lactose is appreciable as the basic broth is enriched with yeast extract at the rate of 1, 2 and 5 % respectively; but the influence of enrichment is not marked until the yeast extract concentration reaches 5 %, an effect which is also reflected definitely in the fermentation of salicin. Cultures EMB₂ 189 and EMB 33 display the same idiosyncrasy to the fermentation of galactose, employing nitrogen source 2 enriched with yeast extract at the rates of 1 and 2 % as is shown by culture EMB₁ 58; and, throughout, mannitol is attacked in like manner by the three cultures.

Whether the total nitrogen content of the peptic caseinogen digest broth supplied as the nitrogen source be 0.5 or 1 %, it would appear that cultures EMB₂ 189 and EMB 33 are more active and vigorous than cultures EMB₁ 58, if activity and vigour may be interpreted in terms of the total titratable acidity produced by each culture from fructose, glucose, mannose, galactose, lactose and salicin; or, it may be that, in the light of our previous results [Sadler *et al.*, 1932, 1], peptic caseinogen digest broth of either 0.5 or 1 % total nitrogen content is a more suitable nitrogen source for cultures EMB₂ 189 and EMB 33 than for culture EMB₁ 58, interpreting suitability in terms of the total titratable

acidity produced by each culture from fructose, glucose, mannose, galactose, lactose and salicin respectively.

An increase of from 50 to 100 % in the total titratable acidity produced by cultures EMB₂ 189 and EMB 33 from fructose, glucose, mannose, galactose and lactose is to be seen, and the increase in the amount of acid produced from salicin is marked when the nitrogen source is peptic caseinogen digest broth containing approximately 1 % total nitrogen rather than the same broth diluted to contain approximately 0.5 % total nitrogen, again confirming previous results.

SUMMARY.

Considering the results as a whole it is seen that for two of the four coccus strains—cultures EMB₁ 173 and 195—enrichment of peptic caseinogen digest broth (approx. 0.5 % T.N. content) with yeast extract at the concentrations defined has only a slight influence on the total titratable acidity produced from fructose, glucose, mannose and salicin even when enrichment is at the rate of 5 %, equivalent in terms of total nitrogen to 0.33 %; and the influence of the nitrogen source on the fermentation of galactose and lactose confirms our previous observation [Sadler *et al.*, 1932, 2].

For two of the coccus strains—cultures EMB₂ 173 and 168—the total titratable acidity produced from fructose, glucose, mannose, galactose and lactose is at least 50 % higher when peptic caseinogen digest broth (approx. 0.5 % T.N.) enriched with yeast extract at the rate of 1 % rather than the same broth without enrichment is employed as the nitrogen source, the increase in the amount of acid produced being more marked as the broth is enriched with higher concentrations of yeast extract.

Of the three rod-forms, culture EMB₁ 58 produces a much higher total titratable acidity from glucose, mannose, galactose and lactose when the nitrogen source is peptic caseinogen digest broth (0.5 % T.N.) enriched with yeast extract at the rate of 0.15 % instead of the same broth without enrichment. Enrichment with yeast extract at the rate of 0.15 % fails to influence the sugar-fermenting abilities of culture EMB 33 and influences only slightly the sugar-fermenting abilities of culture EMB₂ 189: yet both cultures produce a higher total titratable acidity from fructose, glucose, mannose and salicin when the nitrogen is supplied as peptic caseinogen digest broth (0.5 % T.N.) without enrichment than is produced by culture EMB₁ 58 when the same nitrogen source is employed. Increasing the concentration of yeast extract enrichment reflects itself in the amount of acid produced from certain carbohydrates by each of the three cultures; and for the fermentation of such carbohydrates the increase in the total titratable acidity is marked when the yeast extract concentration reaches 5 %.

In the fermentation of mannitol the total titratable acidity produced by cultures EMB₁ 58, EMB₂ 189 and EMB 33 is comparatively low throughout; but comparing the influence of the basic broth and the same broth enriched with yeast extract at the rate of 5 %, there is seen to be an increase of 300 % in the amount of acid produced when the latter nitrogen source is employed. The idiosyncrasy to the fermentation of galactose by each of the three cultures is well defined.

For the fermentation of mannitol, the peptic caseinogen digest broth containing approximately 1 % total nitrogen is no more suitable as a nitrogen source than is the same broth diluted to contain approximately 0.5 % total nitrogen, and, as a nitrogen source, it cannot be compared with the diluted broth

enriched with yeast extract at the rate of 5 % (0.33 % T.N.), the suspicion thereby arising that, as has been shown already for the lactic acid streptococci, the lactic acid-producing rods may, likewise, require a certain moiety of a particular nitrogen fraction for the fermentation of a particular carbohydrate.

For the fermentation of fructose, glucose, mannose, galactose and lactose by the 7 cultures studied—coccus forms and rod forms—the total titratable acidity is increased by 50 to 100 % when employing as the nitrogen source a peptic caseinogen digest broth containing approximately 1 % total nitrogen instead of the same broth diluted to contain 0.5 % (approx.) total nitrogen, previous results being thus confirmed.

In supplying the nitrogen for the fermentation of certain carbohydrates by each of the organisms under study, a stimulating influence equal to that caused by an enrichment of the diluted peptic caseinogen digest broth (approximately 0.5 % T.N.) with yeast extract at the rate of 5 % (0.33 % T.N.) may be assured by employing peptic caseinogen digest broth alone, providing the total nitrogen content is approximately 1 %.

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