CXXI. RACEMIASE, AN ENZYME WHICH CATALYSES RACEMIZATION OF LACTIC ACIDS

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So-called fermentation lactic acids are generally believed to be inactive, since it was ascertained by Phelps & Palmer [1917] and Pederson *et al.* [1926] that optically inactive lactic acid was produced in many natural fermentations.

The lactic acid in *Sake* was found to be the inactive form, although not only inactive lactic acid-producing organisms (*Lactobacillus sake*), but also optically active lactic acid-formers (*Leuconostoc mesenteroides* var. *sake*)(*l*-former) and *d*- or *d*+*dl*-forming varieties of *Lactobacillus sake* were isolated by us [Katagiri *et al.* 1934], from yeast mashes for *sake* manufacture. Pederson *et al.* [1926] and again Tatum *et al.* [1932] observed that acetone-butyl alcohol-producing organisms (*Clostridium acetobutylicum*) affected optically active lactic acid-formers by causing them to form inactive lactic acid, and they suggested that this special property of *Cl. acetobutylicum* would explain the reason why fermentation lactic acids are inactive.

Such a phenomenon would be very peculiar as was pointed out by Stephenson [1930], since a stereochemical specificity is observed in nature in almost all biochemical reactions.

In our previous papers [1935, 1936, 1, 2, 3] it was reported that various kinds of *dl*-lactic acid-formers had the remarkable property of racemizing the lactic acids owing to the presence of an enzyme termed "racemiase" [1936, 3].

Direct racemization of lactic acid was also verified by us [1936, 4] with *Cl. acetobutylicum*. It was therefore concluded that the special effect of the acetone-butyl alcohol-producing organism in rendering the fermentation lactic acid inactive, would also be due to the action of the bacterial racemiase. The enzymic racemization of lactic acid recently has been recorded by Tatum *et al.* [1936] with acetone bacteria.

Thus the production of inactive lactic acid in natural fermentations would be due, in a great measure, to the racemiase of *dl*-lactic acid-formers.

In the present paper the occurrence of racemiase is recorded in *Staphylococcus ureae*. Many authors have discussed the question whether the optical properties of fermentation lactic acids would be modified by the cultural conditions even when pure cultures of lactic acid bacteria were used. From this discussion it would appear that inactive lactic acid can be obtained when optically active lactic acid-formers are used under certain cultural conditions.

In the present work, experiments were carried out with various strains of lactic acid bacteria, in order to ascertain whether any modification of the form of fermentation lactic acid would be produced by varying the conditions of cultivation.

Occurrence of racemiase

In order to detect the presence of racemiase in micro-organisms in addition to the *dl*-lactic acid-formers and the acetone bacteria mentioned in the previous papers [1935; 1936, 1, 2, 3, 4], experiments were at first carried out with various kinds of organisms cultivated on 200 ml. of koji extract at 30° for several days. Fresh cells of these organisms were washed with water and added to 50 ml. of sodium lactate solution containing 1·1–1·5 g. of *d*-lactic acid with 1 ml. of toluene and kept at 30° for 48 hr.

The cells were then collected by filtration or on the centrifuge and the weight determined after being dried under reduced pressure over sulphuric acid. The solution was acidified by sulphuric acid and the lactic acid extracted by ether, neutralized with zinc carbonate and successive crops of zinc lactate, obtained by fractional crystallization. The percentage of optically active form in the total amount of lactic acid was calculated by determinating the water of crystallization of the zinc salts and by the direct observation of the rotatory powers of the salts and of their supernatant liquids.

It will be seen in Table I that racemization of *d*-lactic acid was never found with 20 kinds of organisms including yeasts, moulds and fungi imperfecti, since the first crop of zinc lactate was, in all the experiments, entirely composed of *d*-lactic acid, and almost all the lactic acid was recovered from the solutions in the *d*-form.

Table I. *Action of micro-organisms upon* d-*lactic acid*

Micro-organism	Period of cultivation (days)	Weight of dried cells (g.)	Lactic acid used (g.)	Zinc lactate (1st crop) Yield (g.)	Zinc lactate (1st crop) Water of crystallization (%)
Aspergillus oryzae	4	0·248	1·47	1·330	13·29
Charala mycoderma	8	0·171	1·52	1·166	13·10
Citromyces glaber	6	0·321	1·45	1·411	13·42
Cladosporium herbarum	8	0·540	1·52	1·180	12·96
Debaryomyces membranaefaciens	4	0·312	1·19	1·032	13·10
Monascus purpureus	8	0·324	1·32	1·219	12·79
Monilia candida	6	0·260	1·45	1·260	12·33
Mycoderma cerevisiae	8	0·124	1·24	1·112	13·11
Oidium lactis	8	0·100	1·41	0·776	13·02
O. lupuli	6	0·160	1·52	1·088	13·10
Penicillium glaucum	5	0·322	1·54	1·541	14·12
Pichia rosa	5	0·090	1·36	1·225	13·27
Rhizopus delemar	5	0·175	1·32	1·368	12·68
Saccharomyces cerevisiae	6	0·308	1·34	1·197	12·97
S. sake	4	0·328	1·32	1·378	12·90
Schizosaccharomyces pombe	6	0·424	1·34	1·167	13·53
Torula luteola	6	0·102	1·36	1·144	12·20
Torulaspora fermentati	4	0·173	1·36	1·184	13·28
Willia anomala	4	0·391	1·25	1·260	12·60
Zygosaccharomyces barkerii	5	0·261	1·32	1·366	12·98

Various strains of bacteria were cultured for 5 days at 30° on 200 ml. of nutrient broth; sodium *d*-lactate solution containing nearly 2 g. of *d*-lactic acid and toluene was then added and the cultures were kept at 30° for 4 days.

Staphylococcus ureae was found to be the only bacterium of those tested which produced racemization of *d*-lactic acid, whereas the following were all inactive: *B. butylicus*, *B. coli communis*, *B. fluorescens liquefaciens*, *B. lactis aerogenes*, *B. mesentericus vulgatus*, *B. prodigiosus*, *B. pyocyaneus*, *B. subtilis*, *Sarcina lutea*.

It was found with *Staphylococcus ureae* that racemization occurred not only with *d*-lactic acid but also with *l*-lactic acid, since 53·2 and 36·6 % racemization of 1·6 g. of both *d*- and *l*-lactic acid respectively was produced by the bacteria.

Occurrence of racemiase in *Staphylococcus ureae* was further verified by experiments recorded in Table II in which bacterial cells obtained from nutrient agar culture were suspended in water after being washed twice with water in the centrifuge. To the bacterial suspension, 1·5 g. calcium carbonate and 2 ml. of lactic acid were added, the volume made up with water to 50 ml., 1 ml. of toluene added, and after being kept at 30° for 40 hr. the solution centrifuged and analysed.

Table II. *Action of* Staphylococcus ureae *upon lactic acids*

			Zinc lactate (1st crop)		
Acid	Weight of dried cells (g.)	Lactic acid used (g.)	Yield (g.)	Water of crystallization (%)	Racemization (%)
d-Lactic	0·18	1·45	1·162	15·67	25
l-Lactic	0·19	1·45	1·065	15·44	21

It is of interest that the occurrence of racemiase is not limited to anaerobic bacteria such as the *dl*-lactic acid-formers (*Lactob. sake*, *Lactob. plantarum*, *Lactob. spentoaceticus* and *Strept. lactis bulgaricus*) and acetone bacteria, since *Staphylococcus ureae* is an aerobic organism.

Effect of cultural conditions upon the optical properties of fermentation lactic acids

Nencki [1891] pointed out that the optical properties of the lactic acid produced by bacteria provided a very useful characteristic for the identification of the bacteria, since lactic bacteria always produced the same form of lactic acid. Péré [1892; 1893; 1898] observed remarkable modifications of the form of lactic acid produced by *B. coli* with various types of carbohydrate or sources of nitrogen, and these results were confirmed by Tate [1893] and Kayser [1894].

Gunther & Thierfelder [1895] suggested that *B. lactis acidi* (*d*-former) could spontaneously produce *dl*-lactic acid, while Kozai [1899] denied this, since no modification of the form of lactic acid was ever observed by him under various cultural conditions.

The specificity of the form of lactic acid produced by lactic bacteria was confirmed by Currie [1911] with various sources of nitrogen and of sugar and was again verified by Pederson *et al.* [1926] with various sugars.

In view of these contradictory opinions the effect of cultural conditions upon the lactic acid bacteria isolated by us [Katagiri *et al.* 1934] from yeast mashes for *Sake* manufacture was investigated.

(*a*) *Effect of the type of sugar.* The lactic bacteria were inoculated into 100 ml. of yeast extract containing 2–3 g. of sugar and 2–3 g. of calcium carbonate and incubated at 30° for 7 days. The lactic acid thus obtained was extracted by ether and the optical properties were determined as already described.

It will be seen in Table III that no modification of optical properties of the lactic acids produced by *Leuconostoc mesenteroides* var. *sake* No. 34, 52, 13 and 14 (*l*-formers) and by *Lactobacillus sake* No. 41, 24 and 53 (*d*-formers) was ever observed with various types of sugar.

With arabinose, *d*-lactic acid was always produced by the *dl*-forming strains, Nos. 37 and 42, and by the *dl*+*d*-forming strains, Nos. 45, 57 and 58 of *Lacto-*

bacillus sake, although the form of lactic acid produced by these bacilli did not vary with the hexoses glucose, fructose, mannose and galactose.

Table III. *Effect of type of sugar upon forms of lactic acid*

	Optical property of lactic acid					
No. of strain	Glucose	Fructose	Mannose	Galactose	Arabinose	Xylose
Leuconostoc						
34	*l*	*l*	*l*	.	*l*	*l*
52	*l*	*l*	.	*l*	*l*	*l*
13	*l*	*l*	.	*l*	.	.
14	*l*	*l*	.	*l*	.	.
Lactobacillus						
41	*d*	.	*d*	*d*	.	.
24	*d*	*d*	.	.	*d*	.
53	*d*	*d*	.	.	*d*	.
37	*dl*	*dl*	.	.	*d*	.
42	*dl*	*dl*	.	.	*d*	.
45	*dl*+*d*	.	*dl*+*d*	*dl*+*d*	*d*	.
57	*dl*+*d*	.	.	.	*d*	.
58	*dl*+*d*	.	.	.	*d*	.

It appears that modification of the optical properties of fermentation lactic acids was limited to the *dl*- and *dl*+*d*-formers among the lactic acid bacteria isolated from yeast mashes for *Sake* manufacture.

(*b*) *Effect of the form of nitrogen.* As sources of nitrogen, koji extract and yeast water containing glucose and calcium carbonate were used. Fermentations were carried out at 30° for 7 days and the optical properties of the lactic acids were determined in the same way as was mentioned above.

The results of the experiments are given in Table IV, from which it will be seen that modification of the optical properties of fermentation lactic acids was never observed with the *Leuconostoc mesenteroides* var. *sake* (*l*-former) or with the *Lactobacillus plantarum* sp. (*dl*-former) isolated by us [1935] from fermentation mashes for lactic acid manufacture.

Table IV. *Effect of nitrogen upon forms of lactic acids*

	Optical property of lactic acid		
		Kept for 10 months	
No. of strain	Newly isolated koji extract	Koji extract	Yeast water
Lactob. sake No. 37	*dl*	*d*	*dl*
42	*dl*	*d*	*dl*
57	*dl*	*d*	*dl*+*d* (30%)
41	*dl*+*d* (22%)	*d*	*dl*+*d* (60%)
45	*dl*+*d* (23%)	*d*	*dl*+*d* (30%)
58	*dl*+*d* (40%)	*d*	*dl*+*d* (25%)
24	*d*	*d*	*dl*+*d* (30%)
53	*d*	*d*	*dl*+*d* (40%)
Lactob. plantarum	*dl*	*dl*	*dl*
Leuconostoc No. 14	*l*	*l*	*l*

With newly isolated *Lactobacillus sake* (*d*-, *dl*- or *dl*+*d*-formers), no modification of the optical properties of the lactic acids was observed with various forms of nitrogen, the same forms of fermentation lactic acids being obtained with yeast water as with koji extract.

However, the optical properties of the fermentation lactic acids varied very remarkably with the type of nitrogen when the same bacilli were kept for ten

months on yeast water-maltose agar media. With koji extract only the *d*-form was produced by all the strains of *Lactobacillus sake*, while both forms were obtained with yeast water-glucose cultures.

Thus, a remarkable modification of the optical properties of the lactic acid produced was verified with *Lactobacillus sake*.

(c) *Effect of the temperature of incubation.* Fermentations were carried out for 7 days at 30 or 20° and for 40 days at 6°, with koji extract containing calcium carbonate.

It will be seen in Table V that the effect of the temperature of incubation upon the form of the fermentation lactic acid was negligible with *Leuconostoc mesenteroides* var. *sake* (*l*-former), *Lactobacillus sake* No. 41 (*d*-former) and with *Lactobacillus plantarum* sp. (*dl*-former).

However, with *Lactobacillus sake* No. 42 (*dl*-former), production of the *d*-form was much increased at 6°, and the same phenomenon was observed with *Lactobacillus sake* No. 24 and 45 (*dl*+*d*-former) when these were incubated at 20°.

Table V. *Effect of temperature of incubation upon forms of lactic acid*

	Optical property of lactic acid produced		
No. of strain	30°	20°	6°
Leuconostoc No. 14	*l*	.	*l*
67	*l*	*l*	.
Lactob. sake No. 41	*d*	.	*d*
42	*dl*	.	*dl*+*d* (17%)
24	*dl*+*d* (39%)	*dl*+*d* (59%)	.
45	*dl*+*d* (10%)	*dl*+*d* (47%)	.
Lactob. plantarum	*dl*	*dl*	*dl*

It was thus ascertained that the specificity of the form of lactic acid produced by *Leuconostoc mesenteroides* var. *sake* (*l*-former) and *Lactobacillus plantarum* sp. (*dl*-former) was a fixed character under the experimental conditions employed, whereas very noticeable modifications of the optical properties of the lactic acids were observed with *Lactobacillus sake* (*d*-, *dl*- and *dl*+*d*-formers) where the conditions of fermentation were varied.

The racemization of *d*-lactic acid produced by *Lactobacillus sake* was probably due to the racemiase in the bacterial cells, and it is probable that the modification caused by cultural conditions is due to the amount of racemiase present, rather than to the cause suggested by Orla-Jensen [1919] who concluded that two enzymes, one producing *d*-lactic acid and the other *l*-lactic acid, were present in the bacterial cell but that under unfavourable conditions the activity of one of the enzymes was inhibited and hence an active lactic acid resulted.

Summary

1. Among 30 kinds of micro-organisms tested only *Staphylococcus ureae* was found to produce racemiase.

2. *Leuconostoc* (*l*-former) and *Lactobacillus plantarum* (*dl*-former) were always found to produce specific forms of lactic acids, while very remarkable modifications of the optical properties of fermentation lactic acids were observed with *Lactobacillus sake* (*d*-, *dl*- and *dl*+*d*-formers) when the conditions of cultivation were varied.

3. It is suggested that this modification of the form of the fermentation lactic acid is due to the presence of racemiase in the bacterial cells.

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