CCLXXII. THE PRODUCTION OF CITRIC ACID FROM LACTIC ACID AND FROM ALCOHOL.

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IN the dairy industry moulds are often encountered on media containing lactic acid. According to the literature on the subject, the view appears to be justified that this acid is oxidized by the moulds into CO_2 or transformed into oxalic acid. Wehmer [1929] reports that by the action of *Aspergillus mutantus* calcium lactate is transformed into carbonate and sodium lactate into oxalate. Similarly, Thies [1934] finds that *A. fumigatus* oxidizes calcium lactate to calcium carbonate, whilst its sodium, potassium and ammonium salts are changed into the oxalates of the respective cations. Acklin [1929] states that *Penicillium glaucum* oxidizes lactic acid to water and carbon dioxide via pyruvic acid and acetaldehyde, in agreement with Neuberg's theory. According to Walker & Coppock [1928] *A. niger* acting on calcium propionate first forms lactic acid which is then transformed into pyruvic acid; propionic acid therefore would appear before the lactic acid. These authors accept the view that acetic acid must also be formed as a transient product by dismutation of acetaldehyde, the presence of which was determined by them in these processes.

Botrytis cinerea is a mould which relatively easily transforms acetic into citric acid [Chrząszcz & Zakomorńy, 1936]. If then acetic acid is formed during the decomposition of lactic acid, this would indicate that conditions exist for the relevant mould to form citric acid. The determination of this fact would afford further evidence in support of our hypothesis that citric acid is yielded by acetic acid [Chrząszcz & Tiukow, 1930], whilst this would in addition throw rather a different light upon the problem of the decomposition of lactic acid by moulds. The following research was undertaken in order to elucidate this point.

EXPERIMENTAL.

The spores of the mould *B. cinerea* were inoculated into four or five 750 ml. flasks each containing 100 ml. of 8% sterilized malt extract. After allowing the mould to develop during 10 days at 22°, the extract was decanted, the mycelium was carefully washed with sterilized water, and to each flask 100 ml. of a solution of pure lactate (Merck) free from formic, acetic and propionic acids, were added. The cultures were then kept for a suitable time (see Table I) at 22°, after which they were analysed with the following results.

2.5% solution of calcium lactate with the addition of 1% calcium carbonate.

The developed mycelium was kept in a 2.5% solution of calcium lactate with the addition of 1% CaCO_3 . After 12 days the solution was filtered off and the mycelium carefully washed (solution A). The mycelium with the sediment was treated with 20% acetic acid at room temperature for 24 hours, and then filtered off and washed (solution B). 8% HCl was then poured on to the mycelium and, after 24 hours, filtered off and the residue washed (solution C).

The washed mycelium was then dried at 105°. It was found to weigh 2.45 g. (computed for five flasks, see Table I).

Solution A was divided into three portions. One was treated with dilute sulphuric acid and distilled. In order to neutralize the distillate, 7 ml. of *N*/10 NaOH were used, thus indicating the presence of volatile acids in the distillate. After evaporating to dryness, a small amount of residue was obtained which upon acidification with sulphuric acid yielded a sharp smell, similar to that of acetic acid, and in addition had the odour of rancid butter. Since the reactions for butyric acid [Kline, 1934] and formic acid were negative, it was concluded that this residue contained a mixture of acetic and propionic acids.

The second portion of solution A was concentrated by evaporation during which process a sediment appeared which gave the reaction for citric acid. After filtering, it was washed, purified with bone charcoal and isolated in the form of a calcium salt, which after drying at 130° weighed 0.627 g.

Analysis. Found: CaO, 33.46 %. $(C_6H_5O_7)_2Ca_3$ requires CaO, 33.74 %.

This result is sufficiently concordant to show that the sediment examined is calcium citrate. If it be postulated that three molecules of lactic acid are necessary for one of citric acid, then to secure 0.627 g. of calcium citrate 0.824 g. of calcium lactate are necessary, and therefore the mould in this case used 8.3 % of the calcium lactate for this purpose.

The solution obtained after filtering off the calcium citrate was brought up to 200 ml. in the flask, and of this volume 5 ml. were used for determining lactic acid by means of a Lieb and Zacherl apparatus [1932]. 11.3 ml. of *N*/10 iodine were required, which, computed for the whole of solution A, gives 90.46 ml. of *N*/10 iodine, equivalent to 4.071 g. of lactic acid or 4.931 g. of calcium lactate. As 10.0 g. of calcium lactate were taken for the experiment, 50.7 % of it had been consumed by the mould.

The remainder of the solution was treated with lead acetate. Only a small precipitate was produced, and this, after decomposition with hydrogen sulphide, yielded a small amount of an acid substance, giving a positive result to Denigès and Stahr's reaction for citric acid. Apart from this, the solution of this salt decolorized a solution of $KMnO_4$ at room temperature and yielded a slight precipitate with a solution of $Hg_2(NO_3)_2$ acidified with nitric acid. The precipitate yielded by the lead acetate consisted of small quantities of lead salts of citric and fumaric acids.

The filtrate from the lead precipitate was treated with ammonia. A precipitate formed, which was washed and decomposed by hydrogen sulphide. The substance obtained gave a distinct reaction for malic acid; with a solution of ferric chloride a deep yellow colour was yielded. The reactions for citric and succinic acids were negative. The substance examined was therefore malic acid.

The third portion of solution A was treated with dinitrophenylhydrazine. The resulting precipitate was washed and treated with *N* sodium carbonate. 8 % HCl added to the filtrate produced a small precipitate which after washing and drying had m.p. 216°, indicating that it was the hydrazone of pyruvic acid.

The precipitate left after washing with sodium carbonate was treated with ethyl alcohol. A small amount of residue was left on the filter-paper but it was difficult to identify. The alcoholic filtrate on evaporation yielded a small residue, which melted at 164–165°, thus pointing to the presence of the hydrazone of acetaldehyde.

Solution B in acetic acid was evaporated to dryness yielding 6.433 g. of a substance which, computed in terms of calcium acetate, corresponds to 4.06 g.

of CaCO_3 . Since 4 g. CaCO_3 were used, its quantity remained practically unchanged. After dissolving this residue in water, it was treated with lead acetate and then by hydrogen sulphide. The slight amount of material obtained in this manner gave a distinct reaction for malic acid, but negative reactions for citric and succinic acids.

Solution C in HCl after neutralizing with ammonia yielded a white precipitate of calcium salt weighing 1.007 g.

Analysis. Found: CaO , 38.20 %. $\text{C}_2\text{O}_4\text{Ca}$, H_2O requires CaO , 38.38 %.

0.2024 g. of the substance, required for oxidation 27.6 ml. of $N/10 \text{ KMnO}_4$, corresponding to 0.2016 g. of $\text{C}_2\text{O}_4\text{Ca}$, H_2O .

The substance was therefore calcium oxalate. If it be assumed that two molecules of lactic acid yield two of oxalic acid, then 1.007 g. of calcium oxalate correspond to 1.504 g. of calcium lactate, or, to produce the above amount of oxalic acid, the mould used up 15.0 % of the calcium lactate.

It follows from the above that the mould used up 50.7 % of the calcium lactate, of which $8.3 + 15.0 = 23.3$ % were used for the formation of citric acid and oxalic acid. The remainder (27.4 %) primarily served to form CO_2 and small quantities of malic, fumaric, acetic, propionic and pyruvic acids and acetaldehyde, besides traces of other, unidentified products.

5 % solution of calcium lactate with the addition of 1 % calcium carbonate.

This experiment was conducted as in the preceding series but with the differences that a higher concentration of calcium lactate was used, viz. 5 %, and that the mycelium acted for a longer time, i.e. for 18 days. The results attained were, however, qualitatively identical, only the quantitative relation being different. The mould used up 43 % of the calcium lactate during the experiment, 12.7 of which were used for the formation of volatile acids and citric and oxalic acids, and 30.3 for the formation of CO_2 , the increment of the mycelium and for traces of other products.

5 % solution of ammonium lactate with addition of 3 % calcium carbonate.

The mycelium was kept on ammonium lactate for 21 days. Examination of the liquid showed that the products were almost identical with those obtained from calcium lactate, apart from fumaric acid which was not found. The mycelium used up 55.3 % of the ammonium lactate, of which 13.8 were used for the production of volatile acids and citric and oxalic acids, and 41.5 for CO_2 , increment of the mycelium and small quantities of unidentified products.

5 % solution of ammonium lactate without any additions.

The action of the mould was maintained for 18 days. The products of decomposition were similar to those obtained in the previous cases, apart from oxalic acid which was not found. The consumption of lactate was greater than before, namely 58.3 %, of which 1.4 appeared as volatile acids and citric acid, and the remainder was used chiefly for production of CO_2 and of small quantities of unidentified products.

2.7 % ethyl alcohol with the addition of 4 % CaCO_3 .

In order to get a better idea of the behaviour of the mould *B. cinerea* an experiment with ethyl alcohol was made. With this in view, the mycelium was grown on a 2.7 % solution of ethyl alcohol to which 4 % CaCO_3 had been added. After 18 days the fluid was filtered and solutions prepared as before: A an aqueous solution, B in acetic acid, and C in HCl.

Part of solution A was distilled. The distillate had an alcohol content of 0.4° Tralles, which, computed for the quantity of solution, shows that the mould used up 90.74 % of the alcohol.

During the evaporation of the solution, a precipitate (0.279 g.) was formed which after purifying gave a reaction for citric acid. Lead acetate was then added to the solution and, after decomposing the lead salt, citric acid was obtained. Thus the aqueous solution yielded $0.279 + 0.192 = 0.471$ g. citric acid.

In the filtrate a precipitate was produced by ammonia which yielded malic acid. It weighed 0.039 g. and, assuming that one molecule of malic acid is formed from two of ethyl alcohol, the mould used up 0.036 % of alcohol to form this amount of malic acid. The presence of fumaric and succinic acids was not noted.

The second portion of solution A was acidified with sulphuric acid and steam-distilled. The distillate was neutralized and evaporated to 100 ml., and formic acid was determined in 40 ml. by the method of Fincke [Abderhalden, 1925]; the amount found was 0.0112 g. Assuming that one molecule of formic acid was formed from one of alcohol it appears that 0.131 % of the ethyl alcohol was required.

A residue of barium acetate (0.0315 g.) was obtained by adding baryta water to a second 40 ml. of the distillate and evaporating. It was calculated that the whole solution therefore contained 0.073 g. of acetic acid, which indicates that 0.84 % of the alcohol was used for its production.

Lead acetate added to solution B produced a precipitate from which 0.256 g. of citric acid was obtained. In all, the following amounts of citric acid were obtained: 0.471 g. from solution A + 0.256 from solution B = 0.727 g. of the acid. Taking one molecule of citric acid for three of ethyl alcohol, 0.511 g. of the latter was needed, namely, in our experiment, 5.947 % of ethyl alcohol.

After neutralizing solution C with ammonia, a precipitate was formed which was found to be calcium oxalate weighing 0.215 g. Assuming that for one molecule of oxalic acid one of alcohol is needed, the mould used 0.795 g. of the ethyl alcohol for its production.

Table I. *Results of action of B. cinerea.*

Substrate	Mycelium		% of substrate used by mould to form											CO ₂ and other products %	Total %
	Days	Wt. for five flasks g.	Formic acid %	Acetic acid %	Pro-pi- onic acid %	Suc-cinic acid %	Fumaric acid %	Malic acid %	Citric acid %	Oxalic acid %	Pyruvic acid %	Acetalde- hyde %			
2.5% Ca lactate + 1% CaCO ₃	12	3.06	0.0	Traces	Traces	0.0	Small amount	Small amount	8.3	15.0	Small amount	Small amount	27.4	50.7	
5% Ca lactate + 1% CaCO ₃	18	2.77	0.0	0.6		0.0	Traces	„	4.1	8.0	„	„	30.3	43.0	
5% NH ₄ lactate + 3% CaCO ₃	21	2.48	0.0	0.9		0.0	0.0	„	4.0	7.9	„	„	41.5	55.3	
5% NH ₄ lactate alone	18	2.92	0.0	0.6		0.0	0.0	„	0.8	0.0	„	„	56.9	58.3	
2.7% ethyl alcohol + 4% CaCO ₃	18	5.49	0.131	0.84	0.0	0.0	0.0	0.036	5.95	0.79	—	—	82.9	90.7	

The total consumption of alcohol by the mould was 90.7%, of which 5.947% was used for the citric acid, 0.036% for the malic acid, 0.131% for the formic acid, 0.84% for the acetic acid, and 0.795% for the oxalic acid. The remainder, i.e. 82.95% of the alcohol, was utilized chiefly for the formation of CO_2 and small amounts of unidentified products.

DISCUSSION.

The object of the experiment was primarily to draw attention to the behaviour of *B. cinerea* upon ethyl alcohol as a medium. The alcohol was found to be a good medium since over 90% of it was consumed, of which over 80% was oxidized. This oxidation leads by the simplest route to CO_2 as is shown by the presence of acetic and formic acids.

The observation that acetic, malic and citric acids are found on the ethyl alcohol medium, confirms our previous researches on the formation of citric acid from ethyl alcohol [Chrzęszcz *et al.* 1932].

If we now examine the behaviour of *B. cinerea* on lactate we are first of all struck by the fact that in every case examined during our experiments citric acid is formed from lactic acid. The formation of acetic, fumaric and malic acids from lactic acid on the one hand confirms our view that citric acid is formed from acetic acid [Chrzęszcz & Tiukow, 1930; Chrzęszcz & Zakomorny, 1936], and on the other hand indicates that the views of Wehmer [1929], Thies [1934], and Acklin [1929], that lactic acid under the influence of moulds undergoes transformation only into CO_2 or into oxalic acid, are incorrect. It is also of interest to note that ammonium lactate alone, which Thies states is transformed into oxalic acid by the action of moulds, in our experiments yielded absolutely none of this acid, which, however, appeared on the addition of excess CaCO_3 .

Propionic acid is formed from lactic acid by way of acetaldehyde, as explained by the following reactions, which also cover the formation of the other products:

It follows from the above that the action of *B. cinerea* on lactates first causes the transformation of lactic acid into pyruvic acid which undergoes decarboxylation forming acetaldehyde. The acetaldehyde is either oxidized to acetic acid, or with a molecule of lactic acid undergoes dismutation into propionic acid and acetic acid.

The acetic acid formed is changed via succinic, fumaric and malic acids into citric acid, or is oxidized via glycollic, oxalic and formic acids into CO_2 and water. It is possible that in older cultures the citric acid formed also undergoes decomposition.

SUMMARY.

1. Citric acid can be formed from lactic acid by the action of the mould *Botrytis cinerea* on lactates.

2. Apart from citric acid, various other products are formed from calcium

lactate, viz. acetic, propionic, fumaric, malic, pyruvic and oxalic acids, acetaldehyde and, in addition, CO_2 .

The above compounds are also formed from ammonium lactate, with the addition of calcium carbonate, but the formation of oxalic acid was not observed in the case of ammonium lactate alone without the addition of calcium carbonate.

3. The products yielded indicate that lactic acid passes by dehydrogenation into pyruvic acid, next into acetaldehyde which then undergoes further transformation. With lactic acid, propionic acid and acetic acid are formed by dismutation. Acetic acid is also formed by the direct oxidation of acetaldehyde and changes on the one hand into citric acid, via succinic, fumaric and malic acids, in agreement with our theory, or into CO_2 via formic and oxalic acids on the other hand.

4. *Botrytis cinerea* also easily utilizes ethyl alcohol, transforming it partly into citric acid in agreement with our theory, via glycollic acid and then malic acid. The final oxidation proceeds via formic acid.

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