LXXII.—*A New Iron Bacterium.*

By Ernest Moore Mumford.

Having been recently occupied with a short study of *Clamydothrix ochracea*, the author visited the Bridgewater canal tunnels at Worsley, Lancashire, with the view of examining the growths found there, since the waters of the tunnels and basin are strongly charged with iron, due to the entrance of the colliery pump water.

The basin into which the tunnels debouch is connected to the Bridgewater canal by two short sections of canal, and these, together with the tunnels and basin, are practically disused by traffic. From the two tunnels there issues in one case fresh, clear water without iron, but from the other—the deeper of the two—a yellow, opaque liquid, which colours the whole basin a deep ochre.

The walls of the basin, the woodwork, the grass and trees dipping into the water are covered with a reddish-yellow growth, which at first sight appears to be an aggregation of *Crenothrix* or *Cladothrix*, but it was found that, although the growth and precipitated material were composed of ferric hydroxide, the most careful search failed to reveal more than one or two strands of higher bacteria. Since these were totally insufficient in numbers to account for the mass of ferric hydroxide, a careful bacteriological dilution and plating was carried out which resulted in the isolation of the organism causing the growth, which was found to be a hitherto unnamed bacillus.

Obtained from these sources, the pure culture of the bacillus was used to investigate the specific action of the organism.

For want of a suitable name, the organism will be designated in this paper under its laboratory number, "Bacillus M. 7."

Description.

Growths on :	
Potato	Greenish-brown modules, rising high in the middle, spreading very slowly.
Milk	Coagulation, liquid becomes straw-coloured, no acidity.
Gelatin-peptone-bouillon	Liquefaction from surface and line of stab. Thick, white masses at the bottom of the liquid.
Peptone-bouillon	Opalescence, whitish strings appear.
Glucose in gelatin-peptone-bouillon	Shake culture—no gas.
Peptone-water	No indole reaction.

Optimum temperature, 35—37°.

The organism is a facultative aerobe, and is of the average dimensions: 2·2 microns × 0·4 micron.

It is ciliated and exhibits a very varying motility; it occurs singly, and in short chains of three or four units.

The organism can form an endospore, but the majority of the bacilli under unfavourable conditions form a resistent involution form of varying shape, the general appearance of which is very similar to that presented by *Bacillus subtilis* under similar conditions.

An attenuated medium favours spore formation; a rich medium charged with the products of bacterial life favours involution forms.

Action of the Organism on Iron Solutions.

Since the organism is a truly facultative one, the study of its action falls naturally into two sections, namely, (i) under aerobic conditions, and (ii) under anaerobic conditions.

(i) *Under Aerobic Conditions.*

In both ferrous and ferric solutions it was found necessary to supply a trace of nitrogen. This was done by the addition of 0·005 per cent. of peptone, since the entire absence of nitrogen prevents the growth of the organism.

(*a*) *Ferrous solutions.*—A solution of ferrous ammonium sulphate (0·05 per cent.) was used. Complete precipitation of ferric hydroxide took place in twenty-four to thirty-six hours at 37°. The reaction is essentially aerobic, and proceeds to completion, no iron remaining in solution.

(*b*) *Ferric solutions.*—A solution of ferric ammonium citrate (0·05 per cent.) was used. Complete precipitation took place in twenty-four hours at 37°. In this case also the action was complete, no iron remaining in solution.

With regard to the necessity of nitrogenous material in the media, it is the opinion of the author that this is necessary, not primarily for the metabolism of the organism, but to provide, through the metabolic action, highly basic amino-compounds which unite with the highly ionised acids freed by the precipitation of the metallic bases by the action of the organism, since a small amount of precipitation takes place in the absence of nitrogen, but the action quickly ceases.

In support of this view may be mentioned the oxidation of phenol by *Bacillus helvolus*, which action can proceed without any nitrogenous material in the media, since the acid formed is ionised

only to a small degree. This is also in accordance with the effects of acid concentration in fermentations of cellulose.

(ii) *Under Anaerobic Conditions.*

The organism will not precipitate ferric hydroxide from either ferrous or ferric solutions in presence or absence of peptone. If, however, ferric hydroxide is originally present, it is changed into bog ore. This can be shown with ferric hydroxide precipitated either biologically or chemically. In this case, also, a minute trace of nitrogen is essential, but increasing quantities accelerate the speed of reaction.

This the author regards as being in accordance with the previous observations with regard to nitrogen in relation to aerobic precipitation, since in this case, when there is no acid product formed, the organism can produce the change to completion in the absence of nitrogen outside its own content, but if nitrogen is added, it is used solely in establishing the protoplasm of new organisms, and therefore increases the speed of reaction, but does not affect the completeness. The reaction takes about fourteen days at 37°.

The medium taken consisted of 500 c.c. of tap water, in which were suspended 5 grams of ferric hydroxide chemically prepared, and in which were dissolved 2·5 grams of peptone. This was inoculated, exhausted, and allowed to ferment at 37° for eighteen days. The flask then contained a clear, straw-coloured liquid and a black solid. No gas was evolved. The product was filtered and examined.

The residue, which was black, was dried and analysed. (Found, $FeO = 9{\cdot}00$; $Fe_2O(OH)_4 = 91{\cdot}00$ per cent.) It consisted therefore of bog ore.

The filtrate was a yellow liquid, neutral towards litmus, but basic to acids, which on evaporation in a vacuum gave a yellowish-white solid having an unpleasant odour. On esterification, this solid yielded a mixture of the esters of amino-acids. If this filtrate is added to solutions containing iron (composed as before), precipitation takes place under certain conditions.

This naturally suggests that the organism produces an enzyme which is the cause of these reactions, and it was therefore necessary to determine if an enzyme could be separated and used to produce the effects associated above with the living organisms. It was found that if a solution in which the organism was growing freely was filtered through a Chamberland candle, the filtrate had the power of producing the reactions associated with that living organism.

This property is extended to the filtrate irrespective of the

presence of iron in the medium. It is produced only in minute quantities in the absence of nitrogen, but in order to secure the most reactive enzyme, the latter was produced by the action of the organism on peptone water.

Isolation of the Enzyme.

Peptone water, containing 10 grams per litre, is prepared and sterilised in the usual way. It is then inoculated, incubated at 37° for twenty-four hours, and filtered through a Chamberland candle. Thymol is added to the solution to prevent further bacteriological action.

The most reactive enzyme is obtained after twenty-four hours' incubation. After a longer time it becomes weaker and disappears, being probably broken up by the bacteria as the pabulum becomes exhausted.

The enzyme is not destroyed by boiling or by evaporation in a vacuum, but is destroyed on being kept for forty-eight hours.

Reactions of the Enzyme.—The filtrate is not precipitated by picric acid, alcohol, ammonium sulphate (saturated solution), or sodium phosphotungstate (saturated solution), but is precipitated by Millon's reagent.

A white solid is obtained by evaporation in a vacuum. This white solid was, with the view of an approximate identification, esterified. On distillation and fractionation, several esters were separated, which were esters of the amino-acids usually associated with the partial, bacterial degradation of complex proteins. Some acids containing sulphur were also present.

Experiments carried out with the Filtrate containing the Enzyme.

Qualitative tests showed that the filtrate was able to produce the characteristic actions of the organism.

Solutions of ferrous salts are oxidised and precipitated; solutions of ferric salts are precipitated.

Analysis of the precipitate showed it to consist of ferric hydroxide in all cases. With the view of determining the optimum temperature of the enzyme, the simple precipitating reaction was studied.

Solutions of 10 c.c. of 0·05 per cent. of ferric ammonium citrate and 0·5 c.c. of the filtrate were used with the following result:

Precipitation at	Time.
37°	1·5— 2 hours
60	20—25 minutes
70	10—15 ,,
80	20—25 ,,

On boiling, partial precipitation took place in five to six minutes. If 1, 5, or 10 c.c. of the enzyme are taken, the speed is not increased.

The optimum temperature of the enzyme thus appears to be 70°.

Experiment has also shown that when the ratio between the filtrate and the iron solution varies between one part of filtrate and one part of iron solution, and one part of filtrate and fifty parts of iron solution, the speed or completeness of the precipitation is not appreciably affected. The precipitate in all cases is ferric hydroxide.

Character of Enzyme.—The filtrate was examined in the above manner after having been treated thus: (i) Fresh filtrate; (ii) filtrate kept overnight; (iii) filtrate boiled; and (iv) filtrate evaporated to dryness and extracted with water.

i.	Reactions as above	Active
ii.	No precipitation	Inactive
iii.	Reaction as (i), but slower	Active
iv.	Reaction as (i), but very slow	Active

The boiled solution only decomposes and becomes inactive in the course of several days.

Basicity.

Active.—50 c.c. fresh filtrate required 4·2 c.c. $N/10$-acid.

Inactive.—50 c.c. (kept overnight) required 7·5 c.c. $N/10$-acid.

Active.—50 c.c. boiled liquid required 12·8 c.c. $N/10$-acid.

There is therefore no apparent relation between basicity and enzymatic power. The fresh solution is more active than the boiled solution, although the latter precipitates at 70° in twenty to twenty-five minutes.

With regard to the mechanism of precipitation, the chief points may be summarised as follows:

The precipitation is produced by a crystalloid product formed by the organism from nitrogenous matter.

The optimum temperature of this crystalloid product, known as the "enzyme," is 70°.

It has not been found possible to produce the enzyme in appreciable quantity from media containing no nitrogen.

Starch and dextrose solutions in the presence of salts do not yield the enzyme in any appreciable quantity.

Therefore the organism, although living and reproducing in certain media, cannot produce the precipitating product except under certain conditions.

Since this is so this substance will depend for its nomenclature on the definition of an enzyme. Two broad conceptions of an enzyme are possible, namely, a product of the normal metabolism of the organism, and a product of the metabolism of the organism

dependent for its formation on the nature of the surrounding medium.

This enzymatic substance falls under the second definition, but not under the first, so that this precipitating agent must be described, according to the definition accepted, in the one case as a chemical precipitant produced by the organism under certain conditions, in the other case as a true enzyme.

Summary.

The bacillus is a true facultative organism, preferably an aerobe, and exercises a specific action on iron solutions.

The action of the bacillus on iron solution appears to proceed in two stages, in which the aerobic and anaerobic actions appear to be symbiotic, at any rate in nature.

The aerobic action is to precipitate ferric hydroxide from iron solutions, whilst the anaerobic action is to transform the ferric hydroxide thus precipitated to bog ore with partial reduction of the iron to a ferrous state.

To this organism are probably due the deposits of bog ore hitherto associated with the higher bacteria, since the latter have not the facultative power necessary to dehydrate and reduce the ferric hydroxide to bog ore.

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FRANKLAND RESEARCH LABORATORY,
THE UNIVERSITY, MANCHESTER.