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Further Researches on *Pseudomonas extorquens* Bassalik — A Microorganism Utilizing Oxalic Acid

L. JANOTA

The first fundamental work on bacteria capable of breaking down oxalates was published in 1913 by K. Bassalik who isolated from the excreta of earth-worms a new species of bacteria and described the morphological and physiological properties of these microorganisms which he named *Bacillus extorquens*. The species was the only one among 90 others which utilized oxalates rapidly and completely. Two other species also utilized oxalates but the breakdown was not complete.

In recent years the list of microorganisms capable of breaking down oxalic acid has been greatly enlarged but usually the process is not fully completed. Bhat and Barker (1948) succeeded in isolating a species of bacteria capable of breaking down oxalates which they named *Vibrio oxaliticus*. Khambata and Bhat (1953, a, b) reported the presence in the intestines of earth-worms of as yet undescribed bacteria utilizing oxalates, *Pseudomonas oxalaticus* and *Bacterium oxalaticum*. The ability to decompose oxalates is exhibited too by Streptomycetes (Khambata and Bhat, 1954) and *Mycobacterium lacticola* (Khambata and Bhat, 1955). Other bacteria breaking down oxalates were described by Jayasuriya (1954, 1955) and Stárka (1955).

All these reports indicate that the ability to utilize oxalates is much more common than has been believed at first. The microorganisms breaking down oxalates may be quite distant systematically and because of this the problems involved in their metabolism and primely in the metabolism of oxalic acid are of special interest. In 1949 the authoress (Janota, 1950) succeeded in isolating bacteria which in all respects were exactly similar to *Bacillus extorquens* isolated and investigated by Bassalik. According to Bergey's classification system *Bacillus extorquens* is defined as *Pseudomonas extorquens*. It was found that in accordance with Bassalik's report the bacteria were capable of binding inorganic carbon dioxide (Janota, 1956). The present investigations have been concerned in defining at what dilutions of the bacteria *Ps. extorquens* the rate with which oxalates are utilized is highest.

TWO DIFFERING FRACTIONS OF THE CENTRIFUGATED BACTERIAL MASS

In the experiments the strain *Ps. extorquens* isolated by the authoress in 1949 was used. This strain resembled exactly the strain isolated by Bassalik in 1909.

Ps. extorquens was cultivated on a liquid culture medium containing in every litre of distilled water:

0.5 g. of K_2HPO_4
0.1 g. of $MgSO_4 \cdot 7H_2O$
0.02 g. of $FeSO_4 \cdot 7H_2O$
0.3 g. of NaCl
1.4 g. of $(COO)_2(NH_4)_2 \cdot H_2O$

pH of the medium was $\sim$ 7 and the temperature $28^0 \pm 2^0$C.

A careful macroscopic inspection of the bacteria separated by centrifugation from the culture medium after three days of cultivation revealed that the mass of bacteria was not uniform. It consisted primely of yellow-brown clots and of a smaller amount of a pink precipitate. The former of these fractions will be referred to here as fraction Z and the latter as fraction R.

The fractions were very difficult to separate because when centrifugation was more rapid (4000 r.p.m.) only a part of fraction R was precipitated at the bottom of the test tube, whereas, part of it remained mixed with fraction Z. To obtain nearly complete separation it was necessary to centrifuge the cultures slowly (1000 to 2000 r.p.m.) several times and then wash the fraction Z. Moreover, it was difficult to divide the fraction Z itself and when some of the bacteria from that fraction had to be placed in another vessel the yellow-brown clots had to be torn apart with two needles. When placed in a test tube with distilled water the clots lay loosely at the bottom and when shaken rose in the water which remained quite clear. Not quite clear water proved that the fractions had not been separated accurately.

After centrifugation fraction R was obtained in the form of a very fine pink precipitate adherring to the bottom of the test tube. When shaken the bacteria formed a uniform suspension in which the intensity of the pink colouring depended on the amount of bacteria present. Microscopically the appearance of bacteria from both fractions differred in no respect.

In preliminary experiments the percentage of water in both fractions was established. It was found that in fraction Z the proportion of water was 94 per cent, whereas in fraction R it was 85 per cent. These results are the mean from three measurements the deviation being $\pm$ 2 per cent.

In the course of further experiments only the wet mass of bacteria was determined directly but from the above mean water contents of the dry mass of bacteria was computed.

RELATIONSHIP BETWEEN THE UTILIZATION OF OXALIC ACID BY FRACTIONS Z AND R

The bacteria were cultivated for three days on a liquid medium of the same composition as stated above at a temperature of 28°C, they were then centrifuged to separate the fractions Z and R. After separation bacteria from each fraction were divided into batches of known weight and every batch was placed in a separate Erlenmeyer flask containing each 25 ml. of the medium of the same composition as before. The initial amount of ammonium oxalate was the same in all experiments with an accuracy of ± 0.1 mg. The bacteria were left standing in the culture medium for 24 hours at 28°C and after this time the amount of the oxalate broken down was determined by titrating the medium with a solution of permanganate. The bacteria were transplanted on the medium in amounts corresponding to from 3.5 to 31 mg. of the dry mass. In the course of experiments it was found that there was no relation between the amount of the oxalate utilized and the amount of inoculated bacteria. This was true in the case of both fractions Z and R. On the other hand when the amount of the oxalate utilized was compared to some fixed unit amount of inoculated bacterial mass (in this case the amount was 10 mg. of dry mass) it was found that the rate of the breakdown increased as the amount of inoculated bacteria decreased. The increase was not great at first but then it became very rapid (fig. 1). For instance from the results obtained for the yellow fraction it appeared that when a batch of bacteria corresponding to 30.6 mg. of dry mass was inoculated it utilized in 24 hours only 12.2 mg. of oxalate which made only 4 mg. of oxalate for every 10 mg. of the dry bacterial mass. On the other hand, an amount corresponding to 12 mg. of the dry yellow mass utilized in the same conditions 14.8 mg. of the oxalate which amounted to 12.3 mg. of oxalate for every 10 mg. of the dry mass. It follows therefore that by reducing the amount of inoculated bacteria by 2.5 times the intensity of oxalate breakdown is increased 3 times. By further reducing the amount of the inoculated material this proportion will be further increased.

When comparing the rate at which oxalate was utilized by the fractions Z and R it was found that for small amounts of inoculated material fraction R used more oxalate than fraction Z, whereas, for large amounts of inoculated material this relation was reversed.

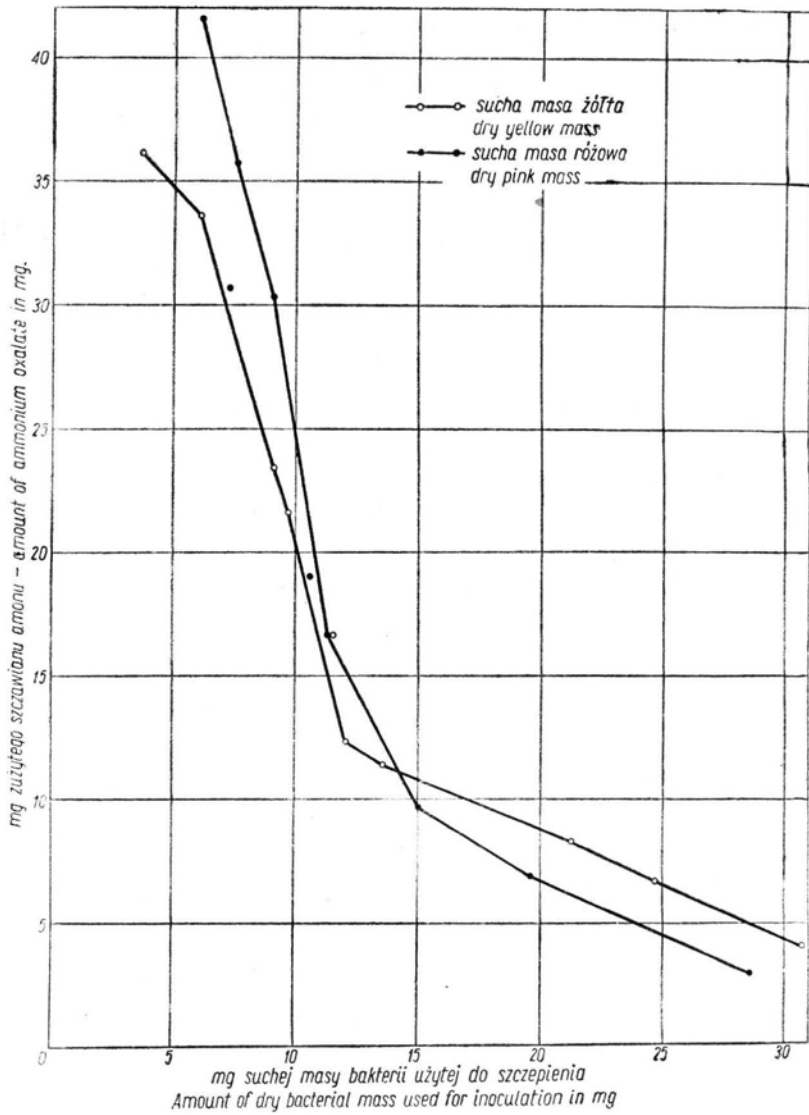

Fig. 1. Amounts of ammonium oxalate utilized by 10 mg. of dry mass of bacteria in relation to the amounts of inoculated material. **Time 24 hours, temperature 28°C.**

RELATION BETWEEN THE RATE OF UTILIZATION OF OXALATE AND THE DILUTION OF BACTERIA IN THE CULTURE MEDIUM

In the course of further experiments an attempt was made at finding the reason for the lack of relationship between the amount of oxalate utilized and the amount of inoculated bacteria. The first supposition was that some toxic products of the bacterial metabolism were introduced to

the medium with large amounts of inoculated bacteria, but this was rejected from lack of evidence (repeated washing of the bacteria did not increase the amount of oxalate utilized). Consequently, it was decided to define the conditions under which the rate of oxalate breakdown was constant regardless of the amount of inoculated bacteria. Fraction R, as

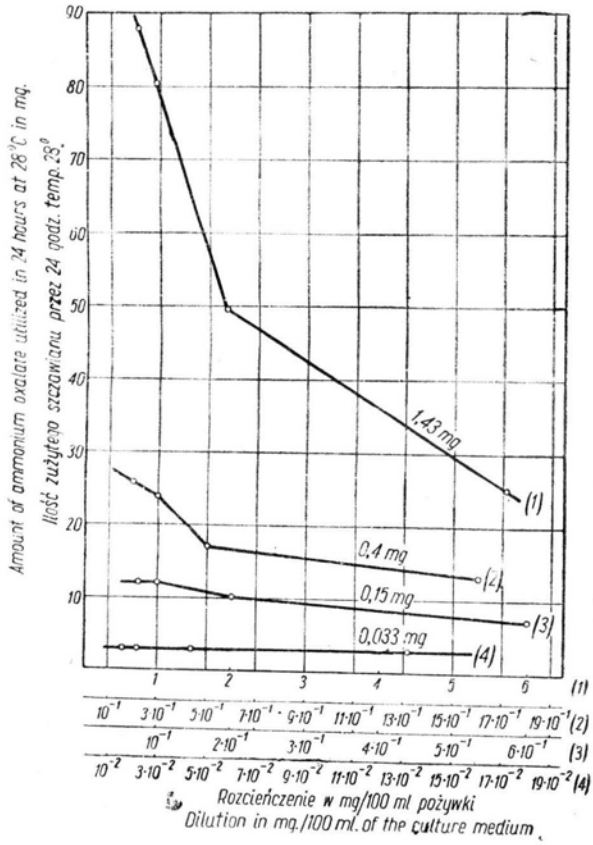

Fig. 2. Relationships between the utilization of oxalate by *Ps. extorquens*, the dilution of bacteria (mg. of the dry mass of pink fraction in 100 ml. of the culture medium) and the absolute amount of inoculated material.

Four scales are marked on the horizontal axis. Scale (1) corresponds to curve (1), scale (2) to curve (2) etc. The numbers alongside the curves denote the absolute amounts of the bacteria used for inoculation in the given experiment (in mg. of dry mass)

the most convenient, was used for these experiments because it could be prepared as a uniform suspension. The bacteria from three-day cultures were separated by centrifugation and a suspension was made in small amounts of water. The suspension was then filtered through a 3G4 Schott's filter. Fraction R passed through the filter, whereas, fraction Z did not. Another suspension was then made of the bacteria from fraction R and this after stirring was added in varying amounts to different

quantities of the liquid culture medium. The composition of the medium was the same as in the first part of the investigation. The amount of oxalate utilized was determined after 24 hours. In the first experiment the amount of bacteria inoculated in every flask corresponded to 1.43 mg. of dry mass and the amounts of the medium were such that the resulting dilutions were 5.7, 1.9, 0.71 mg./100 ml. In the second experiment the amount of inoculated bacteria corresponded to 0.4 mg. of dry mass and the dilutions were 1.6, 0.5, 0.3, 0.2 mg./100 ml. In the third experiment the amount of bacteria for inoculation in every flask was 0.15 mg. of dry mass and the resulting dilutions were 0.6, 0.2, 0.1, 0.075 mg./100 ml. Finally, in the fourth experiment the amount of bacteria inoculated in every flask corresponded to 0.033 mg. of dry mass and the dilutions obtained were 0.132, 0.044, 0.022, 0.0165 mg./100 ml. The results of all the four experiments are shown in fig. 2 where the relationships existing between the utilization of oxalate, the dilution of bacteria and the absolute amount of the inoculated material are demonstrated. It was found that for dilutions greater than 10^{-1} mg. of dry mass of bacteria in 100 ml. of the culture medium the breakdown of oxalate was dependent not on dilution but on the absolute amount of the inoculated material only (curves 3 and 4). In these dilutions the relation between the oxalate utilized and the amount of inoculated bacteria is nearly directly proportional ($0.033 : 0.15 \approx 3 : 12$). At concentrations higher than 10^{-1} mg. of dry bacterial mass in 100 ml. of the culture medium the amount of oxalate utilized by some one given amount of bacteria is smaller the greater the concentration of bacteria in the medium (curves 1,2, and 3).

DISCUSSION

One of the results obtained in the course of this investigation is the separation of the bacteria *Ps. extorquens* into two fractions with the same physiological ability of utilizing oxalates but differring by the water content of the cells, the appearance and the colour of the centrifuged mass. The higher rate of oxalate breakdown by the pink fraction when the amount of the inoculated material is small, i. e. in conditions more resembling natural ones (fig. 1), and the higher proportion of the pink fraction in younger cultures indicate that fraction R contains cells younger than those which compose fraction Z.

The other result reached at in the course of the experiments is the determination at what dilution of bacteria the rate with which oxalates are utilized is highest. The lower limit of dilution is 10^{-1} mg. of the dry pink mass in 100 ml. of the culture medium. At higher concentrations of the inoculated material the intensity with which oxalate is utilized drops, slowly at first and very rapidly later. It is to be noted that in all ex-

periments, even when the concentrations of the inoculated material were highest, the culture medium was always in excess and the lack of oxalate was never the limitting factor. In spite that a satisfactory explanation of the drop of bacterial activity when a larger amount of cells is inoculated to the same amount of the culture medium is still lacking, the discovery of this effect has a great methodological significance. Especially dangerous errors may be committed when large amounts of the bacterial material are used for experiments in batches which are not of the same size and the results are referred to some unit amount. In the previous work on the metabolism of *Ps. extorquens* (Janota 1956), in which Warburg's method was used for measurements and the amounts of inoculated bacteria varied, merely approximating 10 mg. of dry mass per flask, the results were referred to a unit of weight of the dry mass. At that time the circumstance that the concentration of bacteria affects so strongly the intensity with which oxalates are utilized was not yet discovered, but nevertheless, the amounts then used gave in effect results with an error that was undoubtedly smaller than it could be (fig. 1). However, on the basis of the results arrived at in the present work the amounts of bacteria used for measurements should always be equal and the dilutions should be such as to guarantee the most favourable conditions for the breakdown of oxalates by *Ps. extorquens*, i. e. the dilution should be greater than 10^{-1} mg. of the dry mass of bacteria per 100 ml. of the culture medium.

SUMMARY

Bacteria *Ps. extorquens* centrifuged out of the culture medium were separated into two fractions of different colour. One of the fractions, the yellow-brown one, consisted of compact clots difficult to separate. The pink fraction was obtained after centrifugation in the form of a fine precipitate which gave a uniform suspension in water. The water content in the yellow-brown fraction was 94 per cent and in the pink fraction 85 per cent. Microscopically both fractions were exactly alike. The intensity with which oxalates were utilized when the amounts of inoculated bacteria were small was higher in the case of bacteria from the pink fraction.

It was found that when the concentration of inoculated bacteria was higher than 10^{-1} mg. of dry mass in 100 ml. of the culture medium the breakdown of oxalate by *Ps. extorquens* was not proportional to the absolute amount of bacteria. The proportional relationship was exhibitted at lower concentrations, i. e. when conditions resemble more closely natural ones.

The experiments were carried out at the Department of Plant Physiology of the Warsaw University. The authoress wishes to express her most sincere gratitude to the Director of the Department, Professor K. Bassalik for his kindness and the complaisant interest in the progress of the work.

STRESZCZENIE

Bakterie *Pseudomonas extorquens*, odwirowane od pożywki, rozdzielono na dwie frakcje o różnym zabarwieniu. Jedna z nich, frakcja żółtobrązowa składała się z kłaczków trudnych do rozerwania. Druga, różowa, mająca po odwirowaniu wygląd drobnego osadu, dawała w wodzie jednolitą zawiesinę. Frakcja żółtobrązowa zawierała 94% wody, frakcja różowa 85% wody. Wygląd mikroskopowy bakterii należących do obu frakcji był identyczny. Intensywność zużywania szczawianów przy małych ilościach szczepionych bakterii, była większa u bakterii należących do frakcji różowej.

Stwierdzono, że w przypadku gdy stężenie szczepionych bakterii jest większe od 10^{-1} mg suchej masy na 100 ml pożywki, zużycie szczawianu przez *Ps. extorquens* nie jest proporcjonalne do ich bezwzględnej ilości. Proporcjonalność ta jest zachowana przy mniejszych stężeniach, a więc w warunkach bardziej zbliżonych do naturalnych.

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