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NONCELLULAR NITRIFICATION
GROWTH OF CULTURES OF NITROSOMONAS AND FORMATION OF CELL AUTOLYSATES, by
A. A. Imshenetzkii and E. L. Ruban

Two steps are usually followed in studying the biochemical conversion conditioning the activity of the microorganism. The characteristics of the process causing living reproducing microbial cells, are first established. therefore, it is attempted to reproduce the same process without cell presence by the use of culture filtrates and fermentation preparates. I do not consider the important successes of microbe physiology and biochemistry, the many conversions of carbon, nitrogen, sulfur and of other elements known to us as much as the basic studies of dynamic changes, resulting in microorganism cultures. Proof of the fermentative character of a series of processes have not been positive so far. Attempts to describe new ferments, on the basis of tests with diseased microbe cells have been made at times. (5). This is hardly justified, since major differences between microbial culture tests and experiments with diseases cells do not exist. Concerning fermentation action we have a right to speak only after reproduction of a certain chemical process in liquids, not containing microbes. Earlier we often spoke of "extra-cellular" fermentations or "extra-cellular" oxidations. Such a meaning cannot be accepted as accurate, as it stresses that a given chemical process takes place outside the cells, the term "noncellular" fermentation therefore should be given preference.

Sixty years have passed since S. N. Vinogradski (1) completed his brilliant work on nitrification. During this period, our knowledge of the biology, the spread and ecology of nitrification has been greatly completed by new information. In contrast to this, the chemical process of nitrification remains

unclear. It is quite plausible that acidification from ammonia to nitrites takes place in the presence of a number of fermentatives, however we know nothing concerning them. Among these the possibility of ammonia acidification by fermentation, contained in nitrosomonas cells, would open a wide perspective in studying the mechanics of the nitrification process. Research in non-cellular alcohol fermentation sufficiently shows the large part it plays in clearing up the various steps.

Only one attempt is known to have attained nitrification in a medium without nitrosomonas cells. It was conducted by V. L. Omelyanski (4), who ground nitrosomonas cells with washed sand from the sea, and then added distilled water and a solution of ammonia sulphate. During these tests, he did not observe ammonia reduction under the influence of the nitrificate fermentative cell, and therefore came to the conclusion "that the chemical activity of these microorganisms is apparently, indisolubly linked to its living cell". It should be added that Omelyanski's method is not above criticism. The basic tests were conducted with nitrosomonas cells from extremely old cultures (four month), the nitrificate cells before the grinding had dried above sulfuric acid, which could not but affect the fermentative activity.

In our research, because of necessity, we accumulated great quantities of nitrosomonas in cultures, so as to have a possibility of obtaining an active filtrate, not containing cells. Considering the great importance of research methods mainly because a detailed description gives other scientists an opportunity to continue research, this article is consecrated to a description of the methodical manner of operation. The results of the first

series of tests on noncellular nitrification will be given in the next publication.

GROWTH OF NITROSOMONAS CULTURES

Research was conducted with pure nitrosomonas cultures isolated from the soil. The methods of pure nitrosomonas culture production and types of purity tests were previously described (2, 3). An attempt was first made to obtain a large quantity of nitrative cells by placing them in a large number of dishes of gelatinous silicic acid, subsistance of Vinogradski's mineral media. The jelly's surface was covered with a layer of chalk, as usual. A simple tool was used for placing large numbers of drops on the jelly surface, it consisted of a type of cork stopper of large diameter, into which a large number of pins had been pushed in a chess board pattern. The cork with the pins was sterilized, the pin heads were dipped into a live nitrosomonas culture, after which the pin heads were brought into contact with the jelly surface. By this method, a large number of jelly surface areas were seeded simultaneously. It was to be expected that hence the appearance of zones of chalk solution, increasing in size, would thinly cover the entire jelly surface with nitrosomonas cells. But from the large quantities of nitrificated cells obtained by the method of seeding jelly surfaces had to be rejected, because zones were formed only where drops had been placed, cell deposits on the jelly were meager, and taking this deposit from the jelly surface was extremely difficult. Far better results were obtained with deep growing nitrosomonas cultures in large flasks, through which air was circulated. The cultures were conducted in 15-20 l capacity flasks. 3 l of a nutrifying bacteria medium described below were poured into every bottle. The culture layer in the bottle reached 8-9 cm. The bottles,

closed with cotton stoppers, were sterilized for 30 m at 0,5 atm. The following solution was used to replace Vinogradski's medium for nitrosomonas cultivation: $(NH_4)_2SO_4$ - 2,0g; K_2HPO_4 - 1,0g; $MgSO_4$ - 0,5g; NaCl - 2,0g; $FeSO_4$ - 0,4g; microelemental mixture ($LiSO_4$, $CuSO_4$, $ZnSO_4$ $Al_2(SO_4)_3$, $SnCl_2$, $MnCl_2$, $NiCl_2$, $CoSO_4$, $TiCl_4$, KBr) 1 ml; 1000 ml of distilled water, 1,0g completely ground chalk; pH approximately 7,2 - 7,4. As seed material, 10-20 day old nitrosomonas culture was used, which had grown in a 200 ml. Erlenmeyer flask. The flask contained 20 ml nutritive media, the composition of which has been given above. The culture was inoculated at 24°. The contents of one flask, i.e., 20 ml of culture, were placed in every bottle containing 3 l of sterile medium. After insertion of the seed material every bottle was closed with a resinous stopper, through which small glass pipes were placed at 3-4 mm, not reaching the bottom of the bottles. Air penetrating through the tubes aerated the cultures. The air passage velocity being of 15-20 l per minute. The penetrating air had previously been subjected to sterilized tubes and cotton wool filters. The air from the bottles followed short glass tubes, also penetrating the resinous stoppers and furnished at the outside end with cotton wool filters. The culture bottles were incubated at 22-24° for 10-12 days. The culture level was marked on the side of the bottle and sterile distilled water was periodically added to offset evaporation. The presence of chalk was unfortunate, as it hindered the results of cell production when freed of chalk, dilution of the chalk with acids could harm the bacterial virulence. In connection with this an attempt was made to grow nitrosomonas in a nutritive element without chalk, but in the presence of a phosphate buffer (pH - 7,1 ▪ 7,2). But under these conditions nitrificate development was even worse, as chalk is necessary not so much as a

neutralizer, as to produce nitric acids.

Apparently, nitrificates develop more readily, by attaching to the flat chalk particles. During the course of work it was established, that interruption of culture aeration, i.e., air passage through the medium, even for 2-3 hours markedly cuts the intensiveness of nitrification development. The volume of air, passing through the culture during a minute, should as far as possible be steady during the entire operation. During the progress of the cultures quality tests of ammonia (Nessler reaction) and nitrite (Griss reaction), were conducted. After development completion the nitrosomonas cultures were tested for purity by seeding in meat-peptone media and microscopically studying the deposit, taken from every bottle.

OBTAINING OF AUTOLYSATES, FREE OF BACTERIAL CELLS

After 10-12 days approximately 9 l of liquid culture (from 3 flasks, each containing 3 l of culture) were filtered through a Zeitz filter (of 13.5 cm. diameter) with a No. 3 filter membrane. The filter deposit was washed with sterile distilled water until the ammonia and nitrate had vanished. 6-7 l of water were necessary to do this. After this the filter and deposit were placed in a large sterile petri dish, it was taken from the filter by means of a glass spatula, it was placed in a sterile agath mortar and was carefully ground with sterile glass dust for 30 m. The deposit, as was to be expected, was largely composed of chalk and salt crystals (mostly of the phosphate type), and a certain amount was composed of nitrosomonas cells. A usual deposit was of approximately 20.0g, to which were added 0.5-0.8g of glass dust (Shottov glass). 30 ml of sterile water was added to the ground deposit and after mixing it was placed in a dry, sterile Erlenmeyer flask of

100 ml capacity. The flask was closed with cotton wool stoppers and with double cellophane caps, after which it was placed in an incubator at 40° for 24 hours. A certain autolysis of the nutrificate bacterial cells took place during this period. After one day the liquid with the ground deposit was divided into two portions. With an aim to get rid of nitrosomonas cells, a certain portion was filtered through a small Zeitz filter (35 m in diameter) with an SF asbestos filter. The filter was directly connected to a sterile container and the filtrate passed into a sterile Erlenmeyer flask of 50 ml capacity without decanting. Tests were conducted with the first portion of filtrate, used as a control, to determine the nitric contents by the method of colorimetry with Griss reactive, ammonia - by the vacuum elimination process, and also evaluating total nitrogen contents by Kieldal's micromethod. The second portion of filtrate, serving for basic tests, was placed at 37° for 5 days and after this analogous determinations of nitrate, ammonia and nitrogen were produced. By comparing numbers, received through analysis of both test and control portions, one could list the changes in ammonia and nitrate contents which had taken place in the solution which did not contain bacterial cells. The filtrate sterility convinced us of the seeding method on meat-peptone media, and also on Vinogradski media for bacterial nitrification. The latter was necessary, for one could have assumed, that filterable nitrosomonas shapes, passing through filter, could change to vegetative cells, which in turn could cause ammonia acidification.

Autolysis filtrates, obtained through heterotrophic microorganism cultures were studied as a further control element. Pseudomonas sp., musobacterium rubrum and saccharomyces cerevisiae cultures were grown for the latter. The

microorganisms developed in a petri dish with ...A or with agar-wash. The microorganism deposit, growing on the surface of the level medium, was taken and rubbed with sterile glass dust. The following tests and analyses were conducted in the same way as with the nitrosomonas cells. In an aim to simulate the nitrosomonas growing conditions, Pseudomonas sp. were cultivated in flasks, containing Vinogradski's medium. A further facet of normal cellular seeding. The ammonia and nitrate readings in the autolysis filtrate, obtained from heterotrophic bacterial cells, proved to be extremely valuable, as the ammonia volume did not diminish as in nitrosomonas cell autolysates, but rose in proportion to the resulting deaminification. The analytical data concerning this problem will be given in the following number.

CONCLUSIONS

1. A large quantity of bacteria must be obtained if one is to complete an autolysis with nitrifying bacteria.

2. Nitrosomonas growth in a dish with jelly does not give the possibility of obtaining the necessary cell quantity.

3. Nitrosomonas development in deep ventilated culture conditions, allows one to accumulate sufficient biological masses in 10-12 days for autolysis.

4. Tested and described method of completing the nitrosomonas cell autolysis, independent from heterotrophic and nitrosomonas bacteria.