Influence of Corn Steep Liquor on the Oxidation of Ammonia to Nitrite by *Nitrosomonas europaea*

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SUMMARY: The oxidation of ammonia by *Nitrosomonas europaea* is accelerated in mineral liquid media by the addition of small amounts of corn steep liquor or its 'ash'. The amount of nitrite produced in such cultures usually exceeds the controls by 6-7 times after 6 days. However, in the following days nitrite-production becomes slower and after 14 days the controls have reached the same nitrite concentration as the corn steep liquor cultures. Concomitant with the accelerated nitrite-production hydrogen-ion concentration in the cultures increases considerably and may attain values as low as pH 6.3 after 14 days, as compared with c. 7.8 in the controls. The effect of corn steep liquor stimulation is more pronounced the smaller the inoculum used. Microscopical examination of slides immersed in cultures during incubation shows that *N. europaea* adheres to the glass surface only in cultures *without* corn steep liquor. The stimulatory factor in solutions of the 'ash' of corn steep liquor was not removed by dialysis nor by treatment with cation exchange resins. When the solutions were centrifuged both washed sediment and supernatant fluid were active. A possible explanation of the corn steep liquor effect is that colloidal matter in the solutions absorbs ammonia or any other nutrient in the medium in a way more favourable for their utilization by the organism.

The poor growth of autotrophic nitrifying bacteria in conventional simple chemically-defined media has been the subject of much speculation, and the opinion among many students of nitrification seems to be that one or more growth factors or inorganic micronutrients are needed for optimal growth of these organisms. This assumption is supported by two observations: (1) that nitrification usually proceeds faster in enrichment cultures than in pure cultures; (2) that viability of the bacteria in pure cultures tends to decrease. As it would be of great value for the study of the biochemistry of nitrification to obtain larger crops of organisms many attempts have been made to find growth-promoting substances. Unfortunately few of these attempts have met with success. However, Fred & Davenport (1921) reported that Nährstoff-Heyden (a preparation of egg albumin) stimulated nitrate formation by *Nitrobacter* spp.; this was later confirmed by Kingma Boltjes (1935) who found that the material was also effective for *Nitrosomonas* spp. Addition of 0.5% (w/v) Nährstoff-Heyden to a mineral agar medium resulted in larger colonies, and the same compound added to liquid mineral media inoculated with slowly nitrifying strains gave rise to enhanced ammonia- and nitrite-oxidation, respectively. Kingma Boltjes ascribed this effect of Nährstoff-Heyden to its ability to establish more favourable conditions of oxidation-reduction potential in the cultures. Winogradsky & Omeliansky (1899)

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reported that crude and ignited infusion of horse-dung, as well as soil extract, hastened the oxidation of ammonia in cultures. Hes (1937) also found that soil extract was active. Lees (1953) observed an irregular decrease in the lag-phase of *Nitrosomonas europaea* when biotin was added to the media. A stimulatory effect of iron has also been reported; Meiklejohn (1953) found that rather large quantities of ferrous sulphate (optimum at 6 mg. Fe/l.) stimulated oxidation of ammonia by the same organism in liquid mineral media.

The present communication comprises some results from experiments with corn steep liquor which has been found to accelerate the oxidation of ammonia by *Nitrosomonas europaea*. Unfortunately the organism degenerated and almost completely lost its ability to oxidize ammonia before this investigation was completed, and it has not yet been possible to obtain a new and active strain for the continuation of the experiments.

METHODS

The strain under investigation was isolated by Dr H. Lees and identified as *Nitrosomonas europaea*. The purity of the cultures was tested occasionally by transferring material to media able to support the growth of heterotrophic bacteria, viz. asparagine lucerne-extract agar, nutrient broth and a liquid mineral medium enriched with 0.5 % (w/v) glucose and 1 % (w/v) corn steep liquor. In cases where contaminants were found (rarely) the results were rejected.

A medium made up from tap water and the following salts was used throughout (% w/v): $(\text{NH}_4)_2\text{SO}_4$, 0.1; KH_2PO_4 , 0.025; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0125; NaCl , 0.0125; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0025; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.0025. Solid CaCO_3 (sterile) was added at c. 25 mg./5 ml. medium. The initial pH value was 8.0–8.3. This medium in 5 ml. lots in test tubes was autoclaved at 115° for 20 min. In most experiments the cultures were in triplicate, in some cases in quadruplicate. The incubation temperature was 25–28°.

Two different samples of corn steep liquor (CSL) were used. CSL stock solutions containing 1 % (w/v) of the material were prepared in distilled water, neutralized with N-NaOH, filtered, and sterilized by filtration through Seitz bacterial filters. CSL was added aseptically to autoclaved media. Solutions of ignited CSL (designated CSL-ash) were prepared as follows: water and volatile material of 20 g. crude CSL were evaporated in an oven at 105° for 2 days, and the hardened mass ignited at 600° in a quartz crucible. After cooling, 20 ml. distilled water were added and the suspension was boiled carefully for a few minutes, filtered through double filter-papers and the residue washed on the filter with 380 ml. hot distilled water. The filtrate appeared strongly opalescent and was used as stock solution without further clearance. This 5 % (i.e. equiv. 5 % CSL) solution was stored in the cold and, after dilution, added to the basal medium before autoclaving. Concentrations of CSL and CSL-ash referred to in the following paragraphs are expressed on an equivalent basis, namely, as % (w/v) of corn steep liquor.

Nitrate was estimated colorimetrically in a Coleman spectrophotometer, with the Griess-Ilosvay reagent.

RESULTS

Stimulation of nitrite formation by addition of CSL

In several experiments it was found that concentrations between 0.01 and 0.05 % of CSL resulted in a more rapid accumulation of nitrite in liquid mineral media inoculated with *Nitrosomonas europaea* (Table 1). Concentrations lower than 0.01 % had no effect, while 0.1 % and higher concentrations inhibited nitrification completely. Autoclaving a 0.02 % solution of CSL at 120° for 20 min. rendered it inhibitory, but autoclaving at 110° for 15 min.

Table 1. *Effect of corn steep liquor (CSL) on the oxidation of ammonia to nitrite by Nitrosomonas europaea*

Cultures sampled after incubation for 8 days at 25°.

Initial concentration in medium

CSL (%, w/v)	(NH ₄) ₂ SO ₄ (%, w/v)	NO ₂ -N formed (µg./ml.)
0	0	0
0	0.1	7.7
0.02	0	0.8
0.02	0.1	21.1

did not appreciably depress its stimulatory effect below that of a solution sterilized by filtration. Besides harmful organic substances in CSL it was thus evident that the material contained some factor which was able to stimulate either growth or respiration by *N. europaea* and the rather low effective concentrations made it seem probable that some organic growth substance or a mineral micronutrient might be responsible. In order to test the latter possibility a sample of crude CSL was ignited as described above and various concentrations of the resulting ash solution added to liquid media.

Stimulation of nitrite formation by CSL-ash

It was found that concentrations of CSL-ash corresponding to 0.01–1 % of the original CSL exhibited exactly the same stimulation of ammonia oxidation as did CSL itself. Maximum stimulation was found with 0.1 %; 0.001 % was almost without effect and 10 % was inhibitory. No additive effect was observed when CSL-ash (1 %) and CSL (0.02 %) were added simultaneously to the media. These results strongly pointed towards an inorganic stimulant.

The result of one of several identical experiments with 0.1 % CSL-ash is shown in Fig. 1. The stimulation (as measured by nitrite production) results in a decrease in the duration of the lag-phase and is most pronounced at the 6th day of incubation with an excess of nitrite-N in the CSL-ash cultures 6–7 times that of the controls. At the same time pH value in the cultures falls drastically, and when a pH value of c. 6.5 is reached nitrification becomes slower; after 14 days nitrite in the controls reaches the same level as in the CSL-ash cultures.

Role of size of inoculum

It was observed at an early stage that the size of inoculum greatly determined the results of the experiments. A fairly large inoculum (one drop, *c.* 0.05 ml.) containing suspended CaCO_3 from the stock culture gave irregular results and tended to hide the stimulatory effect of CSL. The best results were obtained with uniform droplets of the order of 0.01 ml., containing as little suspended matter as possible. It also proved to be important to use material

Fig. 1

Fig. 2

Fig. 1. Nitrite production and pH value in cultures of *Nitrosomonas europaea*. Δ , 0.1 % CSL-ash added; $\circ$, no addition of CSL-ash.

Fig. 2. Effect of different sizes of inoculum on the nitrite production by *Nitrosomonas europaea*. Inoculum: A, one drop (*c.* 0.01 ml.); B, two drops (*c.* 0.02 ml.); C, five drops (*c.* 0.05 ml.). Hatched columns = 0.02 % (w/v) CSL added; white columns = no addition of CSL.

from young (4–7 days) cultures as inoculum. The effect of different sizes of inoculum is shown in Fig. 2. With extremely small inocula the effect of CSL-ash was most pronounced (Fig. 3). In this experiment probably only a few organisms were carried over in the inoculum, resulting in the production of not more than 3 $\mu\text{g. NO}_2\text{-N/ml.}$ in the controls after 21 days, whereas in the CSL-ash media this amount was produced after only 5 days of incubation.

The role of the stimulating factor

After having established with certainty that crude as well as ignited CSL exhibited a favourable effect on the oxidation of ammonia by *Nitrosomonas europaea* it was of interest to find out whether the active material acted directly on the respiration process or indirectly by increasing the growth rate of the organism. A series of experiments dealt with in the following three paragraphs therefore were carried out.

Effect of CSL in solid media. Solid media were prepared from the basal medium with addition of 0.7–1.5 % (w/v) agar (Difco Bacto-Agar or Difco Special Agar Noble). In several experiments growth and nitrite production in these media usually were more or less retarded when CSL (0.02 %) or CSL-ash (0.1 %) was added. Only in a single case was a favourable effect of CSL-ash observed: after 11 days small colonies *c.* 0.5 mm. diam. had formed on the CSL-ash media, while on the control media colonies were visible only with a hand-lens. Also, the nitrite reaction was stronger in the CSL-ash media. As the cultures grew older the difference between the two series disappeared.

On silica-gel medium covered with a thin layer of CaCO_3 , solution of the carbonate started first in the controls and after 15 days 145 $\mu\text{g. NO}_2\text{-N/ml.}$ was formed as compared with 105 $\mu\text{g./ml.}$ in gels containing 0.1 % CSL-ash.

Experiments with suspensions of organisms + CaCO_3 . Cell material was obtained by growing *Nitrosomonas europaea* in 500 ml. lots of medium in 2 l. Erlenmeyer flasks, with occasional shaking. When all the ammonia had disappeared the cultures were centrifuged and the sediment (mainly consisting of CaCO_3 with adhering organisms) was washed free from nitrite with tap water. Finally, the material was resuspended in diluted (1/20) basal medium (no ammonia) and 50 ml. lots distributed in 300 ml. Erlenmeyer flasks. At zero time 1 % $(\text{NH}_4)_2\text{SO}_4$ was added and nitrite determined immediately and later at regular intervals. With these 'resting' suspensions no stimulation of nitrite formation was noted within 12 hr. with 0.04 % CSL, whereas a small stimulation was found with 0.5 % CSL-ash. The results of these experiments are shown in Fig. 4.

Certain uncompleted experiments

As mentioned above, the strain of *Nitrosomonas europaea* used in this work died before the investigation was completed. However, a few of the uncompleted experiments nevertheless are of some interest and may be described briefly.

Microscopical examination of cultures. It was observed that the part of the test-tube wall in contact with the medium always became more milky or whitish when there was no CSL in the medium than when CSL had been added. As this appearance might have been due to differences in the adhesion of the organism to the glass surface, carefully cleaned slides (18 × 76 mm.) were immersed in ordinary 5 ml. portions of medium, with and without 0.1 % CSL-ash. These culture vessels were autoclaved and inoculated in the ordinary way and the slides allowed to remain undisturbed in the tubes during incubation. The slides showed the same characteristic difference which had been observed on the test-tube walls: slides from CSL-cultures were clearer than those from the controls which showed an increasing whitish coating. After drying and fixation the slides were stained with phenol aniline blue and examined microscopically. Besides regular oval organisms of *Nitrosomonas europaea* evenly distributed over the surface many dividing organisms were seen. In one experiment the counts of organisms were 0.8 ± 2.0 (20 fields) in CSL-ash cultures compared with 48.5 ± 7.2 in the controls after 7 days of

incubation. In another experiment the corresponding figures were 15.8 ± 8.3 (10 fields) with CSL-ash and 135.3 ± 47.6 without CSL-ash after 12 days. These results show that the CSL-ash to a certain degree prevented the adhesion of the organisms to the glass surfaces. It therefore was expected that the number of free-living organisms in the liquid medium would be greater in the CSL-

Fig. 3

Fig. 4

Fig. 3. Effect of a very small inoculum on nitrite production by *Nitrosomonas europaea*. Δ = 0.1 % (w/v) CSL-ash added; $\circ$, no addition of CSL-ash.

Fig. 4. Nitrite production in dense suspensions of *Nitrosomonas europaea*; (two separate experiments). Δ = 0.5 % (w/v) CSL-ash added; $\circ$, no addition of CSL-ash; $\blacktriangle$ = 0.04 % (w/v) CSL added; $\bullet$ = no addition of CSL.

ash cultures than in the controls; but this appeared not to be the case as judged by the examination of drops of culture medium: no macro- or microscopical difference could be observed with certainty.

Ion-exchange treatment of CSL-ash solutions. A 0.5 % (w/v) CSL-ash solution was run six times through an Amberlite IR-100 cation exchange column. Subsequently, the liquid was neutralized with *N*-NaOH, diluted and added to medium to give a final concentration of 0.1 % in the medium. This treatment did not remove the stimulatory factor from the CSL-ash. Samples of the treated CSL-ash solution as well as untreated CSL-ash solution were run through an Amberlite IR 4B anion-exchange column; the solutions thus treated inhibited nitrite formation. Since this was also true with distilled water, the inhibition was probably because of dissolution of small amounts of the phenolic resin.

Dialysis of CSL-ash solutions. CSL-ash solution was dialysed and the two

fractions thus obtained tested for stimulatory effect on nitrite formation. Ten ml. of 5 % (w/v) CSL-ash stock solution (A) were dialysed against 90 ml. glass-distilled water (B) for 48 hr. at room temperature in a Visking bag 18/32. Fraction (B) was added to liquid media to give a concentration equivalent to 0.1 % CSL-ash. The (A) fraction was further dialysed against two lots (1 l.) of glass-distilled water for a total of 96 hr. and finally diluted to give a concentration equivalent to 0.1 % CSL-ash in the media. Only fractions (A) and (A)+(B) stimulated nitrite formation, whereas fraction (B) alone was without effect.

Centrifugation of CSL-ash solutions. CSL-ash solution (equiv. 5 % w/v) was centrifuged and the effect of washed sediment (A) and supernatant (B) tested for stimulatory effect separately and in combination. Centrifugation for 1 hr. at 5000 rev./min. resulted in a water-clear supernatant fluid but both fractions separately and in combination gave the same degree of stimulation as did uncentrifuged CSL-ash solution. A new sample of solution was centrifuged for 90 min. at 13,500 rev./min. The higher speed and the longer centrifugation time somewhat decreased the stimulatory effect of the supernatant fluid (fraction B) indicating that the CSL-factor might be centrifuged down if a sufficiently high value of g were applied.

DISCUSSION

Corn steep liquor (CSL) has found manifold uses in microbiology mainly because of its high content in growth substances (Liggett & Koffler, 1948). The observation that small amounts of this material accelerated the oxidation of ammonia by *Nitrosomonas europaea* therefore made it seem probable that one or more organic growth substances were responsible for the effect. However, this assumption was disproved by the subsequent experiments with ignited corn steep liquor solids. As yet no one has demonstrated that organic substances are necessary, or will stimulate the growth or respiration by *N. europaea*. The effect of Nährstoff-Heyden has been ascribed to its stabilizing effect on the oxidation-reduction potential, and the observation that extracts of horse dung and soil can act as stimulating agents does not necessarily mean that organic substances in the extracts are responsible.

Besides organic growth substances corn steep liquor contains a variety of inorganic materials. By spectroscopic analysis Koffler, Knight & Frazier (1947) found some 20 known biological trace-elements in this material. It seems that it must be the mineral fraction of corn steep liquor which contains the stimulatory factor examined in this present work, but it seems unlikely that cations or undissociated small molecules are responsible for the effect, judging from the experiments with dialysed and centrifuged CSL-ash solution and CSL-ash solution treated in cation-exchange columns. Physically a CSL-ash 'solution' is a rather complex liquid consisting of various ions and salts in true solution, macromolecules in colloidal solution and finally small particles in suspension. Although nothing could be stated with certainty most results point towards the colloidal fraction as being the active agent in CSL-ash.

Possibly macromolecules act as absorbers of ammonia or any other nutrient in the medium in a way favourable for the utilization of these nutrients by the bacteria, or they might change the conditions in other ways when present in cultures of *Nitrosomonas europaea*. In this connexion it should be added that the author has also found a small stimulation with solutions of ignited Nährstoff-Heyden.

The inorganic colloidal matter present in CSL-ash solutions is most probably not present as such in corn steep liquor itself. However, as the latter is a product of corn (maize) seeds it will contain organic colloidal matter which might possibly act in a manner similar to the inorganic macromolecules.

Further support for the 'colloidal theory' might be deduced from experiments made by Meiklejohn (1953) who found that iron stimulated the oxidation of ammonia by *Nitrosomonas europaea*: 6 mg. Fe/l. given as ferrous sulphate to inorganic media (before autoclaving) produced optimal effect. However, it does not seem likely that this stimulation was wholly due to iron since 6 mg./l. is far more than is needed as a nutrient for *N. europaea*. The stimulation by iron must partly be due to other circumstances; either the presence in the ferrous sulphate of a mineral contaminant which is active, or the ferrous sulphate acts unspecifically. It is known that ferrous ion in alkaline solution will soon become oxidized to ferric ion, which in turn will react with water to form colloidal ferric hydroxide. In experiments carried out by the author (unpublished) it was found that even ferric hydroxide (equiv. 50 mg. Fe/l.) when added to the inorganic medium stimulated nitrite formation to the same degree as adding 0.1 % CSL-ash solution. On the other hand, no stimulation was found with aluminium hydroxide or silica gel.

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