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Identification and Physiological Characterization of the Nitrogen Fixing Bacterium *Corynebacterium autotrophicum GZ 29*

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Abstract. The coryneform hydrogen bacterium strain *GZ 29*, assigned to *Corynebacterium autotrophicum* fixed molecular nitrogen under autotrophic (H_2, CO_2) as well as under heterotrophic (sucrose) conditions. Physiological parameters of nitrogen fixation were measured under heterotrophic conditions. The optimal dissolved oxygen concentration for cells grown in a fermenter with N_2 was rather low (0.14 mg O_2/l) compared with cells grown in the presence of NH_4^+ (4.45 mg O_2/l). *C. autotrophicum GZ 29* had a doubling time of 3.7 h at 30° C with N_2 as N-source and sucrose as carbon source and at optimal pO_2. Acetylene reduction reached values of 12 nmoles of ethylene produced/min × mg protein. Although the oxygen concentration in the growing culture was kept constant, the optimal dissolved oxygen tension for the acetylene reduction assay shifted to higher pO_2-values. The overall efficiency of nitrogen fixation amounted to 22 mg N fixed/g sucrose consumed; it reached a maximal value of 65 mg N fixed/g sucrose consumed at the beginning of the exponential growth phase. Intact cells reduced acetylene even under anaerobic test conditions; further anaerobic metabolic activity could not be ascertained so far.

Key words: Nitrogen fixation – Aerobic hydrogen bacteria – Oxygen sensitivity – Efficiency – Aerobic and anaerobic acetylene reduction – *Corynebacterium autotrophicum.*

Nitrogen fixation by aerobic autotrophic hydrogen bacteria has recently been reported by several authors (Ooyama, 1971; Gogotov and Schlegel, 1974; Aragno, 1975; de Bont and Leijten, 1976). Apparently all recently isolated nitrogen fixing hydrogen bacteria belong to *Corynebacterium autotrophicum* (Wiegel and Schlegel, 1976). Since only a few strictly aerobic nitrogen fixing bacteria have been investigated with respect to their physiological properties and their response to environmental conditions (Hill et al., 1972), a study on *C. autotrophicum* strain *GZ 29* was started. The present study aimed at the physiological characterization of this strain, at the optimization of growth under heterotrophic conditions, both in the presence and absence of combined nitrogen, as well as at the development of a reliable standard procedure (i.e. gassing of samples, adjusting oxygen partial pressures) for the measurement of nitrogenase activity by the acetylene reduction assay. Special attention was paid to the influence of oxygen concentration on growth and nitrogenase activity.

METHODS

Culture of Organism and Identification Media. The coryneform bacterium strain *GZ 29* was selected out of about 70 autotrophic (hydrogen oxidising) strains isolated by Auling (1975) from different habitats. Cells were grown autotrophically in a mineral medium of Schlegel et al. (1961) modified for nitrogen fixation conditions as described by Gogotov and Schlegel (1974); sodium molybdate was added at a concentration of about 3 nM. Cells were supplemented with 10% air, 10% H_2, 10% CO_2 and 70% N_2. For heterotrophic growth the mineral medium contained 0.1–0.5% of a suitable carbon source (usually sucrose); it was gassed with 10% air and 90% N_2. Growth on solid media (plus 1.5% agar) or in 100 ml Erlenmeyer flasks (with 10 ml of medium) was performed using a dessicator containing the required gas mixture. Liquid cultures with 10 ml medium were gassed by shaking the dessicator. The ability to utilize different carbon sources was tested by using mineral medium plus carbohydrates, organic acids, ethanol, methanol or hexadecane. In addition, the basal medium employed for assimilation of organic acids by Yamada and Komagata (1972) was used. The media for investigating the physiological characteristics

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were prepared according to the Manual of Microbiological Methods (1957) and to Cowan and Steel (1965). Gram stain was carried out by using Hucker's modification as well as a modification described by Stackebrandt (1974) developed for Gram stain of coryneform *Cellulomonas* species.

Thin sections were made from cells grown with sucrose under nitrogen fixation conditions. Cells were fixed with glutaraldehyde/osmiumtetroxide, stained with uranylacetate and poststained with leadcitrate. Citrate synthase and its mode of regulation as a taxonomic tool (Weitzman and Jones, 1975) was tested with 5.5 dithiobis-(2-nitrobenzoic acid) as used by Spiegelberg (1975). The molecular weight of citrate synthase was estimated by using gel-chromatography (Sephadex G-200, column dimension 2.5 × 40 cm) and Tris-buffer (20 mM, pH 8.0) containing 10 mM $MgCl_2$ and 1 mM ethylenediaminetetraacetic acid (Weitzman and Jones, 1975). All tests were carried out with crude extract (French press).

Growth and Nitrogen Fixation. The nutrient medium contained 0.5% sucrose in the mineral medium free from combined nitrogen. Cells grown in 10 ml medium for 2–3 days at 30° C under 10% air + 90% N_2 in a shaken dessicator were transferred to 250 ml medium in a 2 l round, flat-bottomed flask, stirred magnetically at about 400 rpm (Schlegel et al., 1961). Cultures were gassed with about 300 ml/min of a gas mixture (air and nitrogen) to give a concentration of dissolved oxygen of about 0.55 mg O_2/l. The oxygen concentration was measured by a Clark-type oxygen electrode (Yellow Springs Instruments, model 57). After 2–5 days, cells were harvested and used either for nitrogenase activity measurements or were used as inoculum for fermenters.

Fermenter growth was performed in a 2 l Quickfit fermenter with 1.5 l of medium and an agitation of 700 rpm. The entire fermenter assembly as well as the stirring mechanism used was the same as described by Robra (1971). The dissolved oxygen tension was measured by a Clark-type oxygen electrode (Eschweiler & Co., Kiel, West Germany, model F 51) consisting of a heat sterilisable envelope and an inserted electrode. The gas mixture (O_2 and N_2) was allowed to flow continuously through the culture (400 ml/min) regulated by means of newly designed pressure control modules (Siemens AG, Karlsruhe, West Germany; for details see Berndt and Ecker, 1975) to give an oxygen concentration of 0.55 mg O_2/l. For comparative studies cells were grown in the presence of combined nitrogen (ammonia) under almost identical conditions, however, at an oxygen concentration of 4.45 mg O_2/l and in the absence of nitrogen.

Growth rates with organic substrates were measured using Erlenmeyer flasks (200 ml with 50 ml of medium) shaken in a water bath (30° C, 124 strokes/min, amplitude 5 cm). The cells grew under aerobic conditions in a medium containing 0.5% substrate; when the requirement for growth factors was tested, 0.2 mg of the respective vitamin and 1.0 g yeast extract per litre were added. Growth was followed by measuring the optical density at 546 nm (Zeiss photometer, PM 4).

Nitrogenase activity was determined by the acetylene reduction test (Dilworth, 1966). Intact cells were incubated in serum bottles (about 20 ml volume, exactly determined for each flask) equipped with small cylindrical glass tubes containing pyrogallol to provide anaerobic test conditions (Fig. 3). For aerobic assay conditions, the reaction vessel was flushed for 10 min with argon-oxygen mixtures. The oxygen content in the bacterial suspension at different partial pressures of oxygen was measured by an oxygen electrode (Yellow Springs Instruments Co., Yellow Springs, U.S.A., model 53) immersed in the assay medium, and calculated by a calibration curve, as described by Berndt and Ecker (1975).

After 60 min of incubation (standard assay conditions), 0.2 ml of gas samples were removed by means of disposable plastic syringes and transferred to a Siemens gas chromatograph L 350 equipped with a flame ionization detector. A Porapak R (100–120 mesh) metal column (150 × 0.3 cm; 56° C) was used with N_2 as a carrier gas at a flow rate of 26 ml/min. Quantitative evaluation was based on peak height using a calibration curve prepared with known quantities of ethylene.

For routine nitrogenase assays serum bottles were gassed with oxygen-free argon overnight. Cell suspensions were taken from the fermenter or a round, flat-bottomed vessel and were used immediately or flushed with oxygenfree argon before they were injected into the serum bottle. The serum bottles received 2 ml bacterial suspension of an optical density of 2.0 at 546 nm (≙ 0.49 mg protein/ml). Samples of lower optical densities were used without any treatment, whereas suspensions of higher OD were diluted with an oxygen-free mineral medium containing 0.1% sucrose to give an optical density of about 2. The reaction was started by adding 7% of oxygen-free acetylene (Messer-Griesheim, Kassel, West Germany). All measurements, as well as gassing of samples, were carried out in a water bath (30° C) with reciprocal shaking (105 strokes/min, 3.5 cm amplitude).

The *optical density* of the cell suspension was measured in cuvettes (d = 1 cm) in a Zeiss photometer (PM 4) at a wave length of 546 nm. *Protein* was determined according to Lowry et al. (1951) with crystalline bovine serum albumin as standard. For protein measurement of whole cells 0.5 ml suspension was sonicated by a Braun-Sonic 300 Disintegrator (1 min/ml). After disintegration 0.5 ml NaOH (2 N) and 10 ml of the $CuSO_4/Na_2CO_3$-solution were added and the mixture was incubated for 10 min. Then the Folin reagent was added (1 ml, 1:2 diluted); after further incubation for 10 min at room temperature the extinction was measured in 2 cm cuvettes at 578 nm against a blank.

Sucrose in the growth medium (fermenter) was determined by means of an optical test using β-fructosidase, hexokinase and glucose-6-phosphate dehydrogenase, as auxiliary enzymes. *Glucose* and *fructose* were also measured by coupled optical tests. Ammonia (fermenter-growth) was determined with glutamate dehydrogenase. The methods described by Bergmeyer (1974) were used. Poly-β-hydroxybutyric acid was estimated according to Jüttner et al. (1975).

Nitrogen content of cells was determined by the micro-Kjeldahl technique, followed by microdiffusion and colorimetric analysis of ammonia with Nessler's reagent (Minari and Zilversmit, 1963, modified for microdiffusion).

RESULTS

Identification of Strain GZ 29

The cells of the nitrogen fixing hydrogen bacterium strain *GZ 29* are non-motile, encapsulated, irregular shaped rods with a diameter of 0.7–1.0 µm and 1.8–4.9 µm length (Fig. 1). They contained poly-β-hydroxybutyric acid (PHB), polyphosphate (Fig. 2) and polysaccharide. A snapping type of cell division was observed, however, there was no distinctive pleomorphism. When growing on succinate, branched cells appeared. These observations indicate that strain *GZ 29* belongs to the group of coryneform bacteria (Yamada and Komagata, 1972). On solid media round, glistening, yellow pigmented colonies were formed. No slime formation occurred. The cells grew only under aerobic conditions (with or without

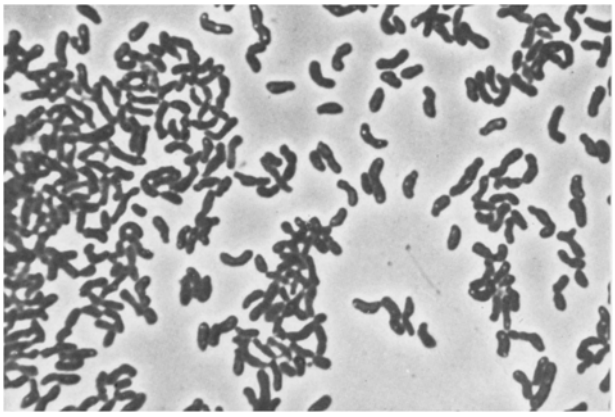

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Fig. 1. Morphology of cells of *Corynebacterium autotrophicum GZ 29*. Cells were grown in gluconate-medium. Magnification about 2000-fold

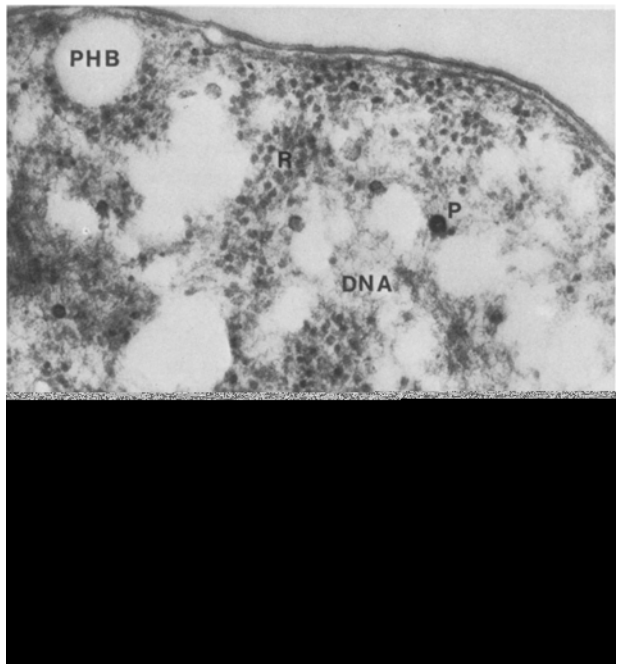

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Fig. 2. Micromorphology of *Corynebacterium autotrophicum GZ 29*. Cells were grown heterotrophically in 0.5% sucrose medium under nitrogen fixation conditions. They were fixed with glutaraldehyde/osmiumtetroxide, stained with uranylacetate and post-stained with leadcitrate. Abbreviations: *CM* cytoplasmic membrane; *OM* outer membrane; *D* dense layer (not distinctive); *DNA* desoxyribonucleic acid; *I* invagination of the cytoplasmic membrane; *P* polyphosphate; *PHB* poly-β-hydroxybutyric acid; *R* ribosomes

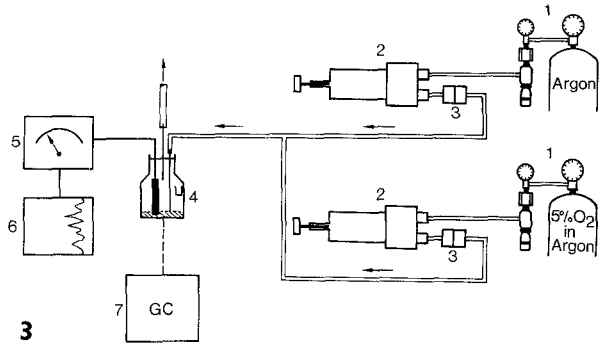

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Fig. 3. Diagram of the acetylene reduction assay set up for measuring nitrogenase activity of intact cells. *1* gas cylinder; *2* pressure control modul; *3* microaperture; *4* incubation vessel with oxygen electrode and small side vessel; *5* and *6* oxygen control unit plus recorder; *7* gas chromatograph

nitrate) and fixed molecular nitrogen both under heterotrophic and autotrophic conditions. In the presence of 8.5% $^{15}N_2$ the cells fixed about 6.7 atom % within 15 h incubation under mixotrophic conditions; in the presence of ammonium the nitrogenase activity was repressed.

The cells utilized carbohydrates only to a small extent, on agar as well as in liquid media. Cells grew well with sucrose, gluconate, lactate, acetate, pyruvate, succinate, malate, adipate, β-hydroxybutyrate, and with a low rate on ethanol, citrate and glutamate (for growth rates see Table 1). On fructose and glucose media growth was observed only after a 7–10 day incubation period. No growth appeared with cellobiose, lactose, maltose, mannose, xylose, arabinose, galactose, ribose, sorbose, trehalose, raffinose, starch, dextran, benzoate and hexadecane.

Table 1. Growth rates with some organic substrates and NH_4Cl as nitrogen source. Growth was measured at 30°C in a shaking Warburg apparatus. Erlenmeyer flasks with suspension were shaken in a thermoconstant water bath (30°C) and optical densities were measured

Substrate	Growth rate μ (h^{-1})	Doubling time (h)
Succinate	0.23	3.01
Malate	0.22	3.15
Pyruvate	0.21	3.30
Gluconate	0.20	3.47
Lactate	0.20	3.47
Sucrose	0.18	3.65
Acetate	0.15	4.62
β-Hydroxybutyrate	0.14	4.95
Ethanol	0.10	6.93
Citrate	0.09	7.30
Glutamate	0.07	9.90

Indole formation, Voges-Proskauer reaction, methyl-red reaction, hydrolysis of starch, gelatine or casein, formation of cellulase, urease, DNase, and hydrogen sulfide were all found to be negative. The cells were not acid-fast and did not grow in the presence of 5% NaCl or high citrate concentrations (s. Yamada and Komagata, 1972). The catalase and oxidase reactions as well as nitrite formation from nitrate were positive.

The GC-content of isolated DNA was found to be 68.7%; the characteristic amino acid of the peptidoglycan was DL-DAP. The Gram reaction was negative when Hucker's modification as well as the technique reported by Stackebrandt (1974) for Gram stain of coryneform *Cellulomonas* species were used. Furthermore, the cell wall consisted of layers typical for Gram negative bacteria (Fig. 2). The regulatory properties of the citrate synthase (Weitzman and Jones, 1975) confirmed the Gram negative character of strain *GZ 29*; the citrate synthase (crude extract) was inhibited by NADH (1 mM, 80% inhibition) and had a molecular weight greater than 250000 ("large enzyme"); no reactivation by AMP (equimolar) was observed.

On the basis of morphological and physiological properties described so far strain *GZ 29* has to be assigned to *Corynebacterium autotrophicum* (Baumgarten et al., 1974).

Growth and Nitrogen Fixation

The rates of growth on some organic substrates under air were estimated in Erlenmeyer flasks shaken in a thermoconstant water bath. Best growth was observed on some organic acids (Table 1). On sucrose an average doubling time of 3.7 h was estimated; addition of thiamine, vitamin B 12, yeast extract and biotin to the sucrose medium resulted in only a slight or no increase of the growth rate.

The influence of the oxygen concentration on heterotrophic growth with sucrose (0.5%) in the presence and absence of combined nitrogen (Fig. 4), was measured in a Quickfit-fermenter. Throughout each fermenter run, the oxygen concentration in the medium was kept constant; it was measured by means of an oxygen electrode. Under nitrogen fixation conditions, (65% N_2 with argon as supplementing gas) a doubling time of 5.0 h was reached at an oxygen concentration of 0.55 mg/l. When this value is compared to doubling times observed in the following growth experiments (3.7 h, Fig. 11), one has to consider that in the previous experiments non-saturating nitrogen concentrations were used (65% instead of about 95% nitrogen). With ammonium as nitrogen source, the maximal growth rate with a doubling time of 3.2 h

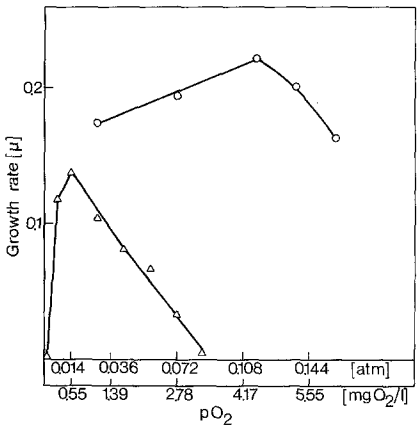


Fig. 4. Growth rates in the presence of ammonium or nitrogen as N-sources at different dissolved oxygen tensions in a 2 l fermenter (Quickfit). For details see "Methods". Oxygen concentrations were measured by means of a Clark-type oxygen electrode (Eschweiler & Co.). Throughout this paper the volumetric portion of oxygen (or acetylene, Fig. 5) of the total gas mixture is expressed in atmospheres (atm). ○, NH_4Cl; △, N_2

was found at 4.45 mg O_2/l. Without oxygen no growth was observed.

Nitrogenase activity was measured throughout almost all experiments in the presence of sucrose as organic substrate (0.1%). The cells had been grown in 2 l round flat-bottomed flasks for 2 days, harvested and washed once by centrifugation at room temperature using an oxygen-free mineral medium containing 0.1% sucrose for resuspending. Care was taken to avoid the contact with air. The pO_2 in the reaction vessel was 0.0036 atm (corresponding to a dissolved oxygen concentration of 0.14 mg O_2/l). Ethylene production was linearly related to the incubation time and the rate of ethylene production showed a linear relationship to the protein concentration. The optimal acetylene concentration in the gas phase was found to be 7% acetylene (Fig. 5); the optimum pH for the acetylene reduction was about 6.9 (Fig. 6). At pH-values lower than 6.8 the rate of ethylene production decreased rapidly.

To prove the validity of the assay system used for determination of nitrogenase activity (acetylene reduction) the oxygen concentration was measured by an oxygen electrode (Yellow Springs Instruments Co.) immersed in the assay medium. While ethylene was produced at a constant rate, oxygen concentration remained unchanged for at least 60 min (Fig. 7). Neither the addition of substrate (C_2H_2) nor the removal of samples (0.2 ml) at intervals of 15 min, caused any change in the oxygen concentration of the suspension.

When cells grown in flat-bottomed round vessels on sucrose as carbon source were used, acetylene

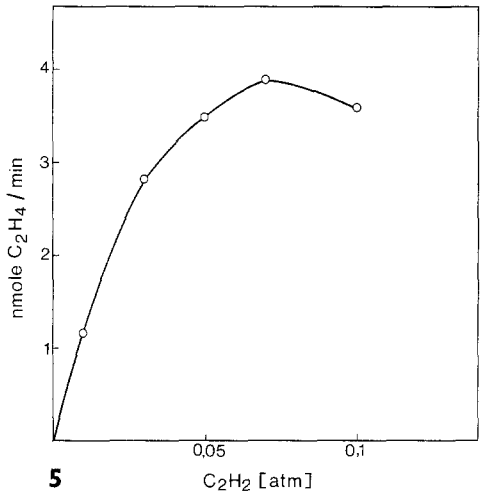


Fig. 5. Dependence of the rate of acetylene reduction upon acetylene concentration. The test was performed using cells grown in a 2 l round flask for 2 days

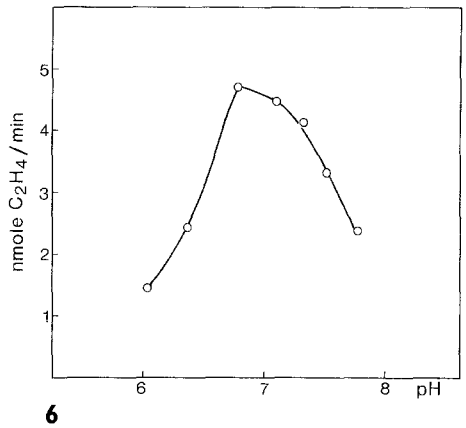


Fig. 6. Dependence of acetylene reduction on pH. The test was performed using standard assay conditions. Cells were washed and resuspended in mineral medium (plus 0.1% sucrose) pre-adjusted to different pH values

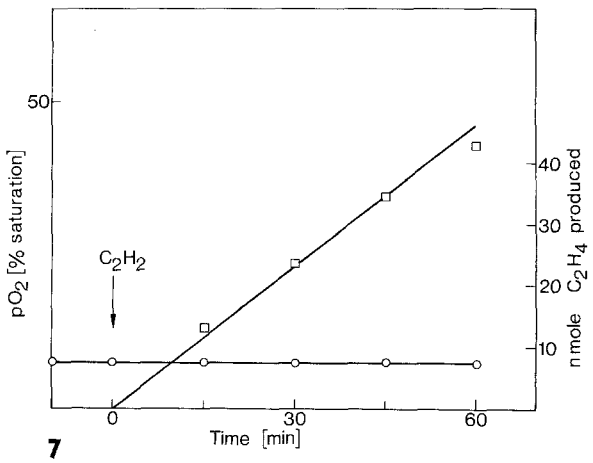
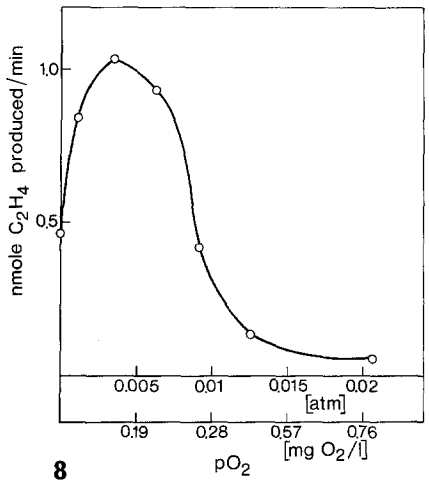


Fig. 7. Acetylene reduction and dissolved oxygen tension during incubation period. 2.5 ml of bacterial suspension were incubated under standard assay conditions. The pO_2 was measured by means of a Clark-type oxygen electrode (Yellow Springs Instruments, model 53; see Fig. 3). □, C_2H_4; ○, pO_2

reduction by washed cells occurred without an added carbon source. Washed cells reduced acetylene in the absence of sucrose almost as fast as in the presence of sucrose. As may be assumed, storage materials served as energy and electron donors for acetylene reduction. For future experiments under autotrophic conditions, the possible interference of storage materials with H_2 as electron donor has to be taken into consideration. Therefore, to perform conclusive experiments under autotrophic conditions, mutants lacking the ability to synthesize storage materials, should be used.

The *pO_2-optimum* for acetylene reduction was measured in serum bottles which were flushed with oxygen-free argon overnight. The desired oxygen concentration was achieved by flushing the reaction vessels with a stream of the appropriate gas mixture for 10 min (see "Methods"). No differences in nitrogenase activity were observed when cells were kept for a short period at low pO_2-values instead of anaerobic conditions before they were used for acetylene reduction measurements. The pO_2-optimum curve (Fig. 8) exhibited an optimum at 0.14 mg O_2/l (0.0036 atm). At higher oxygen concentrations, the reduction rate decreased abruptly. When less than 0.14 mg O_2/l was used, the reduction rate was lower. However, even under strict anaerobic conditions (in the presence of pyrogallol), there was still a measurable rate of reduction.

Obligate aerobic nitrogen fixing bacteria usually need oxygen for energy supply of the nitrogenase system (Yates and Daniel, 1970; Biggins and Postgate, 1969). In order to confirm the observation of an anaerobic acetylene reduction, comparative experiments were carried out using strain *GZ 29* and *Azotobacter vinelandii OP*. As shown in Figure 9, only cells of strain *GZ 29* exhibited nitrogenase activity at a linear rate over 60 min under anaerobic conditions. At an incubation period extended up to 6 h, no decrease of the rate of ethylene production occurred. The anaerobic acetylene reduction reached values of 0.5–1.0 nmole ethylene produced/min × mg protein. This was 10–20-fold less than found with fermenter grown cells under aerobic conditions (about



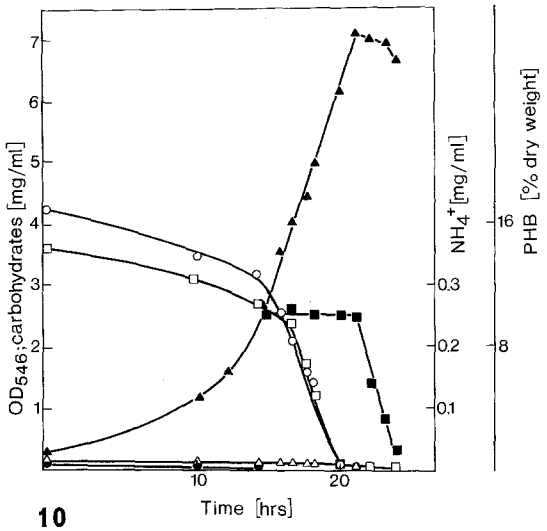


Fig. 8. Dependence of the rate of acetylene reduction on the dissolved oxygen concentration. Cells grown in a round flask for 5 days were used. The optical density at 546 nm was 2.0 corresponding to 0.49 mg protein/ml (standard assay conditions). Dissolved oxygen tensions were calculated by means of a calibration curve

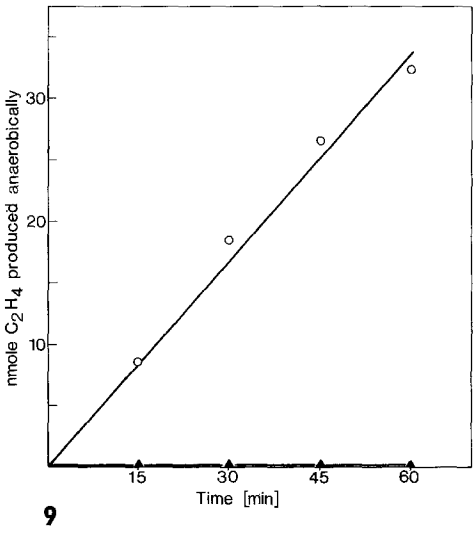


Fig. 9. Acetylene reduction under anaerobic conditions. Cells of *Corynebacterium autotrophicum GZ 29* and *Azotobacter vinelandii OP* were cultivated for 24 h in 2 l round flasks under identical conditions. Assays were performed in the presence of pyrogallol using standard assay conditions. ○ *C. autotrophicum 30,1b*; ▲ *A. vinelandii OP*

Fig. 10. Fermenter growth with ammonium as nitrogen source. A 2 l Quickfit fermenter with 1.5 l of medium was used. The dissolved oxygen tension was kept constant at the optimal value as indicated in Figure 4. ▲ optical density at 546 nm; ○ sucrose; ● glucose; △ fructose; ■ poly-β-hydroxybutyric acid (PHB); □ NH_4Cl

12 nmoles ethylene produced/min × mg protein, Fig. 12). Cells grown in flat-bottomed round flasks exhibited less aerobic acetylene reducing activity (Fig. 8), whereas the anaerobic acetylene reduction by these cells was found to be almost the same as that of fermenter grown cells.

This result indicated that strain *GZ 29* probably contains an energy regenerating system working under anaerobic conditions. To investigate possible anaerobic metabolism as found by Stackebrandt (1974) for coryneform *Cellulomonas* species, gas production and formation of some organic acids were estimated. Under acetylene reduction assay conditions neither CO_2 nor H_2 were produced (measured in Warburg vessels and by gas chromatography). Besides, neither organic acids (lactate, acetate, components of the citric acid cycle) nor ethanol were detected by gas chromatographic measurements.

The *changes of nitrogenase activity* during growth of a batch culture were estimated using a fermenter (2 l Quickfit) with 1.5 l of mineral medium containing 0.5% sucrose. The cells were grown at a constant dissolved oxygen concentration of 4.45 mg O_2/l in the presence of ammonium and at 0.55 mg O_2/l under nitrogen fixation conditions (see Fig. 4).

In cells growing in the presence of combined nitrogen (NH_4Cl), no activity of nitrogenase was observed. Poly-β-hydroxybutyric acid (PHB) was accumulated up to 10%, while NH_4^+ and sucrose were consumed simultaneously (Fig. 10). In cells growing in the absence of combined nitrogen, nitrogenase activity was high. Maximal activity was reached at the end of the lag-phase; in the beginning of the log-phase the activity already decreased. During this fermenter run the logarithmic growth phase was interrupted by a lag indicated by a decrease of the

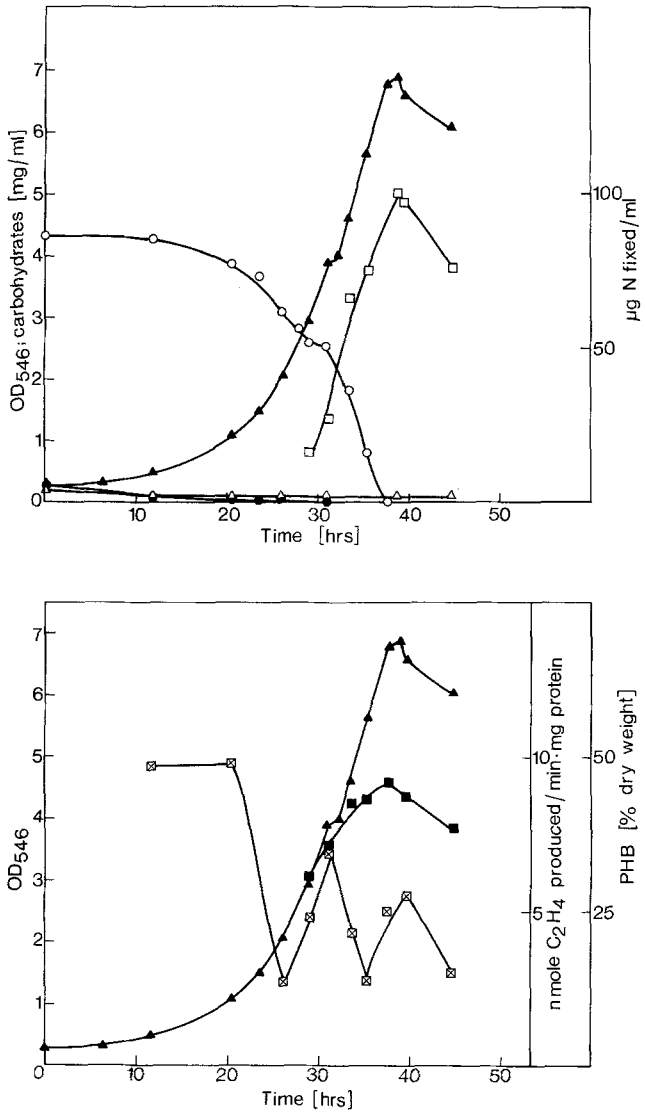


Fig. 11. Fermenter growth with N_2 as nitrogen source. The dissolved oxygen tension was kept constant at the optimal value as indicated in Figure 4. Acetylene reduction was measured as shown in Figure 3; samples with optical densities higher than 2.0 were diluted with anaerobic mineral medium containing 0.1% sucrose to give an optical density (546 nm) of about 2. ▲ optical density at 546 nm; ○ sucrose; □ N fixed; ● glucose; △ fructose; ■ poly-β-hydroxybutyric acid (PHB); ⊠ acetylene reduction

growth rate (OD) as well as sucrose consumption (Fig. 11). Simultaneously, an increase of nitrogenase activity occurred, followed by another decrease when growth accelerated again. It was obvious from this experiment that nitrogenase activity oscillated depending on the growth velocity of the cells. The enzyme was measured throughout the growth experiment by incubating samples with 0.018 atm O_2 (0.68 mg O_2/l) in the acetylene reduction test. PHB was synthesized up to 45% of dry weight at the end of the growth cycle. During the logarithmic growth phase a slight decrease of the rate of PHB synthesis was observed.

Since a decrease of nitrogenase activity at maximal growth (a similar phenomenon was described by Gadkari and Stolp, 1974, cultivating *Azotobacter vinelandii*) seemed to be unreasonable in a phase with maximal synthetic activities, another fermenter was run measuring the acetylene reduction by the cells at different oxygen concentrations. As visible in Figure 12, a shift of the pO_2-optimum of the nitrogenase system appeared during the growth cycle (for pO_2-optimum shift with cells grown at different oxygen concentrations see Drozd and Postgate, 1970). At a pO_2 of 0.0036 atm (0.14 mg O_2/l) acetylene reduction was maximal with cells harvested about 8 h after inoculation of the fermenter. At the 6-fold oxygen concentration (0.018 atm O_2 corresponding to 0.68 mg O_2/l) nitrogenase activity was maximal after about 31 h of growth reaching a value of about 12 nmoles ethylene produced/min × mg protein. For technical reasons, a further possible increase of nitrogenase activity in the late log-phase at higher dissolved oxygen tensions could not be measured.

From these results the conclusion can be drawn that the pO_2-optimum of the nitrogenase reaction depends on the growth state of the cells. Maximal growth rate requires maximal tolerable oxygen tension in the acetylene reduction assay system. The shift of the pO_2-optimum is reversible, as can be seen from Figure 11.

The *efficiency of nitrogen fixation* expressed as mg N fixed/g sucrose consumed was measured during the growth cycle. The nitrogen content of the cells was estimated by Kjeldahl digestion followed by microdiffusion and Nesslerization. A maximum of efficiency (= 65) was found at the beginning of the logarithmic growth phase followed by a decrease at maximal growth rates (Fig. 13). Conditions of growth were the same as described for Figure 11. At the end of the log phase, with small amounts of remaining sucrose, a second increase of the efficiency of nitrogen fixation was observed. The overall coefficient for efficiency was found to be 22 mg N fixed per g sucrose consumed.

DISCUSSION

The nitrogen fixing hydrogen bacterium strain *GZ 29* was identified as a coryneform type of bacterium, and was assigned to *Corynebacterium autotrophicum* (Baumgarten et al., 1974). In contrast to strains *7c* and *14 g* of *C. autotrophicum*, strain *GZ 29* was found to give a negative Gram reaction. Since the type strain *7 c* was described to be Gram positive other criteria such as micromorphology of the cell wall

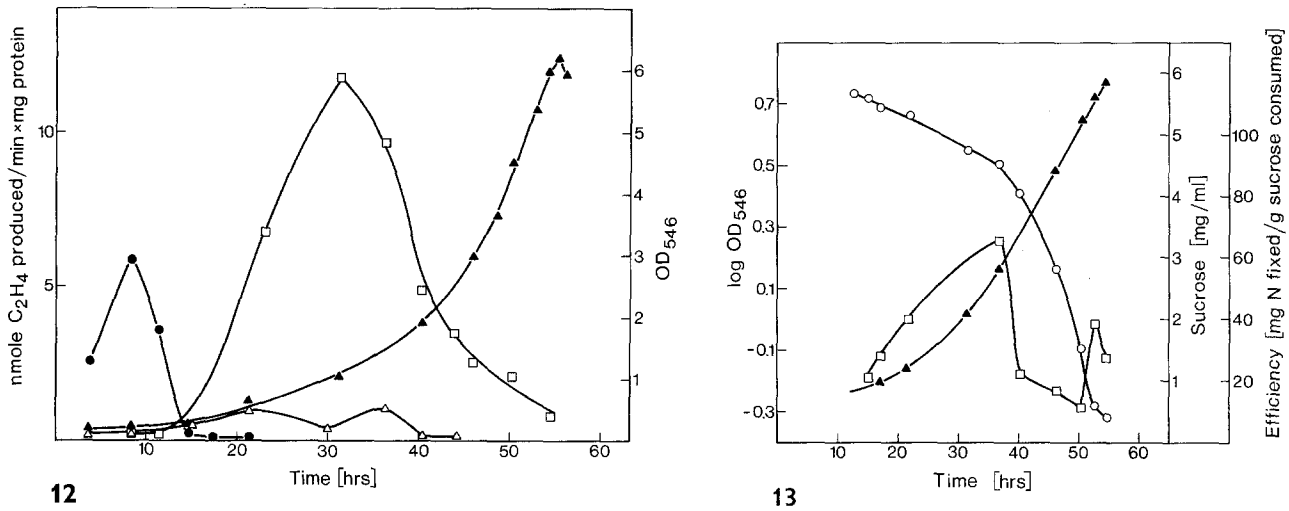


Fig. 12. Fermenter growth with N_2 as nitrogen source. Conditions were the same as described in Figure 11. Rates of acetylene reduction were estimated at different dissolved oxygen tensions. ● acetylene reduction at 0.0036 atm O_2; □ acetylene reduction at 0.018 atm O_2; △ acetylene reduction under anaerobic conditions; ▲ optical density at 546 nm

Fig. 13. Fermenter growth with N_2 as nitrogen source. Measurement of the efficiency of nitrogen fixation. Conditions were the same as described in Figure 11. Nitrogen was measured using Kjeldahl digestion, microdiffusion and Nesslerization. ▲ optical density at 546 nm; ○ sucrose content; □ efficiency of nitrogen fixation

(Costeron et al., 1974) and regulatory properties of citrate synthase (Weitzman and Jones, 1975) of strain *GZ 29* were investigated. The cell wall layers as well as inhibition of citrate synthase by NADH are characteristic for a Gram negative bacterium.

Gram positive *Corynebacterium* species (*C. equi*, *C. fascians*, *C. michiganense*) were shown to possess citrate synthases which were not inhibited by NADH (Weitzman and Jones, 1968). On the other hand thin sections of cells of strain *7 c* and *14 g* indicate that the cell wall of these bacteria resembles more the wall layers of Gram negative bacteria (Mauruschat, 1974), than the Gram positive type. Thus the Gram behaviour of different autotrophic coryneform strains still needs further investigation including additional criteria like micromorphology of the cell wall, properties of citrate synthase and probably the alternative occurrence of ubiquinone or vitamin K (Bishop et al., 1962).

Corynebacterium autotrophicum GZ 29 had a doubling time of 3.7 h under nitrogen fixation conditions, reduced acetylene at a rate of about 12 nmoles ethylene produced/min × mg protein and fixed nitrogen with an overall efficiency of 22 mg N fixed/g sucrose consumed. Comparing these capacities of nitrogen fixation to those of other aerobic nitrogen fixing bacteria such as *Azotobacter* species or *Derxia gummosa* (Jensen et al., 1960; Stewart, 1969; Mulder and Brotonegoro, 1974), the new strain *C. autotrophicum GZ 29* resembles other free-living aerobic nitrogen fixing bacteria very strongly. Besides, strain *GZ 29* exhibited a comparable sensitivity to medium oxygen concentrations when fixing molecular nitrogen.

The efficiency of nitrogen fixation oscillated during the growth cycle (Fig. 13). The highest value (65 mg N fixed/g sucrose consumed) was measured with cells taken from the fermenter at the beginning of the log-phase. Similar variations of efficiency during growth were reported for *Azotobacter chroococcum* (Schmidt-Lorenz and Rippel-Baldes, 1957); in this case the efficiency was maximal at the beginning of the log-phase, too. Using cells grown in continuous culture, Dalton and Postgate (1969) measured maximal efficiency of nitrogen fixation with *Azotobacter chroococcum* at a dilution rate of $D = 0.20-0.22$ (h), whereas at lower or higher dilution rates the efficiency was less.

The oxygen requirement for maximal nitrogenase activity increased with increasing growth rates and vice versa (Figs. 11 and 12). Referring to the results of studies on intact cells of *Azotobacter vinelandii* (Haaker et al., 1974) the increase of dissolved oxygen tensions causes higher intracellular ATP-concentrations and higher nitrogenase activity (until inhibition occurs). Thus, maximal nitrogenase activity is dependent on sufficient supply of energy (and electrons).

In the case of *C. autotrophicum GZ 29* it may be concluded that the shift of the pO_2-optimum for maximal nitrogenase activity is caused by an increasing oxygen requirement, indicative for an increased energy demand to keep the nitrogenase system functioning. A maximum of energy supply for nitrogen fixation is,

therefore, required in the exponential growth phase. At the same time, the efficiency of nitrogen fixation decreased and reached minimal values within the log-phase (Fig. 13). In connection with the high energy requirement of the nitrogenase it may be assumed that a greater portion of sucrose consumed is probably used to maintain the nitrogen fixation process.

Acetylene reduction under anaerobic conditions by aerobically grown cells of strain *GZ 29* (Fig. 9) was unexpected and astonishing. Anaerobic conditions were provided by the presence of pyrogallol to compensate for slight oxygen diffusion through the rubber stoppers into the suspension, as reported by Keister (1975), for long incubation periods. To our knowledge anaerobic acetylene reduction by intact cells of obligate aerobic nitrogen fixing bacteria has not been reported so far. In our experiments using *Azotobacter vinelandii OP* as a reference organism no acetylene reduction was observed under anaerobic conditions (Fig. 9). The results thus agree with those of Yates and Daniel (1970) who used aerobic cells of *A. chroococcum*. Obviously intact cells of the *Azotobacter* group do not have an anaerobic energy regenerating system at their disposal. However, crude extracts of *A. vinelandii* were shown to possess a clostridia-like energy regenerating system. Experiments performed with acetyl-phosphate in the acetylene reduction assay exhibited ethylene formation to nearly the same extent as measurements did with creatine phosphate plus creatine phosphokinase (Haaker et al., 1972).

Anaerobic metabolic activities under the conditions of acetylene reduction assay could not be demonstrated with cells of *C. autotrophicum GZ 29*. Neither CO_2 or H_2 nor acetate, lactate, or ethanol could be detected in the supernatant or in the gas phase by chromatographic methods. Thus, the origin of energy necessary for nitrogenase activity (under anaerobic conditions the C_2H_2 reduction is about 10–20-fold less than under aerobic conditions) is not yet known.

Yet the use of storage material for maintenance of anaerobic acetylene reduction has still to be considered. Concerning polyphosphate the results of Kaltwasser (1962) indicate that polyphosphate is not degraded anaerobically by aerobic hydrogen bacteria (*Hydrogenomonas eutropha*). Thus, it seems unlikely that *C. autotrophicum GZ 29* is able to use this polymer as energy source under anaerobic conditions.

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REFERENCES

Aragno, M.: Mise en évidence d'hydrogénobactéries corynéformes auxo-hétérotrophes pour la biotine dans l'eau d'un lac eutrophe. Ann. Microbiol. (Inst. Pasteur) **126**A, 539–542 (1975)

Auling, G.: Intakte und defekte Phagen von Wasserstoffbakterien. Thesis, Univ. Göttingen (1975)

Baumgarten, J., Reh, M., Schlegel, H.-G.: Taxonomic studies on some Gram-positive coryneform hydrogen bacteria. Arch. Microbiol. **100**, 207–217 (1974)

Bergmeyer, H. N.: Methoden der enzymatischen Analyse, 3. Aufl., Bd. 2. Weinheim: Verlag Chemie 1974

Berndt, H., Ecker, E.: Ein neues System zur Dosierung von Gasen. Anwendung bei der Messung der Sauerstoff-abhängigen Nitrogenase-Reaktion an aeroben Bakterienzellen. GIT, Fachzeitschrift für das Laboratorium **19**, 850–858 (1975)

Biggins, D. R., Postgate, J. R.: Nitrogen fixation by cultures and cell-free extracts of *Mycobacterium flavum 301*. J. gen. Microbiol. **56**, 181–193 (1969)

Bishop, D. H. L., Pandya, K. P., King, H. K.: Ubiquinone and vitamin K in bacteria. Biochem. J. **83**, 606–614 (1962)

de Bont, J. A. M., Leijten, M. W. M.: Nitrogen fixation by hydrogen-utilizing bacteria. Arch. Microbiol. **107**, 235–240 (1976)

Costeron, J. W., Ingram, J. M., Cheng, K.-J.: Structure and function of the cell envelope of Gram-negative bacteria. Bact. Rev. **38**, 87–110 (1974)

Cowan, S. T., Steel, K. J.: Manual for the identification of medical bacteria. London: Cambridge University Press 1965

Dalton, H., Postgate, J. R.: Growth and physiology of *Azotobacter chroococcum* in continuous culture. J. gen. Microbiol. **56**, 307–319 (1969)

Dilworth, M. J.: Acetylene reduction by nitrogen-fixing preparations from *Clostridium pasteurianum*. Biochim. biophys. Acta (Amst.) **127**, 285–294 (1966)

Drozd, J., Postgate, J. R.: Interference by oxygen in the acetylene-reduction test for aerobic nitrogen-fixing bacteria. J. gen. Microbiol. **60**, 427–429 (1970)

Gadkari, D., Stolp, H.: Influence of nitrogen source on growth and nitrogenase activity in *Azotobacter vinelandii*. Arch. Microbiol. **96**, 135–144 (1974)

Gogotov, J. N., Schlegel, H.-G.: N_2-fixation by chemoautotrophic hydrogen bacteria. Arch. Microbiol. **97**, 359–362 (1974)

Haaker, H., Bresters, T. W., Veeger, C.: Relation between anaerobic ATP synthesis from pyruvate and nitrogen fixation in *Azotobacter vinelandii*. FEBS Letters **23**, 160–162 (1972)

Haaker, H., De Kok, A., Veeger, C.: Regulation of dinitrogen fixation in intact *Azotobacter vinelandii*. Biochim. biophys. Acta (Amst.) **357**, 344–357 (1974)

Hill, S., Drozd, J. W., Postgate, J. R.: Environmental effects on the growth of nitrogen-fixing bacteria. J. appl. Chem. Biotechnol. **22**, 541–558 (1972)

Jensen, H. L., Petersen, E. J., De, P. K., Bhattacharya, R.: A new nitrogen-fixing bacterium: *Derxia gummosa* nov. gen. nov. spec. Arch. Mikrobiol. **36**, 182–195 (1960)

Jüttner, R.-R., Lafferty, R. M., Knackmuss, H. J.: A simple method for the determination of poly-β-hydroxybutyric acid in microbial biomass. Europ. J. appl. Microbiol. **1**, 233–237 (1975)

Kaltwasser, H.: Die Rolle der Polyphosphate im Phosphatstoffwechsel eines Knallgasbakteriums (*Hydrogenomonas Stamm 20*). Arch. Mikrobiol. **41**, 282–306 (1962)

Keister, D. L.: Acetylene reduction by pure cultures of *Rhizobia*. J. Bact. **123**, 1265–1268 (1975)

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J.: Protein measurement with the folin phenol reagent. J. biol. Chem. **193**, 265–275 (1951)

Manual of Microbiological Methods: Society of American Bacteriologists. New York-Toronto-London: McGraw-Hill 1957

Mauruschat, A.: Cytologische Charakterisierung von *Hydrogenomonas eutropha H 16* und anderen Knallgasbakterien. Diplomarbeit, Univ. Göttingen (1974)

Minari, O., Zilversmit, D. B.: Use of KCN for stabilization of colour in direct Nesslerization of Kjeldahl digests. Analyt. Biochem. **6**, 320–327 (1963)

Mulder, E. G., Brotonegoro, S.: Free-living heterotrophic nitrogen-fixing bacteria. In: The biology of nitrogen fixation (A. Quispel, ed.), pp. 37–85. Amsterdam: North-Holland 1974

Ooyama, J.: Simultaneous fixation of CO_2 and N_2 in the presence of H_2 and O_2 by a bacterium. Rep. Ferment. Res. Inst. **39**, 41–44 (1971)

Robra, K.-H.: Optimierung der Begasung in Submerskulturen von *Hydrogenomonas eutropha* Stamm *H 16* mit H_2 und O_2 und enzymatische KDPG-Synthese. Thesis, Univ. Göttingen (1971)

Schlegel, H.-G., Kaltwasser, H., Gottschalk, G.: Ein Submersverfahren zur Kultur wasserstoffoxydierender Bakterien: Wachstums-physiologische Untersuchungen. Arch. Mikrobiol. **38**, 209–222 (1961)

Schmidt-Lorenz, W., Rippel-Baldes, A.: Wirkung des Sauerstoff-Partialdrucks auf Wachstum und Stickstoffbindung von *Azotobacter chroococcum* Beij. Arch. Mikrobiol. **28**, 45–68 (1957)

Spiegelberg, T.: Untersuchungen zur Regulation der Citratsynthase bei *Enterobacter aerogenes* und *Candida oleophila*. Thesis, Univ. Göttingen (1975)

Stackebrandt, E.: Biochemisch-taxonomische Untersuchungen an der Gattung *Cellulomonas*. Thesis, Univ. München (1974)

Stewart, W. D. P.: Biological and ecological aspects of nitrogen fixation by free-living micro-organisms. Proc. roy. Soc. B **172**, 367–388 (1969)

Weitzman, P. D. J., Jones, D.: Regulation of citrate synthase and microbial taxonomy. Nature (Lond.) **219**, 270–272 (1968)

Weitzman, P. D. J., Jones, D.: The mode of regulation of bacterial citrate synthase as a taxonomic tool. J. gen. Microbiol. **89**, 187–190 (1975)

Wiegel, J., Schlegel, H.-G.: Enrichment and isolation of nitrogen fixing hydrogen bacteria. Arch. Microbiol. **107**, 139–142 (1976)

Yamada, K., Komagata, K.: Taxonomic studies on coryneform bacteria. IV. Morphological, cultural, biochemical and physiological characteristics. J. gen. appl. Microbiol. **18**, 399–416 (1972)

Yates, M. G., Daniel, R. M.: Acetylene reduction with physiological electron donors by extracts and particulate fractions from nitrogen-fixing *Azotobacter chroococcum*. Biochim. biophys. Acta (Amst.) **197**, 161–169 (1970)

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