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# On culturing Escherichia coli on a mineral salts medium during anaerobic conditions

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**Abstract** The substrate and products of the hydrogenlyase complex, formic acid, carbon dioxide, and molecular hydrogen, are co-operatively implicated in maintaining growth of E. coli under anaerobic conditions. Growth is observed in the presence of a combination of carbon dioxide + molecular hydrogen, or carbon dioxide + formic acid in the medium. The study shows that it is possible to culture E. coli under anaerobic conditions while sparging with nitrogen, without supplementing exogenous carbon dioxide, formic acid or molecular hydrogen. This condition occurs when the strain is allowed an appropriate induction period and is present at a sufficiently high cell density, since the cell density affects the rate of e.g. $CO_2$ production. In a system sparged with nitrogen gas, the removal of $CO_2$ due to this sparging must be balanced with a cell density dependent production rate of $CO_2$.

It is concluded that the "formic hydrogenlyase complex" should be considered as an integral part of the general maintenance of the anabolism of E. coli during anaerobic conditions on a mineral salts medium, as well as being a net producer of end products in E. coli metabolism.

## 1
## Introduction

This study was motivated by the hypothesis that the maintenance of the anabolic metabolism in E. coli during anaerobic conditions is affected by a need for assimilation of $CO_2$ ($HCO_3^-$) as previously suggested by several researchers [1–7]. Thus, the primary purpose of the study was to test if it was possible to culture the chosen E. coli strain during anaerobic conditions on a simple mineral salts medium composition, if considerations to the composition of the gaseous phase was taken. This approach renders data on how the composition of a suitable gas phase could be if culturing of the strain during simultaneous sparging with gas is the objective.

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The sparging of gas through the culture is one way of maintaining an "open" system without allowing a substantial back-diffusion of molecular oxygen through the gas exit [8]. Maintaining an open system and simultaneous sparging does also solve the problem of developing $CO_2$ pressure gradients [9], and allows metabolic adaptation to a well defined culture condition. However, using e.g. nitrogen gas sparging decrease the partial pressure of volatile metabolites in a detrimental manner in cultures of E. coli (ATCC 15224) [6, 10]. It has also been observed that the carbon dioxide pressure was critical during oxygen limitation [6]. Simply adding carbon dioxide to the gas used for sparging in accordance with e.g. [5], did therefore seem to be a "trivial" approach in developing conditions for culturing E. coli during anaerobic conditions.

## 2
## Materials and methods

### 2.1
### Bacterial strain

Studies were performed using E. coli strain CCUG 26462 (previously denoted E. coli LD + [11–13]). All inocula were prepared in a glucose-peptone broth (glucose 10 g/l, peptone [Oxoid L 37] 20 g/l and NaCl 6 g/l), incubated overnight at 21 °C. Prior to harvesting the cells, growth was partially oxygen limited by diffusion. Inocula were washed twice with NaCl 9 g/l prior to inoculation into the fermentor in order to significantly lower the amount of amino acids carried over from the inoculum media. It has previously been shown that the inoculum contains sufficient amino acids to affect the outcome of the fermentation if studies of a functional metabolism on a mineral salts medium is the objective [11]. After inoculation, the cell density was $10^6$–$10^7$ cells/ml.

### 2.2
### Mineral salts medium

The mineral salts medium contained $(NH_4)_2SO_4$ (5 g/l), $KH_2PO_4$ (1.6 g/l), $Na_2HPO_4$ (6.6 g/l), $(NH_4)_2$-citrate (0.5 g/l), and glucose (10 g/l), (glucose solutions were autoclaved separately). A trace element solution was added to supply $CaCl_2$ $2H_2O$. (0.5 mg/l), $FeCl_3$ $6H_2O$, (16.7 mg/l), $ZnSO_4$ $7H_2O$, (0.18 mg/l), $CuSO_4$ $5H_2O$, (0.16 mg/l), $MnSO_4$ $7H_2O$, (0.15 mg/l), $CoCl_2$ $6H_2O$, (0.18 mg/l) and Na-EDTA (20.1 mg/l) in the fermentation medium [14]. Furthermore, 1 ml/l of a 20% $MgSO_4$ solution, and trace elements of importance for the formate hydrogenlyase complex, Nickel (5 μM) [15, 16]. Selenite (1 μM) and Molybdate (1 μM) [17], were added separately.

Table 1. A summary of studies relevant to the presented data. N. s.-(data not shown). Concentrations of gas components given a deviance from concentrations of about 100% are shown in the columns. The carrier gas of $CO_2$ and of $H_2$ are presented with a c for carrier gas. Gas flow rates of 1.6 l/min were used except for the following: 1. 100 ml/min, 2. 60 ml/min, and 3. 230 ml/min

| Study no. | Processtime interval | No gas passage | $N_2$ | Ar | $CO_2$ | $H_2$ | $O_2$ | formate | Growth certain at | Figure number |
|---|---|---|---|---|---|---|---|---|---|---|
| 1. | 0-12 h | | Yes | | | | | | No growth | N.s. |
| 2. | 0-3 h | | Yes | | | | | | | Fig 1. |
| | 3-12 h | Yes | | | | | | | 8 h | |
| | 12-15 h | | Yes | | | | | | | |
| 3. | 0-10 h | | c | | 1% | | | | 1.5 h | Fig 2. |
| 4. | 0-3 h | | Yes | | | | | | | Fig 3. |
| | 3-30 h | | | c | 1% | | | | 23 h | |
| | 21.8-25.6 h | | | c | | 10201 ppm | | | | |
| 5. | 0-3 h | | Yes | | | | | | | Fig 4. |
| | 3-11.1 h | | | c | 1% | | <30 ppm | | 6.5 h | |
| | 11.1-15 h | | Yes | | | | | | | |
| 6. | 0-23.4 h | | Yes | | | | | 3 h | | Fig 5. |
| | 23.4-36 h | Yes | | | | | | | 33 h | |
| | 36-43 h | | Yes | | | | | | | |
| 7. | 0-6 h | | Yes | | | | | 3 h | | Fig 6. |
| | 6-12.5 | | | c | 1% | | | | 9.5 h | |
| | 12.5-19 h | | Yes | | | | | | | |
| 8. | 0-5 h | | Yes | | | | | | | N.s. |
| | 5-26.2 h | | c | | 1% | | | | 20 h | |
| | 6.7- 22.1 h | | | c | | 10002 ppm | | | | |
| 9. | 0-3 h | | Yes | | | | | | | N.s. |
| | 3-10.7 | | | c | 1% | | | 6 h | 7 h | |
| 10. | 0-3 h | | Yes | | | | | | No growth | N.s. |
| | 3-17 h | | | c | | 1040 ppm | | 10 h | | |
| 11. | 0-26 h | | Yes | | | | | 3 h | | N.s. |
| | 26-30 h | | | c | 1% | | | | | |
| | 30-70.8 h | | Yes | | | | | | | |
| | 70.8-77.8 h | Yes | | | | | | | 75 h | |
| 12 | 0-3 h | | Yes | | | | | | | N.s. |
| | 3-15 h | | c | | 1% | | | | | |
| | 6-15 h | | c | | | 9843 ppm | | | 7 h | |

Special fermentations adding peptone (Oxoid L 37)

13. 0-16 h $N_2$ gas passage, peptone added at 5 h process time. Growth was observed at 8.5 h (N.s.).

14. Nitrogen gas passage and additions of different peptones with the inoculum or into the fermenter are further covered in ref. 11.

## 2.3 Fermentor

The apparatus was obtained from Belach AB, Stockholm, Sweden. Culture volume was 2-2.2 l. Gases were obtained from Alfax, Sweden. The gas flow of the major gas used for sparging was always 1.6 l/min. When adding a second gas flow in the bottom of the fermentor directly below the impeller, flow rates of this additional gas flow were as reported in Table 1.

The concentrations of oxygen in the used gases under conditions reported as "strictly anaerobic" were below the reported detection limit (<0.2 ppm). The exit gas was passed through approximately 5 m of tubing ( ∅ 6 mm) before the outlet into an aerobic environment. The pH was, in some cases, kept constant at 6.7–6.8 by use of an automatic alkali/acid dose monitor (NaOH or HCl (1 M)). The cultivation temperature was 37 °C, and the stirrer speed was 300–400 rpm.

## 2.4 Qualitative determination of molecular hydrogen

$H_2$ was followed using a Pd-MOS device (Sensistor AB, Linköping, Sweden) as per [13]. Data are presented in arbitrary units (a.u.).

## 2.5 Determination of bacterial viability

Bacterial viability was determined as colony forming units (cfu/ml) by plating 3 drops (50 µl) of appropriate ten fold dilution's in NaCl solution (9 g/l) on nutrient agar (Oxoid, CM3, 28 g/l). Viability data was compared with optical density data and Bürker counts.

Growth was followed spectrophotometrically by measuring the optical density (OD) at 580 nm.

## 3 Results

The studies performed are summarised in Table 1. In the reference condition (sparging with $N_2$, 1.6 l/min, thus removing metabolically produced $CO_2$ continuously from the culture), the culture was inoculated into strictly anaerobic conditions at a low cell density (approximately $3 \times 10^6$ cells/ml). No growth was observed during the 12 hours period of study and no loss in viability was recorded in viable count (data not shown).

In Fig. 1, the flushing of nitrogen gas was interrupted for about 9 hours. The system was sealed from exogenous gas exchange during that period in a way that back diffusion of air (as would occur using a water column as gas seal) was prevented, thus allowing metabolically produced $CO_2$ and other volatile products to accumulate. Growth was recorded after a time lag of about 7 hours thus indicating that the mineral salts medium allowed growth of the strain.

Introducing the inoculum directly into a medium equilibrated with 1% $CO_2$ resulted in a relatively short time lag before detectable growth, Fig. 2, in accordance with the observations of Lacoursiere et al. [5]. However, if the inoculum was first introduced under conditions of nitrogen gas sparging, followed by sparging with 1% $CO_2$ at 3 hours process time, no growth was observed during a 17 hour long period. $H_2$ was introduced at this stage together with the $CO_2$ gas mixture and this did result in growth.

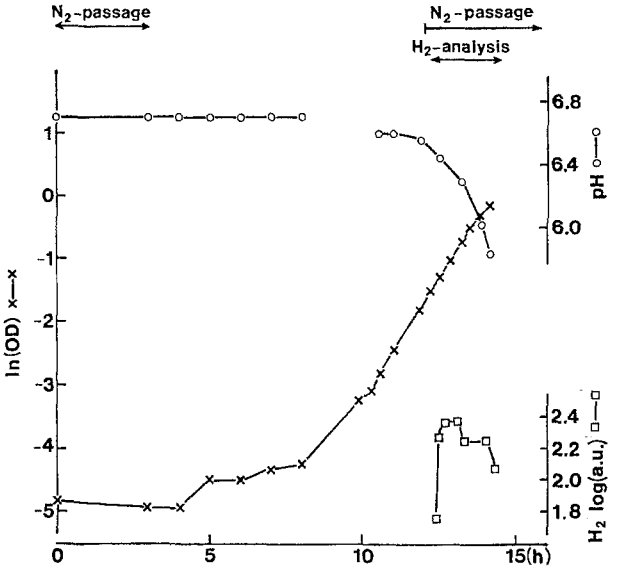


Fig. 1. Fermentation using E. coli (CCUG 26462) was conducted on glucose-mineral salts medium. The sparging with $N_2$ was interrupted, and the gas-phase was sealed of from the environment during the indicated time interval

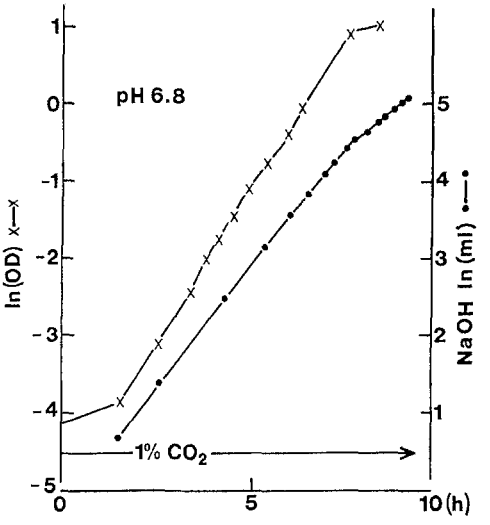


Fig. 2. 1% $CO_2$ in nitrogen was passed through the culture prior to inoculation and throughout the complete fermentation (until glucose was exhausted at about 9.2 hours process time)

In Fig. 4, 1% $CO_2$ in argon containing <30 ppm oxygen was passed through the culture, under conditions initially mimicking Fig. 3. The presence of trace oxygen in combination with carbon dioxide allowed growth. Returning the culture to strictly anaerobic conditions by sparging with $N_2$ at a higher cell density subsequently allowed the continuation of growth. Analysis of the concentration of $CO_2$ in the exit gas during similar conditions showed that the larger production rate of carbon dioxide at this higher cell density reduced the risk of $CO_2$ flush-out. The $pCO_2$ are thus maintained above growth limiting levels (data not shown).

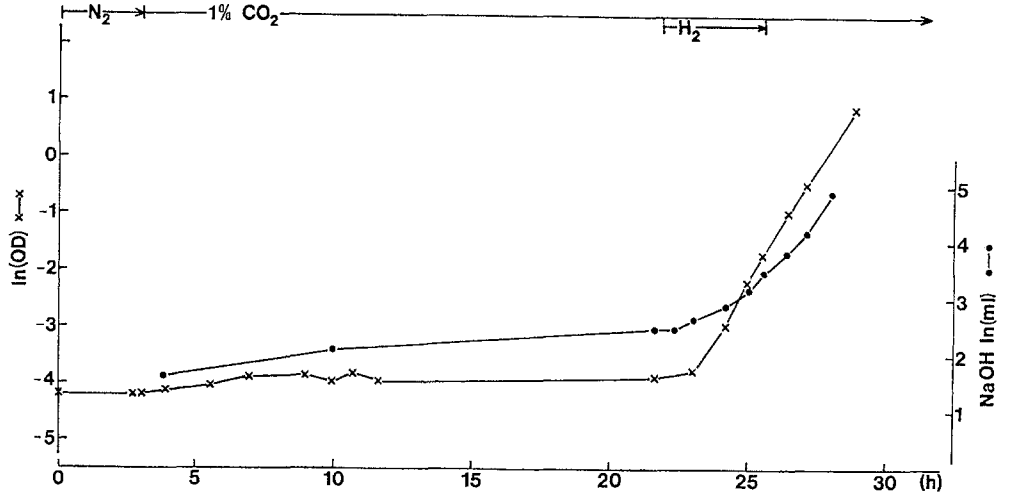


Fig. 3. $N_2$ or a gas mixture of 1% $CO_2$ in argon was passed through the culture as indicated. Induction of growth occurred when an additional gas stream containing 1020 ppm $H_2$ in argon was introduced into the fermentor (gas flow 0.1 l/min)

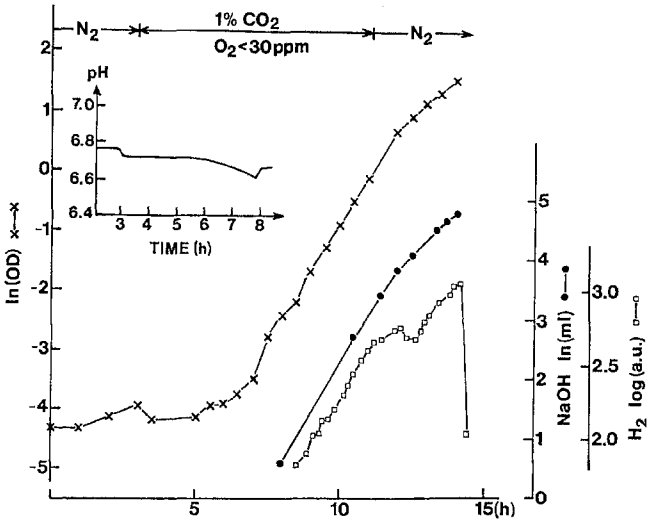


Fig. 4. A gas mixture of $CO_2$ (1%) + $O_2$ (<30 ppm) in argon was passed through the culture as indicated. The production rate of molecular hydrogen was affected at the change of sparging with $CO_2 + O_2$ and $N_2$. The rapid decrease in the production of molecular hydrogen coincided with glucose depletion

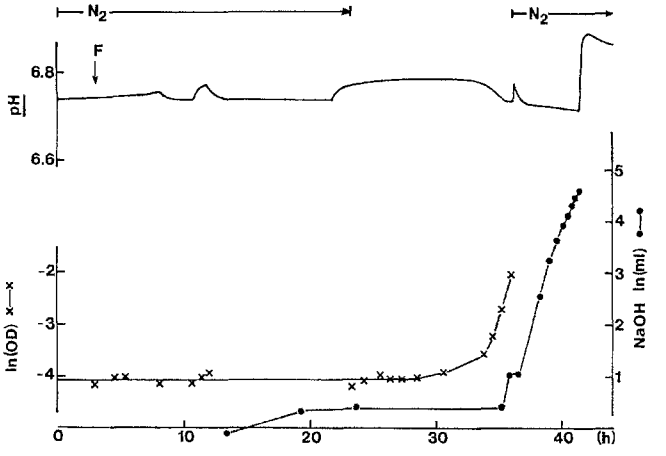


Fig. 5. The nitrogen gas flow was interrupted as shown. F marks an exogenous addition of sodium formate increasing the content of formate by 2.5 mmoles $HCOO^-$ per litre in the fermentor. It is shown that formate in the millimolar range is not sufficient for the induction of growth during simultaneous sparging with $N_2$. At about 23 hours process time the gasphase was sealed of as in Fig. 1. Growth commenced, and sparging with $N_2$ at a higher cell density did not hinder continued growth

A transient in the specific hydrogen production rate was observed in conjunction with the change from sparging with 1% $CO_2$ and pure $N_2$, and the specific growth rate decreased, Fig. 4. It was also observed that the production of $H_2$ decreased rapidly in conjunction with glucose depletion which occurred at about 14 hours process time.

Adding formate while sparging with $N_2$ did not in itself induce growth of the strain (Fig. 5), however sparging the culture with 1% $CO_2$ in argon beginning 3 hours after the addition of formate, did result in growth as seen in Fig. 6. Reversing the order of addition of formate and 1% $CO_2$ did also lead to growth (Table 1).

Sparging of molecular hydrogen coupled with addition of formate did not lead to growth. The time sequence of events during this latter study was; sparging with $N_2$ 0–3 h, $1040 \pm 20$ ppm $H_2$ in argon 3–17 h, and an addition of formate as per Figs. 5 and 6 at 10 h process time. Formic acid

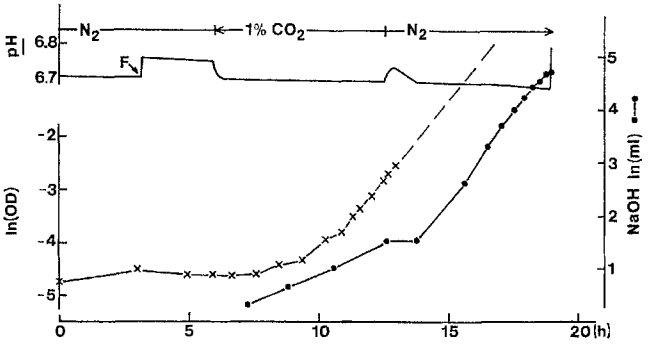


Fig. 6. Fermentation illustrating the simultaneous addition of formate and $CO_2$. F marks the exogenous addition of sodium formate increasing the content of formate by 2.5 mmole $HCOO^-$ per litre in the fermentor. Growth occurred as a response to the presence of both components. After 13 hours process time growth was monitored by the alkali dose added in order to keep a constant pH. The rapid increase in pH at 19 hours process time coincides with the expected cell density at glucose depletion, given a continued growth rate of the same order as during the growth monitored interval (dashed part in OD data)

at 3–6 mM concentration did not replace the presence of $CO_2$ even if molecular hydrogen is exogenously supplied.

Aerobic conditions have also been studied with this particular strain of E. coli, yielding qualitatively similar results with respect to the importance of carbon dioxide. Inoculating the strain at a similar density, into medium sparged with compressor air (containing approximately 470 ppm of carbon dioxide) lead to growth, whereas technical air devoid of carbon dioxide led to a long lag without observable growth (data not shown). This study was only intended as a qualitative indicator, and were not followed in extenso.

Both argon and $N_2$ have been used as carrier gas of carbon dioxide mixtures with similar results. It is therefore suggested that the shown effects of carbon dioxide in combination with molecular hydrogen or formic acid are independent on the used carrier gas.

It was also observed that the growth rate as pictured in ln(OD) data, slowed down at a lower cell density in Fig. 2. It is thus possible that the culture was also partially growth limited, in this case by endogenous components.

## 4
## Discussion and concluding remarks

The primary objective of this study was to define culture conditions allowing growth to be studied under well defined conditions and simultaneous sparging with gases. The components beside the mineral salts medium that were identified in the study ($CO_2$ and formate, or $CO_2$ and $H_2$) are physiologically related with the so called "formic hydrogenlyase complex" which in E. coli are known to degrade formate into $H_2$ and $CO_2$ [18]. Exogenous addition of the two products of the hydrogenlyase complex (molecular hydrogen and carbon dioxide) is able to maintain support of anabolically important pathways such that $CO_2$ is assimilated in e.g. the reactions: phosphoenolpyruvate + carbonate generate oxaloacetate [4], and incorporation of carbonate in the production of carbamoyl phospate [19], e.g. oxaloacetate and carbamoyl phospate may thus be produced following the exogenous addition of carbon dioxide even in the absence of an operative carbon dioxide producing formic hydrogenlyase complex. Molecular hydrogen and formate does both take part in the energy yielding reduction of fumarate to succinate [7].

It is therefore concluded that the hydrogenlyase complex produces metabolites to carbon dioxide and molecular hydrogen consuming reactions in the anaerobic metabolism of E. coli. This enzymatic pathway should therefore, apart from its catabolic role in mixed acid fermentation, also be included as part of the anabolism of anaerobically grown E. coli.

In interpreting the reported data from a physiological point of view, there are potential interfering factors which should be pointed out. The first concerns the very small influx of oxygen through "back diffusion" as reported in [8], using various nitrogen gas flow rates which, in view of their data, must be considered with respect to conditions used in the present study. It is reasonable to assume a rather negligible impact on the interpretation of data using short total process times especially in view of the results from the period of non-growth on 1% carbon dioxide in Ar (Fig. 3).

Secondly, the oxygen concentrations of the used gases were below the commercially reported detection limit (<0.2 ppm) in studies reported as "strictly anaerobic". If sub 0.2 ppm concentrations effectively had an influence on any of the reported observations, it did so only as a "positive" growth promoting factor. Therefore, the report of non proliferation on 1% $CO_2$ in Fig. 3 were not affected by trace oxygen. In studies involving molecular hydrogen and formate in combination with reports of growth, sub 0.2 ppm concentrations of molecular oxygen may have had a contributing impact.

It should also be observed that this study is centred on obtaining a growing culture where a maximum cell concentration in the order of 200–300 times the inoculum size is produced. It is therefore possible that other components of importance for long term culture during strictly anaerobic conditions can limit growth if a larger maximum crop/inoculum size quota is studied. This question is not focused on in this primary study.

The study points out the significance of adding/monitoring both carbon dioxide and formate or molecular hydrogen as required metabolites. Previously it has been known that the $pCO_2$ is a critical factor in anaerobic cultures of E. coli. Formate has also been reported as important in donating electrons to fumarate reduction [13], and as a hypothetical component in signalling anaerobiosis (fnr-regulation) [20].



## References


1. **Swanson, H. D.: Ogg, J. E.:** Carbon dioxide regulation of formate hydrogenlyase in Escherichia coli. Biochem. Biophys. Res. Comm. 36 (1969) 4: 567–575
2. **Repaske, R.: Clayton, M. A.:** Control of Escherichia coli growth by $CO_2$. J. Bacteriol. 135 (1978) 1162–64
3. **Hems, R.; Saez, G. T.:** Equilibration of metabolic $CO_2$ with performed $CO_2$ and bicarbonate. FEBS letters 153 (1983) 2: 438–440
4. **Fujita, N.: Izui, K.: Nishino, T.: Katsuki, H.:** Reaction mechanism of Phosphoenolpyruvate Carboxylase. Bicarbonate-Dependent Dephosphorylation of Phosphoenol-α-ketobuturate. Biochemistry 23 (1984) 1174–1779
5. **Lacoursiere, A.: Thompson, B. G.; Kole, M. M.; Ward, D.; Gerson, D.F.:** Effects of carbon dioxide concentration on anaerobic fermentation's of Escherichia coli. Appl. microbiol. Biotechnol. 23 (1986) 404–6
6. **Hörnsten, E. G.:** Carbon dioxide affects the growth rate of "oxygen limited" Escherichia coli at low concentrations of oxygen. Biotechnol. lett. 14 (1992) 3: 189–194
7. **Clark, D. P.:** The fermentation pathways of Escherichia coli. Microbiol. Rev. 63 (1989) 223–234
8. **Visser, W.: Scheffers, A.W.; Batenburg-van der Vegte, W. H.; van Dijken, P.:** Oxygen requirements of yeasts. Appl. Environ. Microbiol. 56 (1990) 12: 3785–92
9. **Yegneswaran, P. K.; Gray, M.: Thompson, B. G.:** Kinetics of $CO_2$ Hydration in fermentors: pH and pressure effects. Biotechnol. Bioeng. 36 (1990) 92–96
10. **Hörnsten, E. G.:** On using $H_2$ as parameter in studies of mixing. Bioproc. Eng. 7 (1992) 7: 287–290
11. **Hörnsten, E. G.; Danielsson, B.; Elwing, H.; Lundström, I.:** Sensorized on line determinations of molecular hydrogen in Escherichia coli fermentation's. Appl. Microbiol. Biotechnol. 24 (1986) 117–121
12. **Hörnsten, E. G.; Nilsson, L. E.; Danielsson, B.:** The production of molecular hydrogen by Escherichia coli during ampicillin-induced spheroplast formation. Appl. Microbiol. Biotechnol. 32 (1990) 455–60
13. **Hörnsten, E. G.; Lundström, I.; Elwing, H.:** Some biometrical applications of molecular hydrogen and ammonia determination by the use of metal-oxide-semiconductor devices. In, Bio-instrumentation; research, developments and applications. Ed. D. L. Wise, pp 47–91. Boston: Butterworths 1990

14. **Gray, P. P.; Dunnill, P.; Lilly, M. D.:** The effect of controlled feeding of glycerol on $\beta$-galactosidase production by Escherichia coli in batch culture. Biotechnol. Bioeng. 15 (1973) 1179–88
15. **Ballantine, S. P.: Boxer, D. H.:** Nickel containing hydrogenase isoenzymes from anaerobically grown Escherichia coli K12. J. Bacteriol. 163 (1985) 2: 454–459
16. **Waugh, R.: Boxer, D. H.:** Pleiotropic hydrogenase mutants of Escherichia coli K12: growth in the presence of nickel can restore hydrogenase activity. Biochimie 68 (1986) 157–166
17. **Pinsent, J.:** The need for Selenite and Molybdate in the formation of formic dehydrogenase by members of the Coli-aerogenes group of bacteria. J. Biochem. J. 57 (1954) 10–16
18. **Gest, H.:** Oxidation and evolution of molecular hydrogen by microorganisms. Bacteriol. rev. 18 (1954) 43–73
19. **Gottschalk, G.:** In: Bacterial Metabolism, Berlin: Springer-Verlag 1988
20. **Sawers, R. G.; Ballantine, S. P.; Boxer, D. H.:** Differential expression of hydrogenase isoenzymes in Escherichia coli K-12. Evidence for a third isoenzyme. J. Bacteriol. 164 (1985) 3: 1324–1331