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NITRIFICATION: AN OLD PROCESS, A NEW CONCERN

by

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Introduction

Few processes in microbiology have such a long history as nitrification. Pasteur was the first to postulate that the formation of nitrite from ammonium was catalyzed by micro-organisms. In 1890 S. Winogradsky was able to isolate some of the responsible agents. Since then, an exponentially growing amount of research has been devoted to the study of the biological oxidation of nitrogen. Although these studies have yielded considerable insight into the factors and agents governing nitrification in nature, little is known about the basic chemistry and biochemistry underlying these processes. It is exactly the lack of knowledge in these areas which has made us unable to understand and control both the classical and the recent problems caused by nitrification. Since the recent problems affect rather directly both the health and the overall quality of our environment, renewed attention has been focused on nitrification in the hope of gaining some insight in this process which apparently affects man's well-being much more directly than ever was thought before.

Autotrophic Nitrification

Historically, nitrification was defined as the biological oxidation of ammonium to nitrate. This oxidation consists of two steps : ammonium is first oxidized to nitrite by *Nitrosomonas* sp., then nitrite is oxidized to nitrate by *Nitrobacter* sp.. These organisms are autotrophic bacteria. They do not metabolize organic compounds and derive all their energy from the oxidation of the inorganic

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nitrogenous compounds. Hence, in soils and aquatic systems which are relatively low in organic matter, these organisms are the causal agents in the oxidation of ammonium to nitrate. However, in ecosystems rich in organic nutrients, they are repressed by heterotrophs which have a much higher growth rate. An important aspect of autotrophic nitrification is the fact that normally the nitrite formed by *Nitrosomonas* is readily converted to nitrate by *Nitrobacter*. Significant nitrite accumulation happens only when the pH exceeds 8.0, because *Nitrobacter* is inhibited at those pH-levels. Otherwise if conditions favor the first organism, they also favor the second and ammonium ends up as nitrate.

Heterotrophic Nitrification

In the last decennia, it became apparent that a variety of heterotrophic micro-organisms can biologically oxidize nitrogen compounds. These organisms need organic carbon compounds for both energy and carbon supply. They do not only oxidize inorganic nitrogenous compounds as e.g. ammonium and nitrite but also a variety of organic nitrogenous substances as amides, amines, oximes, hydroxamates, nitro-compounds etc. Therefore, M. Alexander (1) redefined nitrification as the biological transformation of nitrogenous compounds from a reduced to a more oxidized state. Since these heterotrophs use organic carbon compounds as their energy sources, they do not oxidize nitrogen so intensively as the autotrophic nitrifiers. However, numerically they are much more abundant than the autotrophs in any ecosystem with a reasonable organic input so that their overall contribution should not be underestimated. In addition, these organisms form a variety of oxidized nitrogenous compounds which are quantitatively of little importance but which qualitatively can be of considerable concern.

The Biochemistry of Nitrification

The oxidation of nitrogen can proceed by means of either an inorganic pathway, an organic pathway, or a combination of both (fig. 1).

Oxidation State of N	−3	−1	+1	+3	+5
Inorganic Pathway	NH_4^+	$\rightarrow NH_2OH$	$\rightarrow [NOH]$	$\rightarrow NO_2^-$	$\rightarrow NO_3^-$
Organic Pathway	$R\text{–}NH_2$	$\rightarrow R\text{–}NHOH$ (R–NOH)	$\rightarrow [R\text{–}NO]$	$\rightarrow R\text{–}NO_2$	$\rightarrow$

Fig. 1 : Biochemical pathways of nitrogen oxidation in microorganisms.

Possible steps in the inorganic pathway are ammonium, hydroxylamine, nitroxyl (which is unstabile), nitrite and finally nitrate. The organic pathway can be visualized in general terms to proceed from an amine or an amide to a substituted hydroxylamine. This compound might be oxidized to a nitroso-compound and further to a nitro-compound. Cleavage of the nitro-group from the carbon moiety may give rise to nitrite and/or nitrate.

Autotrophic nitrification by *Nitrosomonas* and *Nitrobacter* species has been well studied and is thought to proceed according to the inorganic pathway although unequivocal evidence is still not available for the identities of the products with oxidation states of − 1 and + 1.

Biochemical insight in heterotrophic nitrification is virtually lacking. 3-Nitropropionic acid has been shown to be an intermediate in the formation of nitrate by *Aspergillus flavus* (4). *Pseudomonas* species oxidize oximes (R — C = NOH) to nitrite (2). This reaction seems to involve the liberation of hydroxylamine from the oxime. Hydroxylamine is then oxidized to nitrite according to the inorganic pathway. The biosynthesis of various important hydroxamic acids (R-CO-NHOH) has been investigated (13) but the nature and mechanism of the nitrogen oxidation here too are far from elucidated. Overall, the biochemistry of heterotrophic nitrification seems to be very hard to unravel and despite numerous investigations, little insight has been gained into these transformations.

Practical Importance

a) *Fertilizer Losses and Nitrate Pollution*

Since the early breakthrough of microbiology, nitrification has been studied intensively because of its important agricultural implications. In the soil, oxidation of ammonium-fertilizer happens quite readily under normal field conditions. This in itself is not of much concern to the agronomists since most plants can equally well take up the product of oxidation : nitrate. However the nitrate has some considerable drawbacks. Firstly, due to its negative charge, it is mobile in the soil complex and leaches out with the rain. Secondly, plants can accumulate nitrate under certain conditions to such high concentrations that consumation of the plant material by either man or animal results in nitrate toxicity. Finally, nitrate can be used by the facultative aerobic fraction of the soil microflora as an electron-acceptor. In this process, nitrate is reduced to nitrite and volatile nitrogenous gases. This gives rise to a net loss of nitrate-nitrogen from the soil.

In order to reduce nitrogen losses and to forecome possible nitrate-toxicity, the agronomist has tried to inhibit nitrification in the soil. Since autotrophic nitrification is the major mechanism of nitrate formation in the soil, chemicals have been developped capable of inhibiting the causal autotrophic bacteria. Some commercial inhibitors of this process as 2-Cl-6-tri-Cl-methyl pyridine (N-serve) have been used successfully in practical agricultural management. However, since in developed countries the price of nitrogen fertilizer has become relatively low, most farmers apply some extra-amount of fertilizer rather than bother to control losses. This then has given rise to the accumulation of alarming amounts of nitrate, both in the crops and in the drainage water from arable land. High nitrate concentrations are dangerous in two ways. First of all, high nitrate levels in drainage and runoff water are a major cause of eutrofication of rivers and lakes. Secondly, high nitrate levels in food and drinkwater are a potential threat to the health of man and animal consuming them. We will discuss this more in detail further on.

b) *Formation of environmental toxicans*

Although heterotrophic nitrification is of much less importance in terms of quantitative transformation of nitrogen, qualitatively it can not be overlooked. First of all, a number of inorganic and organic nitrogenous compounds formed by these heterotrophic micro-organisms are toxic to man and animal. Hydroxylamine is a potent mutagen. Verstraete and Alexander (14) have shown that this compound can be formed and accumulated by *Arthrobacter* sp. in natural ecosystems if a suitable carbon source (acetate) and ammonium source (amides, amines) are provided.

Nitrite is toxic in many ways. It is a potent mutagen just as hydroxylamine. In addition, in babys and ruminants it causes methemoglobinemia. Finally nitrite can give rise to much more potent toxicants as the N-nitrosamines. The formation of nitrite is widespread among heterotrophic micro-organisms. Eylar and Smidt (5) tested over 1000 heterotrophic organisms isolated from different ecosystems and found that 26 per cent of the bacteria, 17 per cent of the fungi and 27 per cent of the actinomycetes were able to produce nitrite from reduced nitrogenous compounds.

Nitrate accumulation can also be brought about by reductive microbial metabolism. Many heterotrophic micro-organisms can form nitrite by reducing nitrate. It is in this perspective that the high levels of nitrate in food and drinkingwater, due to excessive use of nitrogen fertilizers, are hazardous. Microbial reduction of nitrate, either in the habital itself or in the gastro-intestinal tract upon consumption, gives rise to nitrite. Baby's and ruminants are

especially sensitive to this ion because it reacts with their hemoglobin to form methemoglobin, which is not able to transport oxygen.

One of the most important groups of toxic nitrogenous compounds are the nitroso-compounds. Many of the N-nitrosamines are potent carcinogenic compounds. Dimethylnitrosamine has been shown to be carcinogenic in sheep (9). This very compound has been detected in certain fish products, cereals, tea and tobacco. Although the origin and formation of this compound is far from clear, current evidence suggest microbial involvement. Indeed, the nitrosamines are formed through a reaction of nitrite with secondary amines. Micro-organisms seem to be directly involved in the formation of the precursors. The reaction of nitrite with secondary amines proceeds rapidly in acid conditions as in the stomach but might also be catalyzed directly by micro-organisms themselves. Accidentely, at least one fungus has been reported to form a nitrosamine (7) : p-methylnitrosaminobenzaldehyde has been isolated from the mushroom *Clitocybe suaveolens*.

The issue that nitrosamines might be important environmental carcinogens has only been raised recently by Lijinsky, W. and S. S. Epstein (9). Undoubtedly, now the warning is out, many more of naturally occuring nitrosamines will be detected. It is absolutely essential from environmental health point of view that the toxicology, the formation, and the occurence of these hazardous chemicals is carefully investigated.

Various nitroso- and nitro-compounds are also toxic to mammals. 3-Nitropropionic acid hase been identified as the toxic component of *Indogofera endecaphylla* Jacq (10). This compound has also been detected in a number of fungi and actinomycetes. *Arthrobacter* sp. have been shown to accumulate 1-nitrosoethanol (14). Toxicological studies about this compound are lacking. However, the fact that this molecule is accumulated, but does not seems to be readily degraded in a natural system is of concern.

c) *Pharmaceutical chemicals*

Fortunately, heterotrophic nitrifiers form, besides this variety of toxins also some compounds of more constructive nature. A first group of compounds, which are quite promissing as therapeutic agents, are the hydroxamic acids. A variety of microorganisms form these organic nitrogenous molecules (Table 1). The functional group in these molecules is the hydroxamic acid : R-CONHOH.

TABLE 1.

Organism	*Hydroxamic acid*	*Reference*
Ustilago sphaerogena	Ferrichrome	(11)
Mycobacterium tuberculosum	Mycobactin	(12)
Streptomyces sp.	Albomycin, ferrimycin	(12)
Penicillium frequentans	Hadacidin	(8)
Arthrobacter sp.	Terregens factor	(11)
Actinomyces sp.	Coprogen	(11)
Aerobacter aerogenes	Aerobactin	(6)

The role of these metabolites in microbial metabolism is not clearly established. However, the fact that hydroxamates are strong metal chelators suggest that these compounds are involved in mineral transport.

Hadacidin (N-formyl-N-hydroxy-glycine) has been shown to inhibit effectively human tumor growth. Albomycin and ferrimycin are antimicrobial agents. In addition, some of the hydroxamic acids which are potent urease-inhibitors as e.g. acetohydroxamic acid exhibit a marked synergistic effect when used together with other antibiotics (3).

A second group of compounds with medical applications are of course the antibiotics containing nitro-groups as chloramphenicol, azomysin, aureothin, etc. This group of products has been isolated from *Streptomyces* and *Nocardia* species.

SUMMARY AND CONCLUSIONS

The microbial oxidation of nitrogen is an extremely importact proces in nature. Bot autotrophic and heterotrophic micro-organisms take part in these transformations. In recent years, it has become apparent that the nitrification phenomenon affects much more directly man's well-being than was thought before. To the agronomist, nitrification is of concern because it influences the efficient use of nitrogen fertilizers. The ecologist has to study the impact of nitrification on eutotrification. Many oxidized nitrogenous compounds directly affect the environmental health; some even seem to be important environmental carcinogens. Finally, a variety of hydroxamates and nitro-compounds formed by micro-organisms are, or might be, potent therapeutic agents.

Although we do have some insight into the chemistry and biochemistry underlying oxidative nitrogen transformations, the basic knowledge about these phenomena is lacking. In view of the overall impact and importance of nitrification, we can only hope that this gap will be filled rather quickly in the years to come.

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SOME FACTORS AFFECTING THE PRODUCTION OF L-ASPARAGINASE BY *ERWINIA AROIDEAE*

by

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It has been proved by Broome (2) in 1963 that the L-asparaginase of guinea pig serum is responsible for its antilymphoma effects. In recent years Mashburn and Wriston (3) and Oettgen et al. (4) have shown in clinical experiments that L-asparaginase is also a promising compound in the treatment of some forms of neoplastic disease in man. For their clinical trials these authors and Adamson and Fabro (1) used L-asparaginase preparations produced by the bacterium *Escherichia coli*. However Oettgen et al. (4) observed antigenic reactions in patients treated for leukemias with L-asparaginase produced by *E. coli*.

Looking for other bacterial sources of L-asparaginase, Peterson and Ciegler (5,6) have shown that some bacteria produced larger quantities of L-asparaginase than *E. coli*. Of these an *Erwinia aroideae* strain seems to be a most promising organism.

In the present paper some factors influencing the production of L-asparaginase by *Erwinia aroideae* were investigated.

Materials and methods

Microorganism. Erwinia aroideae (Towsend) Holland NRRL B-138 was kindly supplied by the Northern Regional Research Laboratory, Peoria, Illinois (USA) and used throughout this investigation. The culture was maintained on tryptone-glucose-yeast extract agar GTY (glucose, 1.0 g; K_2HPO_4, 1.0 g; yeast extract, 5.0 g; tryptone, 5.0 g; agar, 20.0 g; distilled water, 1.0 liter. The pH was adjusted to 7.0 before sterilisation).

L-Asparaginase Assay. A 0.1 ml sample of a cell suspension was mixed with 0.9 ml of a 0.1 M sodium borate buffer (pH=8.5) and 1 ml of a 0.04 M L-asparagine solution. The mixture was incuba-

ted for 15 minutes at 37°C. The reaction was stopped by adding 0.5 ml of a 15% (w/v) trichloroacetic acid solution. After centrifugation at 5.000 rpm during 10 minutes, 1.0 ml of the supernatant fluid was diluted with 7.0 ml distilled water and 2.0 ml of Nessler's reagent. The color reaction was allowed to proceed for 15 minutes before the OD at 500 nm was determined. The OD was then compared to a standard curve prepared from solutions of ammonium sulfate as the ammonia source. One international unit (IU) of L-asparaginase is that amount of enzyme which liberates 1 μmole of ammonia in 1 minute at 37°C.

Dry weight of bacterial suspensions. The dry weight of bacteria suspensions is determined by drying 2 ml at 105°C, added to calcinated sand, during 4 hours and weighing.

Results and discussion

Influence of glucose on production of L-asparaginase

Inocula were prepared in 300 ml Erlenmeyer flasks each containing 50 ml of tryptone-glucose-yeast extract broth. After 24 hours of incubation on a rotary shaker (200 rev/min) at 28°C, 5.0 ml of the cell suspensions were transferred aseptically to 500 ml Erlenmeyer flasks each containing 100 ml of the following basic medium; tryptone, 5.0 g; yeast extract, 5.0 g; K_2HPO_4, 1.0 g; distilled water 1 liter (pH=7.0). To these media, increasing amounts of glucose were added. The glucose solution (1.0% w/v) was sterilized by filtration before addition.

After 24 hours of incubation on a rotary shaker (200 rev/min) at 28°C the cultures were centrifuged and the L-asparaginase activity was measured in the supernatant fluid. The results obtained are summarized in Table 1.

TABLE 1

mg glucose added to 100 ml medium	pH after growth	Dry weight of the bacteria in 2 ml medium	L-asparaginase activity in IU/g dry weight of cells
0	8.3	63.1 mg	546
25	8.3	67.2 mg	515
50	8.3	73.6 mg	413
75	8.3	84.8 mg	408
100	8.3	78.3 mg	362
125	8.2	88.0 mg	355

In the same time, the medium containing 0.1% glucose was used to determine the growth curve of the microorganism. After different incubation times, the turbidity of the culture was measured at 608 nm against the sterile culture medium (Figure I).

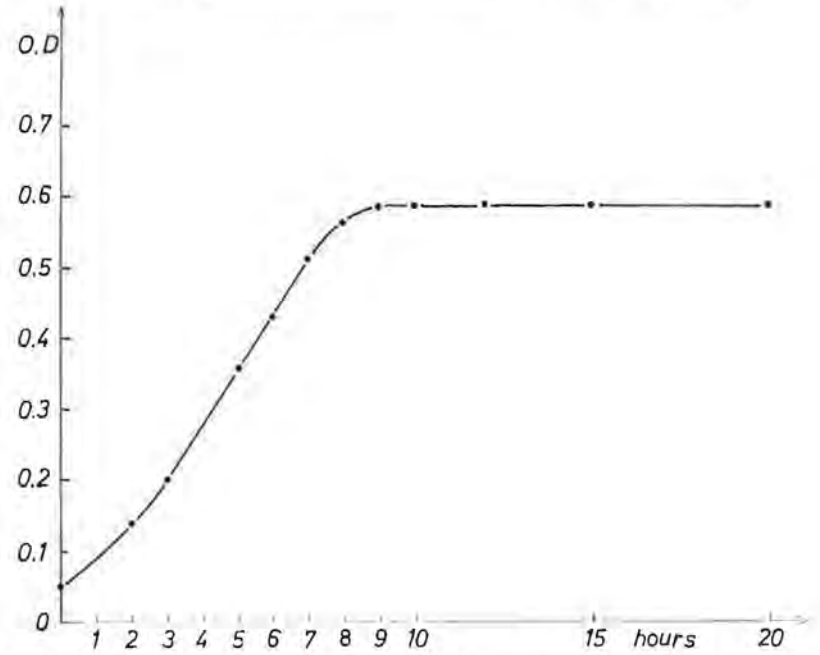

Fig. 1. Growth curve of *Erwinia aroideae* in glucose-tryptone-yeast extract broth (GTY).

Cell growth was maximum in 9 to 10 hours in 300 ml Erlenmeyers containing 100 ml GTY. Yields of L-asparaginase were highest in the medium without glucose. Addition of glucose to the medium decreased the yields considerably. The pH of the media of all cultures increased from 7,0 to 8.3.

To determine the influence of buffering the fermentation medium on the L-asparaginase production, comparative experiments were carried out. Tryptone, 5.0 g; yeast extract, 5.0 g were dissolved in 1 liter of a M/15 phosphate buffer. The pH of the medium was 7.0. To this medium glucose, sterilized by filtration, was added in concentration of 0.1% and 1.0%. These two media were compared with the GTY medium containing also 0.1 and 1.0% glucose. 100 ml of these four media were sterilized in 500 ml Erlenmeyer flasks. The media were inoculated with 5 ml of a cell suspension grown on GTY enriched with 0.1% glucose. After 24 hours of incubation on a rotary shaker (200 rev/min) at 28°C, the cultures were centrifuged and the L-asparaginase activity was

measured in the supernatant fluid. The results obtained are given in Table 2.

TABLE 2

Culture media	pH before sterilization	pH after growth	Dry weight of the bacteria in 2 ml medium	L-asparaginase activity in IU/g dry weight of cells
GTY + 0.1 % glucose	7.0	8.3	79.8 mg	354
GTY + 1.0 % glucose	7.0	4.9	78.8 mg	36
GTY buffered + 0.1 % glucose	7.0	7.5	96.6 mg	253
GTY buffered + 1.0 % glucose	7.0	5.7	83.2 mg	131

Buffering the GTY (0.1% glucose) medium resulted in a much smaller increase of the pH after growth and produced a higher dry weight of bacterial cells in the medium. However the production of L-asparaginase decreased significantly in the buffered medium. Increasing the glucose concentration in the culture medium to 1.0% resulted in an appreciable decrease of the L-asparaginase. From these experiments it seems clear that a high production of L-asparaginase is accompagnied by an increase of the pH from 7.0 to approximately 8.3. Furthermore an increase of the dry weight of bacteria in the medium is not always followed by an increase in the L-asparaginase activity.

Influence of different nitrogen sources on L-asparaginase production.

To investigate the influence of different nitrogen sources on the L-asparaginase production, the following medium was used: glucose, 1.0 g; yeast extract, 1.0 g; K_2HPO4, 1.0 g; the nitrogen source, 5.0 g; distilled water 1 liter (pH=7.0). As nitrogen sources were used: tryptone, peptone, neopeptone, proteose pepton nr 2, proteose peptone nr 3, proteose peptone nr 4 (all Difco products). 100 ml of each medium were sterilized in 500 ml Erlenmeyer flasks. 5 ml of a cell suspension, grown for 24 hours in GTY, were inoculated in the different media. After 24 hours of incubation on a rotary shaker (200 rev/min) at 28°C the cultures were centrifuged and the L-asparaginase activity was measured in the supernatant fluid. The results obtained are given in Table 3.

TABLE 3

Nitrogen source	Dry weight of the bacteria in 2 ml medium	L-asparaginase activity in IU/g dry weight of cells
Tryptone	78.4 mg	360
Peptone	76.7 mg	341
Neopeptone	60.4 mg	264
Proteose peptone nr 2	81.8 mg	382
Proteose peptone nr 3	77.2 mg	380
Proteose peptone nr 4	79.3 mg	336

From these results it can be concluded that tryptone, proteose peptone nr 2 and nr 3 are the best nitrogen sources for the L-asparaginase production under these experimental conditions.

In a second series of experiments the nitrogen sources were tested again in the same growth medium but *without* addition of glucose. The different L-asparaginase activities measured are summarized in Table 4.

TABLE 4

Nitrogen source	Dry weight of the bacteria in 2 ml medium	L-asparaginase activity in IU/g dry weight of cells
Tryptone	63.0 mg	550
Peptone	61.2 mg	545
Neopeptone	51.8 mg	475
Proteose peptone nr 2	86.6 mg	592
Proteose peptone nr 3	81.0 mg	585
Proteose peptone nr 4	83.3 mg	523

The results obtained indicate once more that in the medium without glucose, and under the given experimental conditions, tryptone, proteose peptone nr 2 and nr 3 are the best nitrogen sources for the L-asparaginase production.

L-Asparaginase activity of some simple bacterial preparations

Cultures of *E. aroideae*, grown on 100 ml GTY medium in 500 ml Erlenmeyers during 24 hours at 28°C, were centrifuged at 5,000 tpm. The cells were suspended in a borate buffer (pH=8.5). On an aliquot of this suspension the dry weight and the L-aspara-

ginase activity were determined. To another 20 ml of the suspension, 5 ml of toluene were added. The mixture was shaken for 44 hours at 28°C. After centrifugation no L-asparaginase activity could be detected in the water fase of the supernatant fluid. Furthermore the L-asparaginase activity of the autolysed cells was decreased to 7.5 IU/g dry weight of cells.

The treatment with toluene of *E. aroideae* resulted practically in a complete loss of the L-asparaginase activity. This enzyme seems to be very sensitive to toluene.

Acetone dried cells were also prepared from *E. aroideae* cultures. Cultures grown on 100 ml GTY medium in 500 ml Erlenmeyers during 24 hours at 28°C, were centrifuged at 5,000 tpm. The precipitated cells were suspended in 30 ml of a borate buffer (pH=8.5). On an aliquot of this suspension the dry weight and the L-asparaginase activity were determined. The rest of the suspension was mixed and stirred with ice cold acetone during 10 minutes. The cells were then filtered on a Gooch filter and washed with ether. L-asparaginase determinations were carried out on freshly prepared acetone dried cells and on these preparations stored at room temperature (+ 20°C). The activity of the acetone dried preparations are given in Table 5.

TABLE 5

Cell preparations	Dry weight of the bacteria in 2 ml medium	L-asparaginase activity in IU/g dry weight of cells
Living cells	78.7 mg	390
Freshly prepared acetone dried cells	70.1 mg	384
Acetone dried cells stored during 4 days	55.0 mg	407
Acetone dried cells stored during 14 days	60.0 mg	342

It is clear from these results that the procedure of acetone drying is not affecting the L-asparaginase activity of *E. aroideae* cells. Storage of acetone dried cell preparations during 4 days has no influence on the L-asparaginase capacity. Only after 14 days of storage the L-asparaginase activity has decreased.

SUMMARY

Some factors affecting the L-asparaginase activity of *E. aroideae* were investigated. Increasing concentrations of glucose in the culture medium had an inhibiting effect on the production of L-asparaginase by this microorganism. Buffering of the culture medium in order to stabilize the pH during growth resulted in a decrease of the L-asparaginase activity. From the different nitrogen sources examined, tryptone, proteose peptone nr 2 and nr 3 stimulated the L-asparaginase production. Toluene treatment of the cells practically destroyed the L-asparaginase. Acetone dried cells showed an L-asparaginase activity comparable with the activity of living cells.

RÉSUMÉ

Etude de quelques facteurs qui influencent la production de la L-asparginase par *Erwinia aroideae.*

L'influence de quelques facteurs sur la production de la L-asparaginase par *E. aroideae* fut étudiée. Il fut constaté que la présence de concentrations croissantes de glucose dans le milieu diminue la production de cet enzyme par le microorganisme. La présence d'un tampon phosphaté dans la milieu provoque une diminution dans l'activité de la L-asparaginase. Il ressort des résultats que le tryptone, le protéose peptone nr 2 et nr 3 sont les meilleures sources d'azote pour la production de la L-asparaginase. Le traitement des cellules avec du toluène détruit pratiquement toute activité de la L-asparaginase. Les cellules séchées à l'acétone présentent une activité enzymatique comparable à celle des cellules vivantes.

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