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ABSTRACT

Title of Thesis: The Effects of Oxygen Tension on Nitrate Reduction by a Pseudomonas Isolate

Hao-Jan Hsing, Master of Science in Environmental Science, 1991

Thesis directed by: Dr. David Kafkewitz
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It is a generally accepted concept that nitrate reduction only occurs under anaerobic conditions. Oxygen inhibits denitrification. However many studies of nitrate reduction under low oxygen tension have shown what has been called aerobic denitrification. This project examines the process of aerobic denitrification.

Oxgyen affects cell growth and the amount of nitrate utilized. When oxygen is absent, the cell growth rate is lower and nitrate utlization is higher then when oxygen is present.

Nitrate is reduced to nirite under anaerobic conditions. Nitrite accumulation did not continue increase. But increased then decreased producing a "Z" shaped graph when plotted.

THE EFFECTS OF OXYGEN TENSION ON NITRATE REDUCTION

BY

PSEUDOMONAS ISOLATE

BY

HAO-JAN HSING

Thesis submitted to
the Faculty of the Graduate School of
the New Jersey Institute of Technology
in partial fulfillment of
the requirment for the degree of
Master of Science in Environmental Science

1991

APPROVAL SHEET

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Chapter One : Introduction

We can find nitrate and nitrite in nature. In air, soil, marine, or spring water, nitrate and nitrite may combine with other elements to form different compounds. Nitrate can fertilize the soil and increase agricultural production. But too much nitrate or nitrite is a problem. High concentration of nitrate and nitrite will affect the environment and is harmful to human. Therefore, the EPA established standards for nitrate and nitrite concentrations in drinking water to protect public health.

There are large groups of nitrate reducers in nature. Included are the green plants, algae, many fungi, and some bacteria that can reduce nitrate to nitrite and then to ammonia or nitrogen. Some higher plants and microorganisms use nitrate to yield energy or produced anaerobic respiration. Many microorganisms utilize nitrate as an electron acceptor under anaerobic conditions. They take nitrate into their respiration cycle. At the same time, the anaerobic energy-yielding process in which the electron transport chain acceptor (nitrate) is an inorganic molecule other than oxygen is called anaerobic respiration.

Under anaerobic conditions, some microorganisms such as pseudomonas are denitrifiers which can reduce nitrate to nitrite, nitrous oxide, or dinitrogen. This process is

been termed denitrification.

The process of nitrate reduction involves three different pathways. The first one is *assimilation*, in which nitrate reduced only for the building up of cell materials; the second one is *incidental dissimilation*, in which nitrate is used as a non-essential electron acceptor; the third is *true dissimilation*, in which nitrate acts as the essential electron acceptor which enables the microorganisms to grow (Fewson & Nicholas; 1961).

Under aerobic conditions, microorganisms use oxygen instead of nitrate for aerobic respiration because nitrate reductase synthesis is repressed by oxygen (Prescott, Harley, and Klein; 1990). The other reason is that the oxygen respiration is more efficient.

Most of the presently recognized denitrifiers are chemotrophic, and heterotrophic. The genus of *Pseudomonas* has more denitrifier species than any other genus in bacteria. In addition, these bacteria are probably the most widely distributed of all the denitrifiers.

In this project, I focused on the relationship between cell growth and nitrite production under aerobic and anaerobic conditions.

Chapter Two : Paper Review

In 1886, Gayon and Dupetit demonstrated that nitrite is the first product of nitrate reduction. Nitrite has been identified as a product of nitrate reduction in bacteria, fungi and higher plants. In recent years, many studies demonstrated nitrate reduction occurs under anaerobic conditions generally.

Many microorganisms and higher plants utilize nitrate. The overall process whereby nitrate is reduced to ammonia with the subsequent formation of amino acids, protein and other nitrogenous cell constituents is known as "nitrate assimilation". Under certain conditions, however, several microorganisms can use nitrate or some of its reduction products as terminal hydrogen acceptors in place of oxygen. This process has been called "nitrate respiration" or "dissimilatory nitrate reduction".

Any denitrifiying system has four basic components: (1) electron donors in aqueous solution (normally is the carbon source, such as methane, acetate) ; (2) one of the appropriate nitrogen oxides as electron acceptor (such as nitrate, nitrite); (3) the microorganisms (denitrificans); (4) a lowered oxygen tension. Mineral nutrients, pH value, and temperature are influential as well.

The micoorganisms consume carbon source (electron donor) and produce CO_2 and electrons. They use the electrons to reduce nitrate (electron acceptor) to nitrogen oxides or nitrogen. The electrons are transfered from the carbon source to a nitrite forming a electron transfer chain and respiration. Under aerobic conditions, oxygen is reduced instead of nitrate.

The anaerobic reduction of nitrate and nitrite to nitrous oxide or element nitrogen is termed, as we already knew, denitrification, and the physiological process is called anaerobic respiration (Williams, Rowe, 1978). The process of denitrification is thought to occur in a stepwise manner as follows: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$ (Payne, 1973).

Several themes run constant. The absence of oxygen depresses the reductases necessary for denitrification. Once depressed, the quantity of these reductases is directly affected by the initial nitrate concentration in the culture (Elliot , Gilmour, 1971). Finally, nitrite accumulates in the culture media, usually before the onset of visible gas production. Nitrite may or may not inhibit further reduction of nitrogen oxides, depending on the species of bacteria and the culture conditions (Bovell, 1967).

Nitrite accumulates in the medium during early exponential phase when nitrate is the terminal electron

acceptor and then decreases to extinction before mid-exponential phase under anaerobic conditions (Williams, Romero, 1978).

Under anaerobic conditions, either nitrate or nitrite is used as the terminal electron acceptor, but nitrite is not as satisfactory as an electron acceptor to support the growth of *Pseudomonas denitrificans* under anaerobic denitrifying conditions as is nitrate (Williams,Rowe, 1978).

Oxygen is known to prevent the development of the true nitrate dissimilatory pathway with its nitrogen production in several bacteria, e.g. *Pseudomonas denitri-ficans* , *P. Stutzeri* , *Micrococcus denitrificans*, and *P. aeruginosa* . Oxygen is clearly preferred over nitrate as terminal electron acceptor in the respiration of the denitrificans. Denitrification is clearly a "second choice" mechanism for even the most active of the denitrifiers, which vary over a significant range in their demands for rigorousness of exclusion of oxygen before turning to an alternative form of respiation (Payne, 1981). An inhibitory effect of oxygen on nitrate reduction by washed suspensions of organisms previously adapted to nitrate utilization has been observed. On the other hand, there have been reports of loss of nitrate in the form of gaseous nitrogenous products from aerobically growing cultures (Chang & Morris, 1962).

It has been accepted that denitrification will proceed under anaerobic conditions and many studies have shown when cultures exposured to oxygen the denitrification stops completely. But there was something that had often been ignored since the dissolved oxygen was not monitored (Watahiki et al, 1983). However, in more recent experiments the dissolved oxygen was measured, and the cultivation concentrations were such that dissolved oxygen was present in homogeneously suspended bacterial cultures at concentrations ranging from 10% to twice air saturation. The results of the experiements clearly indicate that aerobic denitrification does indeed occur (Krul & Veeningen, 1977; Meiberg et al., 1980).

Chapter Three : Materials and Methods

Medium. The medium contained(The medium was adjusted to pH 7.5 with NaOH solution) : 7.9 g of $Na_2HPO_4.7H_2O$; 1.5 g of KH_2PO_4; 0.3 g of NH_4Cl; 2 g of KNO_3; and diluted to 1 liter (B. Taylor, W. Campbell, 1970). All the chemicals mentioned above were obtained from Fisher Scientific Co..

Trace metal solution (5 ml per liter medium): 200 ml 10% KOH; EDTA, 50 g; $ZnSO_4.7H_2O$, 22 g; $CaCl_2$, 5.54 g; $MnCl_2.4H_2O$, 5.06 g; $FeSO_4.7H_2O$, 5.99 g; $(NH_4)_6Mo_7O_{24}.4H_2O$, 1.1 g; $CuSO_4.5H_2O$, 1.57 g; $CoCl_2.6H_2O$, 1.61 g; adjusted to pH = 6 with NaOH then diluted to 1 liter.

Vitamin solution. (1 ml per liter medium): Biotin, 0.002 g; Folic acid, 0.002 g; Pyridoxine Hydrochloride, 0.01 g; Riboflavin, 0.05 g; Thiamine, 0.005 g; Nicotinic acid, 0.005 g; Pantothentic acid, 0.005 g; Vitamin B12, 0.001 g; P-aminobenzonic acid, 0.005 g; Thioctic acid, 0.005 g; diluted to 1 liter and filtered.

PS-1 salt solution (5 ml/L medium) and *Agar* : $MgSO_4.7H_2O$, 20 g/L. Agar was made by Special agar-Noble, 1.5 to 2% (w/v), obtained from DIFCO Lab..

Materials. Culture tubes, 16*150 mm, Fisher Sci. Co.; Robber stopper #20, Bellco Biotech. Co.; Standard seals (

Alumium opened-hole cap), 20 mm, ALLTECH Co.; Sterilized needles, B-D Co.; Sterilized syringes, B-D Co.; Alum Seal tube, 18*150 mm, Bellco Biotech. Co.; Cameo IIS, 25 mm nitrofilter, MSI Co.; Petri dishes, 100*15 mm from Fisher Co.. 2% oxygen-98% nitrogen gas and 100% nitrogen gas, from Matheson Co.. The medium, trace metal solution, and magnesium sulfate solution were sterilized separately.

Isolation and Purification. The microoganisms were isolated from garden soil by standard microbiological techniques (Prescott, Harley, & Klein, 1990). The medium was solidified with Noble-agar in the concentration of 1.5 to 2.0 % (w/v), and sterilized by autoclave. Pour the agar to the petri dishes and store for 24 to 48 hours. Screw-cap tubes were filled with the medium without nitrate, and put the soil samples into the tubes and shaking it. After several minutes, take 1 ml of the top layer of the sample solution and spread on prepared agar plates. These agar plates were incubated at 30^{o}C for one to two days in the incubator. After several times isolation and purification under 2% oxygen- 98% nitrogen mixture gas and 100% pure nitrogen conditions with the non-nitrate agar plates which had the sole substract (succinate, from Fischer Scientific Co.). The cultures were transfered to some prepared MR-VP medium agar plates with half strength.

Assay for nitrate and nitrite. *Buffer solution*: Mix 50 ml

of 0.2M KCl with 34 ml of 0.2M HCl, and dilute to 400 ml. Adjust the pH to 1.5 with HCl. Prepared 2% (w/v) of Ammoniun chloride. The reagents for detecting nitrite were sulfanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride. Sulfanilamide solution was prepared by 5 g of sulfanilamide (from Nutritional biochemicals Co.) adding to 300 ml of distill water containing 50 ml of concentrated HCl. Adjust the volume to 500 ml with distill water. N-(1-naphthyl)-ethylenediamine dihydrochloride was prepared by 500 mg of N-(1-naphthyl)-ethylenediamine dihydrochloride (from Sigma Co.) in 500 ml of distill water.

Nitrite assay procedure: Add 0.2 ml sulfanilamide to 10 ml sample, allow it to react at least 2 minutes, but not longer than 8 minutes. Then add 0.2 ml of N-(1-naphthyl)-ethylenediamine solution, and mix immediately. If there has nitrite exist, the solution may shown pink to red color. After 10 minutes, measure the absorbance at 543 nm by reading colormeter (Gerhardt, Murray, 1986). The absorbances of the culture were determined by Spectrophotometer (Gilford Instrument Co.).

Nitrate assay procedure: Add 0.4 ml of 2% NH_4Cl and 0.8 ml of buffer solution to 10 ml sample, and bring to 20 to 25^{o} C. Add 0.08 g of zinc metal dust (Mallinckordt Chem. Co.), and let stand for 30 min with occasional shaking. Filter out the zinc powder and follow the procedure of

nitrite assay (Gerhardt, Murray; 1986).

The Best Denitrifier Selection. All of the isolated microorganisms were streaked on prepared agar plates which contained nitrate and succinate, succinate, and nitrate separately. Those plates were stored in two different atmospheres, one was incubated in anaerobic conditions the other was in 2% oxygen-98% nitrogen conditions.

Use of the nitrite assay procedure to identify the denitrifiers. Transfer of the denitrifiers were made to culture tubes which were filled with succinate-nitrate medium, succinate medium, nitrate medium, and medium only. Those tubes were incubated in 100% nitrogen and 2% oxygen-98% nitrogen conditions at $30^{o}C$ for 4 days. After that, followed the nitrite assay to find out the best denitirfier.

The Growth of Culture. The selected microorganism was transfered to five different nitrate contain media, 4mM, 8mM, 12mM, 16mM, and 20mM, representively, which had 1 umole of succinate in each 10 ml medium. Each nitrate contained medium was incubated separately in anaerobic and aerobic conditions, with shaking at speed of 60 r.p.m.. Take samples three times a day. Observe the optical density and do nitrite assay after every sampling. The optical density of culture was determined by Spectronic 20 spectrophotometer at 560 nm (Bausch & Lomb Co.).

Chapter Four : Results

The isolated microorganisms are in the genus *Pseudomonas*. As many basic experiments had been done before, the isolated microorganisms belonged to the *genus Pseudomonas*. They had roughly ball shapes, grain-negative, and existed as an individual cells. After several times testing, they were facultative bacteria. That means they can survive under aerobic or anaerobic conditions. They grew well both in nitrate-succinate medium and succinate medium under aerobic conditions. They also can survive in nitrate-succinate medium under anarobic conditions.

The nitrate concentrations would not affect the optical density. Under aerobic conditions, isolate *Pseudomonas* reached to their steady-state after around 64 hours. In designed concentrations of nitrate, 4mM, 8mM, 12mM, 16mM, 20mM, there are no significant differences among them. It is the same that occured in the condition of anaerobic. When we compare the optical density of aerobic and anaerobic conditions, we find out the absorbances of aerobic cultures are larger than in anaerobic cultures. From thermodynamic calculations of energy yield, the maximum cell yield under denitrifying conditions was only 77 % of the maximum aerobic yield (Payne, 1981). Refer to Table 1. and Figure 1. to 5.

Aerobic denitrification has occured. There is a

controversy concerning aerobic denitrification. The evidence was obtained from many individual experiments. The figures (Fig. 1 to 5) showed that nitrite kept accumulating in the media. When the nitrate amount equaled 40 and 80 umoles, had large amounts of nitrite accumulated comparatively.

Table 1. Optical Density under aerobic conditions

Time (hr)	O.D. (4 mM)	O.D. (8 mM)	O.D. (12 mM)	O.D. (16 mM)	O.D. (20 mM)
0	0.0154	0.0154	0.0072	0.0072	0.0112
7	0.0728	0.1038	0.1276	0.0616	0.0886
15	0.2864	0.3076	0.2792	0.3060	0.3194
22	0.3122	0.3474	0.3646	0.3534	0.3574
28	0.3898	0.4628	0.4922	0.4316	0.4426
36	0.4882	0.6004	0.6616	0.5962	0.5924
43	0.5930	0.7162	0.7638	0.7074	0.6978
51	0.6712	0.8102	0.8718	0.7870	0.7972
60	0.7054	0.8712	0.9380	0.8658	0.8374
67	0.8338	1.0368	1.0818	0.8838	0.9756
74	0.8470	1.0438	1.0826	0.8794	0.9810

* The data were multiplied by 10 when showed on figures

Table 2. Nitrite accumulation under aerobic conditions

Time (hr)	umol (4 mM)	umol (8 mM)	umol (12 mM)	umol (16 mM)	umol (20 mM)
0	0	0	0	0	0
7	0.3	0.505	0.636	0.407	0.571
15	0.728	0.765	0.965	0.837	1.038
22	0.770	0.892	1.034	0.933	0.973
28	1.331	1.557	1.714	1.534	1.579
36	1.882	2.158	2.383	2.208	2.208
43	1.732	2.094	2.260	2.149	2.049
51	2.205	2.421	2.701	2.514	2.606
60	2.761	2.932	2.829	2.723	2.795
67	3.337	3.375	3.375	3.185	3.260
74	4.049	3.965	3.761	3.271	3.244

* The data were transfered from absorbance to umoles

The maximum nitrite production occured before reaching mid-exponential phase and decreased after that. After 7 hours of incubation time, some nitrite accumulated. It continued to increase up to a maximum amount after 43 hours. After that, the amount decreased (at 60 hours), then, increased again. It looked like a zigzag shape. Refer Fig. 6 10 and Table. 4.

Table 3. Optical Density under anaerobic conditions

Time (hr)	O.D. (4 mM)	O.D. (8 mM)	O.D. (12 mM)	O.D. (16 mM)	O.D. (20 mM)
0	0.028	0.032	0.035	0.029	0.035
7	0.116	0.110	0.116	0.090	0.077
15	0.256	0.142	0.168	0.125	0.103
22	0.304	0.174	0.168	0.137	0.141
28	0.356	0.200	0.283	0.178	0.164
36	0.400	0.288	0.314	0.267	0.213
43	0.420	0.418	0.408	0.367	0.375
51	0.468	0.488	0.588	0.481	0.462
60	0.540	0.598	0.604	0.548	0.548
67	0.584	0.600	0.608	0.561	0.571
74	0.644	0.600	0.608	0.582	0.574
83	0.668	0.618	0.609	0.581	0.578

* The data was multiplied by 10 when showed in figure

The maximum nitrite production amount occured when [NO_3^-] equals to 16 mM (160 umol/ml) under anaerobic conditions. In the anoxic system, the denitrifier utilize nitrate as an electron acceptor and followed the denitrification pathway. In 5 designed nitrate concentrations media, we found out that there was up to 110 umoles of nitrite accumulated in the solution with 16 mM (160 umol/ml) of nitarte. In the mean time, the gas pressure of sealed culture tubes were

larger than 24.7 psia (1 atm equals 14.7 psia, data not shown).

Table 4. Nitrite Accumulation under anaerobic conditions

Time (hr)	umol (4 mM)	umol (8 mM)	umol (12 mM)	umol (16 mM)	umol (20 mM)
0	0	0	0	0	0
7	0.242	0.341	0.193	0.193	0.160
15	0.728	0.783	0.381	0.418	0.454
22	1.780	0.892	0.872	1.338	1.196
28	6.535	3.314	3.314	8.270	4.778
36	8.840	9.441	9.266	108.690	11.518
43	2.455	12.510	11.690	92.480	24.870
51	2.668	6.222	1.803	49.610	11.784
60	3.241	1.216	1.628	79.470	4.512
67	4.557	3.328	2.231	76.930	6.423
74	7.143	4.596	3.201	78.270	8.035
83	6.405	4.473	4.431	71.240	8.089

* The data were transfered from absorbance to umoles

Under anaerobic conditions, the nitrate reduction is faster than under aerobic conditions. By reading the sample absorbances, we could find out the colorimetric readings of anaerobic were higher than the aerobic.

The highest nitrite accumulation occured at time 36 to 43 hours. From Table. 4, at the time around 36 to 43 hours, it was near mid-experiment point and the nitrite generation up to 108.69 umoles among five different nitrate concentration media.

Under aerobic conditions, it needed more than 60 hours to get to the stationary phase. But it needed more than 67

hours to get to the stable under anaerobic conditions. From Table. 1 and 3, compared with the optical densities in the different systems, there was almost 7 hours delay for the anaerobic conditions to reach the stationary phase comparing with the aerobic conditions.

Chapter Five : Discussion

As was mentioned before, most of the denitrifiers in nature belong to the genus of *Pseudomonas*. So it is not surprising to find that, the isolated microorganism is a *Pseudomans* . We performed ten cell growth curve experiments involving five different nitrate concentrations under anaerobic and aerobic condtions. Under anaerobic conditions, five growth curves were almost the same (refer Fig. 6 to 10). Under aerobic conditions, the cell growth curves were very similar. From this point of view, we could say nitrate concentration would not affect the cell growth when $[NO_3^-]$ lower than 20 mM.

No matter of oxygen present or not, the cell growth curves were similar to each other. The only difference between them that was the aerobic growth rate was faster than the anaerobic. It was easy to understand because the activity of oxygen is larger than nitrate and it is, then, used by microorganisms. In this situation, microorganisms preferred to use oxygen than nitrate if oxygen was present.

It is a generally accepted concept that denitrification just works under oxygen absent system. It will shut down when oxygen appears. But many studies show the different results, aerobic denitrification phenomenon indeed occur in some microorganisms studies, please refer to Table. 2 and

Fig. 1 to 5.

In this project, aerobic denitrification was observed. Robertson and Kuenen reported in 1984 that they found that aerobic denitrification indeed occured in some organisms. Their report supports my data. From my experiments, the headspace oxygen amount of sealed tubes are exceeded two times of the sole carbon source, succinate, totally oxidation needs. And there should have oxygen remain after succinate was used up. Some oxyegn will dissolved in solution by shaking. So, the dissolved oxygen concentration may keep saturated in solutions. Both oxygen and nitrate present in solution, microorganisms might use some oxygen and a little nitrate to proceed their aerobic respiration. So far, that might explain the higher growth rate of microorganisms in the presence of oxygen and nitrate than in anaerobic systems.

There are some special data in the experiment that needs to be examined furhter. One is the nitrite accumulation curves show zigzag shape in anaerobic systems, refer to Table. 4 and Fig. 6 to 10. We supposed that nitrate had been used as an electron acceptor and itself was reduced to nitrite initially. The nitrate reductase was very active until nitrite accumulated to an concentration or when nitrite reductase synthesized. The nitrate reductase and nitrite reductase might inhibit each other. The nitrite

concentration might induce nitrite reductase generate when the nitrate reductase was active. Nitrate reductase was depressed when nitrite reductase showed up. So the nitrite accumulation curves showed like a zigzag shape.

The other finding is gas production under anaerobic conditions. Although we did not measure the amount of gas produced. As we know that the end product of denitrification is nitrogen. And, the tubes pressure were larger than the initial pressure (24.7 psia). From the chemical mechanism of microbial denitrification, Averill and Tiedje reported in 1981, it was impossible that would have other than nitrogen and carbon dioxide generated. Also according to the pathway of denitrification, we believed that the extra gas produced might have a large portion of nitrogen in it.

Fig.1 Cell Growth in Aerobic Conditions
(Nitrate = 40 umoles)

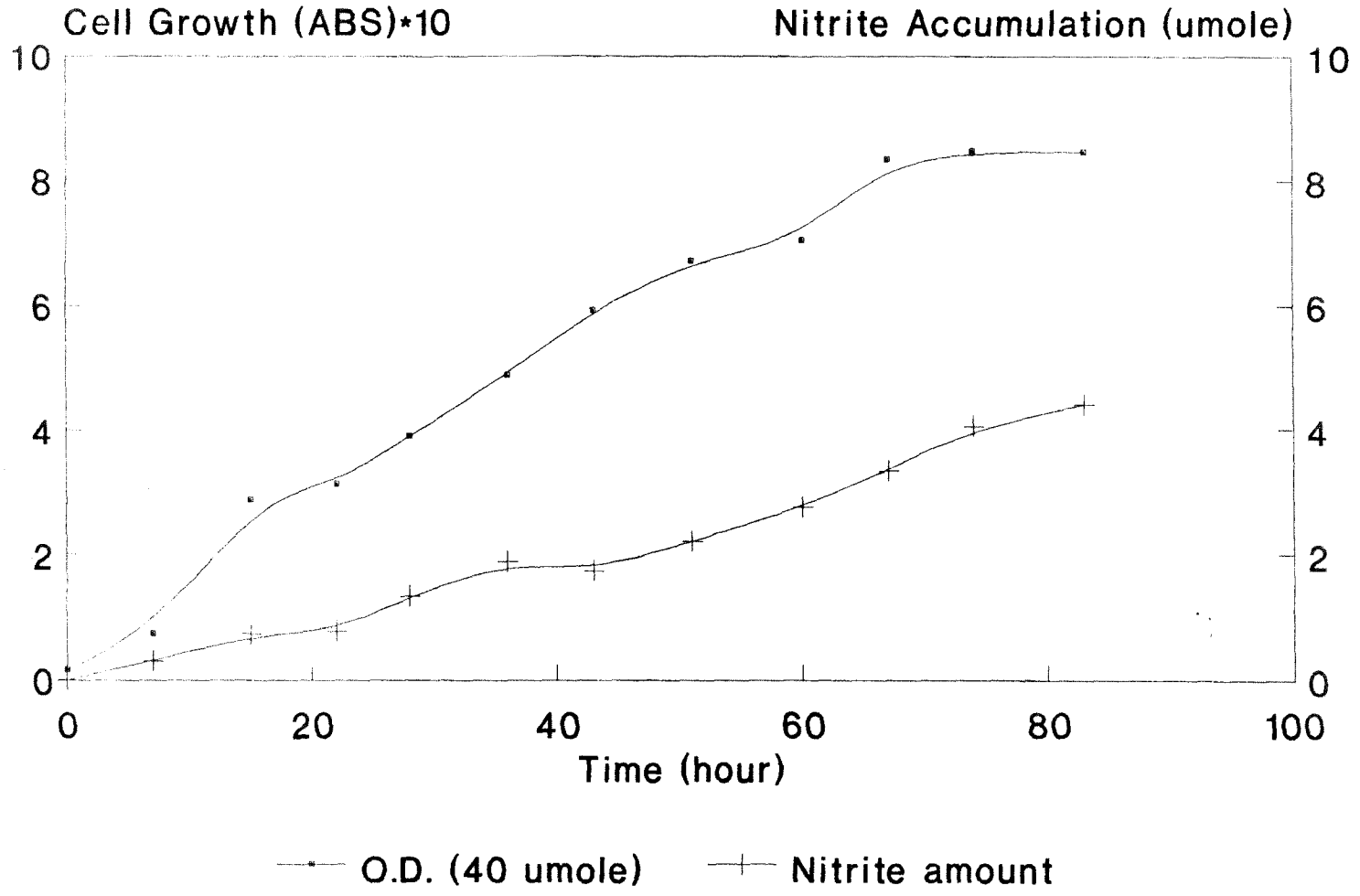


Fig.2 Cell Growth in Aerobic Conditions

(Nitrate = 80 umoles)

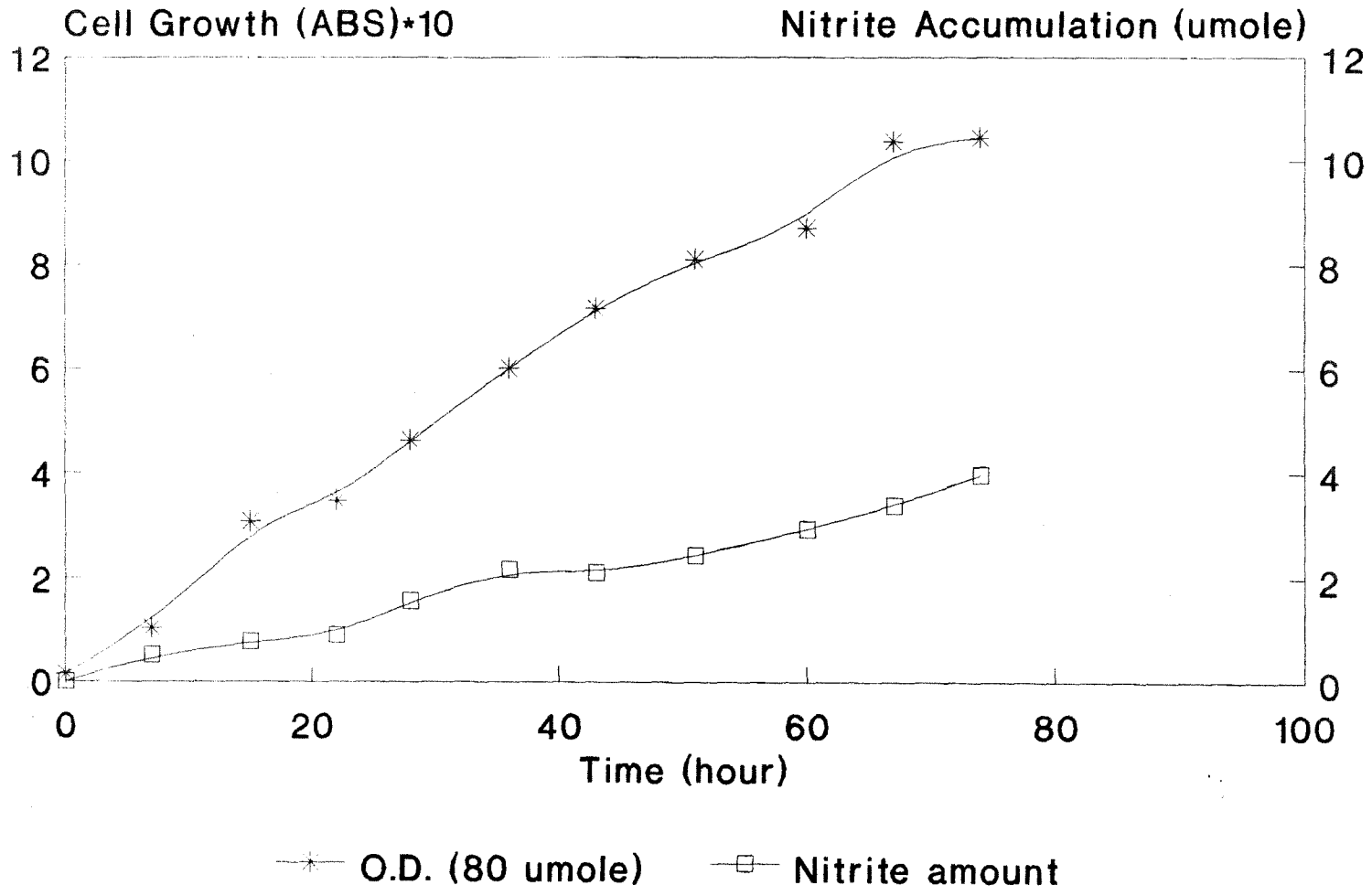


Fig.3 Cell Growth in Aerobic Conditions

(Nitrate = 120 umoles)

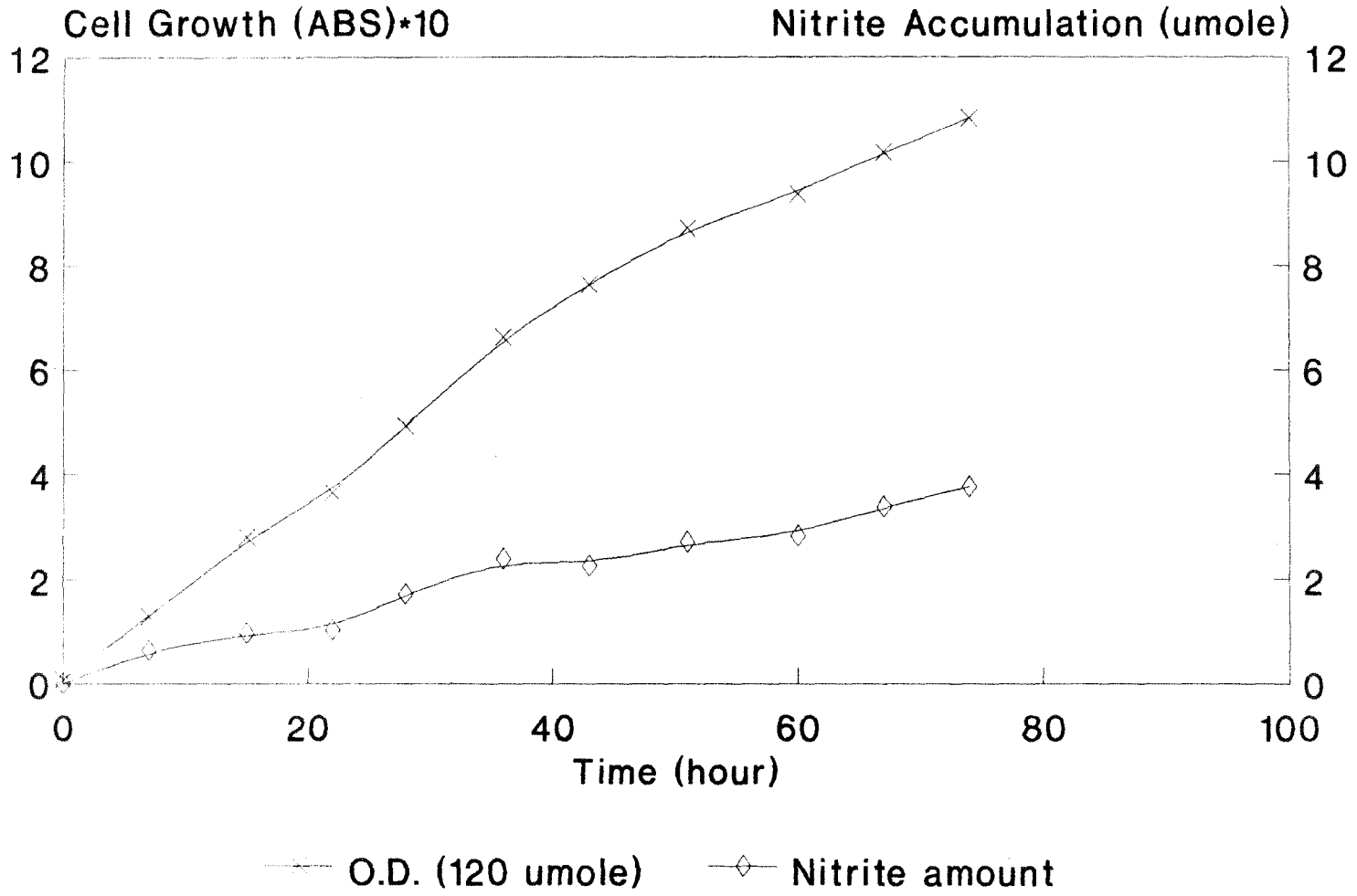


Fig.4 Cell Growth in Aerobic Conditions

(Nitrate = 160 umoles)

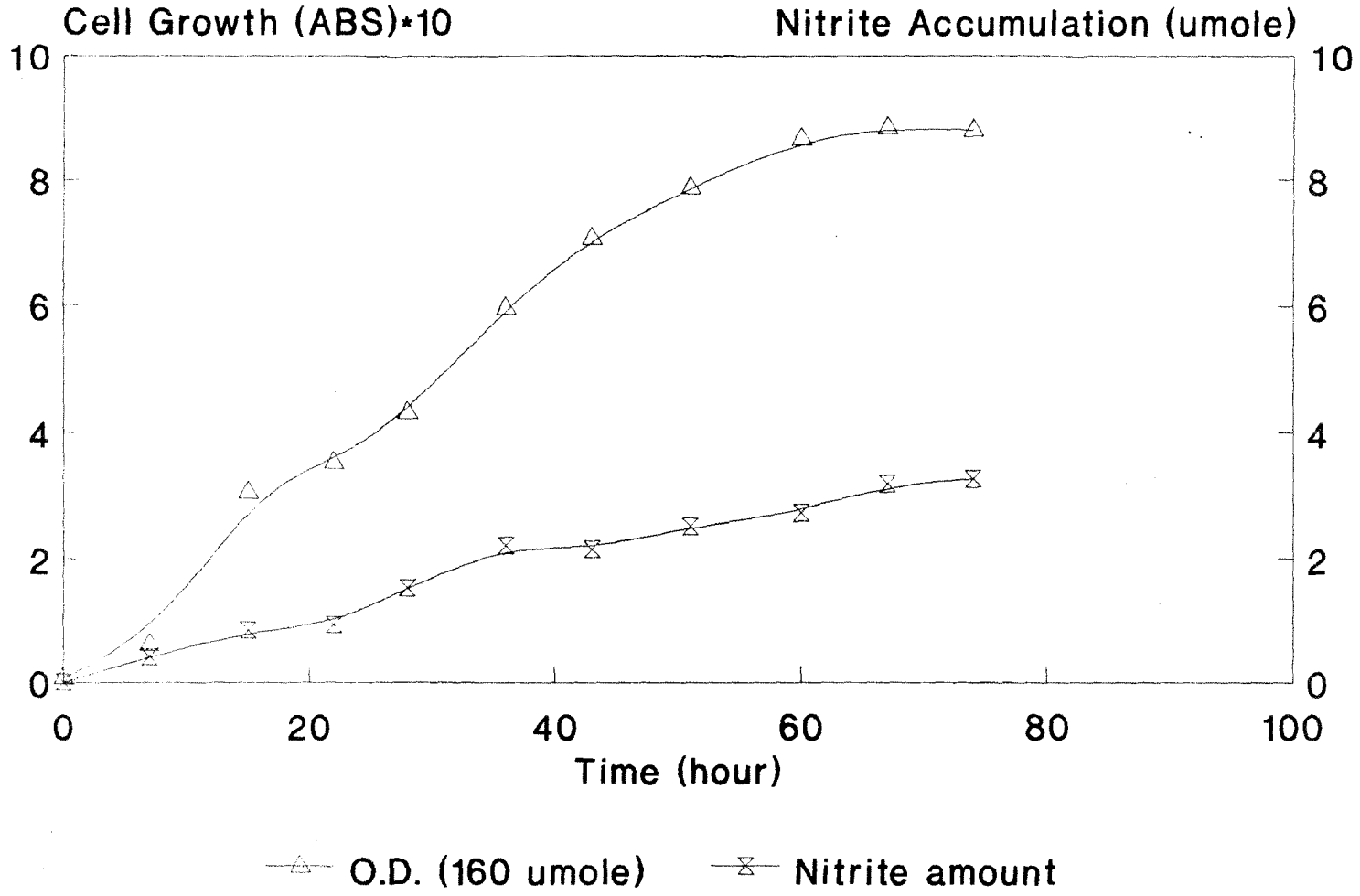


Fig.5 Cell Growth in Aerobic Conditions
(Nitrate = 200 umoles)

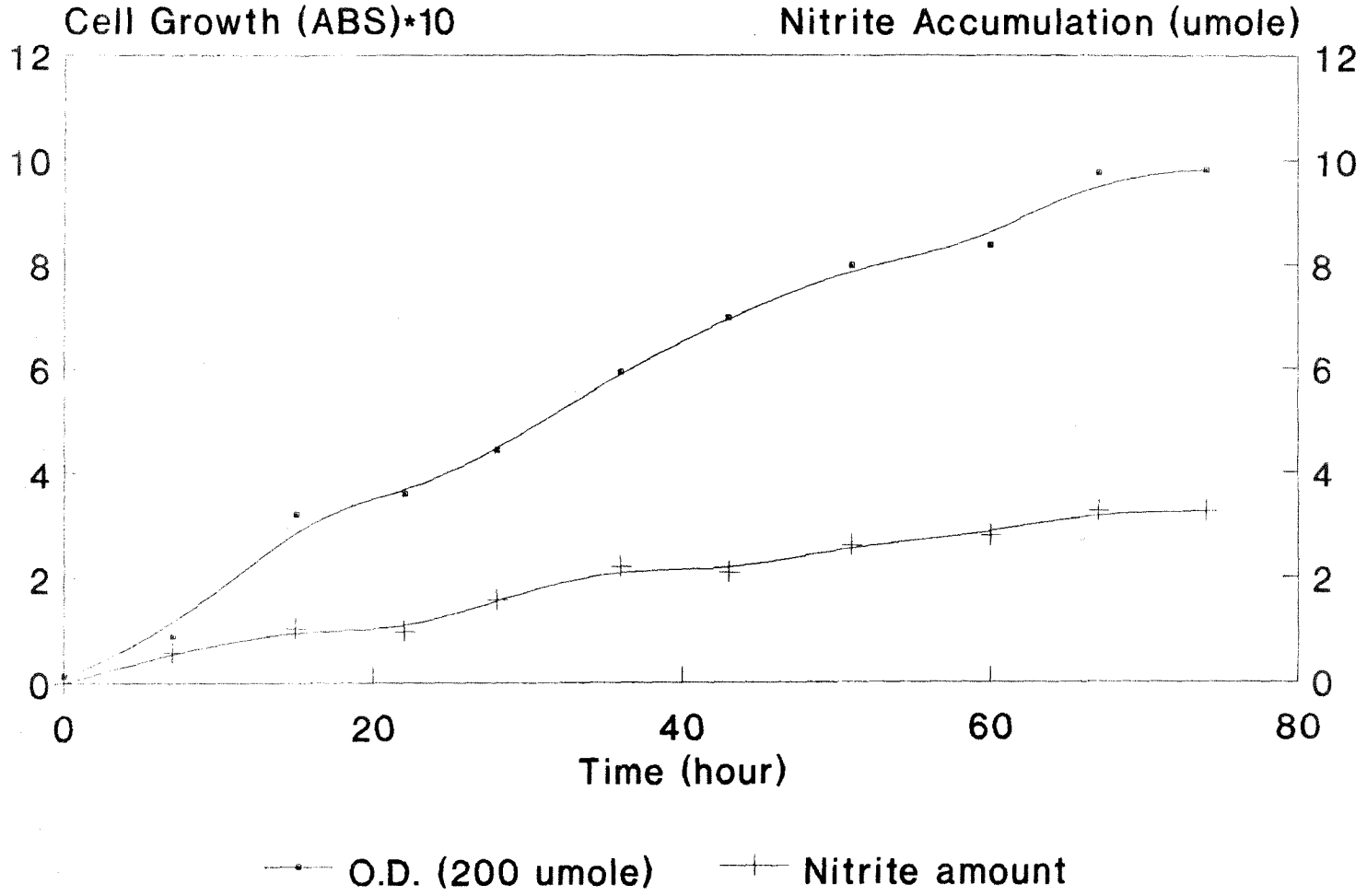


Fig.6 Cell Growth in Anaerobic Conditions

(Nitrate = 40 umoles)

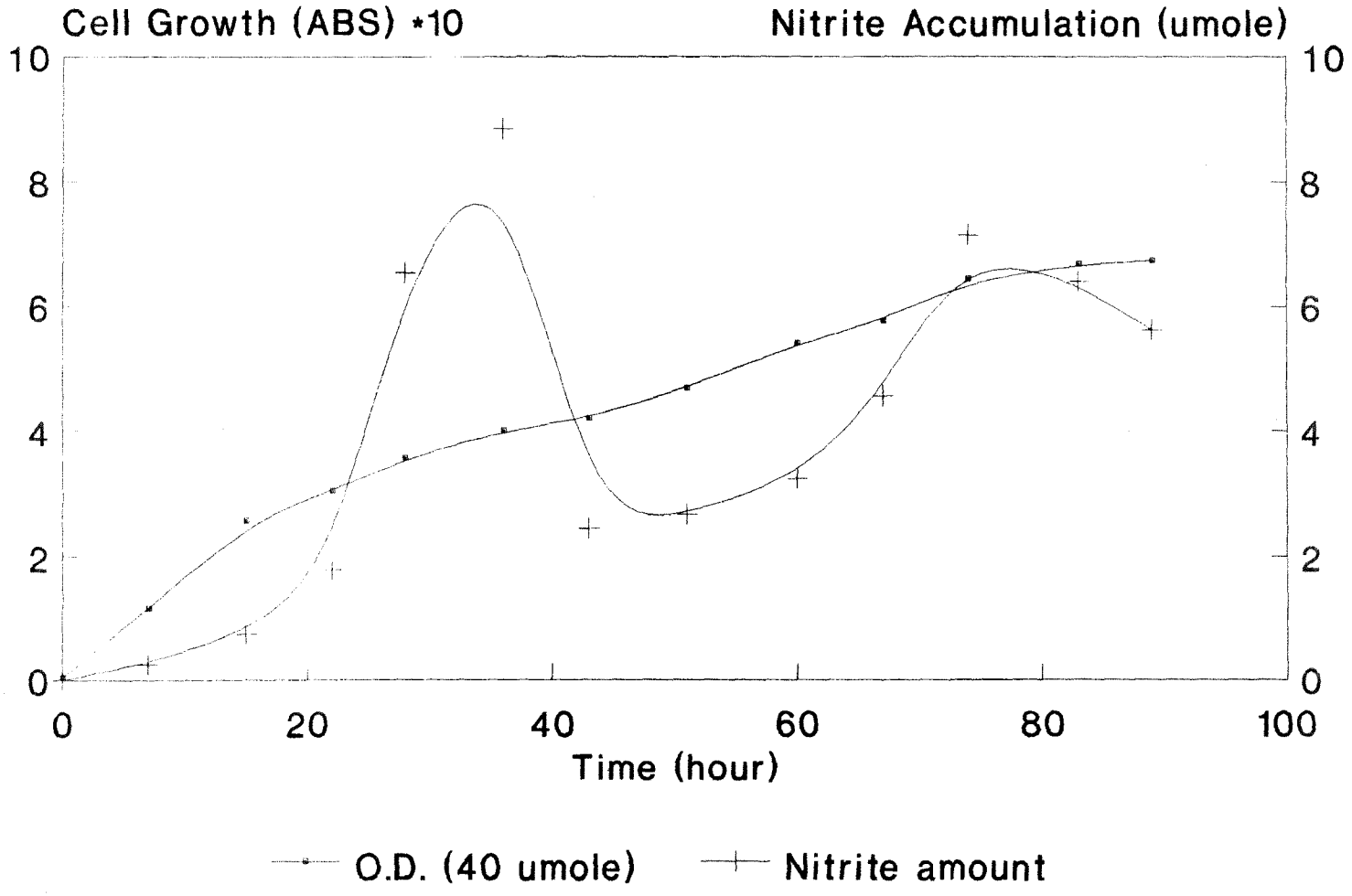


Fig.7 Cell Growth in Anaerobic Conditions

(Nitrate = 80 umoles)

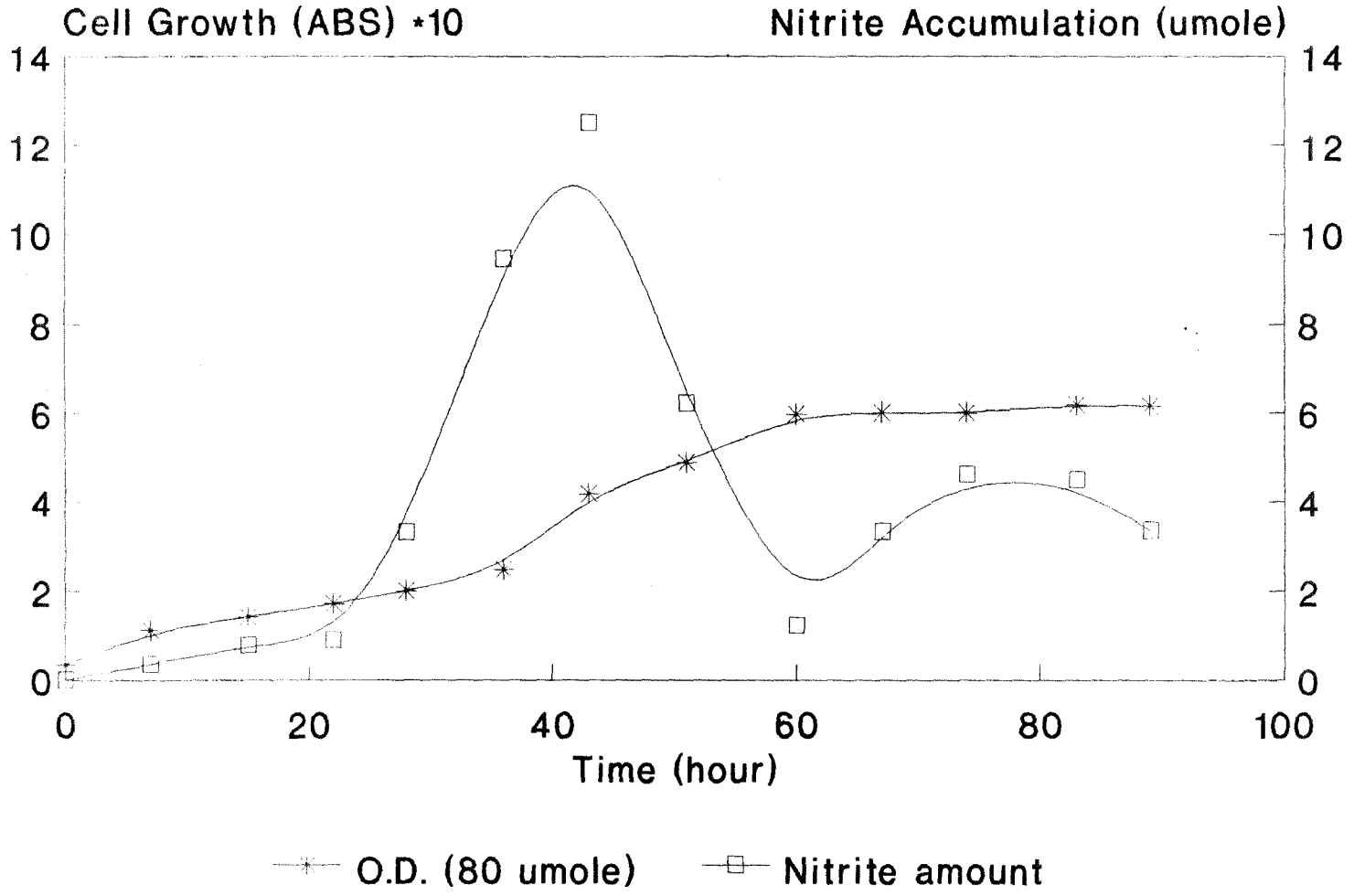


Fig.8 Cell Growth in Anaerobic Conditions

(Nitrate = 120 umoles)

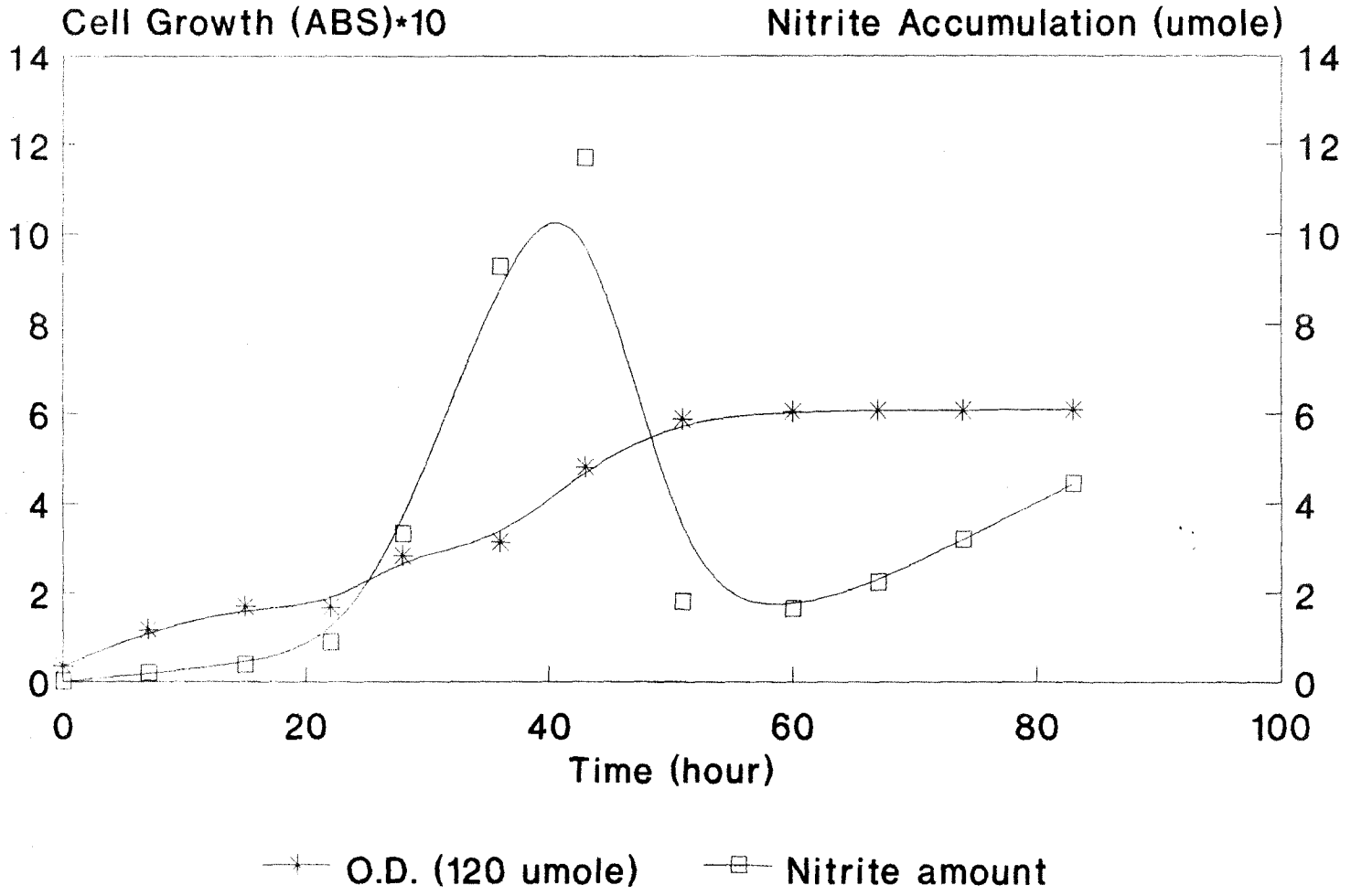


Fig.9 Cell Growth in Anaerobic Conditions

(Nitrate = 160 umoles)

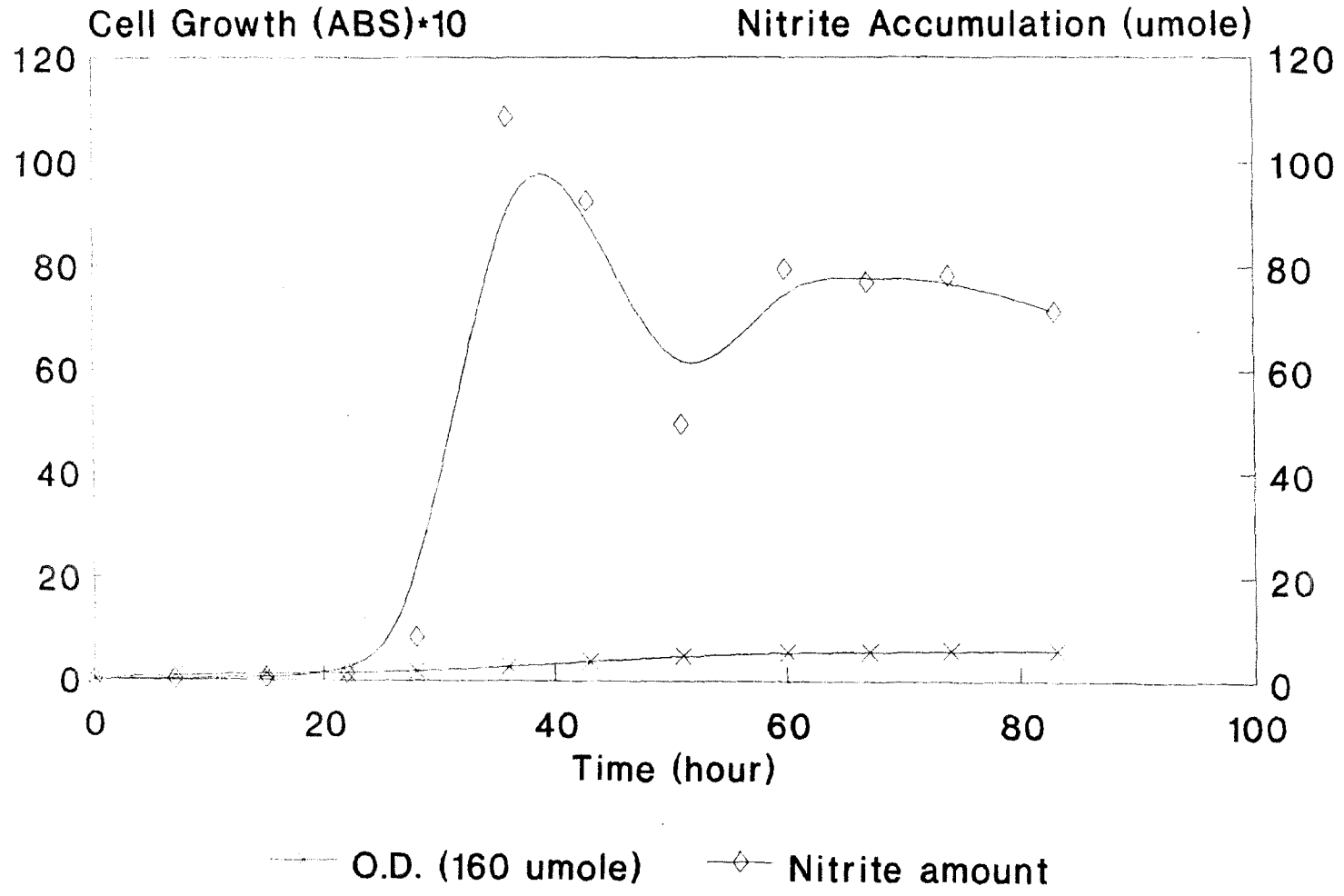
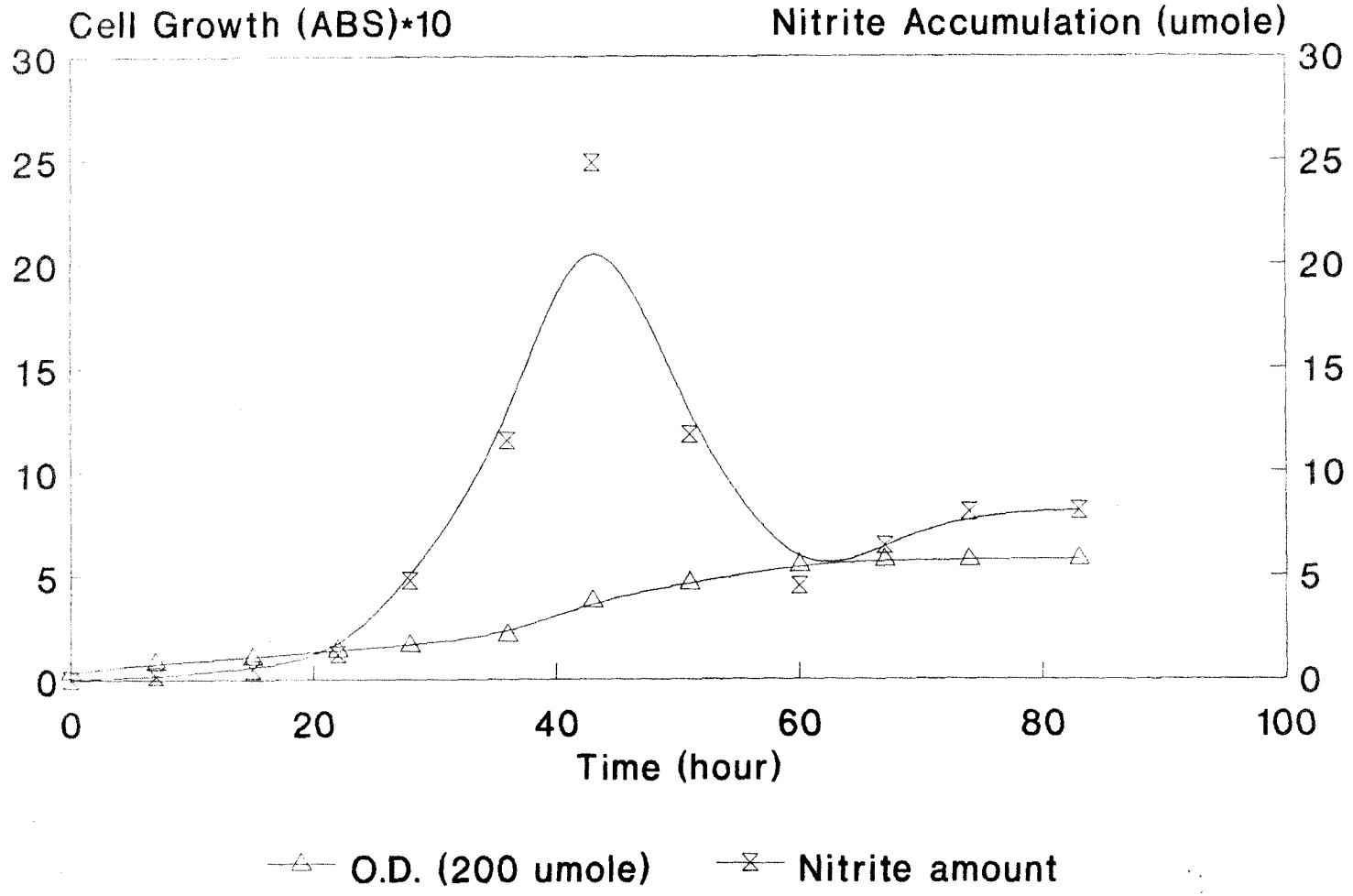


Fig.10 Cell Growth in Anaerobic Conditions
(Nitrate = 200 umoles)



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