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TOXICITY COMPARISON OF SELENIUM OXYANIONS WITH A
PROPOSED BIOMETHYLATION INTERMEDIATE DIMETHYL
SELENONE IN A MINIMAL MEDIUM
ACCOMPANIED BY SELENIUM DISTRIBUTION ANALYSIS

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By

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Abstract

Yu, Rui, Toxicity Comparison of Selenium Oxyanions with a Proposed Biomethylation Intermediate Dimethyl Selenone in a Minimal Medium Accompanied by Selenium Distribution Analysis.

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Purpose

The purpose of this research was to compare the relative toxicity of selenite and selenate with a proposed biomethylation intermediate, dimethyl selenone, and analyze bacterial media to determine the distribution of selenium species in bacterial cultures.

Methods

Pseudomonas fluorescens K27 was used and a minimal medium (DM-N medium) was applied for anaerobic growth of this bacterium. Bacterial cultures were amended with selenate, selenite and freshly synthesized dimethyl selenone; growth inhibition and doubling time methods were used to determine the toxicity.

The distribution of selenium species in amended cultures was measured in a series of time course experiments. Volatile methylated selenium species produced by the bacteria were detected by gas chromatography coupled with a fluorine-induced chemiluminescence detection. Elemental selenium precipitates and total selenium oxyanions in the supernatant were determined by atomic absorption spectroscopy. The selenite anions in the solution were determined by a colorimetric test via UV/VIS spectrophotometry over time. Furthermore, the change of nitrate

concentration over time in bacterial cultures was quantified by using UV/VIS spectroscopy.

Findings

The relative toxicity of the three selenium species examined using both growth inhibition and doubling time methods with *Pseudomonas fluorescens* K27 increased in the order of selenite < selenate < dimethyl selenone. However, the maximum concentration at which this bacterium was observed to survive is in an increasing order of dimethyl selenone (0.7 mM) < selenite (35 mM) < selenate (200 mM).

The growth rates of K27 were dramatically slowed when cultures were amended with selenate in the range of 1 mM to 5 mM; they were even two times slower than 100 mM selenate amended cultures. The higher the concentration of selenate amended, the longer the lag phase observed for this bacterium. However, in selenite amended cultures, growth rates were smoothly decreased with the increasing of the concentration of selenite, and the higher the concentration of selenite amended, the more elemental selenium was produced. Most interesting, more than one exponential growth phase was observed in this case.

Nitrate is the limiting reagent for the anaerobic growth of K27 in our DM-N medium according to these experiments. Nitrate reduction does not inhibit selenite reduction while selenite reduction does inhibit nitrate reduction: selenite was reduced simultaneously with nitrate reduction and only about 3/4 of added nitrate was consumed in the 10 mM selenite amended culture even 120 hours after stationary phase was achieved. For the selenate amended cultures, on the other hand, nitrate inhibits selenate reduction but selenate does not inhibit nitrate reduction: only when nitrate

was almost consumed, (less than 1 mM nitrate in the solution in 10 mM selenate amended culture), could K27 start to reduce selenate.

The reduction of selenate was accompanied by the production of volatile selenium and sulfur compounds with little elemental selenium being produced. However, the reduction of selenite mainly involved the production of elemental selenium and volatile selenium compounds; much less organosulfur compounds were observed in selenite amended cultures than selenate amended cultures. No dimethyl selenenyl sulfide was observed in selenite amended culture; while this is one of the major volatile organosulfur compound present in the headspace of selenate amended cultures of K27.

Thomas G. Chasteen
Thesis Director

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Chapter I

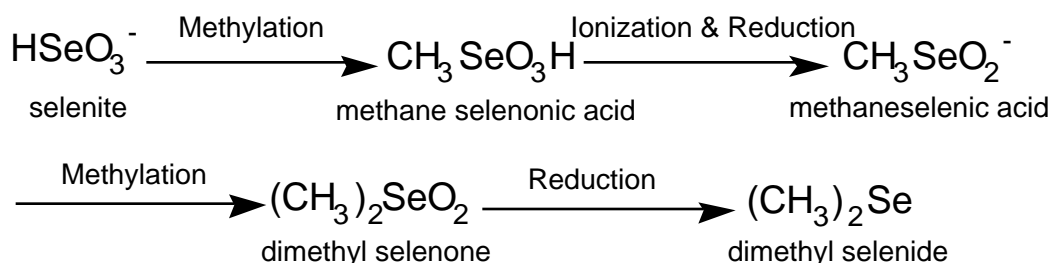
Introduction

Part 1. Bioreduction of Selenium Oxyanions

"Selenium toxicity was first confirmed in 1933 to occur in livestock that consumed plants of the genus *Astragalus*, *Xylorrhiza*, *Onuses*, and *Stanleya* in the western regions of the United States" [Spallholz, 1994]. About 40 years later, the discovery of selenium in glutathione peroxidase of mammalian species established the requirement of this element for mammalian and human metabolism [Flohe *et al.*, 1973; Rotruck *et al.*, 1973]. Because the difference between the nutritional levels of selenium and levels toxic to human health is only a small margin [Schroder *et al.*, 1970], researchers have been focused on the potential environmental toxicity of this element [Chau *et al.*, 1976].

As early as 1934, Challenger and North observed biomethylation of selenite by a selenium poisoned fungal culture. In the 1970s, relatively high concentrations of selenium compounds were found in the atmosphere above remote areas of the earth [Zoller *et al.*, 1974; Duce *et al.*, 1975; Maenhaut *et al.*, 1979], possibly due to natural biomethylation of this element. Unlike other heavy metals such as lead, arsenic, tin and mercury, whose volatile organic compounds are more toxic than their inorganic forms [Reamer and Zoller, 1980], methylation products of selenium such as dimethyl selenide and some other organic products were verified to be less toxic in comparison to the inorganic oxyanion selenite [Spallholz, 1994]. Therefore, one subject for the research of selenium compounds was the study of the detailed process of biomethylation of this element.

The first mechanism of bioreduction and methylation of selenium oxyanion was proposed by Challenger in 1945 through the study of live bacteria and fungi:



In this mechanism, dimethyl selenone, $(\text{CH}_3)_2\text{SeO}_2$, was assumed to be an intermediate and dimethyl selenide was the final product. The discovery of dimethyl diselenide $(\text{CH}_3\text{SeSeCH}_3)$, and dimethyl selenenyl sulfide $(\text{CH}_3\text{SeSCH}_3)$ as volatile products led to a further extension of Challenger's mechanism [Reamer and Zoller, 1980; Chasteen *et al.*, 1990; Chasteen, 1993; McCarty *et al.*, 1993]. Based on the observation of the production of elemental selenium in bacterial culture, Doran [1982] suggested a different pathway for selenium Biomethylation: where the selenium oxyanions are first reduced to elemental selenium and then further reduced and methylated to organoselenium forms. However, the biochemical pathway of the biomethylation of selenium oxyanions is still under investigation. Many of the proposed mechanisms suggest that either biologically produced elemental selenium precipitates or volatile organoselenides are released by bacterial cultures, decreasing the concentration of the inorganic salts and thereby decreasing the toxicity of these compounds to microorganisms [Spallholz, 1994].

Through comparing the production of organoselenium by bacterial strain *Pseudomonas fluorescens* K27 amended with SeO_4^{2-} and SeO_3^{2-} and

$(\text{CH}_3)_2\text{SeO}_2$, Zhang and Chasteen [1994] first discovered the biological reduction of dimethyl selenone. This discovery indicated that $(\text{CH}_3)_2\text{SeO}_2$ might indeed be a viable intermediate of the reduction and methylation pathway first proposed by Challenger. However, they used an enriched, complex medium, TSN (tryptic soy broth/nitrate), for their research. Their efforts to cultivate this bacterial strain under anaerobic conditions on well defined, minimal media were not successful.

At the beginning of this project, we obtained 2 recipes (called DM and F of defined media from Ray Fall at University of Colorado, Boulder. Aerobically, *Pseudomonas fluorescens* K27 was successfully cultivated in both media [Yu, *et al.*, 1996]. For anaerobic cultivation of K27 (under nitrate-reducing conditions), KNO_3 was added to both media. Just inadvertently not removing the test tubes from the water bath for more than a week during our first experiment, we fortunately obtained a very simple medium for anaerobic cultivation of K27 strain. It was called DM-N medium.

Starting at this point, several series of toxicity tests involving the study of growth and growth inhibition of *Pseudomonas fluorescens* K27 in DM-N medium amended with selenium oxyanions (selenate and selenite) and dimethyl selenone were performed. One goal of this research was to follow the efforts of Zhang [1993] by comparing toxicity of these three selenium compounds to K27 grown in DM-N minimal medium in order to further investigate the viability of dimethyl selenone as a possible intermediate of the reduction pathway.

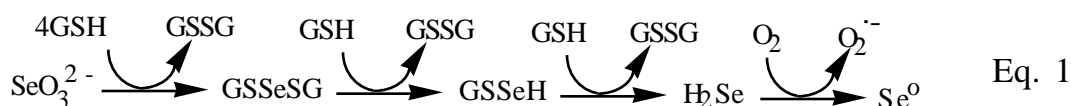
Similar to many workers who have observed that some selenite reducing bacterial cultures yield more elemental selenium than that of selenate reducing cultures, we can also easily distinguish the differences

between the two poisoned cultures by simply observing whether a reddish color is present after a period of time or not. However, all of the mechanisms reported above assume that selenate and selenite have similar biomethylation pathways, and that the first step of selenate reduction is its conversion to selenite [Reh and Macy, 1992; Lortie *et al.*, 1992; Spallholz, 1994]. Therefore, much more extended research has been carried out to study the bioreduction of selenite than selenate [Tomei *et al.*, 1992]. Based on this point, another interest of ours is trying to differentiate between the pathways of selenite and selenate reduction.

Part 2. Toxicity of Selenium Oxyanions

Since the 1930s when people started to recognize that selenium was toxic to livestock and humans, one theme of the research on selenium has been its toxicity. Lots of work has been conducted to compare the relative toxicity of various organic and inorganic selenium compounds. Although the results are somewhat variable depending on the organism tested, the general consensus is that selenate is less toxic than selenite, and organic selenium compounds exhibit widely different toxicities [Wilber, 1980; Ingersoll *et al.*, 1990; Sandholm, 1993; Spallholz, 1994]. However, until recently there has been little additional understanding about why this essential element is toxic.

In 1989, Seko *et al.* proposed that the toxicity of selenite was due to its induction of the production of superoxide ($O_2^{\cdot-}$) which was suspected to be the cause of damage to the rat eurythrocyte (red blood) cell membranes and membranes of *E. coli* and *B. subtilus*. The reaction suggested by Seko *et al.* is shown in the following equation 1:



In this equation, GSH represents glutathione. This reaction series was confirmed by Yan and Spallholz [1991] through the application of a chemiluminescence technique for the detection of superoxide. Only selenite and selenium oxide were confirmed to be able to undergo this reaction.

However we are cultivating bacteria under anaerobic conditions without any oxygen present, and thus the mechanism above is obviously not suitable for explaining the phenomena of selenium oxyanion toxicity; what is the toxic effect of selenium under anaerobic conditions? *Pseudomonas fluorescens* K27, "one of several hundred selenium resistant bacteria isolated from sediment at Kesterson Reservoir (California) after a selenium pollution episode" [Chasteen *et al.*, 1990], was used anaerobically throughout this research. This strain is a facultative bacterial strain; it can grow under either anaerobic or aerobic conditions. In our research, growth inhibition was used as a measure of oxyanion toxicity of selenium compounds to *P. fluorescens* K27.

We also want to investigate whether components of the growth medium affected the toxicity of selenium oxyanions. Therefore, the growth of bacteria was determined through a total growth period along with the measurement of concentration change of selenium and nitrate in the medium.

Part 3. Inhibition of sulfate and nitrate assimilation

The similar chemical properties of selenium and sulfur led early research about the metabolism of selenium to the inhibition of sulfate reduction in living systems [Postgate, 1952]. The complete replacement of some sulfur nutrients with their selenium analogs was confirmed in the 1950s. [Cowie and Cohen, 1957; Mautner and Gunther, 1959]. Furthermore, the major products of plant selenium metabolism are either non-proteinaceous and proteinaceous amino acids: selenomethionine or selenocystine. [Olson *et al.*, 1970; Shibata *et al.*, 1992]. In *E. coli* K-12 cells, selenate and selenite were found to be assimilated by the enzyme systems responsible for the sulfate metabolism [Huber *et al.*, 1967; Lindblow-Kull *et al.*, 1985]. The Michaelis-Menten kinetic analysis of sulfate, selenite and selenate of *E. coli* K-12 cells revealed substrate specificities and the affinities of the enzymes for the following order of sulfate>selenate>selenite. It was also found that the sulfate uptake was inhibited by selenate and selenite, with selenate being more effective in *E. coli* K-12.

The results of the inhibition of sulfate reduction by selenium oxyanions vary depending on the different living system examined. On the other hand, the effects of sulfate on selenium toxicity have been widely investigated, too. In 1993, Maier and Knight reported that an increase in sulfate concentration greatly decreased the toxicity of selenate for *Daphnia Magna*. However, for selenite, toxicity increased with the increase of sulfate until sulfate reached a certain higher concentration; then the selenite toxicity decreased with increasing sulfate concentration. For seleno-DL-methionine, results show that the change of sulfate concentrations does not

affect the toxicity. In *Pseudomonas stutzeri* strain, Lortie *et al.* [1992] found that sulfate concentration did not have an affect on either selenite or selenate reduction in this strain. However, higher concentrations of sulfite inhibited both growth and selenium reduction in *Pseudomonas stutzeri*.

Recently researchers have investigated different effects of selenate and selenite in living cells [Oremland *et al.*, 1989]. The production of superoxide and hydrogen peroxide, which was assumed to be the reason for the toxicity of selenium compounds, was only observed by amending cultures with selenite and selenium oxide. Selenate started to show toxic effects only after being reduced to selenite or a selenol. [Spallholz, 1994]. At the same time, Yan and Frenkel [1994] reported that the exposure of tumor cells to selenite exhibited in a decrease in fibronectin receptors which are present at the cell surface. However, selenate, selenomethionine and selenocystine do not have any effect on these cell surface fibronectin receptors.

The discovery of new forms of anaerobic respiration which use selenate as the terminal electron acceptors [Maiers *et al.*, 1988; Macy *et al.*, 1989; Steinberg *et al.*, 1992] opened a new area for distinguishing selenate and selenite reduction. Toxicity effects of both selenate and selenite on nitrate reduction started to be reported. Nitrate reductase was found serving as the terminal reductase for respiration with both nitrate and selenate for *vibrio* and the *P. stutzeri* strains [Steinberg *et al.*, 1992]. However, through studying a selenium resistant bacterial strain, *Thauera selenatis*, Rech and Macy [1992] reported that selenate and nitrate respiration were due to two distinct terminal reductases; furthermore, DeMoll-Decker and Macy [1993] reported that selenite reduction of this strain was probably catalyzed by another reductase: periplasmic nitrite

reductase. In addition to the study of bacterial cells, Aslam *et al.* [1990] reported on the different effects of selenite and selenate on nitrate assimilation in barley seedlings: they found that 0.1 mM selenite in solution severely inhibited the induction of nitrate uptake and active nitrate reductase, and 1 mM selenate had little effect on induction of nitrate reduction until after 12 hours. After the seedlings were pretreated with selenite, sulfate had no effect on alleviating the inhibition while sulfate still could partially alleviate the inhibitory effect of selenate. Furthermore, selenite inhibited the nitrate uptake but did not inhibit nitrate reduction. In contrast, selenate inhibited nitrate reduction but did not inhibit nitrate uptake.

Because inhibition of nitrate reduction by selenium oxyanions has not been reported for the *Pseudomonas fluorescens* K27 (a strain which we are interested in studying for toxicity effects), we directed some of our research to studying the inhibition of nitrate reduction by selenate and selenite under anaerobic conditions.

The research reported in this thesis involves the further investigation of the selenium resistant *Pseudomonas fluorescens* strain isolated from the San Joaquin Valley's Kesterson Reservoir ten years ago [Burton *et al.*, 1987]. Previous work involving the investigations of the headspace components of cultures amended with selenate, selenite, and later, dimethyl selenone has herein been broadened to include a measure of the toxic effects of these selenium species on this microorganism. Finally we have pursued a very different avenue of research with this microbe, that of determining the time course consumption of nitrate as a function of selenium oxyanion concentration.

Chapter II

Experimental Methods and Procedures

Part 1. Synthesis of Dimethyl Selenone

1-1. Apparatus and Reagents

All chemicals used in our synthesis were of analytical reagent grade and used without further purification. Dimethyl selenide was purchased from Strem Chemicals, Inc. (Newburyport, MA, USA). 3-chloroperoxybenzoic acid (65%) was acquired from the Spectrum Chemical Mfg. Corp. (Gardena, CA, USA). Methylene chloride and HPLC grade methanol were obtained from the Aldrich Chemical Company, Inc. (St. Louis, MO, USA).

1-2. Synthesis of Dimethyl Selenone

The method used for the synthesis of dimethyl selenone followed Zhang and Chasteen [1994] but was slightly modified. Dimethyl selenone was synthesized by oxidizing dimethyl selenide with an excess of 3-chloroperoxybenzoic acid in methylene chloride solution. One mL dimethyl selenide (0.013 moles) was dissolved in 5 mL methylene chloride. Three mole equivalents of MCPBA (3-chloroperoxybenzoic acid, 65%, 10.35 g) were added into 25 mL methylene chloride to form a white cloudy solution (fresh MCPBA was dissolved in CH_2Cl_2 to obtain a clear solution). Dimethyl selenide was dropwise added into the MCPBA solution and the reaction was stirred for two hours at 20°C. Then the white cloudy solution was dried using a rotary evaporator. Forty mL aliquots of ethyl ether were added individually three

times to rinse the obtained white powder. The by-product, 3-chlorobenzoic acid, which dissolved in the ether solution, was separated from the crude solid dimethyl selenone by vacuum filtration. The crude selenone was dissolved in boiling methanol (ratio: 1 g product in 12 mL HPLC grade CH₃OH) and recrystallized two times to obtain white, odorless, leaf-shaped crystals.

1-3. Melting Point Analysis of Dimethyl Selenone

The measurement of the melting point of dimethyl selenone was performed on an Fisher-John melting point apparatus (Fisher Scientific, Inc. Fair Lawn, NJ, USA) in our lab. The thermometer for this measurement was not recalibrated.

Part 2. Microbiology of *Pseudomonas fluorescens* K27

2-1. Growth of K27 in Minimal Media

2-1.1. Apparatus and Reagents

A reciprocal water bath shaker, model R76 (New Brunswick Scientific Co. Inc., Edison, NJ, USA) was used to aerobically cultivate K27. A water bath, model 83 (Precision Scientific Co., Chicago, IL, USA) was used to anaerobically cultivate K27. The growth of bacteria was measured by a Klett-Summerson Photoelectric Colorimeter (Klett Mfg. Co., New York, NY, USA) through measuring the optical density at 526 nm with a green filter (Filter Number 54). A 716-liter autoclave (Wisconsin Aluminum Foundry Co., Inc., Maniowoc, WI, USA) was used to sterilize all of the materials for the experiments.

The bacterial strain used in this project was *Pseudomonas fluorescens* K27, which was supplied by Ray Fall, University of Colorado, Boulder.

2-1.2. Microbial Incubations

In this project, F medium and a series of modified DM media were used to aerobically cultivate *Pseudomonas fluorescens* K27 bacterial strain; the corresponding F-N medium and DM-N media (with added nitrate) were used to anaerobically cultivate K27.

F minimal medium contained the following compounds: K_2HPO_4 , 7 g/L; KH_2PO_4 , 3 g/L; NH_4Cl , 1 g/L; , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g/L; sodium citrate, 0.5 g/L; trace elements stock solution, 10 mL/L; glycerol, 10 g/L. Glycerol was added as 50% (w/w) water solution.

The 1 liter trace elements stock solution used for F medium contained the following compounds: $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 125.0 mg; CaCl_2 , 5.5 mg; $\text{FeCl}_2 \cdot 6\text{H}_2\text{O}$, 13.5 mg; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1.0 mg; ZnCl_2 , 1.7 mg; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.43 mg; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.6 mg; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.6 mg.

DM minimal medium contained the following compounds: K_2HPO_4 , 7 g/L; KH_2PO_4 , 3 g/L; $(\text{NH}_4)_2\text{SO}_4$, 1 g/L; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g/L; sodium citrate, 0.5 g/L; glycerol, 10 g/L. Glycerol was added as 50% (w/w) water solution. The pH was adjusted to 7.4.

Anaerobic analogs of these media were prepared as following:

F medium + 0.1% KNO_3 (1 g/1 L) -----> F-N Medium

DM medium + 0.1% KNO_3 (1 g/1 L) -----> DM-N medium

A 1.5% (w/w) agar was added to the media recipes to prepare solid media for plates.

Other media used for the study of growth include a series of modified DM media, which are shown in the following table.

Table I.

DM culture media used in this research

Anaerobic medium	DM-I	DM-II	DM-III	DM-IV
K ₂ HPO ₄	7g/L	7g/L	7g/L	7g/L
KH ₂ PO ₄	3g/L	3g/L	3g/L	7g/L
NH ₄ Cl	-	1g/L	1g/L	1g/L
(NH ₄) ₂ SO ₄	1g/L	-	-	-
MgCl ₂ ·6H ₂ O	-	-	0.096g/L	0.096g/L
MgSO ₄ ·7H ₂ O	0.1g/L	0.1g/L	-	-
L-cysteine·HCl·H ₂ O	-	-	1.2g/L	-
L-(-)-methionine	-	-	-	<1.5g/L
Sodium Citrate	0.5g/L	0.5g/L	0.5g/L	2g/L
glycerol	10g/L	10g/L	10g/L	-
KNO ₃	-	1g/L	1g/L	-

2-2. Toxicity Experiment with *Pseudomonas fluorescens* K27

2-2.1. Apparatus and Reagents

The apparatus used in this experiment were the same as the growth experiment described in 2-1.1. of this chapter.

Na₂SeO₄ and Na₂SeO₃ used in the toxicity studies were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA) and Chemical Procurement Laboratories (College Point, NY, USA). Dimethyl

selenone was synthesized using the method described in Part 1 of this chapter.

2-2.2. Preparation of Toxicants

Stock solutions of Na_2SeO_4 , Na_2SeO_3 and dimethyl selenone were prepared by adding the chemicals to DM-N medium. While Na_2SeO_4 (0.5M) and Na_2SeO_3 (0.5M) stock solutions were sterilized by autoclaving, dimethyl selenone stock solutions were freshly prepared immediately before each experiment and sterilized by 0.2 μm sterile filtration.

All test tubes, caps, pipettes, DM and DM-N media were also sterilized by autoclaving.

2-2.3. Culture Growth and Amendment

A preculture I was prepared by inoculating 20 mL DM medium with one bacterial colony from a petri dish. The preculture was aerobically cultivated in a 50-mL Erlenmeyer flask in a 30°C water bath at a shaking speed of 105 rpm.

After aerobic cultivation for 24 hours, 2.5 mL of preculture I were added to 250 mL of DM-N medium in a screw cap flask to prepare an anaerobic preculture II. After 10 hours of anaerobic incubation at 30°C, preculture II reached the early exponential phase of growth and was used in growth and growth inhibition experiments. Different amounts of the three selenium toxicants were added to 16-mL screw cap test tubes (used to measure culture growth in a Klett meter; see section below). Preculture II was added to each tube until the final volume was 10 mL in each. Control samples only contained 10 mL of preculture II. All concentrations of the three compounds tested were replicated at least 3 times.

2-2.4. Determination of Growth Inhibition

The method used to determine growth inhibition was based on a procedure described by Bringmann & Kühn [1975] and Strotmann *et al.* [1994].

Generally, growth inhibition (GI) is expressed as:

$$\% \text{GI} = \frac{\text{OD}_{526(\text{control})} - \text{OD}_{526(\text{test})}}{\text{OD}_{526(\text{control})}} \times 100\% \quad \text{Eq. 2}$$

where OD_{526} of all poisoned samples was determined when the control reached the late exponential phase of growth [De Wever *et al.*, 1994; Strotmann *et al.*, 1994]. Since the bacteria were poisoned in the early exponential phase of growth, and the experiment was terminated just before the control reached its stationary phase, the following formula was used to calculate the percentage of growth inhibition in our experiments:

$$\% \text{GI} = \frac{\Delta \log(\text{OD}_{526(\text{control})}) - \Delta \log(\text{OD}_{526(\text{test})})}{\Delta \log(\text{OD}_{526(\text{control})})} \times 100\% \quad \text{Eq. 3}$$

where $\Delta \log(\text{OD}) = \log(\text{OD}_{\text{final}}) - \log(\text{OD}_{\text{initial}})$. $\text{OD}_{\text{initial}}$ was measured at the point when the bacteria were amended with the toxicant, and OD_{final} was measured just before the control reached the stationary phase. The EC_{50} values were determined for toxicant concentrations at which $\text{GI} = 50\%$. The growth inhibition experiment needed approximately 24 hours.

2-2.5. Determination of the Doubling Time of Bacterial Growth

The doubling time (DT) of bacterial growth was calculated by using the following expression:

$$DT = \frac{\log 2}{GR} \quad \text{Eq. 4}$$

The growth rates (GR) of bacterial samples were obtained by calculating the slope of exponential growth phase of growth curves by using the following formula:

$$GR = \frac{d\{\log(OD)\}}{dT} \quad \text{Eq. 5}$$

Here the unit of GR is hour⁻¹.

In this case, the EC₅₀ values were determined as the toxicant concentration at which the doubling time was doubled.

2-2.6. Colony Counting Method

Another direct and simple method for the measurement of bacterial growth is the colony counting of bacteria cells [Ingraham *et al.*, 1983].

Solid agar-based medium was prepared before these experiments. A flask covered with a piece of aluminum foil containing 1.5% (w/w) agar with medium was autoclaved at 10 lb/in² for 15 minutes. Then the hot sterilized flask was swirled until all of the agar solid dissolved to form a yellowish semi-transparent solution. Approximately 20 mL solution was poured into each sterilized petri dish. The plates were cooled to room temperature to obtain a solid agar medium.

In 9 sterile test tubes, nine mL of DM medium or NaCl sterile solution (concentration: 9 g/L) was added into each test tube. One mL of original bacterial culture was inoculated to the first test tube; thus a ten times diluted suspension was obtained. Then 1 mL of this suspension was transferred into the second test tube to obtain a 100 times diluted suspension. Gradually, a 10^9 diluted suspension was obtained in the ninth test tube.

A Drigalsky glass rod was used to spread 0.1 mL suitably diluted suspension on each agar plate. After 24 hours, grown colonies were counted on the plate. The optimal numbers of colony forming units were between 30 to 70 per plate.

2-3. Analysis of Selenium Distribution and Nitrate Consumption in Time Course Experiments of Bacterial Growth

2-3.1. Apparatus and Reagents

A Perkin Elmer 603 Atomic Absorption Spectrophotometer (Norwalk, CT, USA) was used to analyze the digested elemental selenium and selenium in the form of Na_2SeO_4 and Na_2SeO_3 species. A UV/VIS spectrophotometer, model V-550 (Jasco Corp., Tokyo, USA) was used for quantitative analysis of selenite and nitrate. A Hewlett Packard 5890 Series II gas chromatograph coupled with a Sievers Research model 300 sulfur chemiluminescence detector (SCD) was used to analyze the headspace compounds above bacterial cultures.

A Sorvall RC-5 Superspeed Refrigerated Centrifuge (Dupont Instruments, Wilmington, DE, USA) was used for centrifugation. A 250-mL autoclavable polycarbonate filtration apparatus with diameters of 47

mm and removable 0.2 μm membranes were purchased from VWR Scientific, Inc. (Sugarland, TX, USA). Ten-mL syringes with Schleicher & Schuell Uniflo-25 mode 0.2 μm disposable syringe filters (Keene, NH, USA) were also used to filter sterilize solutions.

2-3.2. Procedure of Time Course Experiments

The medium used in all time course experiments was DM-N. After aerobic cultivation of one bacterial colony in DM medium at 30°C for 24 hours, a volume of 25 mL of this preculture was transferred into 1 liter of sterile DM-N medium. The diluted culture was anaerobically cultivated for 10 hours at 30°C; then 200 mL was used in order to add 10 mL portions into 20 test tubes with screw caps. These were our control cultures. Specific amounts of sterile selenite or selenate stock solutions were added to the remaining 800 mL of preculture; this 800 mL preculture was further separated into 80 test tubes; each test tube contained 10 mL of the poisoned culture. For those samples which needed headspace analysis, Teflon[®] lined silicone septa (13 mm) with open-top screw caps (Alltech Associates, Inc., Deerfield, IL, USA) were used to seal the 16-mL test tubes instead of screw caps.

All poisoned samples and controls were incubated in a 30°C water bath. One control and 4 samples were taken to measure optical densities every 2 to 12 hours depending on the growth rate of the bacteria. Accompanying the OD measurement, headspace gas analyses were carried out by injecting 1 mL of the headspace gas from each test tube into the hot injector of the GC/SCD, followed by the analysis describe in the instrumental section. Then the 5 test tubes were put into the refrigerator (-2°C) before conducting further separation and analysis.

Four individual time course experiments were carried out by amending *P. fluorescens* K27 with 1 mM or 10 mM of selenite or selenate.

2-3.3. Preparation of Samples

The samples were taken from the refrigerator and the cells were collected by either centrifugation or filtration. Samples were usually separated by filtration using a 0.2 μm filter paper. If the sample amount was more than 50 mL, centrifugation was used for the further separation.

The samples were centrifuged at 7000 rpm for 20 minutes in the Sorvall RC-5 Superspeed Refrigerated Centrifuge to spin down the elemental selenium precipitate and cells at 10°C. The clear supernatant was stored in the refrigerator at -2°C to stop bacterial growth and await further analysis. The precipitate was washed with DI water and centrifuged two times before being transferred to a 75-mL beaker.

2-3.4. Measurement of dry weights

The 0.2 μm filter papers were dried at 105°C for 24 hours in an oven; then they were weighed and used to vacuum filter 10-mL samples. The filtrate of each sample was transferred to a test tube for further analysis. The precipitate was rinsed several times with DI water before being dried in an oven. The dried paper and precipitate were weighed on an analytical balance to 4 place precision.

2-3.5. Digestion of the Precipitate

The dried precipitate was added into a 75-mL beaker with filter paper, and 5 mL of concentrated nitric acid was added. A hot plate placed in the hood was used to heat the beaker at medium rate until more than half of the

solution was evaporated and the red precipitate disappeared from the filter paper. Then the filter paper was rinsed with up to 3 mL DI water into the beaker. The beaker was reheated until all water and nitrogen oxide fumes were given off and a yellowish solid was left. The beaker was allowed to cool for about 2 minutes and the digestion was repeated by adding an additional 2 mL of concentrated nitric acid; when the yellowish precipitates appeared again and all of the brown nitric oxide fumes were given off, the beaker was cooled for about 1 minute and then 1 mL of concentrated hydrochloric acid (J. T. Baker Co.) was added. The solution was then heated for half a minute. After this it was cooled to room temperature; the solution was transferred to a 10-mL volumetric flask and diluted with DI water to a final volume of 10 mL.

2-3.6. Quantitative Analysis of Components

The digested precipitate and supernatant were at first analyzed by atomic absorption spectroscopy to determine the amount of elemental selenium and the concentration of selenium oxyanions (See Section 3 and 4 of this chapter).

The concentration of SeO_3^{2-} in the supernatant solution was measured in a UV/VIS instrument at a wavelength of 420 nm. Nitrate concentrations were measured by UV/VIS at both 220 nm and 275 nm. All of these UV/VIS procedures are detailed in section 3-2 of this chapter.

Part 3. Instrumental Methods

3-1. Measurement of Optical Density

3-1.1. Instrument

A Klett-Summerson Photoelectric Colorimeter was used to measure optical densities of bacterial cultures in our experiments. A green filter (Filter Number 54) was used to obtain a maximum absorption at 526 nm wavelength.

3-1.2. Operation

Before turning the lamp on, the light filter was placed properly between the lamp housing and the instrument, and the pointer was adjusted to coincide exactly with the line on the blank pointer scale. A fitted test tube containing DI water was used as the blank. Then the lamp could be turned on and warmed up for 15 minutes before switching on the measurement circuit. A test tube containing DI water was used to zero the optical density of the instrument before measuring the optical density of bacterial cultures.

3-2. Determination of Nitrate by UV/VIS Spectrophotometry

3-2.1. Instrument and Reagents

A Jasco model V-550 UV/VIS spectrophotometer was used for quantitative analyses of selenite and nitrate.

The selenite complexation reagent, 3,3-diaminobenzidine, 99%, was obtained from Aldrich. HPLC grade toluene was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Potassium nitrate (certified A.C.S. grade) was obtained from Fisher Scientific.

3-2.2. Quantification of Nitrate

The method used for nitrate analysis followed the Standard Methods for the Examination of Water and Wastewater [Greenberg, *et al.*, 1992] with a critical correction; a matched pair of fused silica cells of 1-cm light path was used in all of the experiments.

3-2.2.1. Preparation of Standards and Samples

The linear range for the quantification of nitrate in DM medium lies between 0 and 35.6 ppm nitrate. This range was obtained from our experimental data. Eight standard solutions of varying nitrate concentrations were prepared within this range for calibration.

Potassium nitrate was dried in an oven at 105°C for 24 h. A mass of 0.1805 g was dissolved in DM medium (not DM-N medium!) and diluted to 250 mL to obtain stock solution I. Ten mL of stock solution I was diluted to 100 mL with the same DM medium to obtain stock solution II.

All samples were diluted with DM medium to the concentration range between 0 ppm to 35.6 ppm: volumes from 0.00 to 8.00 mL of stock solution II in 1.00 mL increments were individually diluted with DM medium to a final volume of 10 mL in each. Therefore, standard solutions with concentrations ranging from 0 ppm to 35.6 ppm were obtained.

3-2.2.2. Determination of the Nitrate Concentrations

The UV/VIS instrument was turned on and warmed up for 20 min before starting any sample measurement. Wavelength settings were 220 nm and 275 nm. The reference cell contained DM medium blank and DM medium was added into the sample cell to autozero the absorption at

220 nm; then the absorption at 275 nm was read. Absorption of all the standards and samples were read at both 220 nm and 275 nm. No sample blank was needed for this experiment. Because dissolved organic matter, especially proteins from dead bacterial cells absorbing at 275 nm interfere with the absorption of nitrate at 220 nm, the absorption at 275 nm must be subtracted before plotting calibration curves and calculating nitrate concentrations.

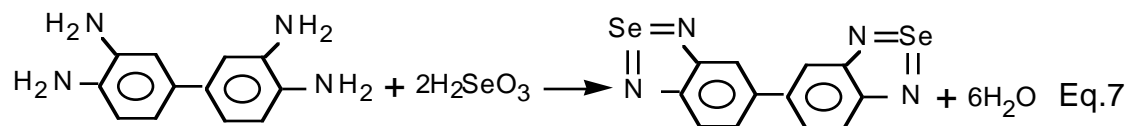
The net absorption used for calibration curves and calculation of nitrate concentration of samples was obtained from the following formula:

$$\Delta A = A_{220} - 2A_{275} \quad \text{Eq. 6}$$

3-3. Colorimetric Determination of Selenite (SeO_3^{2-}) Using UV/VIS

Spectrophotometry

A sensitive and widely used method for the spectrophotometric measurement of SeO_3^{2-} [Cheng, 1956] was applied. The method is based on the chemical reaction between SeO_3^{2-} and the photosensitive dye reagent 3,3-diaminobenzidine (DAB reagent) under acidic conditions. (Eq. 7)



Unlike the DAB reagent, the product dipiazselenol is a photo stable yellowish compound with an absorbance at 420 nm which varies linearly with the concentration of SeO_3^{2-} . Therefore, it could be used for quantitative analysis of SeO_3^{2-} .

3-3.1 Standard Preparations

For this method the linear range for the quantification of SeO_3^{2-} by this method lies between 10 μg and 140 μg selenium in 10 mL toluene.

Sodium selenite was dried in an oven at 105°C for 24 h. A mass of 0.2189 g was dissolved in DI water and diluted to 100 mL; thus 1000 ppm SeO_3^{2-} stock solution I was obtained. Stock solution I was further diluted with DI water individually to 10 ppm, 30 ppm, 60 ppm, 90 ppm, 120 ppm and 140 ppm stock solutions. One mL from each of the above six stock solutions was used as a standard to react with DAB (See Section 3-3.4 of this chapter). One mL DI water was used as a standard blank.

3-3.2. Sample Preparation from Selenite (SeO_3^{2-}) Amended Cultures

Selenite samples were diluted to the concentration range of 10 ppm and 140 ppm with water. Then 1 mL of each sample was used to react with DAB. One mL DM medium was used as sample blank.

3-3.3. Sample Preparation from Selenate (SeO_4^{2-}) Amended Cultures

In order to detect SeO_4^{2-} by this method, SeO_4^{2-} had to be reduced to SeO_3^{2-} before analysis. Sample containing SeO_4^{2-} were diluted to a concentration range between 10 ppm and 140 ppm with DI water. Then 1 mL of each diluted sample was added into a 16-mL test tube with 7 mL DI water and 8 mL concentrated HCl. A screw cap sealed with a Teflon lined silicone septum was used to tightly close the test tube. Only the Teflon face had contact with the solution. The test tube was placed in a 91°C water bath

for 30 min to carry out the reduction reaction. Then the test tube was cooled to room temperature for further analysis.

3-3.4. Reaction between DAB and SeO_3^{2-}

One mL of each standard, SeO_3^{2-} samples and blanks were added into 75 mL beakers and diluted with 23 mL DI water and 8 mL concentrated HCl individually. The reduced SeO_4^{2-} samples were transferred from test tubes to 75 mL beakers by rinsing the test tubes with 16 mL DI water; the 16 mL water was combined with the corresponding sample in a beaker; therefore, a total volume of 32 mL was obtained. These 32 mL solutions result in a 3 molar HCl matrix.

A mass of 0.5 g DAB solid was added into 10 mL concentrated HCl and further diluted with DI water to 100 mL solution. Five mL of this freshly prepared 0.5% DAB acidic solution were immediately transferred into the above 3 molar HCl matrix solutions which lead to a maximal DAB color development [Brimmer *et al.*, 1987].

These mixtures were immediately put into the dark for 50 minutes for the formation of the selenite containing complex: dipiazselenol. Then the samples were neutralized with concentrated ammonium hydroxide to pH 7-8, leading to the development of a yellowish color. The neutralized solutions were extracted with 10 mL HPLC grade toluene in a separatory funnel; the top layers with the yellowish color were used for UV/VIS analysis.

3-3.5. UV/VIS Determination of SeO_3^{2-}

The UV/VIS instrumentation operation was the same as for nitrate measurement; the wavelength was set at 420 nm. Toluene was used as reference. When the sample cell contained toluene, the absorption was set to zero. Standards were run to make a calibration curve in each experimental period.

3-4. Quantification of Selenium Oxyanions by Atomic Absorption

Spectroscopy

3-4.1. Instrument and Reagents

A Perkin Elmer 603 Atomic Absorption Spectrophotometer was used to analyze the digested elemental selenium and both Na_2SeO_3 and Na_2SeO_4 species. A Se-As-Te hollow cathode lamp, purchased from Fisher Scientific, Inc., was used as light source for the atomic absorption spectrometer.

A selenium standard (SeO_2 in HNO_3) for atomic absorption spectroscopy was obtained from Ricca Chemical Company (Arlington, TX, USA). Concentrated HCl was obtained from J. T. Baker Inc. (Phillipsburg, NJ, USA).

3-4.2. Standard Preparation

The linear range for selenium detection using AAS lies between 5 ppm and 50 ppm at 196 nm wavelength with a 0.2 nm slit width. Standard solutions, usually five concentrations, were prepared within this range. The standard solutions were prepared by adding a certain volume of a 1000 ppm standard SeO_2 solution into 100-mL volumetric flask and diluting with

DI water and 10 mL concentrated HCl (36.5-38%) to a final volume of 100 mL. The final concentration of HCl in the solution was 10% (v/v).

3-4.3. Sample Preparation

Samples prepared from supernatants and digested solutions of precipitates were diluted with DI water and concentrated HCl to approximate Se concentrations between 5 ppm and 50 ppm. The concentration of HCl in the solution was 10% (v/v) in order to mimic the matrix of the standards.

3-4.4. Detection of Selenium Oxyanion Species

The flame atomic absorption spectrometer was set at 196.0 nm wavelength with slit width of 0.2 nm. The electronic current of the Se-As-Te hollow cathode was set at 10 mA. The flame was air-acetylene (oxidizing) with the fuel to air ratio set at 20 to 60. A lean blue flame was obtained with this fuel to air ratio which was recommended by the instrument manufacturer [Perkin Elmer Cook Book, 1984]. The AAS instrument and lamp were turned on for at least 15 min to warm up before igniting the flame. Before measuring, wavelength setting, position of the lamp, linearity and height of burner head, flow rate of sampling and flow ratio of air and acetylene were optimized. Ten percent HCl acidic DI water was used as a blank to zero the absorption at 196.0 nm. Absorption of samples was read using the integration setting. The integration time of each reading was 4 seconds. Five readings for each sample were recorded and an average absorbance value was calculated. Before turning off the acetylene, oxygen and power of the lamp and instrument, DI water was

aspirated for several minutes instead of acidic DI water in order to clean out the nebulizer and mixing chamber.

3-5. Detection of Volatile Headspace Compounds by GC/SCD

3-5.1. Instrument and Reagents

A Hewlett Packard 5890 Series II gas chromatograph coupled with a Sievers research model 300 sulfur chemiluminescence detector (SCD) was used to analyze the headspace compounds above bacterial cultures. A length of 30 m with 0.32 mm internal diameter capillary chromatographic column (Alltech Associates, Inc., Deerfield, IL, USA) was utilized with 1 μm 5% phenyl, 95% methyl polysilicone as the stationary phase. Technical grade helium (Bob Smith Gas Products) was used as carrier gas with flow rate at 1 mL/min. The injector temperature was 275°C.

3-5.2. Sample Preparation

The preparation of samples was described in 2-3.2 of this chapter. For samples in which headspace compounds were analyzed, open-top screw caps with 13 mm Teflon lined silicone septa were used to seal the 16-mL test tubes instead of regular screw caps.

3-5.3. Experimental Procedures

One mL of headspace gas was taken from the tubes by a gas tight syringe (Dynatech Precision Sampling Co., Baton Rouge, LA, USA) for GC analysis from cultures incubated at 30°C for a specific time. After the column oven temperature of the instrument was cooled down to -20°C by liquid N₂ through a cryogenic temperature control program, the 1-mL headspace sample was injected into the hot injector inlet and cryogenically

trapped on the capillary column at -20°C for one minute; then the oven temperature was increased at a rate of $20^{\circ}\text{C}/\text{min}$ to a final temperature of 200°C where the temperature was kept for 1 minute. The same temperature program was used for all standard and headspace sample analyses.

Dimethyl sulfide (DMS), dimethyl disulfide (DMDS), dimethyl selenide (DMSe) and dimethyl diselenide (DMDS₂) standards were analyzed under the same conditions by Mr. Hakan Gürleyük in our research group [Gürleyük, 1996]. The retention times of standards were used for peak identification. The identity of dimethyl selenenyl sulfide (DMSeS), which is not commercially available, was verified by GC-MS [Gürleyük, 1996].

The concentration of each compound in the headspace was calculated by using the corresponding peak area calibrated for each compound [Gürleyük, 1996]. DM-N medium blanks and K27 bacterial culture growing in DM-N medium without any amendment were run along with poisoned samples as controls.

A syringe cleaning device was used to clean the gas syringes after each headspace analysis [Gürleyük, 1996]; one mL of lab air in the gas tight syringe was analyzed by GC to check whether or not each syringe was clean before each sample run.

Chapter III

Data

1. Anaerobic growth of *Pseudomonas fluorescens* K27 in the following minimal media (Table II)

Table II.

Results of Starting Optical Density and Maximum Optical Density of K27 Observed in Minimal Media.

OD _{start} - OD _{max.}	DM-I	DM-II	DM-III	DM-IV
Control	9-70	27-130	20-96	10-30
1 mM SeO ₃ ²⁻	9-129	N.A.	20-87	9-28
10 mM SeO ₃ ²⁻	9-225	N.A.	*40-60	9-55
1 mM SeO ₄ ²⁻	9-44	28-110	20-70	8-11
10 mM SeO ₄ ²⁻	9-81	28-114	*35-40	8-10

* The high OD_{start} were due to reddish color of the medium which was caused by chemical reactions between selenate or selenite with medium.

2. Growth curve of *P. fluorescens* K27 in DM-N minimal medium (Figure 1)

3. Growth curve of *P. fluorescens* K27 in DM-N minimal medium containing 100 mM selenate (Figure 2)

4. Growth curve of *P. fluorescens* K27 in DM-N medium containing 30 mM selenite (Figure 3)

5. Change of *P. fluorescens* K27 bacterial population in DM-N minimal medium in a time course growth experiment (Figure 4a), and in 10 mM selenite amended DM-N minimal medium (Figure 4b)

6. Growth curve of anaerobic cultivation of *P. fluorescens* K27 in nitrate free DM-I medium (Figure 5)

7. Comparison of EC₅₀ for selenate, selenite and dimethyl selenone amended *P. fluorescens* K27 cultures (Table III)

8. Growth inhibitions of *P. fluorescens* K27 amended with selenate, selenite and dimethyl selenone in DM-N medium (Figure 6)

9. Doubling times of growth of *P. fluorescens* K27 amended with selenate, selenite and dimethyl selenone in DM-N medium (Figure 7)

10. A typical calibration curve for nitrate analysis by UV/VIS (Figure 8)

11. A typical calibration curve for selenium analysis by AAS (Figure 9)

12. A typical calibration curve for selenite analysis by UV/VIS (Figure 10)

13. The chromatogram of the headspace of DM-N sterilized medium after 120 hours incubation (Figure 11a), and DM-N medium inoculated with *P. fluorescens* K27 after 15 hours incubation (Figure 11b)

14. The chromatograms of the headspaces of *P. fluorescens* K27 in DM-N medium after 120 hours incubation (Figure 12a and 12b)

15. The chromatogram of the headspace of *P. fluorescens* K27 amended with 1 mM selenate in DM-N medium after 15 hours incubation (Figure 13a), and after 120 hours incubation (Figure 13b)

16. The chromatogram of the headspace of *P. fluorescens* K27 amended with 1 mM selenite in DM-N medium after 15 hours incubation (Figure 14a), and after 120 hours incubation (Figure 14b)

17. The chromatogram of the headspace of *P. fluorescens* K27 amended with 10 mM selenate in DM-N medium after 15 hours incubation (Figure 15a), and after 120 hours incubation (Figure 15b)

18. The chromatogram of the headspace of *P. fluorescens* K27 amended with 10 mM selenite in DM-N medium after 15 hours incubation (Figure 16a), and after 120 hours incubation (Figure 16b)

19. A typical time course measurements of growth of *P. fluorescens* K27 in DM-N minimal medium: the change of nitrate concentration (Figure 17a); and the production of volatile compounds in headspace (Figure 17b)

20. Time course plots of *P. fluorescens* K27 amended with 1 mM selenate in DM-N medium: the change of concentration of selenate in supernatant (Figure 18a); the change of nitrate concentration (Figure 18b); and the production of volatile compounds in headspace (Figure 18c)

21. Time course plots of *P. fluorescens* K27 amended with 1 mM selenite in DM-N medium: the change of concentration of selenite in supernatant (Figure 19a); the change of nitrate concentration (Figure 19b); and the production of volatile compounds in headspace (Figure 19c)

22. Time course plots of *P. fluorescens* K27 amended with 10 mM selenate in DM-N medium: the change of concentration of selenate in supernatant (Figure 20a); the change of nitrate concentration (Figure 20b); and the production of volatile compounds in headspace (Figure 20c)

23. Time course plots of *P. fluorescens* K27 amended with 10 mM selenite in DM-N medium: the change of concentration of selenite in supernatant (Figure 21a); the change of nitrate concentration (Figure 21b); and the production of volatile compounds in headspace (Figure 21c)

24. Results of selenium distribution in selenate and selenite poisoned *P. fluorescens* K27 cultures (Table IV)

25. Results of final nitrate concentrations in selenate and selenite poisoned *P. fluorescens* K27 cultures and control cultures (Table V)

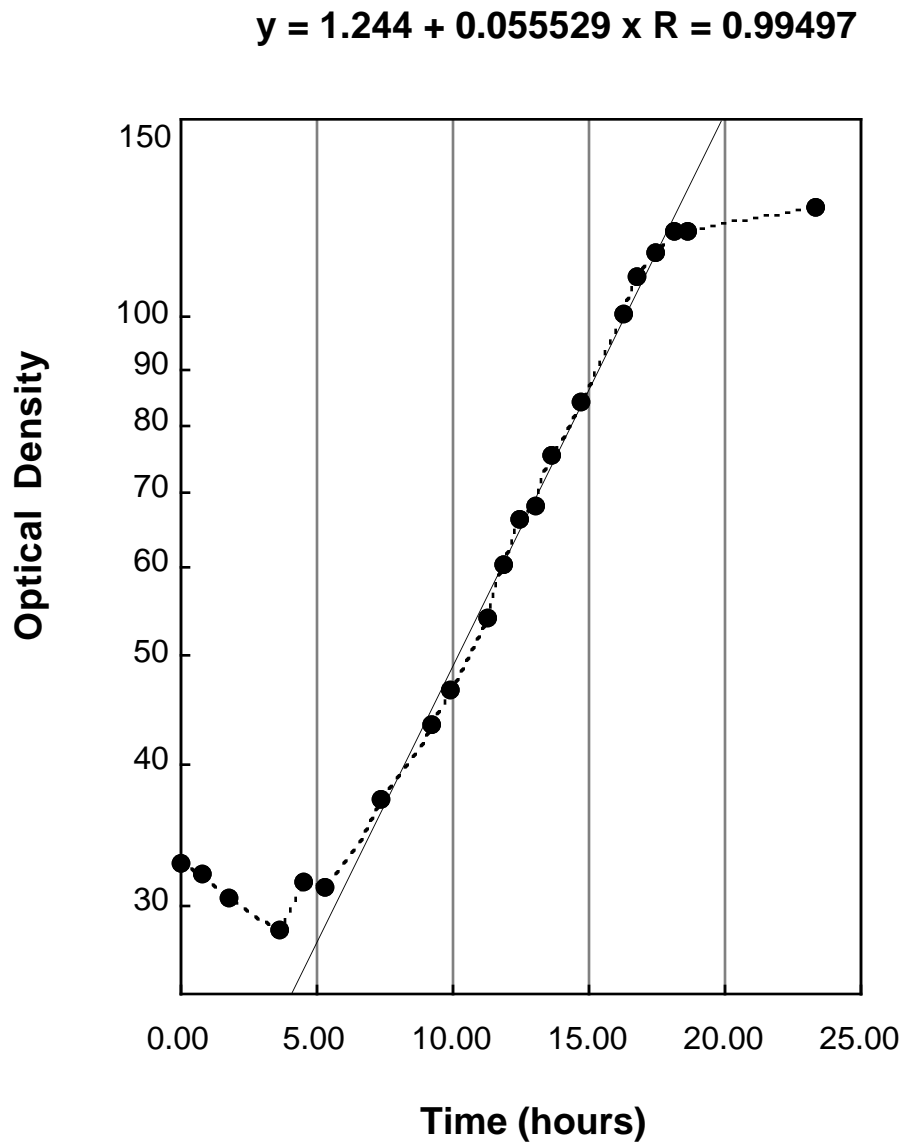


Figure 1. A typical growth curve of *P. fluorescens* K27 in DM-N minimal medium. It has a linear exponential growth phase. Growth rate = 0.05529; doubling time $DT = (\log 2 / GT) = 5.42$ hours.

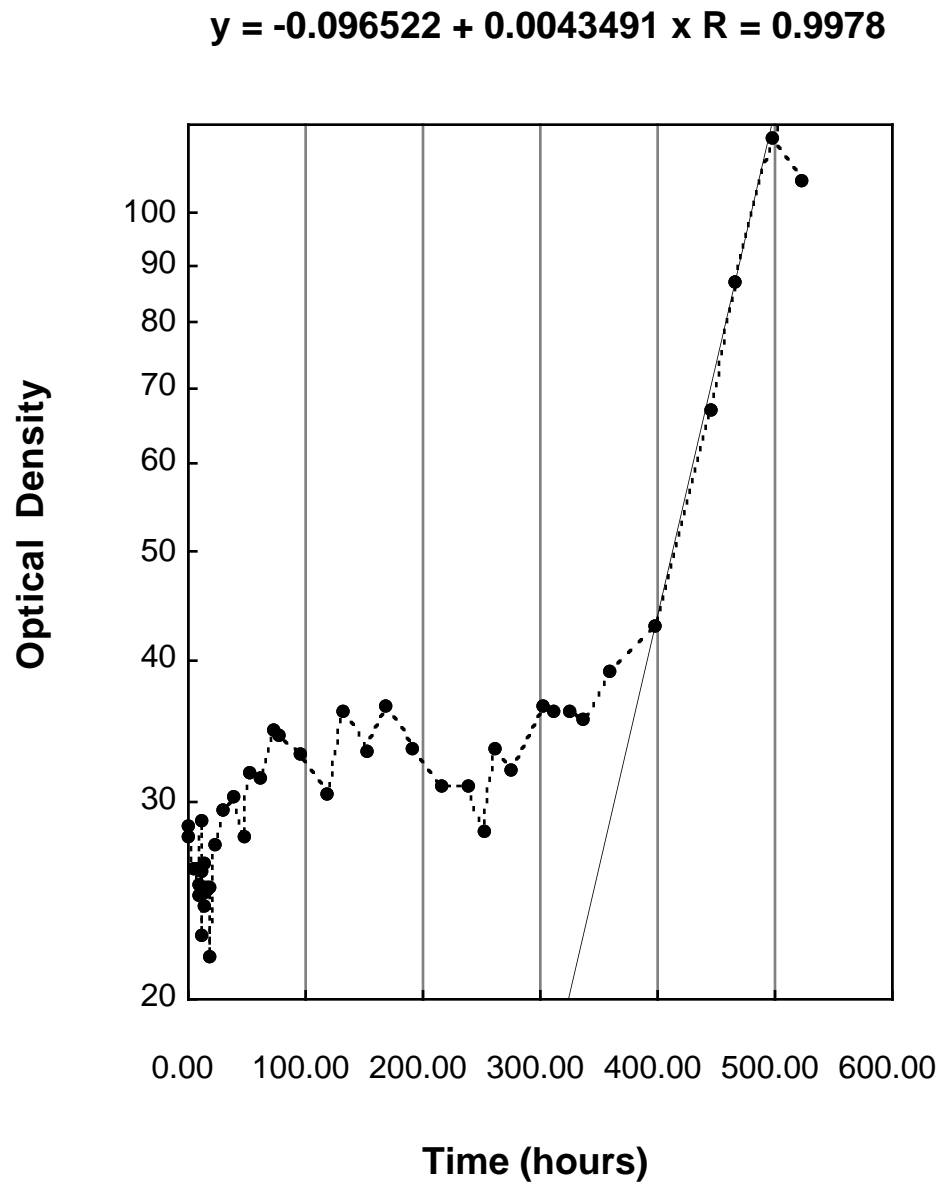


Figure 2. A typical growth curve of *P. fluorescens* K27 in 100 mM selenate amended DM-N minimal medium.

$$(1) y = -1.3585 + 0.012902xR = 0.99657$$

$$(2) y = 0.37741 + 0.0063863xR = 0.99823$$

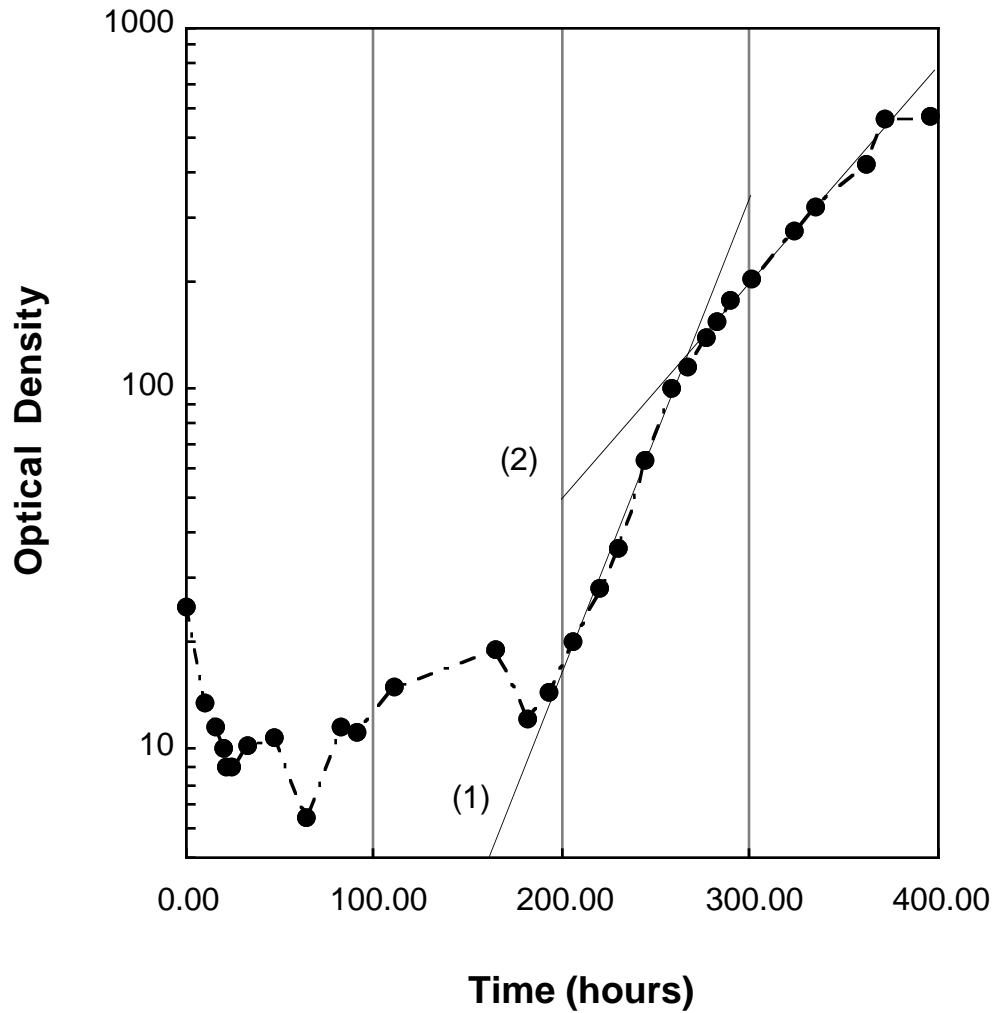


Figure 3. A typical growth curve of *P. fluorescens* K27 in 30 mM selenite amended DM-N minimal medium. Lines marked 1 and 2 represent two different exponential growth phase.

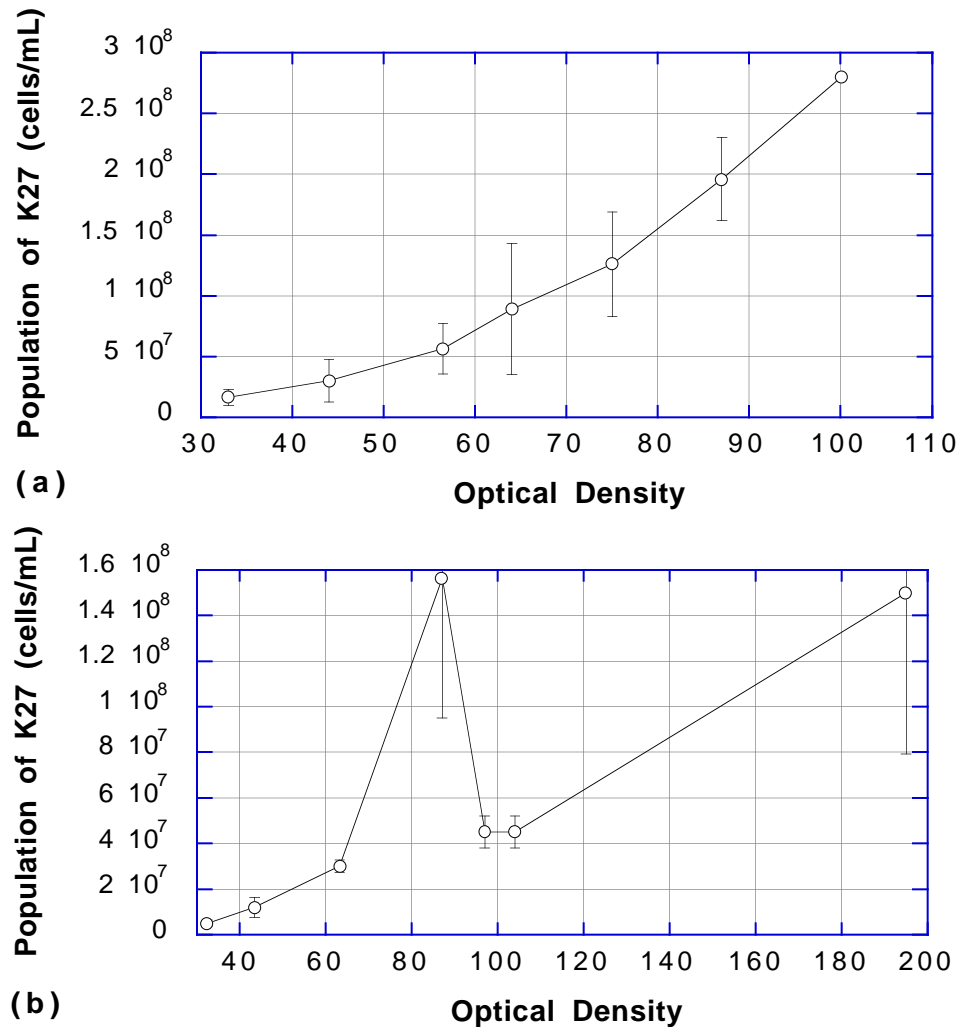


Figure 4. (a) Change of *P. fluorescens* K27 bacterial population in DM-N minimal medium in a time course growth experiment. (b) Change of *P. fluorescens* K27 bacterial population in 10 mM selenite amended DM-N minimal medium in a time course growth experiment. Error bars represent standard deviation for triplicate samples.

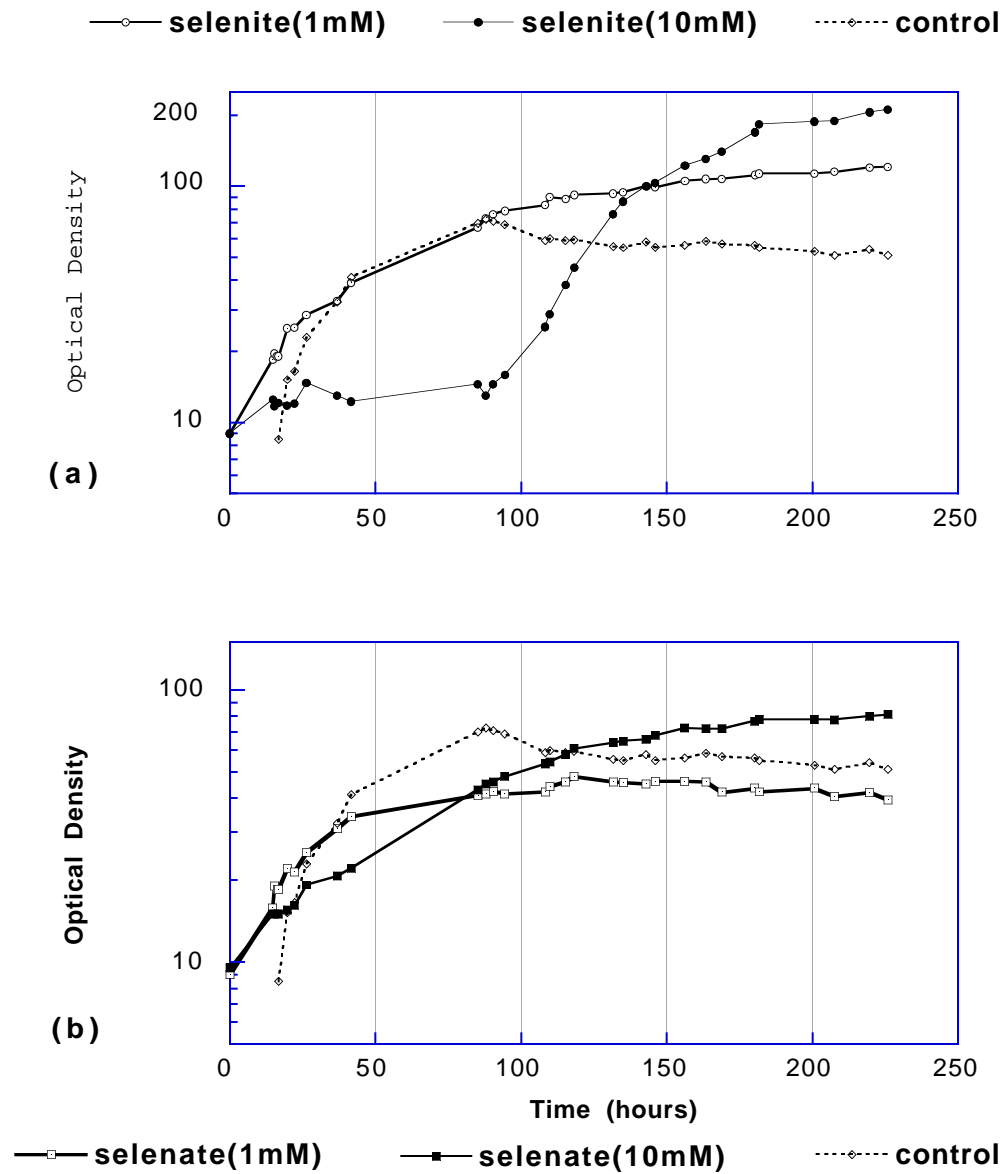


Figure 5. *P. fluorescens* K27 anaerobically grown in nitrate free DM-I medium: (a) amended with selenite, (b) amended with selenate.

Table III.

Toxicities of seleniferous compounds towards *P. fluorescens* K27 given as EC50, the toxicant concentration that leads to a 50% growth inhibition or to a 50% increase in specific doubling time.

Method	(CH₃)₂SeO₂	SeO₄²⁻	SeO₃²⁻
Growth Inhibition	EC₅₀ (ppm Se)	21	61
	r	0.999	0.988
	range (ppm Se)	log(7.9-39.5)	log(39.5-79)
	n	6	4
			log(39.5-1185)
			276
			0.998
Doubling Time Measurement	EC₅₀ (ppm Se)	14	49
	r	0.990	0.997
	range (ppm Se)	3.95-39.5	39.5-158
	n	6	6
			79-1580
			10

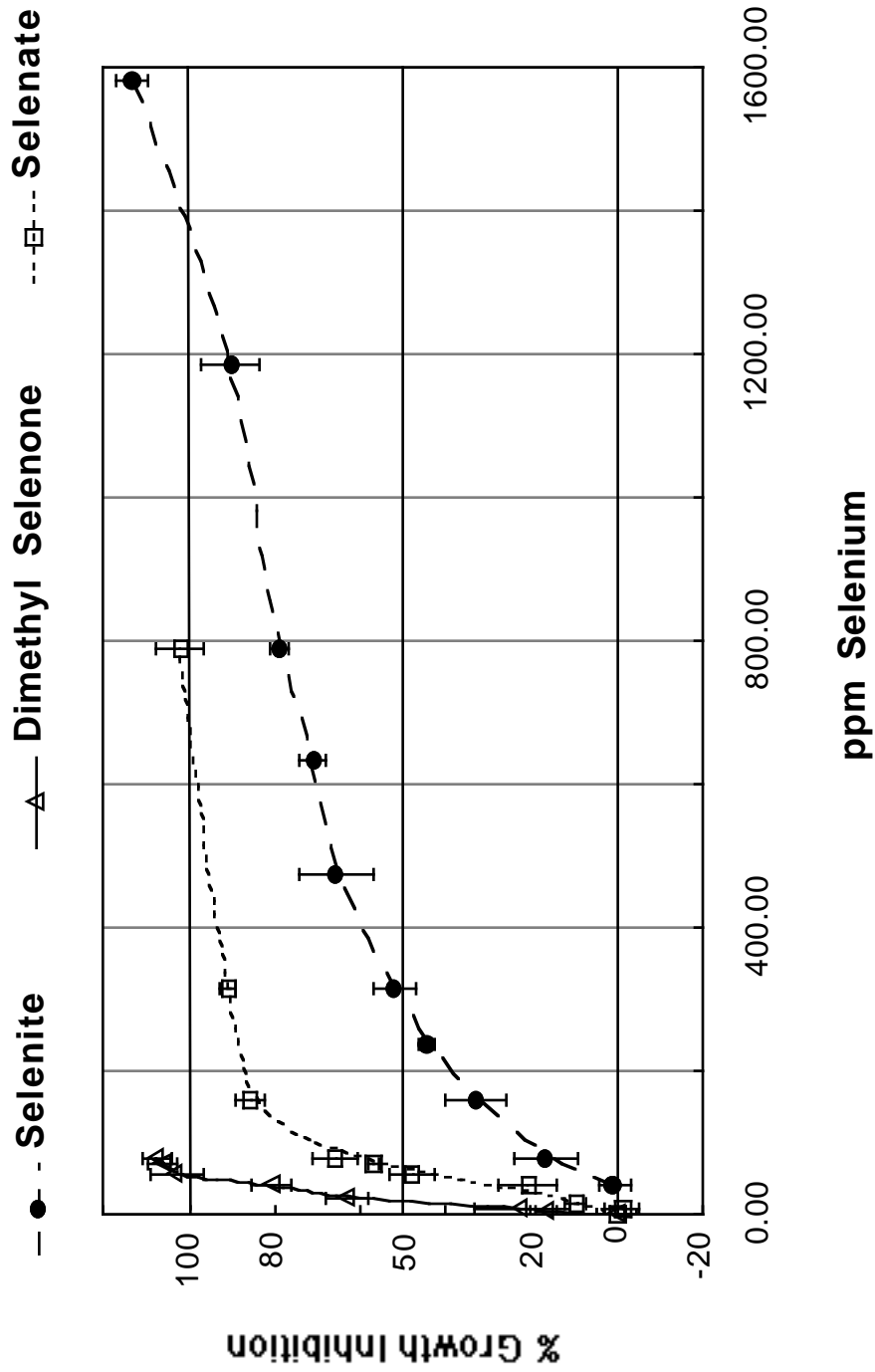


Figure 6. Percent growth inhibition of *Pseudomonas fluorescens* K27 cells amended with selenium compounds in DM-N medium. Error bars represent standard deviation of replicate samples.

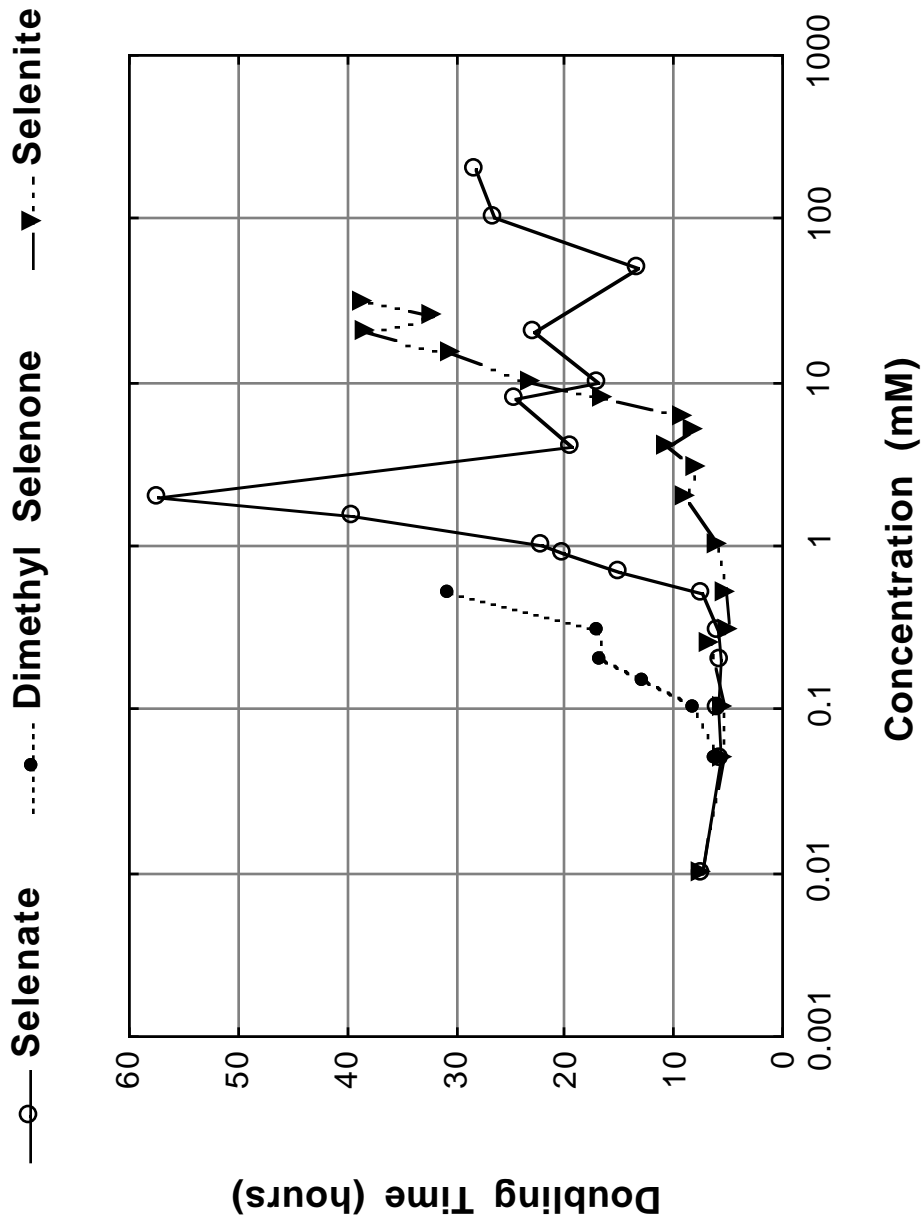


Figure 7. Doubling Times of *P. fluorescens* K27 cells amended with selenium compounds in DM-N medium.

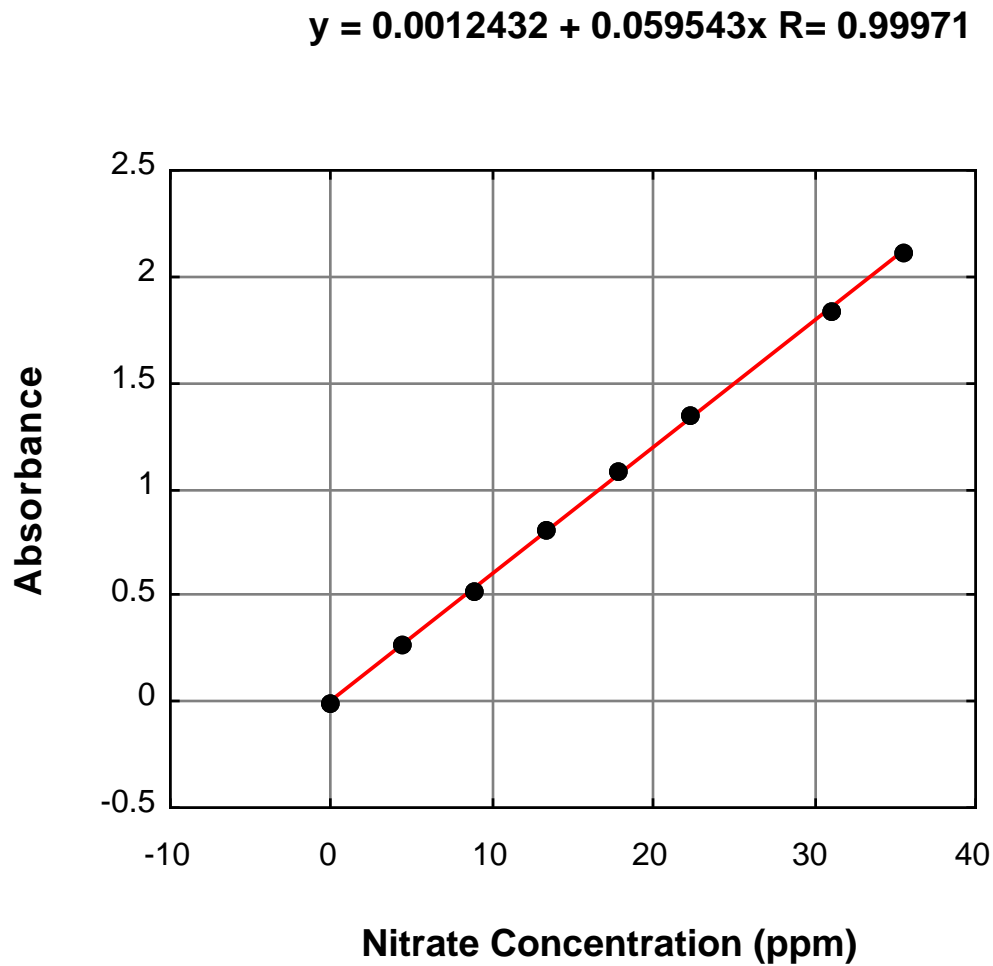


Figure 8. A typical calibration curve for nitrate analysis by UV/VIS spectrometry. The linear range is from 0 ppm to 35.6 ppm nitrate.

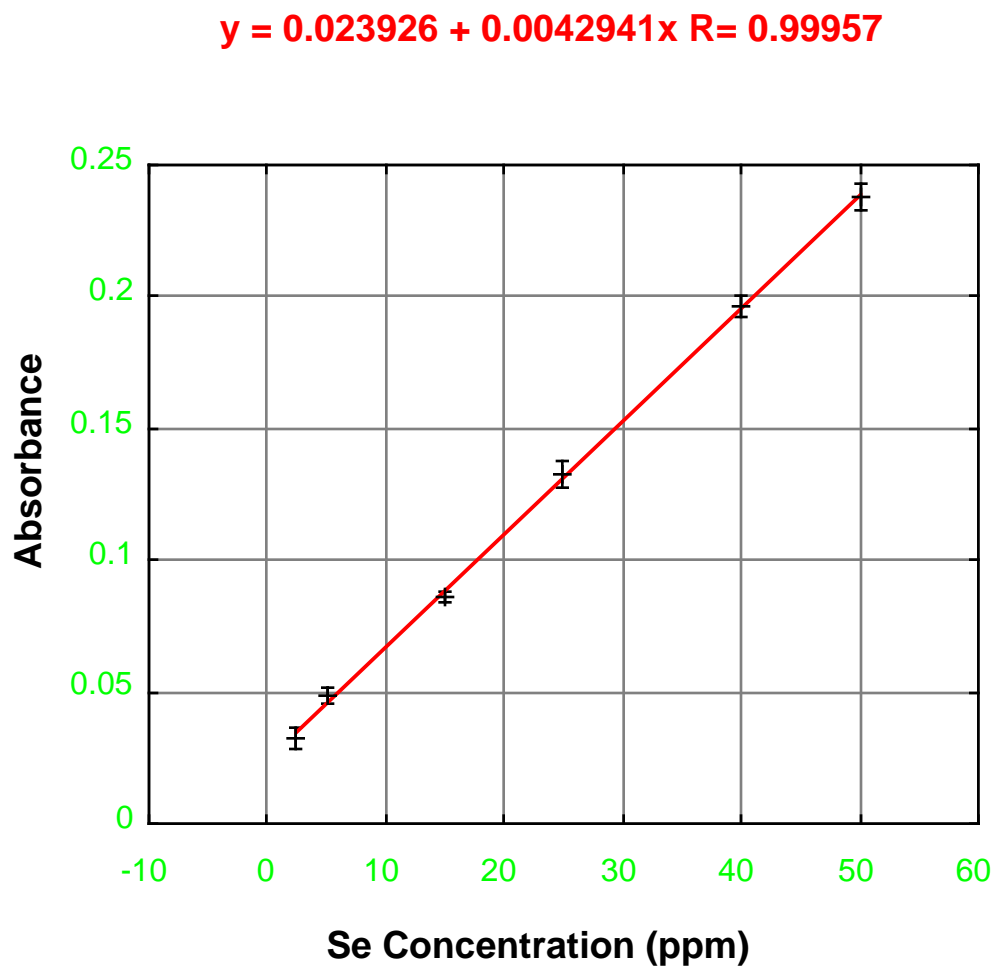


Figure 9. A typical calibration curve for selenium analysis by AAS. The linear range is from 2.5 ppm to 50 ppm. Error bars represent the standard deviation of five replicate readings.

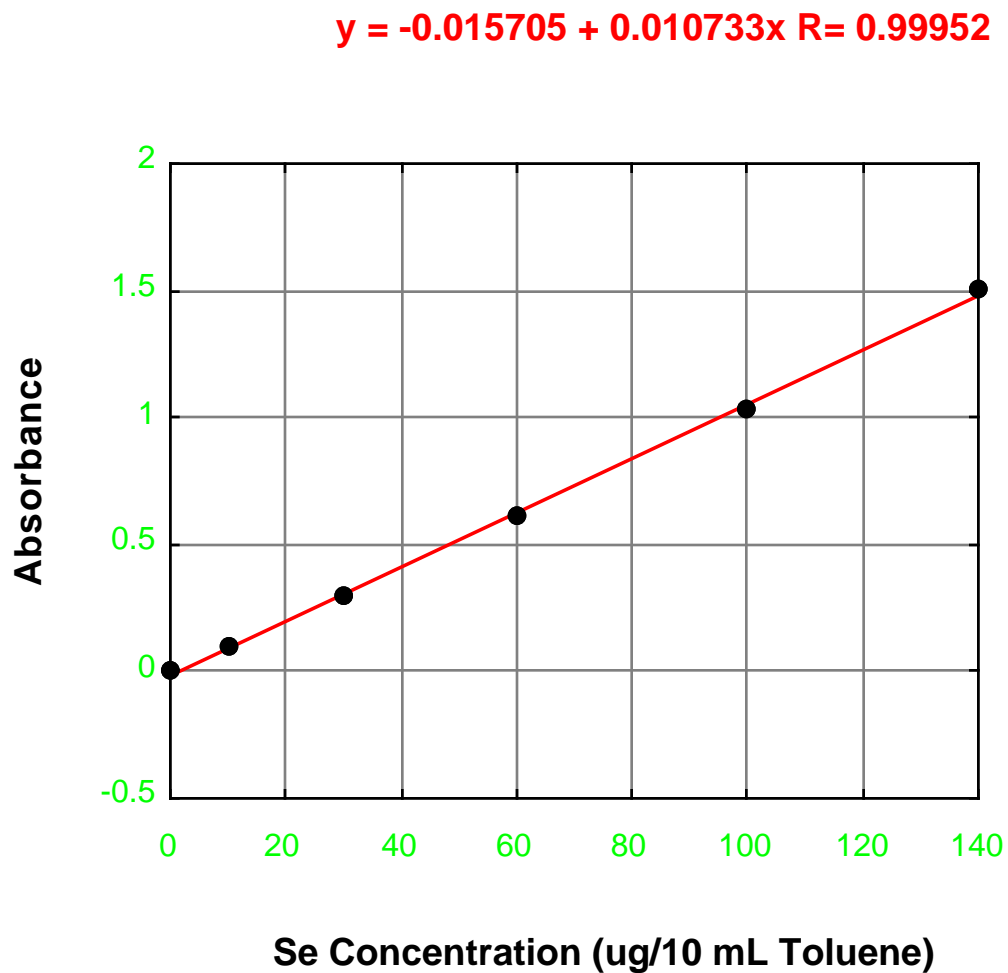


Figure 10. A typical calibration curve for selenite oxyanion analysis by UV/VIS spectrometry. The linear range is from 10 micrograms to 140 micrograms of selenium in 10 mL of toluene solutions.

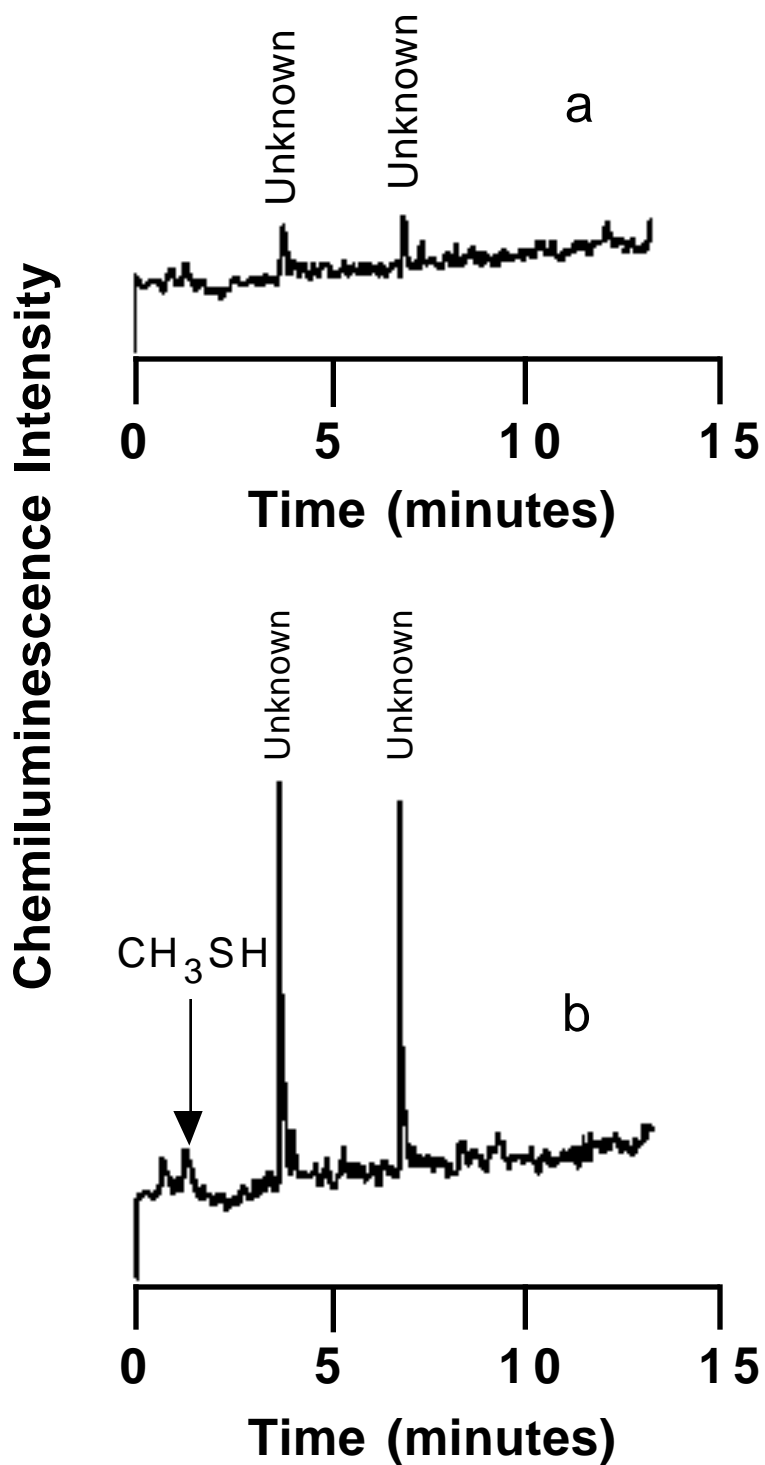


Figure 11. The chromatogram of the headspace of (a) sterilized DM-N medium after 120 hours. (b) *P. fluorescens* K27 in DM-N medium after 15 hours incubation at 30°C.

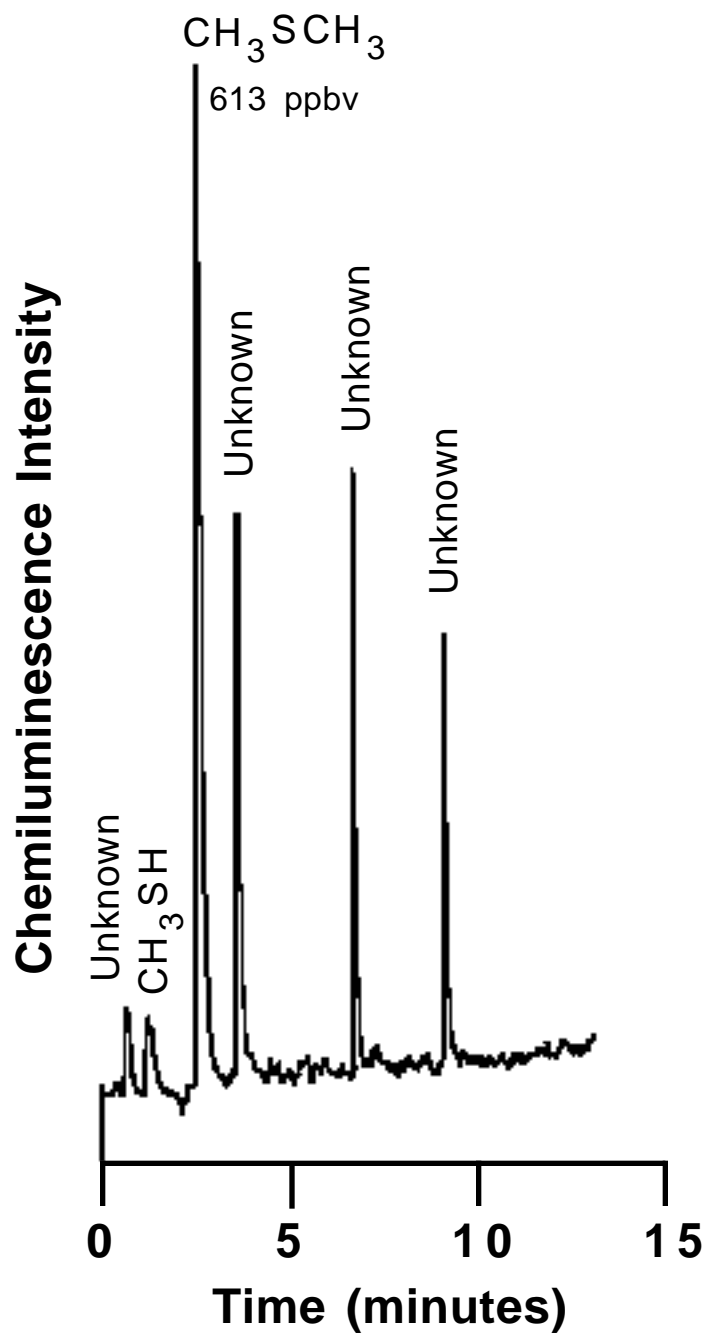


Figure 12(a). One of the chromatograms of the headspace of *P. fluorescens* K27 in DM-N medium after 120 hours incubation at 30°C.

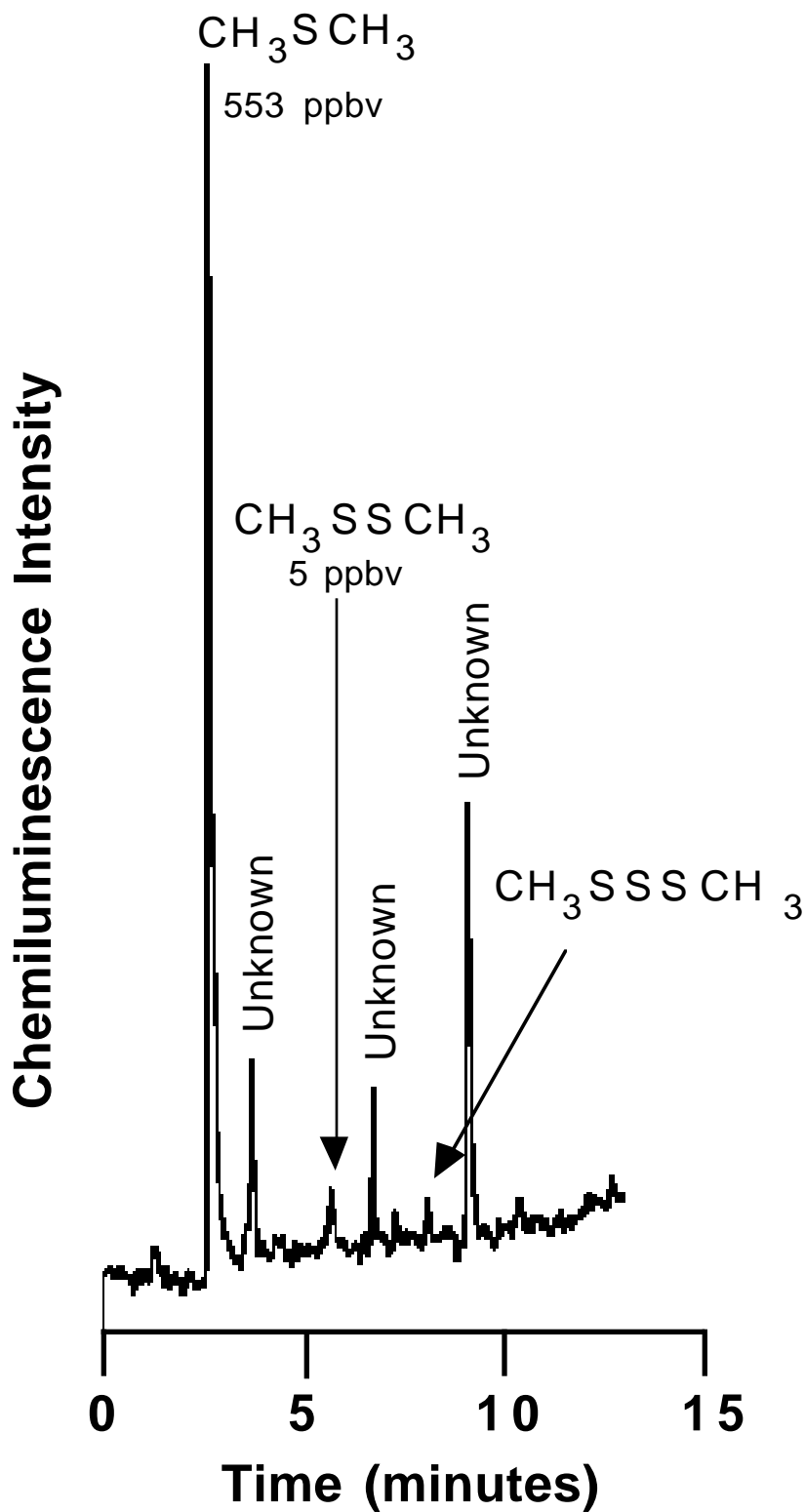


Figure 12(b). One of the chromatograms of the headspace of *P. fluorescens* K27 in DM-N medium after 120 hours incubation at 30°C.

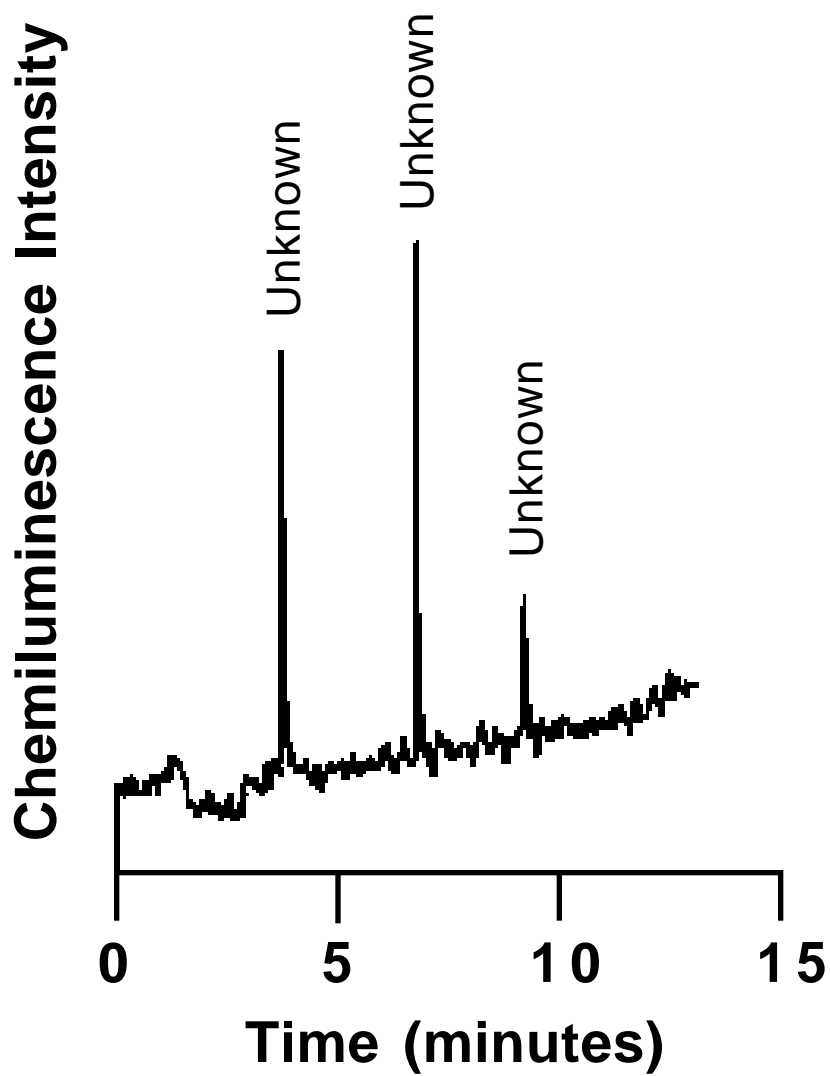


Figure 13(a). The chromatogram of the headspace of *P. fluorescens* K27 amended with 1 mM selenate in DM-N medium after 15 hours incubation at 30°C.

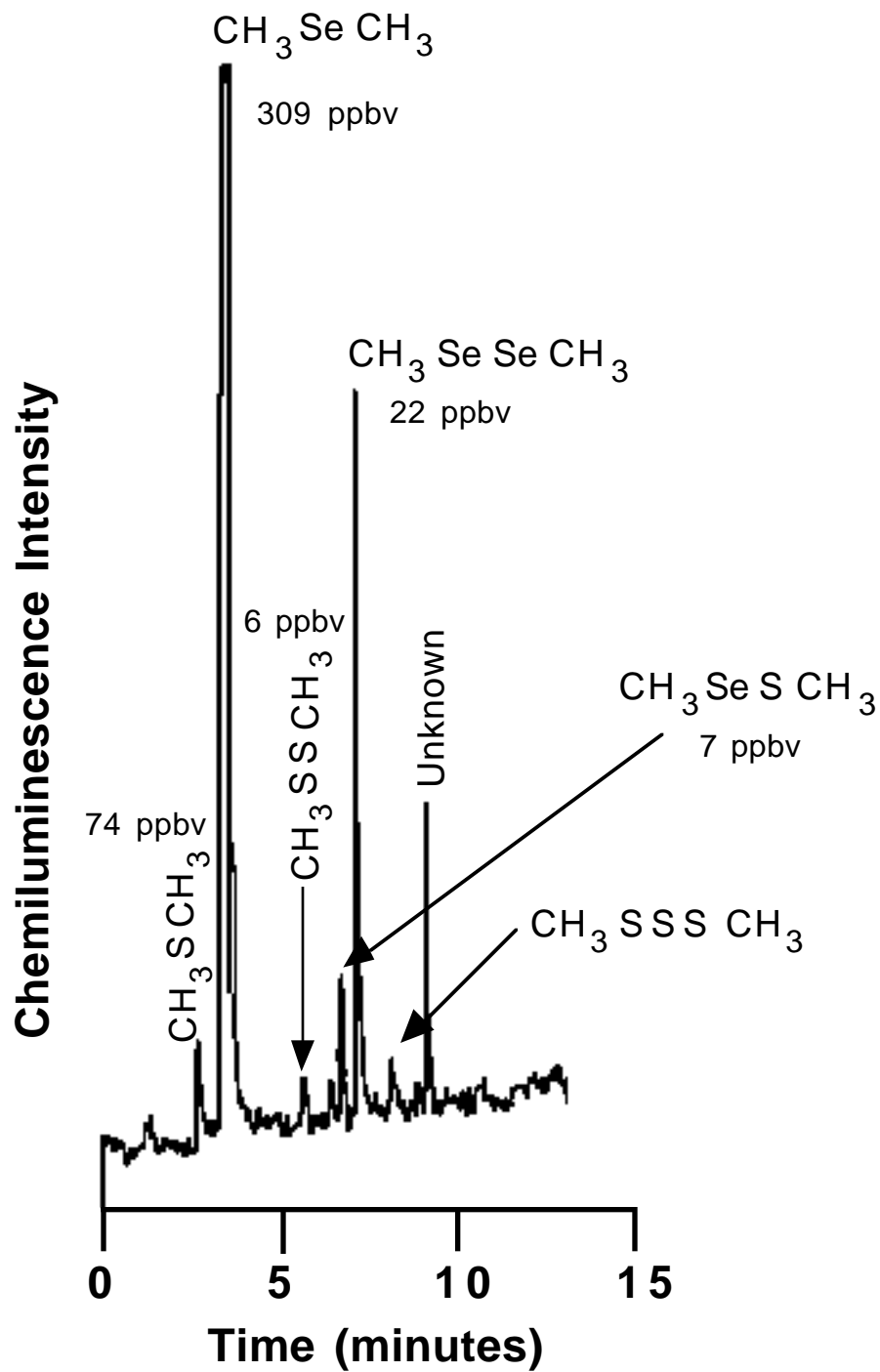


Figure 13(b). The chromatogram of the headspace of *P. fluorescens* K27 amended with 1 mM selenite in DM-N medium after 120 hours incubation at 30°C.

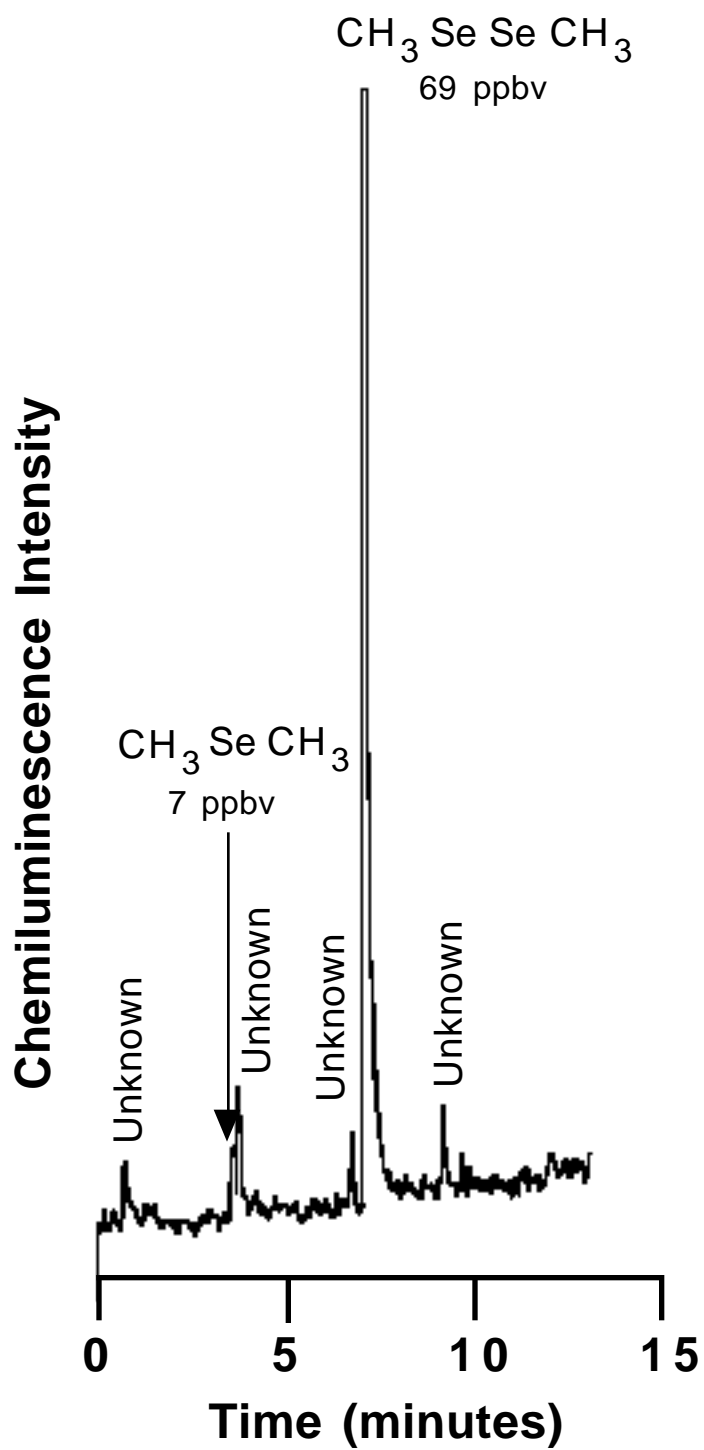


Figure 14(a). The chromatogram of the headspace of *P. fluorescens* K27 amended with 1 mM selenite in DM-N medium after 15 hours incubation at 30°C.

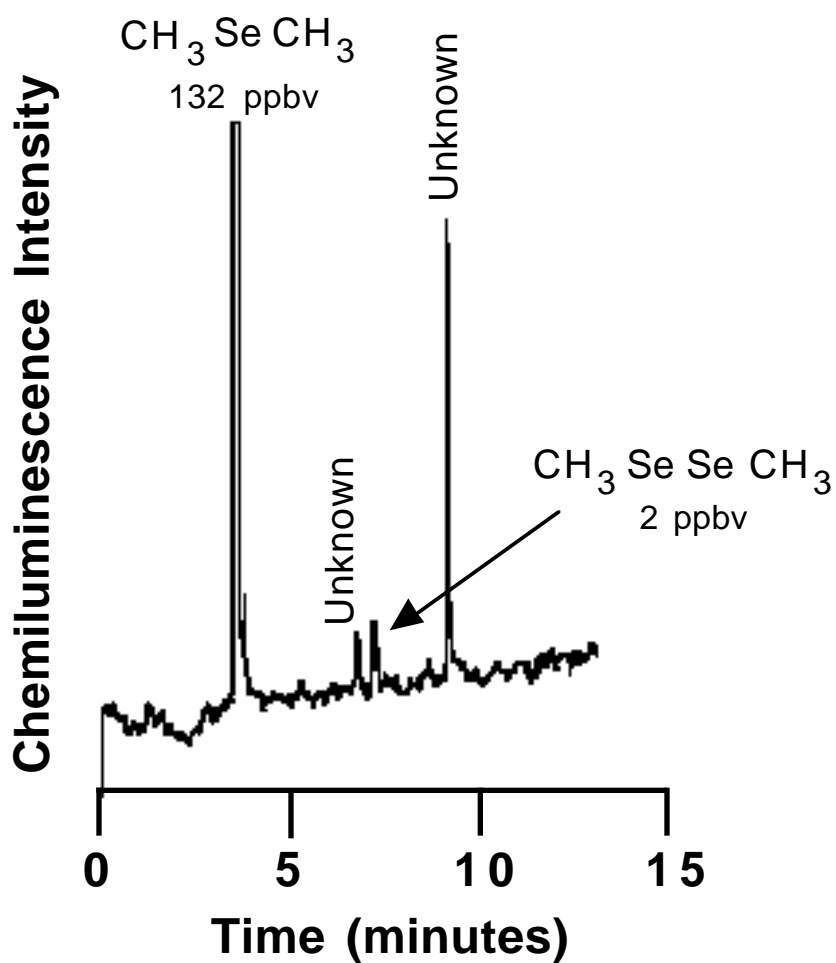


Figure 14(b). The chromatogram of the headspace of *P. fluorescens* K27 amended with 1 mM selenite in DM-N medium after 120 hours incubation at 30°C.

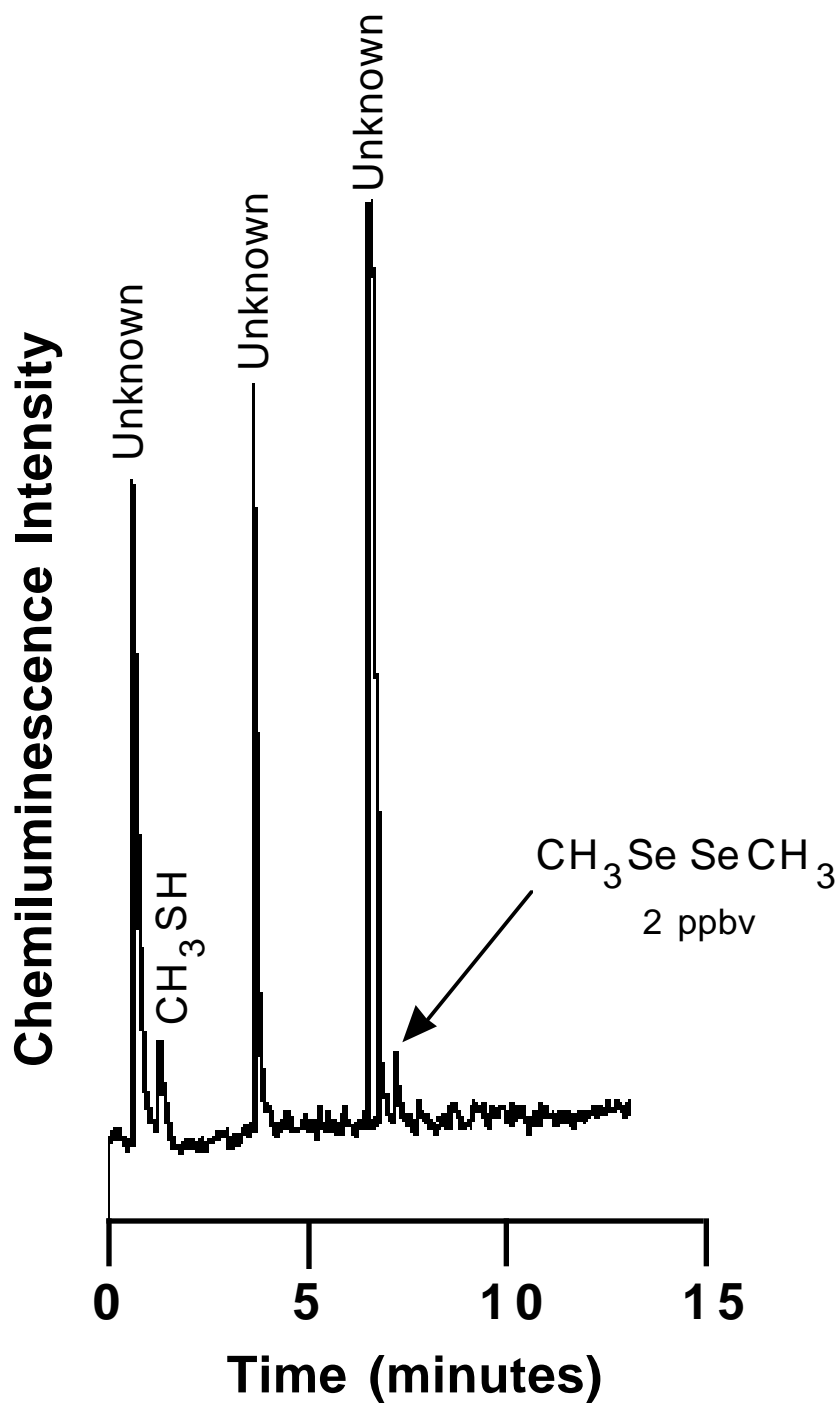


Figure 15(a). The chromatogram of the headspace of *P. fluorescens* K27 amended with 10 mM selenate in DM-N medium after 15 hours incubation at 30°C.

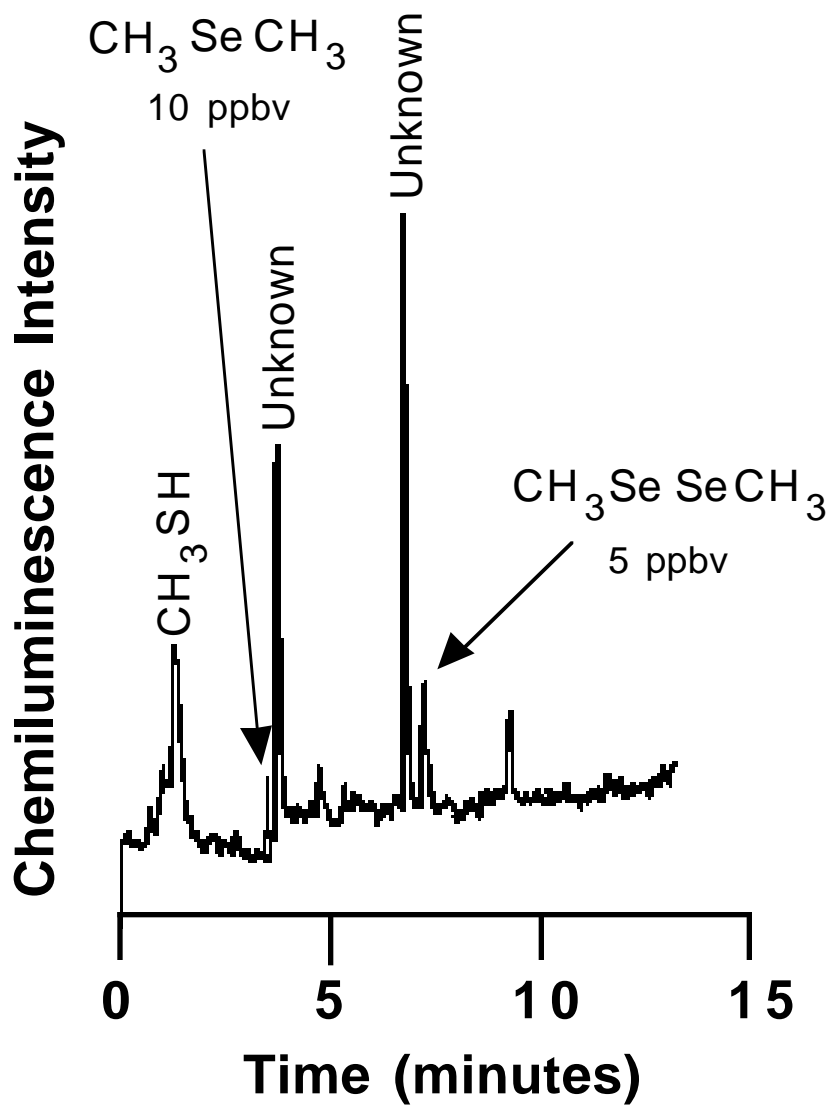


Figure 15(b). The chromatogram of the headspace of *P. fluorescens* K27 amended with 10 mM selenate in DM-N medium after 120 hours incubation at 30°C.

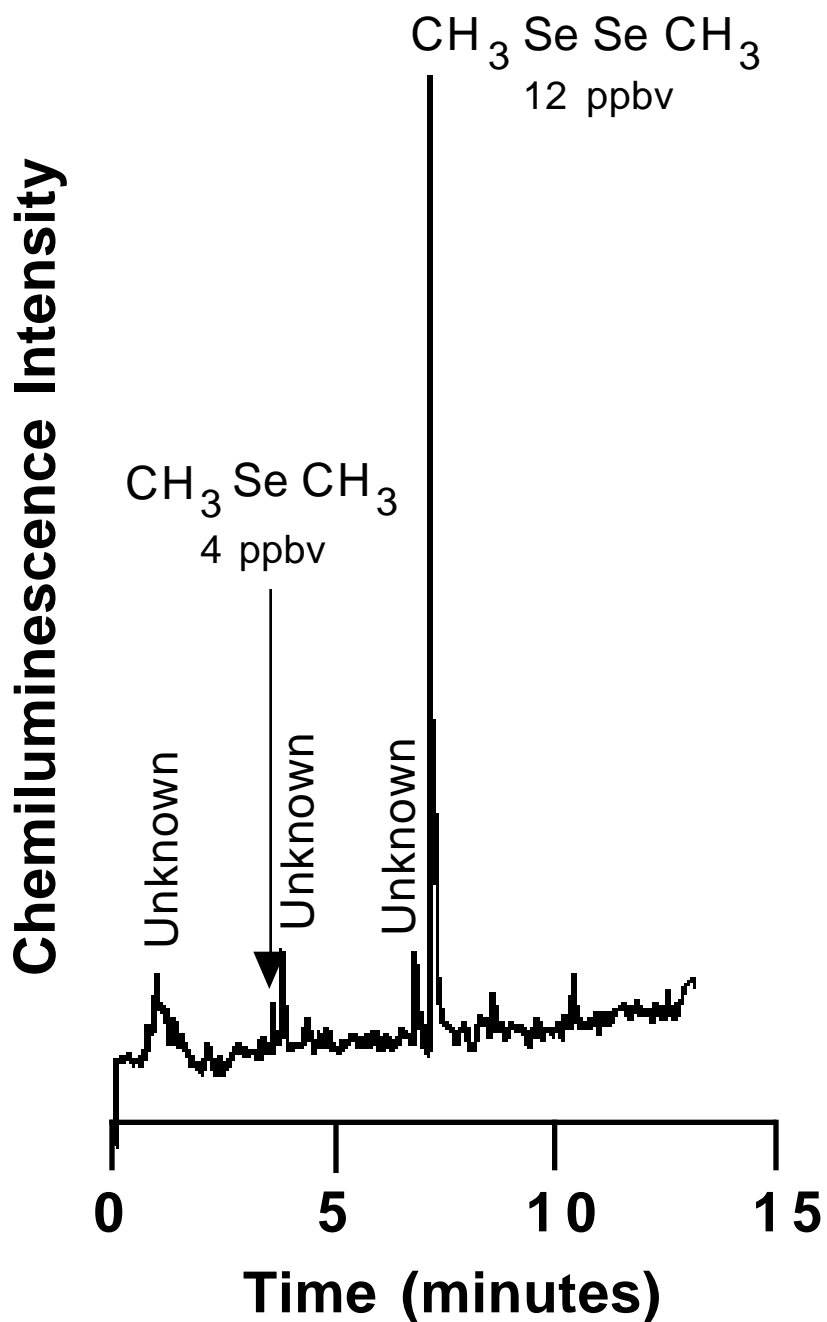


Figure 16(a). The chromatogram of the headspace of *P. fluorescens* K27 amended with 10 mM selenite in DM-N medium after 15 hours incubation at 30°C.

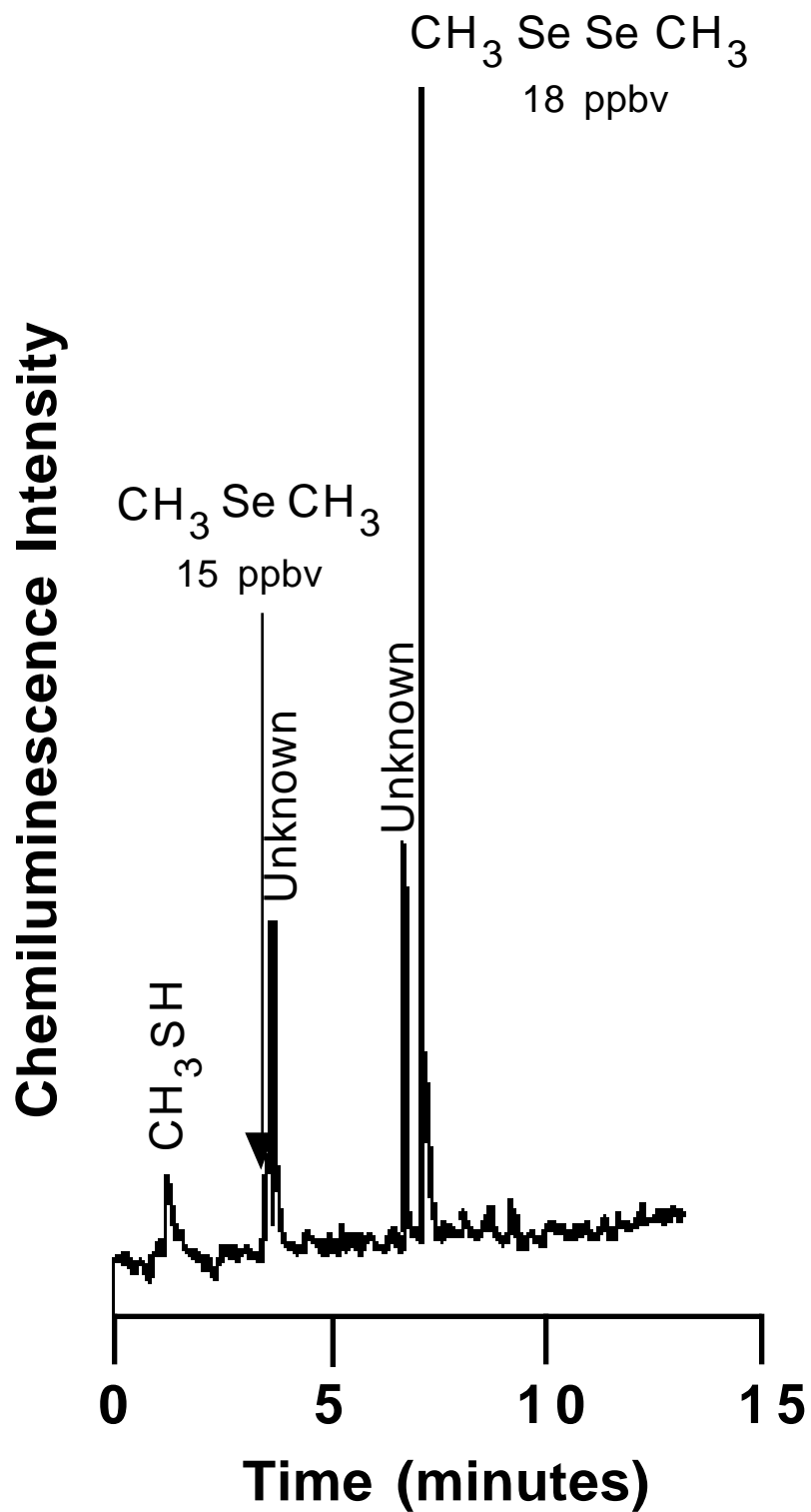


Figure 16(b). The chromatogram of the headspace of *P. fluorescens* K27 amended with 10 mM selenite in DM-N medium after 120 hours incubation at 30°C.

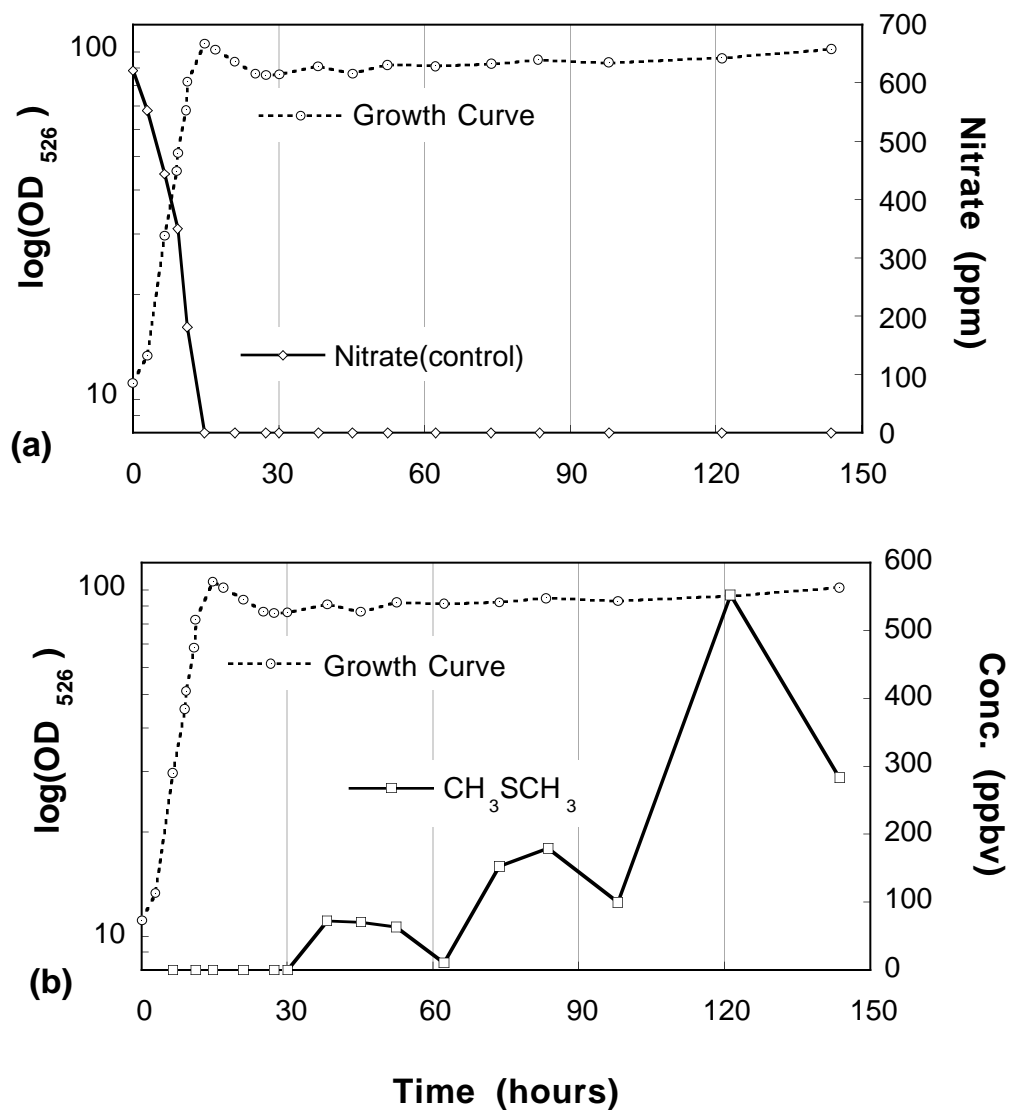


Figure 17. Time course of the growth of *P. fluorescens* K27 control in DM-N minimal medium. (a) The change of nitrate concentration. (b) The headspace production of the volatile compound dimethyl sulfide.

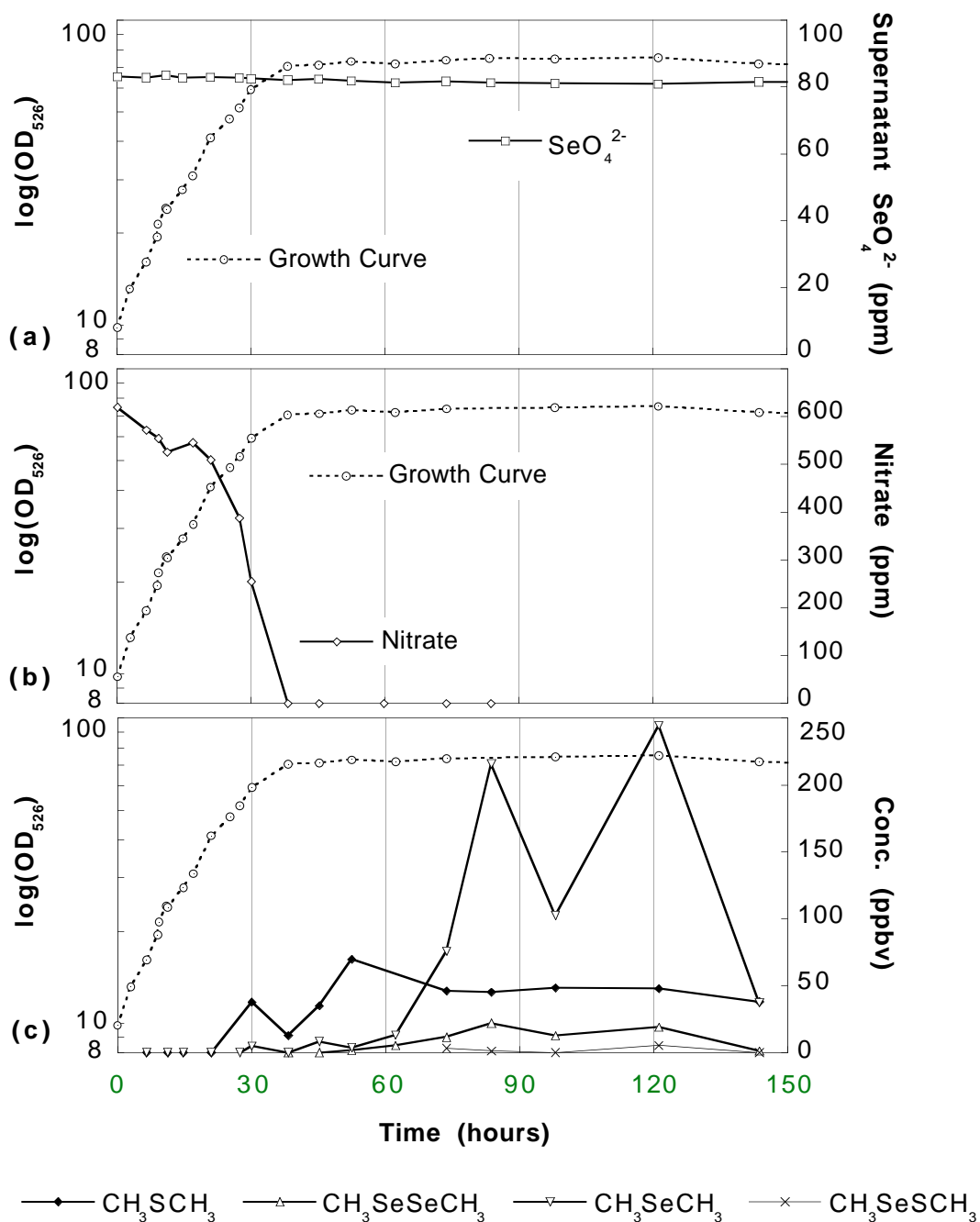


Figure 18. Time course of *P. fluorescens* K27 amended with 1 mM selenate in DM-N medium. (a) The change of selenate concentration in solution. (b) The change of nitrate concentration. (c) The production of volatile compounds.

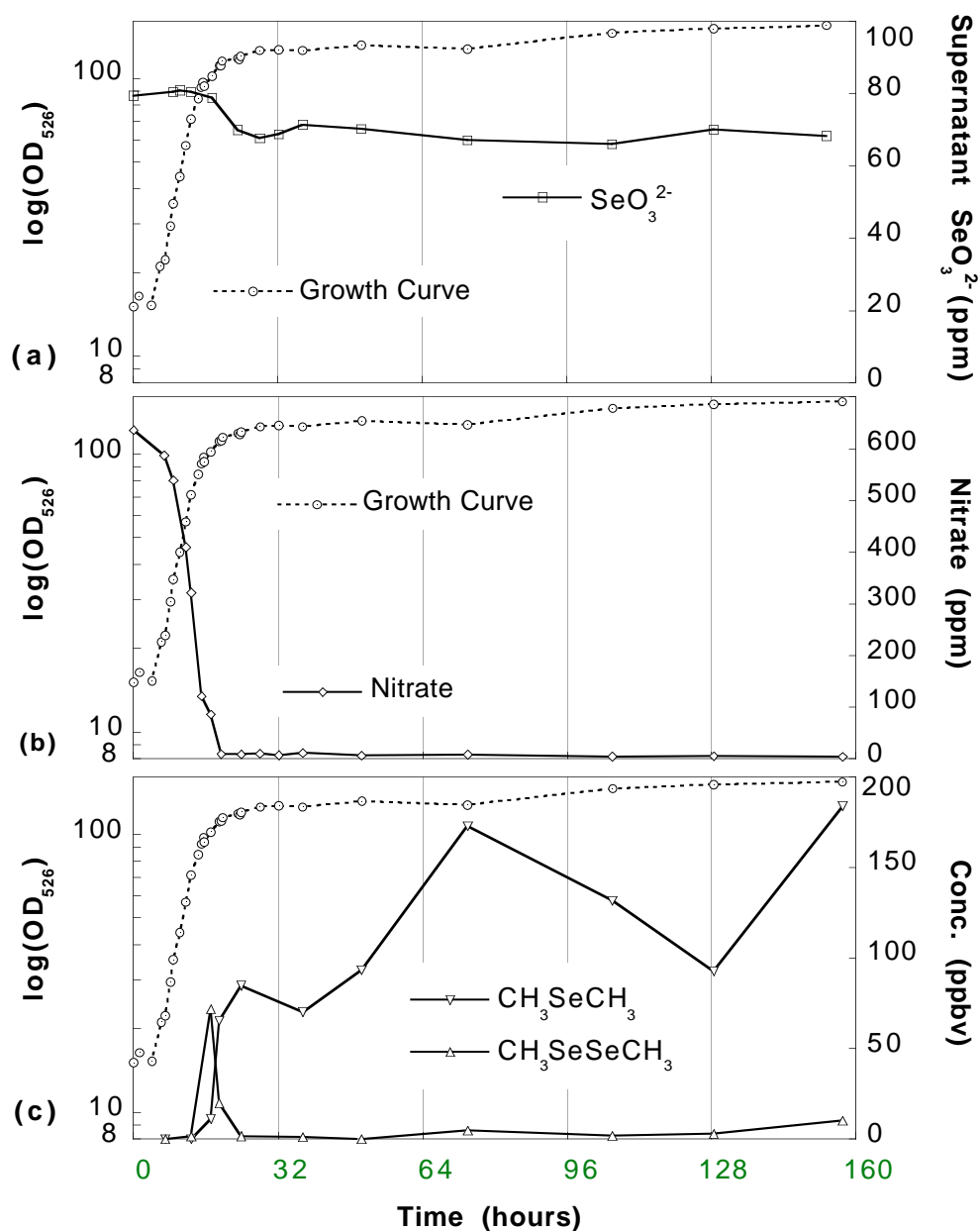


Figure 19. Time course of *P. fluorescens* K27 amended with 1 mM selenite in DM-N medium. (a) The change of selenite concentration in solution. (b) The change of nitrate concentration. (c) The production of volatile organoselenium compounds in headspace.

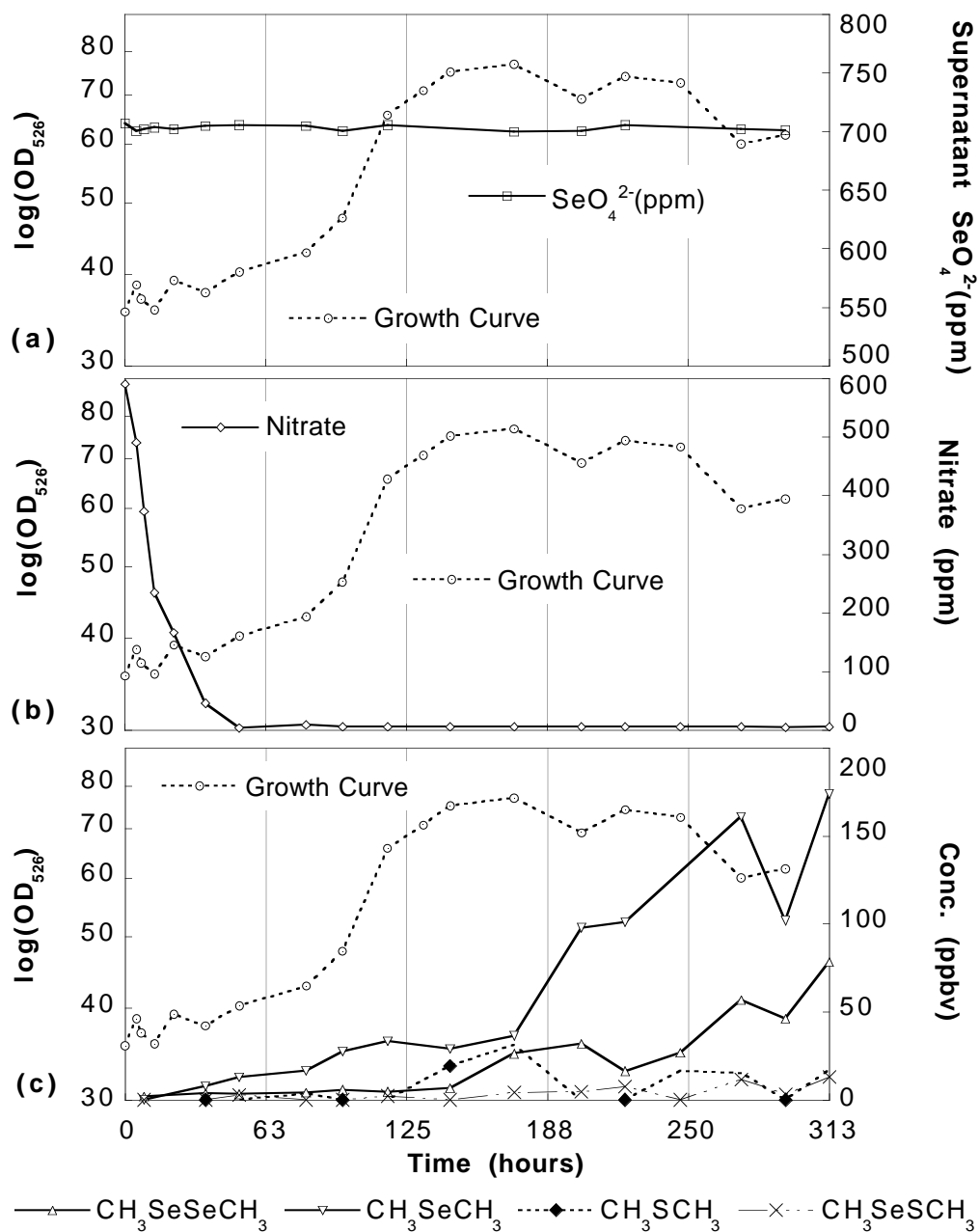


Figure 20. Time course of *P. fluorescens* K27 amended with 10 mM selenate in DM-N medium. (a) The change of selenate concentration in the solution. (b) The change of nitrate concentration. (c) The production of volatile compounds in the headspace.

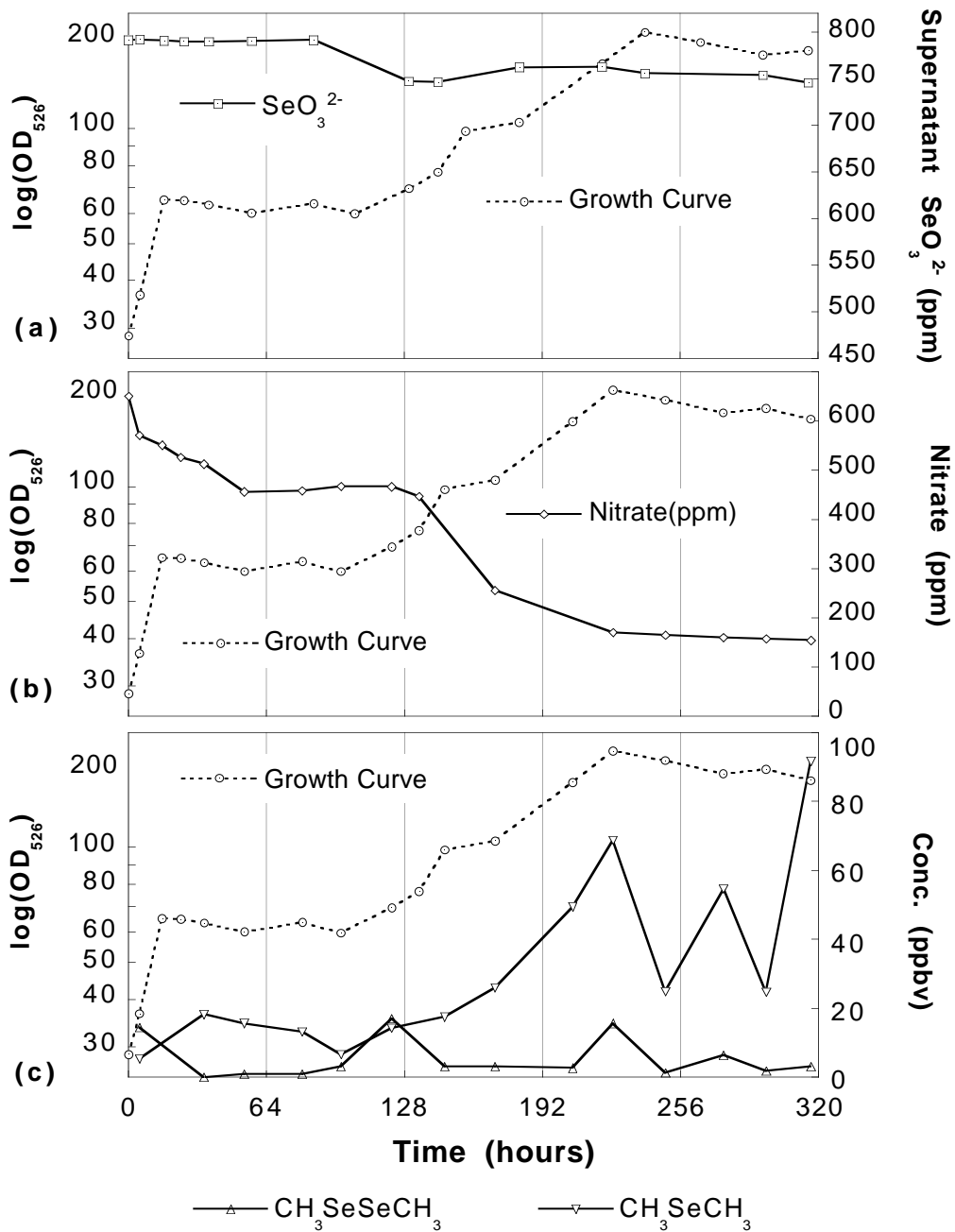


Figure 21. Time course of *P. fluorescens* K27 amended with 10 mM selenite in DM-N medium. (a) The change of selenite concentration in solution. (b) The change of nitrate concentration. (c) The production of volatile compounds in headspace.

Table IV.

Results of selenium distribution in selenate and selenite amended *P. fluorescens* K27 cultures and control cultures. One mM selenite and selenate amended cultures and controls were measured at 120 hours after inoculating and amending; while 10 mM selenite and selenate amended cultures were measured at 240 hours after amending.

SAMPLE NAME	VOLATILE SELENIUM (ppb)			VOLATILE SULFUR (ppb)			ELEMENTAL SELENIUM (c)		
	DMSe	DMDSe	DMSeS	DMS	DMDS	DMTS	SeO ₄ ²⁻ (c)	SeO ₃ ²⁻ (c)	SeO ₃ ²⁻ (c)
K27 & DM-N	none	none	none	>38000	>30	++(a)	none	none	none
K27 & DM-N +	>1854	>132	>42	>444	>36	++	<0.6%	97.8%	none
SeO ₄ ²⁻ (1mM)									
K27 & DM-N +	>966	>336	>71	>92	none	none	≈0.6%	99.1%	none
SeO ₄ ²⁻ (10mM)									
K27 & DM-N +	>792	>6	none	none	none	none	6.1%	none	90.2%
SeO ₃ ²⁻ (1mM)									
K27 & DM-N +	>412	>93	none	none	none	none	3.1%	none	94.1%
SeO ₃ ²⁻ (10mM)									

(a), ++ Positive results. (b), Percentages of initially added selenium counted to elemental Se. (c), Percentages of added selenium found in this form.

Table V.

Results of final concentration of nitrate in *P. fluorescens* K27 cultures and corresponding incubation times.

SAMPLE NAME	Final Concentration of NO ₃ ⁻ (ppm)	Final Concentration Reached after
K27 + DM-N	0	15 (hours)
K27 + DM-N + SeO ₄ ²⁻ (1 mM)	0	30 (hours)
K27 + DM-N + SeO ₄ ²⁻ (10 mM)	4-6	50 (hours)
K27 + DM-N + SeO ₃ ²⁻ (1 mM)	5	20 (hours)
K27 + DM-N + SeO ₃ ²⁻ (10 mM)	154-160	250 (hours)

Chapter IV

Results and Discussions

Part 1. Synthesis of Dimethyl Selenone

Dimethyl selenone was successfully synthesized in our lab following the method described by Zhang [1994] with slight modification. In our method, the two reactants were combined in the reverse order of Zhang's method after we determined that adding dissolved dimethyl selenide *to* the oxidant increased the yield of dimethyl selenone. The purified freshly synthesized DMSeO_2 has a melting point of 147-148°C, the same as reported in the literature [Paetzold and Bochamann, 1968]. A 65% yield of dimethyl selenone was achieved.

Part 2. Growth of *P. fluorescens* K27 in Minimal Media

2-1. DM Medium and F Medium

At the beginning of this project, we inoculated one *P. fluorescens* K27 colony into both DM medium and F medium respectively and aerobically cultivated the precultures; *P. fluorescens* K27 grew well in both media. However, after the aerobic cultures were transferred to anaerobic DM-N and F-N medium (containing nitrate as electron acceptors), *P. fluorescens* K27 showed very weak growth in F-N medium. By amending with 0.1 mM selenite or selenate, the growth of this bacteria was completely inhibited in this medium. On the other hand, *P. fluorescens* K27 grew very well in DM-N medium even when amended with 1 mM selenite and selenate.

Three differences were found through comparing the two media. At first, F-N medium contained 1 g/L NH_4Cl instead of 1 g/L $(\text{NH}_4)_2\text{SO}_4$ which was a major component for DM-N medium. Therefore, sulfate concentration in F medium was only 39 ppm comparing to that in DM medium which was 770 ppm. Secondly, pH of DM medium was adjusted to 7.4, but that of F-N medium was not adjusted (pH about 6.7). More over, F medium contained trace elements; DM medium did not.

Based on the above facts, we decided to use the simpler DM media to do our research. At the same time, we still wanted to know why K27 can not grow in F-N medium which contains major components similar to DM-N media. Therefore, we performed the following two experiments to compare the bacterial growth. First, we adjusted the sulfate concentration of DM-N to 39 ppm (same as in F-N medium) to obtain a low sulfate DM-II medium (Table I). We observed the same growth rate of K27 as that in DM-N medium. Therefore, we concluded that sulfate concentration did *not* affect anaerobic growth of *P. fluorescens* K27 in both DM-N and F-N medium.

Secondly, we adjusted the pH of F-N medium to 7.4 with potassium hydroxide. We did not observed better anaerobic growth by adjusting pH. Therefore, the weak growth of *P. fluorescens* K27 in F-N medium was not due to a pH differences. So the weak growth of K27 in F-N medium was possibly caused by the trace elements in the solution, probably due to high concentration of Mg^{2+} (10 g/L) in F-N medium, which could inhibit the growth of K27. We did not do further related experiments because DM-N medium was chosen as the growth medium for all subsequent experiments.

The doubling time for K27 growth in DM-N medium was about 5.5 hours. A typical growth curve is shown in Figure 1.

2-2. Anaerobic Cultivation of K27 in Nitrate - Free DM Medium

Based on an assumption that this bacteria can use sulfate as electron acceptor, we designed an experiment in which we anaerobically cultivated K27 in DM-I medium (without adding KNO_3 as electron acceptor) (Table I). Corresponding poisoned cultures were obtained by amending 1 mM or 10 mM of either selenite or selenate solutions individually into the above control culture. All of the growth curves are shown in Figure 5. The results show that both control and poisoned cultures were able to grow even without nitrate present. Therefore the growth of the control cultures was possibly due to sulfate reduction or fermentation (which is using a carbon source as an electron acceptor). Unlike the corresponding growth curves in DM-N medium (Figures 1, 2 and 3), one mM selenate amended cultures did not show unusually slow growth rates in this medium, and the 10 mM selenate amended culture had relatively shorter lag phase than that in DM-N medium. On the other hand, the behavior of selenite amended cultures also displayed a different growth patterns from that in DM-N medium. Ten mM selenite amended cultures exhibited much longer lag phases than those in DM-N medium.

2-3. Anaerobic Cultivation of K27 in Sulfate-Free DM-N Media

The media used for this experiment were DM-III and DM-IV which were shown in Table I. Instead of sulfate as a component in DM-N, sulfur containing amino acids L-cysteine·HCl·H₂O and L-(-)-methionine were added to DM-III and DM-IV media respectively. The purpose is not only to prevent this strain from utilizing sulfate as electron acceptor but also to

supply enough sulfur for the bacteria to synthesize necessary sulfur containing proteins.

K27 was successfully aerobically cultivated in both media. Better growth was obtained in L-(-)-methionine containing cultures; and both cultures presented much stronger fluorescent color than sulfate containing cultures. Although the higher population of *P. fluorescens* K27 will result in stronger fluorescent production, the fluorescent light is directly energy related, which is strongly depended on the components of media.

Under anaerobic conditions, growth was observed in both DM-III and DM-IV media with and without amending with 1 mM selenite. However, reddish color was present when L-cysteine·HCl·H₂O was added into bacteria-free 10 mM selenate or selenite solutions. An oxide-reducing reaction may happened between S²⁻ (from cysteine) and Se⁴⁺ or Se⁶⁺, and the red color was possibly due to the formation of elemental selenium. Therefore, we suggest that further experiments need be carefully designed, such as control of pH of solution when using L-cysteine *etc.*, We did not do further work on these aspects.

Part 3. Toxicity Experiments of K27

The two toxicity assays of growth inhibition and doubling time measurements are similar; both are based on measuring the optical density of the bacterial cultures. The difference is that the growth inhibition method only measured the first 24 hours of the culture's growth and the toxicant was added at the time that the bacteria culture reached the exponential growth phase, while doubling time experiments measured the

whole growth process and required amendment with toxicant when the bacterial cultures were inoculated.

Figure 6 shows results of growth inhibition experiments with K27 amended with selenate, selenite, and dimethyl selenone. Linear regression analyses of growth inhibition versus $\log(\text{concentration})$ were used to determine EC_{50} values reported in Table III; range, number of data points (n), and linear regression coefficients (r) are also given in Table III. These growth inhibition curves are typical for measuring 4 replicate samples.

Following the growth of *P. fluorescens* K27 in anaerobic cultures amended with various concentrations of selenate, selenite and dimethyl selenone allowed for the determination of the corresponding doubling times as displayed in Figure 7. The average doubling time of unpoisoned cultures of *P. fluorescens* K27 grown anaerobically at 28°C in DM-N medium was determined to be 5.5 hours (n=50). EC_{50} values, where K27 doubling times doubled, were determined using equation 4; corresponding growth rates were determined by linear regression of $\log(\text{concentration})$ (shown in equation 5). At concentrations as low as 79 ppm (1.0 mM) dimethyl selenone or as high as 3950 ppm (50 mM) selenite, no K27 bacterial growth was observed. In contrast, even at concentrations of 15800 ppm (200 mM) selenate, *P. fluorescens* K27 was still able to grow, but with extended lag phases of 4 to 8 weeks.

Comparing growth inhibition and doubling time of K27 (Table III), the EC_{50} values lie in the same order of magnitude and close to each other. Determined by doubling time measurement, EC_{50} values of these three compounds show relatively wider ranges with higher values. However, one of the conclusions of this work is that the term growth inhibition was found

to be somewhat misleading: 100% growth inhibition was determined at concentrations of 10 mM selenate and 15 mM selenite, even though growth occurred far beyond these concentrations. The limitation in this method is the definition of growth inhibition which depends on the relative growth of a control and does not, in any way, take extended lag phase into account.

In addition, comparing the highest concentrations where growth was observed, dimethyl selenone (39.5 ppm) is still the most toxic to K27 among the three selenium compounds investigated; selenite (2370 ppm) is more toxic than selenate (15800 ppm), as could generally be expected from the literature [Ibrahim & Spacie, 1990]; but these results were opposite to the EC₅₀ values obtained by the above two assays.

Towards *P. fluorescens* K27, the results of the growth inhibition and doubling time measurement show that dimethyl selenone is more toxic than either of the selenium oxyanions. Based on these results alone one might rule dimethyl selenone out as a possible intermediate of the selenium reduction pathway. However, Zhang and Chasteen [1994] reported that dimethyl selenone is converted into less toxic and less water soluble volatile organoselenides at much higher rates than selenite and selenate by *P. fluorescens* K27 in cultures amended with the species. This means that if dimethyl selenone *is* an intermediate in the bioreduction process for bacteria that have developed a resistance mechanism, the rate limiting step in the formation of volatile organoselenides from selenite or selenate would probably lie between selenite (or selenate) and dimethyl selenone. Thus, relative toxicity aside, high concentrations of dimethyl selenone would not be allowed to build up and do damage in the cell since DMSeO₂ is so readily converted to more reduced, volatile, and less soluble forms.

Colony Count Experiments

These series of experiments were affected by several factors; therefore, errors were easily produced. The more colony formation units were counted, the less error was produced; however, the more colonies formed, the more difficult it was to count the colonies. Our experiments using this method were not reproducible.

Because these experiments were carried out under aerobic conditions, the results are not comparable with the above anaerobic growth inhibition and doubling time measurement experiments. These series of experiments show that even on 1 mM selenite poisoned plates, K27 colonies produced brick red color, and colonies forming units decreased rapidly with the increase of concentration of selenite. On 30 mM selenite amended plates, no colonies were observed at all.

In selenate amended plates, colony formation exhibits a very strange phenomena. With the concentration increasing to 20 mM, colony forming units gradually decreased, and yellowish colonies were produced. With the further increase of selenate concentration on the plates from 40 to 200 mM, colony forming displayed two stages; in the first stage, only a few colonies formed; but a few days later, many colonies emerged immediately. Therefore, we always obtained two different sizes of colonies after the second growth phase was reached. In addition, both small colonies and big colonies produced a brownish color instead of a reddish color which was produced in selenite amended culture.

Part 4. Analysis of Selenium Distribution and Nitrate Reduction in Time Course Experiments of Bacterial Growth

4-1. Nitrate Analysis

The nitrate reductions by *P. fluorescens* K27 were shown from figure 17 to figure 21 for the control culture and the cultures amended with various concentrations of selenite and selenate.

The nitrate analysis results show that nitrate was the limiting reagent for the growth of bacterial controls and selenate poisoned cultures. As Figure 17, 18 and 20 showed that as soon as nitrate had been consumed, the bacteria reached the stationary phase of growth. The initial nitrate concentration in our DM-N medium is 10 mM, and the increase of nitrate concentration in this medium directly resulted in the increase of bacterial population (optical density) in stationary phase .

Unlike selenate amended culture in which nitrate was consumed before selenate was reduced, selenite amended cultures tell a different story: nitrate was reduced simultaneously with selenite reduction. In 10 mM selenite amended solutions, nitrate reduction was affected by the production of elemental selenium caused by selenite reduction; the formation of elemental selenium inhibited nitrate reduction. In those test tubes which produced more elemental selenium when the culture reached stationary phase, the final concentration of nitrate in the culture was higher; nitrate consumption in 10 mM selenite amended cultures were not more than 3/4 of the original nitrate of the DM-N medium in our experiments.

Based on data shown in Table V, we conclude that selenite inhibits nitrate reduction more than selenate.

4-2. Analysis of Selenium distribution

After amending *P. fluorescens* K27 with various concentrations of selenite and selenate, time course experiments were performed to measure the consumption of selenate and selenite. The productions of both volatile organoselenides and elemental selenium were shown in the Figure 19 to Figure 23. The concentration changes of the remaining selenate and selenite in these solutions are also shown in the same figures.

In comparing the productions of volatile compounds, volatile organosulfur compounds, dimethyl sulfide, dimethyl disulfide and dimethyl selenenyl sulfide were only observed in selenate amended cultures. Except for methanethiol (CH_3SH), no additional organosulfur compounds were observed in selenite amended cultures grown anaerobically on minimal DM-N medium (Table IV). On the other hand, the production of *organoselenides* was observed at the early exponential growth phase by amending with selenite while at the early stationary phase when amending with selenate. Furthermore, selenite amended cultures produced more dimethyl diselenide than dimethyl selenide, but over time, the dimethyl diselenide production decreased while dimethyl selenide production gradually increased, Selenate amended culture did not produce more dimethyl diselenide than dimethyl selenide during any of the time course experiments. As shown in Table IV, selenate amended culture produced more volatile organoselenium compounds than that of selenite amended culture for comparable selenium amended concentrations.

In comparing the production of precipitates in selenite and selenate amended cultures, ten mM selenite amended cultures turned brick red after reaching the stationary phase; while the selenate amended cultures only turned to a very light pink. As shown in Table IV, much more elemental selenium was produced in selenite amended cultures than in selenate amended cultures.

When comparing the consumption of selenate or selenite in the supernatant of these solutions as shown in Table IV, selenite consumption were more than that of selenate amended cultures as measured by AAS. In addition, selenite concentrations were detected in the selenate amended cultures using UV/VIS detecting the DBA/Se complex at 420 nm (detection limit is 1 ppm). Therefore, we can conclude that the bioconversion of selenate to selenite was very small in this cultures. On the other hand, no oxidized selenite was observed in selenite amended cultures handled anaerobically.

All of the above experimental results were also affected by the bacterial populations of the precultures. By amending selenite with lower initial bacterial population, such as starting at optical density at about 10, the bacteria produced much more elemental selenium when compared with starting at OD about 30; and only one exponential growth phase was observed under these conditions.

Based on all of above observations, we may conclude that selenite and selenate reduction have different pathways. Selenite reduction may use nitrate reductase; therefore nitrate reduction did not inhibit selenite reduction. But the production of elemental selenium and dimethyl diselenide (Figure 14 and 16) by selenite reduction may inhibit nitrate reduction by damaging the functions of the cell membrane through radical

reaction of dimethyl selenide or formation of selenium granules [Seko *et al.*, 1989; Yan and Frenkel, 1994]. However, there are no reports in the literature that selenite reduction and nitrate reduction used the same enzyme system.

Based on our observations, we also may conclude that selenate reduction may be due to different reductases; and under this situation, nitrate was the preferred electron acceptor. Therefore, only after nitrate was almost completely consumed in 1 mM selenate amended culture and down to a concentration less than 10 ppm in 10 mM selenate amended culture, were volatile organoselenium compounds detected. Moreover, dimethyl sulfide, dimethyl disulfide and dimethyl selenenyl sulfide were observed in these cultures. There have been reports that nitrate reduction and selenate reduction use different enzymatic systems on another selenium resistant bacterial strain *Thauera selenatis* [Reh and Macy, 1992]. However, comparison of nitrate reduction with selenate and selenite reductions by this *P. fluorescens* bacterial strain is first reported by this project; no previous reports have been made, probably due to the difficulty of the cultivation of K27 in minimal medium under anaerobic conditions.

In order to clearly differentiate selenate and selenite reductions, further enzyme isolation and analysis following this project may throw even more light on the possible mechanisms of reductions and bioremediation of selenium oxyanions.

Chapter V

Conclusions

All of the following conclusions can be made for anaerobic cultures of *P. fluorescens* K27 examined in this research:

- EC_{50} values for *P. fluorescens* K27 increase from dimethyl selenone (0.23 mM, ~30 ppm) to selenate (0.67 mM, ~130 ppm) and selenite (3.7 mM, ~630 ppm).
- The maximum concentrations of toxicants in which *P. fluorescens* K27 can grow in the DM-N medium is in an increasing order of dimethyl selenone (~ 0.7 mM), selenite (~35 mM) and selenate (~ 200 mM).
- In the concentration range from 1 mM to 5 mM, selenate amended K27 cultures exhibit extremely slow growth even compared with 100 mM selenate culture. However, the higher the concentration of selenate amended, the longer the lag phase of growth. The 200 mM selenate amended cultures had a lag phase as long as 6 to 8 weeks.
- Selenate amended cultures produced more volatile organoselenium compounds than selenite amended cultures; and only in selenate amended cultures were dimethyl sulfide (DMS), dimethyl disulfide (DMDS) and dimethyl selenenyl sulfide (DMSeS) ever observed. However, except for methanethiol, no additional organosulfides were produced in the selenite amended culture.

- Selenite amended cultures produced more elemental selenium than selenate amended cultures: in 10 mM amended solutions, 5 times more Se was produced when the cultures incubated for 250 hours were compared; while in 1 mM amended solutions, even 10 times more elemental Se was produced during anaerobic growth for 120 hours.
- Bioconsumption of selenite is more than that of selenate when comparing cultures amended with the same concentration. In 10 mM amended solutions, reduction of selenite is 5.9%, while that of selenate is 0.9% during anaerobic growth for 250 hours.
- Nitrate reduction by this bacterium is inhibited more in selenite amended solutions than in selenate amended solutions. On the other hand, nitrate does not inhibit selenite reduction but does inhibit selenate reduction. Therefore, bacteria produced volatile compounds in the exponential phase when amended with selenite while in the early stationary phase by those amended with selenate.
- Less than 1% of added selenate is reduced in the 10 mM selenate amended culture over a time period of 240 hours. However, about 5% of added selenite is reduced in the 10 mM selenite amended culture.

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