Chemolithotrophic growth of fungi including *Fusarium solani* on elemental sulfur

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List of abbreviations

2-DE: Two dimensional electrophoresis
APS: Ammonium persulfate
BPB: Bromophenol blue, 3′,3″,5′,5″-tetrabromo phenolsulfonphthalein
CHAPS: 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
DAPI: 4',6-diamidino-2-phenylindole
DNA: Deoxyribonucleic acid
DTT: Dithiothreitol
GSB: Green sulfur bacteria
GSG: Glutathione disulfide
GSH: Glutathione
GSSG: Glutathione persulfide
GS_nG: Higher homologues of glutathione persulfide
HPLC: High performance liquid chromatography
IEF: Isoelectric focusing
IPG: Immobilized pH gradient
PCR: Polymerase chain reaction
PMSF: Phenylmethylsulfonyl fluoride
PNSB: Purple nonsulfur bacteria
PSB: Purple sulfur bacteria
RNA: Ribonucleic acid
SDS: Sodium dodecyl sulfate
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA: Trichloroethanoic acid
TEMED: Tetramethylethylenediamine
Tris: (Hydroxymethyl) aminomethane
UV-Vis: Ultraviolet and visible

Abstract

Lithotrophic sulfur oxidation, one of the major reactions of global sulfur cycle, is thought to be an ancient metabolic process carried out exclusively in prokaryotes. Some literatures exist concerning oxidation of elemental sulfur (S^0) and thiosulfate by organotrophic fungi. Previous study about *Fusarium solani* THIF01, harboring an endobacterium, showed that it could grow chemolithotrophically using S^0 as a sole energy source. However, lithotrophic sulfur oxidation by endobacteria-free fungi has not been unequivocally documented. Given that chemoorganotrophic sulfur oxidation by fungi has been found existing in diverse niches including nutrient-limiting environments, it is reasonable to doubt about the "fact" that eukaryotes are not capable of lithotrophic sulfur oxidation. Therefore, the aims of this study are to investigate the distribution of lithotrophic sulfur-oxidizing fungi and the physiological characteristics of sulfur oxidation by fungi.

Our group has found that 13 named fungal strains from culture collections oxidized S^0 on an organics-free medium. In this study, 7 of these fungal strains, forming larger area of clearing zones on S^0-containing plates, were screened for absence of endobacteria and ability of lithotrohpic sulfur oxidation. No 16S rRNA gene was amplified from the genomic DNA, indicating that these seven fungal strains did not harbor endosymbionts. All of the strains grew in an S^0-containing

mineral-salts submerged media. *Fusarium solani* f.sp. *pisi* NBRC9425, one of these fungi, exhibited the intensest sulfur oxidation when it formed the largest clearing zone on agarose-solidified medium and the culture pH showed the most dramatic decrease.

Activity of sulfur-oxidation of strain NBRC9425 was examined after the fungus was cultured in organics-free, S^0-containing submerged medium for 15 days. Neither culture filtrate nor the concentrate of filtrate incubated with sulfur substrates, such as GSSG/GS_nG, S^0, $S_2O_3^{2-}$, and SO_3^{2-}, exhibited any activity of sulfur oxidation, indicating that sulfur oxidation occurred intracellularly. However, cell-free extract also did not show any activity of sulfur oxidation. It looked that the integrity of the fungal cell was vital to maintain the activity of sulfur oxidation, and the cell disruption when preparing cell-free extract caused the loss of activity.

The proteomic profiling in response to oligotrophic growth condition on sulfur and heterotrophic growth condition on maltose was analyzed on two dimensional electrophoresis (2-DE) gels. In this study, optimization of the conditions of protein extraction and isoelectronic focusing was performed. 2D display of soluble proteins of strain NBRC9425 grown on the different energy sources suggested greatly distinctive metabolism. This study paves way for comparative proteomic study of the identification of proteins participating in fungal sulfur-oxidation.

Physiological characteristics of chemolithotrophic fungal growth on reduced inorganic sulfur

compounds were investigated. Chlamydospores collected from organics-free medium served as inoculum. When grown in mineral-salts medium containing S^0, strain NBRC9425 produced thiosulfate, and the produced thiosulfate decreased after 20 days cultivation. According to the decrease of thiosulfate, sulfate was produced, and the concentration was kept increasing during a period of 50 days. Neither tetrathionate nor sulfite was detected in the culture. These phenomena suggest that S^0 was oxidized to thiosulfate, and then to sulfate eventually. When grown with thiosulfate in mineral-salts medium, strain NBRC9425 also produced sulfate, but the concentration of sulfate decreased from the 5th day. Notably, sulfate production and biomass yield were greatly enhanced when thiosulfate was supplemented into the S^0-containing medium.

In order to study the effects of organic compounds on the sulfur oxidation by fungi, different concentrations of yeast extract (0–200 mg L^{-1}) were added into S^0-containing medium. As the supplemented yeast extract increased, thiosulfate and sulfate production and fungal biomass increased accordingly. However, the values of sulfur compounds equivalent, the quotient of sulfate/thiosulfate divided by ergosterol, showed that the highest sulfur oxidation ability was achieved at 15 mg L^{-1} yeast extract. Fungal hypha were observed to attach to the S^0 particles when growing with less than 100 mg L^{-1} yeast extract, but no such association was evident when incubated with 200 mg L^{-1} or more of yeast extract. Although high concentrations of organic compounds shifted the metabolism from chemolithotroph to chemoorganotroph, strain NBRC9425 oxidized more S^0 because of higher yield of biomass.

Chapter 1. Introduction

1-1 Sulfur-oxidizing microbes

Since discovered in 1880s, the sulfur cycle, the first biogeochemical cycles ever unveiled, has been in the spotlight in the scientific community. The oxidation states range from -2 (in sulfide) to +6 (in sulfate) (Table 1-1). Sulfide, S^0, and thiosulfate are the main sulfur species utilized by microbes. Hydrogen sulfide gas, as a cause of inhalational deaths, exists in soils and rock, springs, shelf water, and sour gas which is petroleum or natural gas with a high percentage of hydrogen sulfide. Metal sulfides are found in minerals in rock ores and mine waste. Like sulfide, (poly)sulfides have a great affinity for transition metal ions and is essential in the formation of sulfide minerals (Luther, 1991). In various anoxic and oxic environments, (poly)sulfides have been found (Kristiana et al., 2010; Kamyshny et al., 2014). S^0 can be found in hot springs and volcanic regions. The natural occurrence of thiosulfate is in springs and geysers with a smaller amount compared to sulfate, which naturally occurs in combination with metals in the form of salts.

The variety of oxidation states of sulfur is reflected by the fact that a variety of redox reactions drive the global cycling of this element (Steudel, 2000). Ecologically and taxonomically diverse microorganisms participate in the sulfur cycle: photolithotrophic bacteria, chemolithotrophic bacteria and archaea conduct such redox reactions and act together to drive the oxidative half of the sulfur cycle (Brüser et al., 2000). Anaerobic phototrophic purple bacteria, a phylogenetically highly diverse group of bacteria, extensively oxidize H_2S and other reduced inorganic sulfur

compounds produced in anoxic water layers and sediments of lakes (Imhoff et al., 2017). Chemolithotrophic sulfur-oxidizers occupy a variety of habitats. *Thiobacillus* in activated sludge could utilize thiosulfate and S^0 as energy sources (Katayama et al., 1995). Sulfur-oxidizing bacteria *Halothiobacillus* in wasterwater biofilms featured with microaerophilic conditions (Ito et al., 2004). *Thiomicrospira* in intertidal mud flat utilized thiosulfate as energy source and CO_2 as carbon source (Brinkhoff et al., 1999). Gamma- and Epsilon-proteobacteria oxidize sulfide in Namibian shelf water (Lavik et al., 2009).

Table 1-1 Biologically relevant inorganic sulfur compounds and their oxidation states (Dahl et al., 2008).

Compound	Chemical formula	Sulfur oxidation state
Sulfide	HS^-/S^{2-}	-2
(Poly)sulfides	S_n^{2-}	-1 (termical S)/ ±0 (inner S)
Elemental sulfur	S_n (S rings), S_μ or S_8 (polymetric S)	0
Sulfite	HSO_3^-/SO_3^{2-}	+4
(Poly)thionates	$-O_3S(S)_nSO_3^-$	+5 (sulfone S) ±0 (inner S)
Thiosulfate	$S_2O_3^{2-}$	+5 (sulfone S) −1 (sulfane S)
Sulfate	SO_4^{2-}	+6

Compared to that of prokaryotic sulfur oxidation, researches about sulfur oxidation by fungi have a shorter and briefer history. It was in1920's that sulfur oxidation by fungi was first discovered (Waksman, 1918; Armstrong, 1921; Abbott, 1923). However, the importance of sulfur oxidation by fungi in biotechnology and environment was not realized until the last decades. To date, numerous fungal genera have been found to be able to oxidize reduced inorganic sulfur compounds: *Absidia*, *Acremonium*, *Alternaria*, *Amanita*, *Aspergillus*, *Aureobasidium*, *Cephalosporium*, *Epicoccum*, *Fusarium*, *Geosmithia*, *Hymenoscyphus*, *Hypholoma*, *Monilia*, *Mortierela*, *Mucor*, *Myceliophthora*, *Paecilomyces*, *Paxillus*, *Penicillium*, *Phanerochaete*, *Pisolithus*, *Rhizopogon*, *Rhodotorulla*, *Suillus*, *Trichoderma*, and *Zygorhynchus* (Czaban and Kobus, 2000; Wainwright, 1989).

1-2 Agricultural and biotechnological importance of microbial sulfur-oxidizers

Owing to its importance in agriculture, wastewater treatment, biomining and biological metal processing, lithotrophic sulfur oxidation by prokaryotes has been extensively studied (Dahl et al., 2008, Rawlings, 2005; Liljeqivst et al., 2013). Whereas sulfur-oxidizing fungi, to some extent, have been neglected.

Sulfur-oxidizing microorganisms play an important role in agriculture. Farmland soil is a functionally complex ecosystem. Because the reactions in the cycling of carbon and nitrogen are

the major energy flow systems (Tourna et al. 2014), C and N budgets in farmlands have been emphasized with great importance and studied extensively (Meisinger et al., 2008; Kirk et al., 2015). Although occurs as a tracer element in agricultural ecosystem, sulfur, together with other elements, constitutes the essential amino acid (such as methionine, cysteine and cystine) involved in chlorophyll production. Clearly, sulfur is required for protein synthesis and plant function and structure. As a result, plant productivity in farmland relies on this element. It has also been found that sulfur affects the rates of processes of C, N and P cycling (McGill and Cole, 1981; Chapman, 1997; Kirkby et al., 2011). Sulfur deficiency has been found on a worldwide basis (Kanwar and Mudahar, 1986; Westermann, 1974). Bunemann and Condron (2007) found that sulfur fertilizers greatly improved the primary production in S-deficient soils. It is a universal activity to use S^0-fertilizer to alleviate the S deficiency in soils. These fertilizers must be converted to sulfate before the crop can utilize. This conversion is performed by soil microbes. The biochemistry of prokaryotic sulfur oxidation mediating such conversion has been studied well and the ecology of sulfur-oxidizing bacteria in soils was analyzed (Tourna et al., 2014). Despite of the significance of S cycling for soil fertility, little is known concerning the fungal taxa involved in the process.

Sulfur-oxidizing microbes have proved their value in treatment of wastewater. Reduced inorganic sulfur compounds in wastewater, if not removed, would do harm to natural system by means of chemical oxygen demand and biochemical oxygen demand. In addition, the oxidation of reduced inorganic sulfur compounds in nature can cause pH decrease because of the produced sulfuric acid

(Millano and Sorber, 1986). An internal sulfur cycling driven by wastewater microbes consists of sulfate reduction and subsequent sulfide oxidation (Okabe et al., 2005). The ecology and *in situ* ecophysiology of sulfate-reducing bacteria and sulfur-oxidizing bacteria in wastewater have been studied (Okabe et al., 2005; Belila et al., 2013). The major sources of sulfide in nature are volcano eruptions, diffusion from geothermal springs and the hydrothermal vent of the sea (Kuenen and Robertson, 1992). Human activities have been disturbing the sulfur cycling by burning off fossil fuels, and boosting biological products of reduced sulfur compounds. Sulfide in industrial wastewater is an undoubtedly serious problem (Table 1-2). Thiosulfate is another important sulfur compound in industrial wastewater. This compound comes from photographic processing laboratories in which silver thiosulfate is used as a photographic fixer. In nature, thiosulfate occurs as a product by chemical or biological oxidation of sulfide (Millano and Sorber, 1986). Sidpietersite, as a thiosulfate mineral, occurs very rarely in nature. Sulfide and thiosulfate can be used as not only energy source but also sulfur source by prokaryotes. Biochemical, ecological and physiological studies of sulfur-oxidizing bacteria applied to wastewater treatment have drawn much attention. But the application of sulfur-oxidizing fungi in wastewater treatment does not interest scientific community so much as bacteria do.

Table 1-2 Estimates of sulfide emissions due to industrial activity (Ryaboshapko, 1983).

Process	Sulfide	Emission (as TgS $year^{-1}$)
Paper making	DMS, H_2S, mercaptans	0.1-0.65
Coke production	H_2S	0.07-0.7
Smelting of iron	H_2S	0.1-0.5
Cement production	H_2S	1.2

Microbial sulfur oxidizers contribute greatly to mining industry. Dating back to Roman times in the first century BC or even the Phoenicians, human started to employ the natural capability of microorganisms to mining activities without realizing it (Siddiqui et al., 2009). Like other industries, mining industry faces the challenge of efficient, low-cost and environmentally friendly process. Biomining permits less energy-intensive, less polluting procedures, and high efficiency of metal recovery (Drewniak and Sklodowska, 2013). It is thought that biomining is mainly driven by acid-tolerant autotrophs in tank or heap processes (Rawlings and Johnson, 2007). For the oxidation of acid-insoluble metal sulfides, prokaryotes use and gain energy via a "thiosulfate mechanism", and in the case of acid-soluble metal sulfide, a "polysulfide mechanism" is employed (Schipper and Sand, 999):

Thiosulfate mechanism (FeS_2, MoS_2, and WS_2)

$$FeS_2 + 6\,Fe^{3+} + 3\,H_2O \rightarrow S_2O_3^{2-} + 7\,Fe^{2+} + 6\,H^+$$

$$S_2O_3^{2-} + 8\,Fe^{3+} + 5\,H_2O \rightarrow 2\,SO_4^{2-} + 8\,Fe^{2+} + 10\,H^+$$

Polysulfide mechanism (e.g., ZnS, $CuFeS_2$, or PbS)

$$MS + Fe^{3+} + H^+ \rightarrow M^{2+} + 0.5\,H_2S_n + Fe^{2+}\ (n \geq 2)$$

$$0.5\,H_2S_n + Fe^{3+} \rightarrow 0.125\,S_8 + Fe^{2+} + H^+$$

$$0.125\,S_8 + 1.5\,O_2 + H_2O \rightarrow SO_4^{2-} + 2\,H^+$$

A variety of sulfur-oxidizing prokaryotes in biomining tank or heap have been identified, and the application of these oxidizers in biomining industry has also been extensively studied (Rawlings and Johnson, 2007). Although the autotrophs are the main contributors to biomining processes, a complex microbial consortia comprising of both autotrophs and heterotrophs exists in biomining operators. The heterotrophs feed on the organic compounds provided by the autotrophs and boost the processes of extracting metals (Rawlings, 2005). The eukaryotic sulfur oxidizers in stirred-tank or large-scale heap are clearly of importance, but to date the biochemistry and physiology of fungi in biomining processes still remain unveiled.

Table 1-3 Sulfur-oxidizing microorganisms and the established pathways of sulfur oxidation

Domain	Nutrition	Pathways or enzymes of sulfur oxidation
Archaea	Lithotrophic	Sulfur oxygenase reductase, thiosulfate:quinone oxidoreductase
Bacteria	Lithotrophic, Phototrophic	The sulfur oxidation pathway encoded by *sox* gene cluster, The S4 intermediate pathway
Eukarya	Organotrophic	Unknown

1-3 Reactions, enzymes, and pathways of microbial sulfur oxidation

Eukaryotes and prokaryotes use sulfur to build up cell constituents. It is believed that some members of bacteria and archaea can harness the produced reductant (such as NADPH) and ATP molecules in sulfur oxidation to respiration and/or CO_2 assimilation (Gosh and Dam, 2009; Mattes

et al., 2013), but fungi can not. Dissimilatory sulfur oxidation in *Eukarya* is mediated by lithotrophic bacterial endosymbionts (Dahl et al., 2008). Although the molecular mechanism of CO_2 fixation associated with sulfur oxidation by fungi has not been elucidated, chemilothotrophic growth of fungi on reduced inorganic sulfur compounds was observed (Li et al., 2010).

The diversity of reduced inorganic sulfur compounds oxidized by microbes (Table 1-1) is also reflected by the multiplicity of enzymes of sulfur oxidation. The wide distribution of sulfur available to sulfur oxidizers is also in accordance with the ecological and taxonomical diversity of sulfur-oxidizers. Various photo- and chemolithotrophic sulfur-oxidizing bacteria and archaea, which live in a diversity of extreme and moderate environments, have evolved different pathways of sulfur oxidation (Table 1-2).

Basic research has unveiled the mystery of the biochemical basis of prokaryotic sulfur metabolism in the past decades. Reactions, proteins and genes involved in sulfur oxidation by prokaryotes have been extensively studied. New methods to study microbial metabolism are emerging such as genomics and proteomics of sulfur-metabolizing bacteria (Weissgerber et al. 2013; 2014). Although the importance of sulfur oxidation by fungi has been realized, enzymes and pathways of sulfur oxidation in fungi are yet to be investigated.

1-4 Sulfur oxidation found in fungi

Given the "fact" that fungi are heterotrophs, chemolithotrophic sulfur oxidation has been thought existing exclusively in prokaryotes but not in fungi. Only one prior study discovered a chemolithotrophic sulfur oxidation by a fungal isolate *Fusarium solani* THIF01 which harbored an endobacterium. To the best of our knowledge, in all of the existing literatures about sulfur oxidation by endobacteria-free fungi, the eukaryotic sulfur-oxidizers depended on organic compounds as the energy sources. For example, *Trichoderma harzianum* oxidized S^0 in a medium added with 10–100 μg glucose-C g^{-1} (Wainwright and Grayston, 1988). Grayston and Wainwright (1987) proposed a chemolithoorganotrophic sulfur oxidation by *Aspergillus niger* and *T. harzianum* which utilized both thiosulfate and sucrose as energy substrates. A soil yeast, *Rhodotorula* sp. oxidized S^0, thiosulfate and sulfide in the presence of maltose (Kurek, 1978).

However, fungi have been found growing in various oligotrophic environments in nature such as cave rock, sub-seafloor basalt and sandstones, and interaction between fungi and rock has been observed (Cunningham et al., 1995; Ivarsson et al., 2016; Li et al., 2008). Microbes are key in the regulation of the global cycles of materials, and they are also responsible for the stability of materials, no matter natural or man-made. It is believed that microorganisms interact with materials in two ways: microbes utilize the materials as a nutrient source or secrete degrading enzymes or metabolic compounds; physical forces generated by microbial growth can damage the

material (Beimforde, 2011). Bacteria, cyanobacteria, archaea, algae, lichens, and fungi have been found as the biodeteriorating agents of stones (Perry IV et al., 2005). Decomposition of cave rocks and minerals by fungi is believed to be processes of mycelia invasion and organic acid excreting (Gorbushina et al., 1993; Gómez-Alarcón et al., 1994). Pedersen thinks that autotrophs form a chemosynthetic base through CO_2 fixation to support the heterotrophs in oligotrophic environments (2000). However, recent researches on biodeterioration of sandstones in Angkor temples showed that fungi also could utilize inorganic compounds as energy substrates and survive in oligotrophic environments. Kusumi et al. (2011) observed chemolithotrophic sulfur oxidation by *Mycobacterium* spp. isolated from sandstones in Angkor Wat, Bayon, and Phnom Krom temple. Nineteen fungal strains isolated from deteriorated sandstones of Angkor temples were able to oxidize S^0. One of them, *F. solani* THIF01, is able to use S^0 as a sole energy source (Li et al., 2010). These discoveries raise questions: whether chemolithotrophic sulfur-oxidizing fungi distribute widely; which reduced inorganic sulfur compounds can be used as energy sources by fungi; how do organic compounds affect sulfur oxidation by fungi.

1-5 Symbiotic association of fungi with bacteria

Fungal-bacterial endosymbiosis represents the mutualistic relationship between a fungal host and its intracellular bacterial symbiont(s). Bacterial endosymbionts were overlooked for a long time, and once were thought to be rare. Until 2009, only *Burkholderia endofungorum*, *Burkholderia*

rhizoxinica, and *Candidatus* Glomeribacter gigasporarum as endobacteria were found in fungi (Castillo and Pawlowska, 2009). Recently, however, fungal-bacterial endosymbiosis have been reported in a growing number (Moebius et al., 2014). Symbiosis with endofungal bacteria usually has a profound effect on the host's lifestyle (Moebius et al., 2014).

F. solani THIF01, capable of chemolithotrophic growth on S^0, harbors an endobacterium *Bradyrhizobium* sp. (Li et al., 2010).The endosymbiot might mediate the sulfur oxidation because *Bradyrhizobium* is able to oxidize sulfur (Masuda et al., 2010; Xia et al., 2014). As a result, endobacteria are a concern in studies of fungal sulfur-oxidation.

1-6 Purposes of this dissertation

Sulfur oxidation by fungi is of agricultural, environmental and industrial importance. Most researches concerning interaction between microorganisms and sulfur have focused on bacteria and archaea, whereas the knowledge about fungal sulfur-oxidation is limited. The neglect of sulfur oxidation by fungi is partly due to the long-existing preconceived view that fungi are not capable of oxidizing sulfur lithotrophically which is the key reaction in the biotechnology of microbial sulfur-oxidation. With the aim to explore chemolithotrophic sulfur oxidation by fungi, the present study has been designed with the following objectives:

Firstly, to screen lithotrophic sulfur-oxidizing fungi from named fungal strains;

Secondly, to examine activity of sulfur-oxidation in fungi;

Thirdly, to characterize physiological properties of sulfur-oxidizing fungi.

The obtained results are described in the following five chapters:

Chapter 2 represents the way that the model fungal strain was screened.

Chapter 3 concerns about the *in vitro* and *in vivo* sulfur-oxidizing activity of fungi.

Chapter 4 is mainly about the physiological study of lithotrophic sulfur oxidation by fungi.

Chapter 5 deals with the effects of organic compounds on sulfur oxidation by fungi.

At last, chapter 6 sums up and discusses the major findings of the present study.

Chapter 2. Screening of lithotrophic sulfur-oxidizing fungi from named strains

2-1 Introduction

Prior studies found that most fungi oxidize reduced inorganics sulfur compounds in the presence of organic compounds, especially organic carbon, except for the one that Li et al. (2010) previously reported about *F. solani* THIF01. Strain THIF01 was isolated from deteriorated sandstones in Angkor temples and it was able to grow chemolithotrophically on S^0 (Li et al., 2010). *F. solani* THIF01 harbored an endobacterium *Bradyrhizobium* sp., and Li et al. (2010) pointed out the possibility that the endosymbiont mediated the sulfur oxidation because a bacterium in the genus *Bradyrhizobium* is able to oxidize sulfur (Masuda et al., 2010; Xia et al., 2014). *F. solani* fungi, a complex group of more than 50 fungal species, are capable of growing in a diverse range of habitats, and can cause disease on more than 100 genera of plants and opportunistic infections in humans (Coleman et al., 2009). As a pathogen isolated from Cambodia, strain THIF01 is under the regulations of the Plant Protection Station, Ministry of Agriculture, Forestry and Fisheries of Japan, and it has not been deposited in any microbial culture collections currently. As a result, strain THIF01 cannot be used further for the study of fungal sulfur-oxidation. Our group previously found that 13 fungal strains, belonging to the genera *Aspergillus*, *Trichoderma*, *Fusarium*, *Rhizopus, Amylomyces,* and *Gliocladium*, out of 18 named fungal strains showed oxidation of S^0 on mineral-salts agar plates (Table 2-1). Seven of these strains, NBRC9425, AOKB1466, NBRC6790, NBRC9462, AOK1606, AOK12-2, JCM22676, exhibited relatively high activities of sulfur-oxidation, with clearing zones more than 0.5 cm in radius (Table 2-1). As a result, these 7

strains make ideal candidates for studies of lithotrophic sulfur oxidation by fungi. This discovery also indicates that lithotrophic sulfur oxidation by fungi may exist widely in nature.

On the other hand, endobacteria are a concern in the study of fungal sulfur-oxidation. Although overlooked for a long time, fungal-bacterial endosymbiosis has been reported recently in a growing number (Moebius et al., 2014). Many endofungal bacteria are important to the function and growth of their hosts: the endosymbionts controlled the asexual reproduction of *Rhizopus microspores* (Partida-Martinez et al., 2007); the bacterium "*Candidatus* Glomerbibacter gigasporarum" greatly affected the presymbiotic growth and sporal morphology of its host *Gigaspora margarita* (Lumini et al., 2007); the obligate *Ca.* Glomerbibacter gigasporarum might be implicated in the vitamin B12 provision for its host (Ghignone et al., 2012); *Rhizopus microsporus*, a rice seedling blight fungus, and its bacterial symbiont *Burkholderia rhizoxinica* cooperated to produce toxins that killed rice plants (Moebius et al., 2014). As a result, in this study, endobacteria are examined to ensure no involvement of bacteria in fungal sulfur-oxidation. As the aim of this dissertation is to investigate lithotrophic sulfur oxidation by fungi, selecting a fungal model strain which harbors no endobacteria is the first task. 16S rRNA gene-targeted polymerase chain reaction (PCR) has been employed to analyze the successful elimination of endofungal bacteria from fungi, such as *Gigaspora margarita* (Lumini et al., 2007) and *Rhizopus microsporus* (Partida-Matinez et al., 2007). In the present study, the existence of endobacteria was analyzed using this method.

Table 2-1 Fungi, their sulfur oxidation capability and sources related to this study.

Strain No.[a]	Species names	Sulfur oxidation activity[b]	Notes
THIF01	*Fusarium solani*	+ +	Isolated from deteriorated stones in Angkor Wat, Cambodia (Li et al., 2010)
THIF01BF	*Fusarium solani*	+ +	The cured THIF01
NBRC9425	*Fusarium solani* f.sp. *pisi*	+ +	
AOKB1466	*Gllocladium virens*	+ +	
NBRC6790	*Gliocladium deliquescens*	+ +	
NBRC9462	*Fusarium graminearum*	+ +	Accession No. as received in this study: IFO 9462
AOK1606	*Trichoderma viride*	+ +	
AOK12-2	*Aspergillus oryzae*	+ +	
JCM22676	*Trichoderma reesei*	+ +	
NBRC31095	*Fusarium equiseti*	-	Accession No. as received in this study: IFO 31095
NBRC32209	*Fusarium roseum* f.sp. *cerealis*	-	Accession No. as received in this study: IFO 32209
NBRC32000	*Fusarium oxysporum* f.sp. *raphani*	+	Accession No. as received in this study: IFO 32000
NBRC31660	*Fusarium decemcellulare*	-	Accession No. as received in this study: IFO 31660
CBS438.76	*Amylomyces roouxii*	+	
NBRC5442	*Rhizopus javanicus*	+	
NBRC4707	*Rhizopus oryzae*	-	
NBRC30499	*Rhizopus chinensis*	+	

JCM1876	*Trichoderma harzianum*	+ +	
NBRC30467	*Fusarium oxysporum* f.sp. *spinaciae*	-	Accession No. as received in this study: IFO 30467
AOKB650	*Aspergillus niger*	-	

[a]NBRC: Biological Resource Center, National Institute of Technology and Evaluation, Tokyo, Japan
JCM: Japan Collection of Microorganisms, Tsukuba, Japan
IFO: Institute for Fermentation, Culture Collection of Microorganisms, Osaka, Japan. All cultures preserved in the IFO have been transferred to the NBRC, in July 01, 2002.
AOK: Stock collection in Akita Konno Co., Ltd.
[b] Sulfur oxidation activity is expressed according to the radius of clearing zone: -, <0.1 cm; 0.1 ≤ + < 0.5 cm; + + ≥ 0.5 cm

2-2 Materials and Methods

All chemicals used in this dissertation were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), except when mentioned specifically.

2-2-1 Examination of the existence of endobacteria

Hyphal discs of the 7 fungal strains growing on PDA plates were transferred to 60 mL submerged culture of Potato Dextrose Broth (Difco Laboratories, Detroit, MI, USA) dispensed in 300 mL Erlenmeyer flasks and incubated at 30°C and 120 rpm rotary shaking for 4 days. The collected mycelia were subjected to DNA extraction followed by RNAase A (0.02 μg μL^{-1}) using ISOPLANT DNA extraction kit (Nippon Gene, Toyama, Japan). The obtained DNA solutions were analyzed using UV-Vis spectrophotometer (Nano Drop 2000, Thermo Fisher Scientific, Wilmington, DE, USA). In this study, PCR was performed in 20 μL reaction volumes with 2.0

μL 10 × PCR Buffer (HotStarTaq Plus, QIAGEN, Germantown, MD, USA), 0.2 M dNTPs, 0.5 μM Forward-primer SF, 0.5 μM Reverse-primer 520R, 0.5 unit HotStarTaq Plus DNA polymerase, 1 μL DNA template (0.5 ng, 5 ng, or 50 ng), and 12.9 μL MiliQ water. Cycle sequencing was achieved with an initial denaturation step of 5 min at 95°C; 10 cycles of 1 min at 94°C, 1 min at 65°C to 55°C (decease by 1°C/cycle), 1 min at 72°C; 20 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C; and 10 min at 72°C. The integrity of the extracted DNA and the PCR products were analyzed using electrophoresis with 1.5% agarose gel and staining with ethidium bromide.

2-2-2 Screening of sulfur-oxidizing fungi on organics-free S^0-containing media

Sulfur-oxidation activity of fungi was examined with clearing zone formation on plates. Instead of agar that contains traces of available carbons (Payton et al. 1976), pure agarose (SeaKem, Lonza Rockland, Rockland, ME, USA) was used to solidify the medium that was prepared according to the procedure described by Wieringa (1966) with minor modification. In brief, sulfur-free agarose-solidified medium (g L^{-1}, 3.0 KH_2PO, 0.16 NH_4Cl, 0.41 $MgCl_2 \cdot 6H_2O$, 0.007 $FeCl_2 \cdot 4H_2O$, 0.25 $CaCl_2 \cdot 2H_2O$, pH 5.0, Li et al., 2010) was used as the basal medium. Into sterilized Petri dishes with a diameter of 9 cm, 1 mL sterilized 0.1 M HCl solution was added. Then, 20 mL autoclaved aforementioned agarose medium (60°C) was slowly poured in to the Petri dishes. After mixing, the medium was allowed to solidify. A thin layer of 10 mL of the agarose medium, supplemented with filtration-sterilized 20 μL polysulfide, was poured on top of the first layer. The polysulfide solution was prepared by saturating a solution of sodium sulfide in distilled water with S^0: add S^0 into the

saturated sodium sulfide solution until no more S^0 can be dissolved. After the solidification of the top layer, the agarose plates were put above a 60°C water bath without contact of water to allow the steam to heat the bottom layer medium. In this way, the HCl from the bottom layer diffuses through the top layer and precipitates the sulfur from the polysulfide. In this way, very fine granules of S^0 were thoroughly suspended in the medium. At the same time, hydrogen sulfide formed in this process was removed because of heating steam from the water bath.

The 7 fungal strains were grown on PDA (Potato-Dextrose-Agar, DAIGO, NIHON SEIYAKU Co., Tokyo, Japan) plates at 30°C for 7 days. Fungal hyphae were pick from the PDA plates and inoculated to the above-mentioned agarose medium plates. After culturing at 30°C for 7 days, area of the clearing zones were measured.

The 7 candidate strains were cultivated in an organics-free S^0-containing liquid medium (WS5-S (g L^{-1}); 3.0 KH_2PO, 0.16 NH_4Cl, 0.41 $MgCl_2 \cdot 6H_2O$, 0.007 $FeCl_2 \cdot 4H_2O$, 0.25 $CaCl_2 \cdot 2H_2O$, 10 S^0, pH 5.0, Li et al., 2010) to test the pH decrease in culture. Five mL medium (three biological replicates) was put in 15 mL screw capped tubes (16.5 mm diameter × 130 mm length) and were subjected to autoclave for 20 min at 120°C. Granules of crystal S^0 (KANTO CHEMICAL CO., INC., Tokyo, Japan) were autoclaved at 105°C for 30 min for three times, and added in the medium before inoculation. A mycelial disc (3 mm diameter) cut from the growing edge of a PDA plate stock culture served as the inoculum. After culturing the 7 fungal strains at 30°C for 7 days, sulfur

oxidation indicated by pH decrease was analyzed using a pH meter (HORIBA F-52, HORIBA Ltd., Kyoto, Japan).

2-3 Results

2-3-1 Examination of the presence of endobacteria

As shown in Fig. 2-1, genomic DNA was extracted after treated with RNAase. In the PCR experiment, different amount of DNA template (0.1ng, 0.5 ng, 5 ng in 20μL reaction mixture) were used, but no 16S rDNA was amplified (Fig. 2-2). Thus, it can be concluded that these 7 fungal strains harbored no endobacteria.

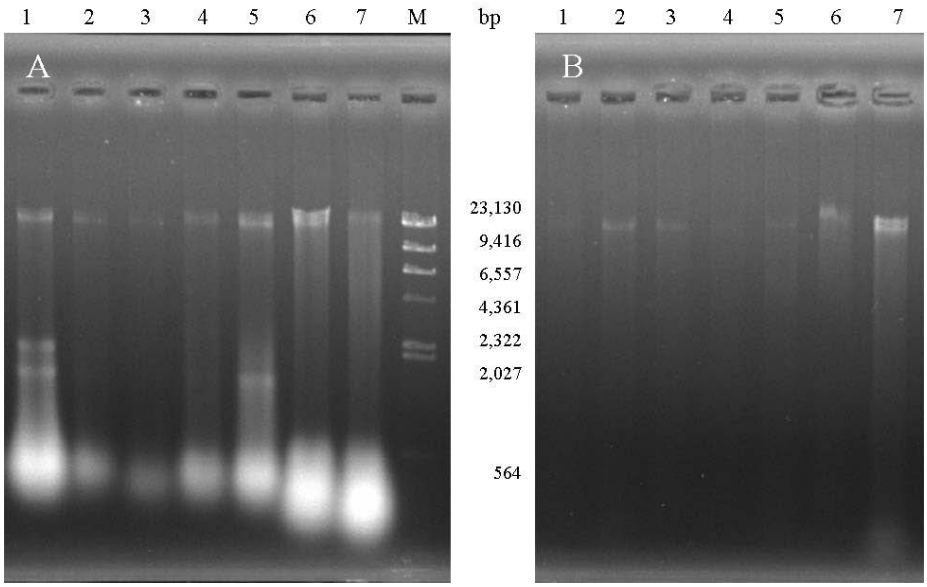

Fig. 2-1 DNA isolation. A: Lane 1, 2, 3, 4, 5, 6, 7 were DNA solutions of strain AOKB1466, NBRC6790, NBRC9462, AOK12-2, JCM22676, NBRC9425, and AOK1606, respectively. B: Lane 1, 2, 3, 4, 5, 6, 7 were DNA samples in Fig. A treated with RNase A. Lane M is DNA standard marker.

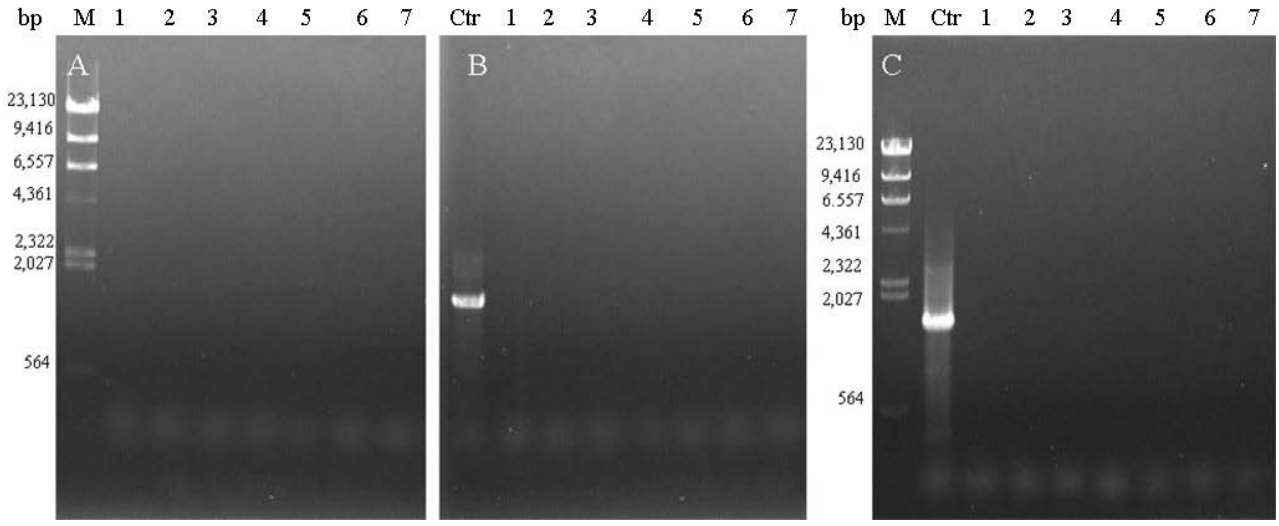

Fig. 2-2. 16S rRNA genes amplification from different amount of DNA temple: 0.1 ng (A), 0.5 ng (B), 5 ng (C). Lane 1, 2, 3, 4, 5, 6, 7 were amplification products using genomic DNA as templates of AOKB1466, NBRC6790, NBRC9462, AOK12-2, JCM22676, NBRC9425, and AOK1606, respectively. Lane Ctr was amplification product using genomic DNA of *E. coli* as template. Lane M, marker.

2-3-2 Screening of sulfur-oxidizing fungi on organics-free S^0-containing media

Fig. 2-3 shows *F. solani* f.sp. *pisi* NBRC9425 formed a clearing zone beneath and beyond the periphery of the colony on the plate: insoluble S^0 granules were oxidized to soluble sulfur compounds. As shown in Fig. 2-4A, clearing zones were formed by all of the examined seven fungal strains.

The pH decrease found in WS5-S medium (Fig. 2-4B) is probably mainly due to the sulfuric acid produced from the oxidation of S^0. The lowest pH (4.04) was observed for the culture of strain NBRC9425, which is in accordance with the biggest clearing zone (12.5 cm^2) formed by the same strain. These findings indicate that strain NBRC9425 exhibited outstanding ability of lithotrophic sulfur oxidation. Thus, strain NBRC9425 was selected for further study.

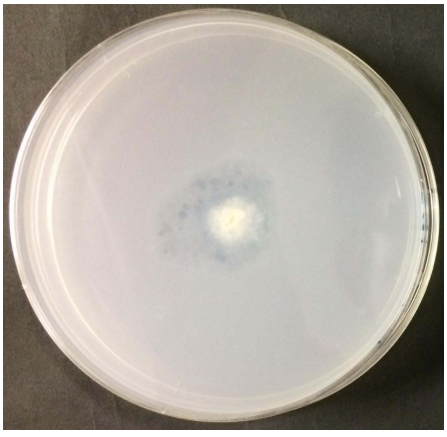

Fig. 2-3 *F. solani* f.sp. *pisi* NBRC9425 oxidized S^0 and formed a clearing zone on an agarose-solidified mineral-salts medium after cultivated 7 days at 30°C.

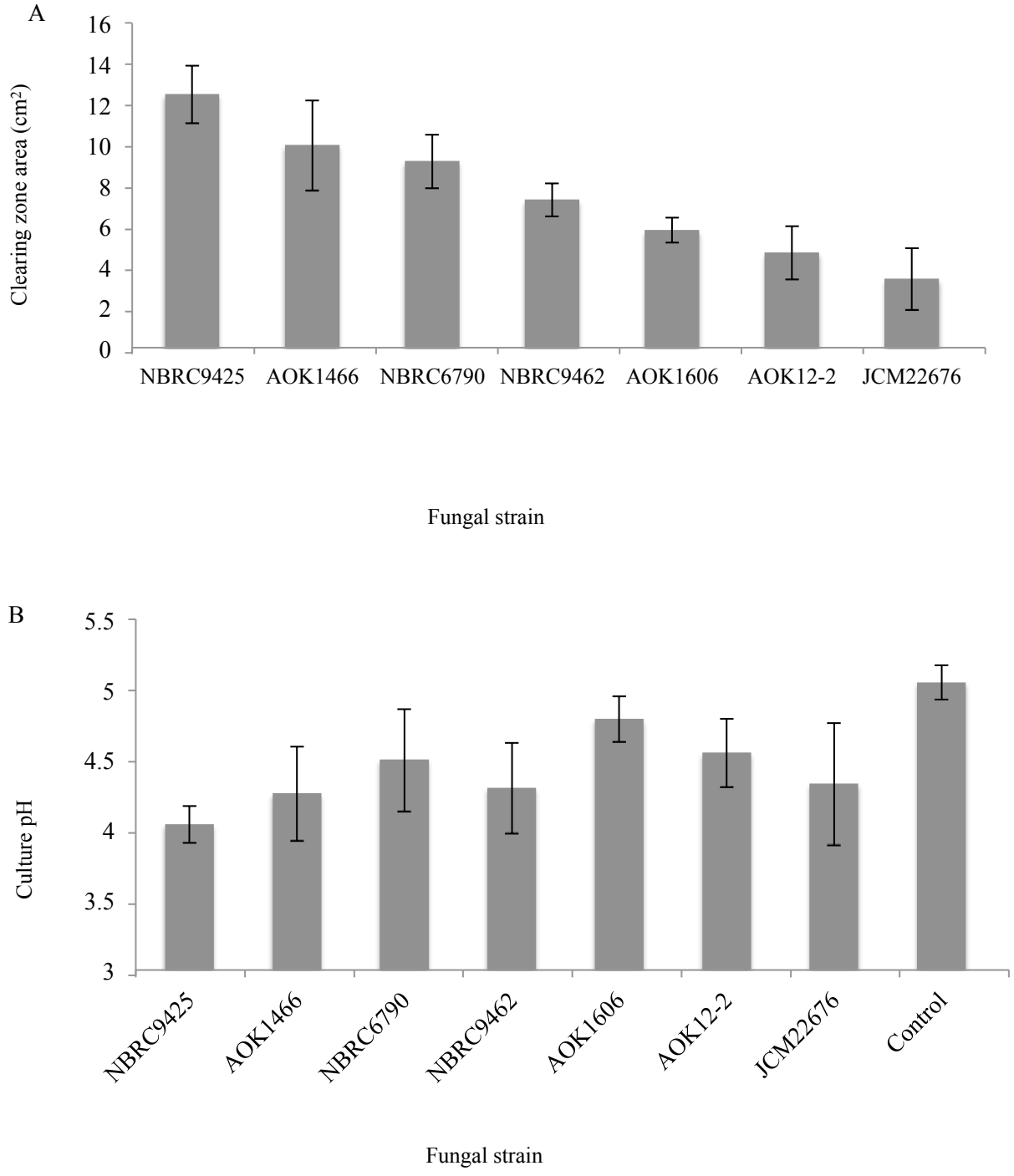

Fig. 2-4. Clearing zones formation on powdered sulfur dispersed agarose medium (A) and pH decrease of WS5-S liquid medium (B) inoculated with 7 named fungal strains after 7 days growth at 30°C (3 biological replicates). The basal salts medium containing elemental sulfur was solidified with pure agarose. Error bars correspond to s.d.

2-4 Discussion

Dahl et al. (2008) think that sulfur oxidation in eukaryotes is limited to degradation of sulfur-containing amino acids and membrane-components, and that dissimilatory sulfur oxidation in eukaryotes is taken charged by the endobacteria. Endobacteria were found in various classes of fungi, including a sulfur-oxidizing fungus (Li et al., 2010). As a consequence, before examining the ability of sulfur-oxidation by fungi, it is necessary to check the endosymbionts.

ISOPLANT DNA extraction kit is designed for the isolation of botanical and bacterial genomic DNA. However, it proved efficient in the isolation of fungal DNA in this study. *F. solani* is a group of more than 50 phylogenetic species known as the "*Fusarium solani* species complex" (O'Donnell, 2000). Some of the members are capable of colonizing a wide range of environments. Coleman et al. (2009) found that *Nectria haematococca*, a member of *F. solani*, has a large genome of 54.43 Mb. The large genome size is consistent with this species' diversity of habitats. Clearly, as shown in Fig. 2-1, genome of strain NBRC9425 is not as large as 54.43 Mb. *F. solani* f.sp. *pisi* NBRC9425 was first isolated from soils. In this study, this strain utilized organic compounds efficiently like other examined strains, and also grew faster than other tested strains on organics-free S^0-containing plates. These properties of utilizing both organic and inorganic energy substances reflect the ability of living in diverse environmental niches.

The endobacterium, *Bradyrhizobium* sp., was eliminated from *F. solani* THIF01. The complete elimination of the symbiont was analyzed using PCR method. Interestingly, the amount of DNA template used in PCR affected the outcome greatly: after strain THIF01 was cultured in the presence of rifampicin, the 16S rRNA gene of *Bradyrhizobium* sp. was amplified from 5 ng genomic DNA extracted from fungal cells. On the other hand, when 0.5 ng or 50 ng template was used, the agarose electrophoresis gel showed no binds of any 16S rRNA genes. As a result, in the experiment of checking endobacteria in the named 7 fungal strains, 0.1 ng, 0.5 ng, and 5 ng genomic DNA extracted from fungi hyphae was subjected to PCR analysis.

It was intriguing to find that all the 7 fungal strains, belonging to the genera of *Fusairum*, *Gllocladium*, *Trichoderma* and *Aspergillus*, could grow on organics-free medium by oxidizing S^0 in culture. It pointed out a possibility of the abundantly natural occurrence of fungi capable of oxidizing reduced inorganic sulfur compounds lithotrophically. It is safe to conclude that sulfur oxidation by fungi has long been underrated in nature and anthropogenic environments. On one hand, the distribution of sulfur-oxidizing fungi may be much wider than we are currently aware of. One the other, in nutrient-deficient environments sulfur-oxidizing fungi may act as a precursor by "digesting" inorganic sulfur and synthetize organic compounds which support other forms of lives.

Li et al. (2010) used fine S^0 granules-suspended agar medium to show the sulfur oxidation by fungi. Thiosulfate and sulfate were detected from the extract of the agar located within the clearing zone.

In this study, agarose plates containing the same medium were employed. Table 2-1 shows that all the fungal strains examined in this study formed large clearing zones on agar-solidified S^0-containing medium plates (radius $\geq$ 0.5 cm). When growing on agarose-solidified S^0-containing medium plates, as shown in Fig. 2-4A, much smaller clearing zones formed by the same strains were observed. These findings suggest that organic compounds, even in trace amount, can greatly affect the sulfur oxidation by fungi, and they warn us to remove organic contaminants in the following study of lithotrophic growth of fungi on reduced inorganic sulfur compounds.

Chapter 3. Activity of sulfur-oxidation of *F. solani* f.sp. *pisi* NBRC9425

3-1 Introduction

Lithotrophic sulfur oxidation is an energy-yielding process, usually associated with bioenergetics metabolism. The emerging biochemical and molecular studies of microbial sulfur metabolism show that archaea and bacteria use different pathways. Given fungi belong to domain *Eukarya*, enzymes and pathways of sulfur oxidation in fungi may be different from those in archaea and bacteria. The academic community thinks reduced inorganic sulfur compounds are exclusively oxidized by prokaryotes (Friedrich et al., 2001), and has extensively explored the molecular basis of prokaryotic sulfur-oxidation and the proteome and genome of sulfur-oxidizing prokaryotes. However, few researches have been conducted to investigate the fungal enzymes and proteins participating in sulfur oxidation.

Protein mass spectrometry, genomics, bioinformatics have enabled the advance of fungal enzymes researches. However, the assignment of function to fungal proteins, encoed by *in silico* annotated and unannotated genes, is still a challenge (Doyle, 2011). Recently, fungal proteomics studies have progressed greatly as an efficient tool of high-throughput protein identification and functional assignment. A comparative quantitative proteomic study by Weissgerber et al. (2014) enabled the identification of new proteins relevant for sulfur oxidation in the purple sulfur bacterium *Allochromatium vinosum*. Now that the genome of *Nectria haematoocca*, a member of *F. solani* species complex, is available (Coleman et al., 2009), the comparative proteomic study of strain

NBRC9425 might be promising to reveal the molecular basis of sulfur oxidation. The protein extraction is vital to proteomic study, and the isoelectric focusing (IEF) conditions are sample-specific. In this study, efforts have been made to optimize protein extraction and IEF conditions to investigate the proteomic profiling of strain NBRC9425 grown on organics/sulfur.

3-2 Materials and Methods

3-2-1 *in vitro* activity of sulfur-oxidation of *F. solani* f.sp. *pisi* NBRC9425

3-2-1-1 Fungal strain, media and growth conditions

S^0-grown cells of strain NBRC9425 were prepared as the inoculum in this experiment. After the fungus grew for 10 days on WS5-S agarose plates, its spores were harvested and rinsed with 2 mL sterilized saline onto a plate. One mL spores suspension was then inoculated into 200 mL Erlenmeyer flasks containing 40 mL WS5-S medium and incubated at 30°C without shaking. After 15 days, 20 μL culture was pipetted into the fresh medium, and incubated for another 15 days. This reinoculation procedure was repeated 3 times. The obtained culture was then transferred into 5 L Erlenmeyer flasks which contains 500 mL autoclave-sterilized medium: 3.0 g L^{-1} KH_2PO, 0.16 g L^{-1} NH_4Cl, 0.41 g L^{-1} $MgCl_2 \cdot 6H_2O$, 0.007 g L^{-1} $FeCl_2 \cdot 4H_2O$, 0.25 g L^{-1} $CaCl_2 \cdot 2H_2O$, 10 g L^{-1} S^0, 2 g L^{-1} $Na_2S_2O_3 \cdot 4H_2O$ pH 5.0. After inoculation, the flasks (three biological replicates) were incubated at 30°C without shaking for 15 days in the dark.

3-2-1-2 Harvest of culture supernatant and fungal cells, preparation of cell-free extract

The culture supernatant and cell-free extract were prepared to examine the activities of sulfur-oxidation. After cultivation, fungal cells were harvested by filtration. The obtained filtrate was collected and centrifuged at 12,000 × *g*, 4°C for 10 min to remove the fungal spores and small S^0 particles. The collected cells were washed against phosphate buffer (50 mM, pH 6.0) on the filter paper and then subjected to beads beating milling. After centrifuged at 20,000 × *g*, 4°C for 10 min, cell-free extract was obtained.

In order to concentrate the proteins in culture filtrate, two methods were employed. The culture filtrate was subjected to 80% ammonium sulfate precipitation. The precipitated proteins were collected and dialyzed against 20 mM phosphate buffer (pH 6.0) for 12 h at 4°C. Centrifugal filtration was also used for concentration. Ten mL culture filtrate was put in 15 mL Amicon Ultra centrifugal filter untis (10,000 NMWL, Merk Millipore Ltd., Co. Cork, Ireland), and centrifuged at 5,000 × *g*, 4°C for 30 min.

3-2-1-3 Enzyme assays

Activities of sulfur-oxidizing enzymes were tested according to the assays described by Rohwerdert and Sand (2003) with some modifications. The reaction mixtures contained 30 mM

phosphate buffer (pH 6.0), enzyme solutions or fungal cells, and various substrates (see below). The reactions were started by an addition of substrate. To determine non-enzymatic reactions, assays without substrates were used. After an incubation at 30°C for 60 min, samples were collected and analyzed for sulfur species as described below.

3-2-1-4 Substrates tested in the enzyme assays

S^0 solution was prepared by saturating S^0 in acetone with a concentration of approx. 20 mM S^0. Ten mL acetonic sulfur solution was mixed with 10 mL distilled water and then dialysed against 5 L distilled water (at 4°C, 24 h, exchanging the water three times) to remove the acetone. The milk-like dialysis product had a final volume of 30 mL and contained about 6 mM S^0. The sulfur formed droplets of 2–10 mm in diameter and was used for experiments within 10 h after preparation at a concentration of 4 mM.

In order to make solutions of glutathione persulfide (GSSG) and higher homologues (GS_nG), 500 mM elemental sulfur (powder) was mixed with 100 mM glutathione (GSH) at pH 7.5 (adjusted with KOH) under stirring and anaerobic conditions until the solution became lemon-colored within 1–2 days (Rohwerdert and Sand, 2003). Then the pH was adjusted to 5.0 with concentrated HCl. The produced H_2S was removed by evacuating the gas phase. After degasing H_2S, the pH was adjusted to 6.5. The final solution contained GSSG and higher homologues up to the pentasulfane

homologue (GS_5G).

In addition, 1 mM Na_2SO_3 and 1 mM $Na_2S_2O_3$ were tested as substrates for sulfur-oxidizing enzymes.

3-2-1-5 Analysis of sulfur compounds

Thiosulfate and sulfate ions were measured by ion chromatography as described by Li et al. (2010) with minor modification: the culture was sampled and subjected to centrifugation at 10,000 ×*g*, 4°C for 10 min. The obtained supernatant was diluted ten times with distilled deionized water and loaded onto an ion chromatography (861 Advanced Compact IC, Metrohm Ltd., Switzerland) with a Shodex IC I-542A column (4.6 mm in diameter × 100 mm in length, working temperature at 40°C) and an IC IA-G guard column (4.6 mm in diameter × 10 mm in length). A solution of 1.7 mM $NaHCO_3$/1.8 mM Na_2CO_3 was used as eluent. The flow rate was set as 1.0 mL min^{-1} with a pressure at 7.0 MPa. Thiosulfate and sulfate were detected based on the conductivity.

Tetrathionate was analyzed according to a cyanolysis and spectrophotometric method established by Kelly et al. (1969). Into glass tubes (15.5 mm diameter × 105 mm length), 400 μL NaH_2PO_4-NaOH buffer and 500 μL distilled water were added. After the mixture was cooled on ice for 2 min, 500 μL 0.1 M KCN was added and mixed rapidly. The mixture was maintained on ice for 20 min. Afterwards, 300 μL of 1.5 M $Fe(NO_3)_3$ in 4 M $HClO_4$ was added and mixed. The

mixture was then warmed to room temperature to allow for redissolving any precipitate. At last, distilled water was added to give a total volume of 2.5 mL. The ferric thiocyanate color was read at 460 nm with a UV-VIS spectrophotometer (JASCO V-630, JASCO INTERNATIONAL CO. LTD., Tokyo, Japan). NaH_2PO_4-NaOH buffer was prepared by adding 0.2 M NaOH to 0.2 M NaH_2PO_4 until pH reached to 7.4.

Sulfite was examined as described earlier (Kletzin, 1989): 50 μL of reagent (40 mg of fuchsin dissolved in 87.5 mL of double-distilled water and 12.5 mL of concentrated H_2SO_4) and 195 μL of double-distilled water were added to 250 μL of sample. After 10 min at room temperature, 5 μL of commercial Formalin was added. Readings were taken after at least a 90-min incubation at 570 nm against a reagent blank.

3-2-1-6 Protein determination

Protein concentration was measured according to the method established by Lowry et al. (1951) using bovine serum albumin as the standard.

3-2-2 Two dimensional electrophoresis of the proteome of strain NBRC9425

3-2-2-1 Media and culture conditions

Spore suspension of strain NBRC9425 was prepared in the same way in section 3-2-1-1.

Organics-grown fungal cells and elemental sulfur or inorganic sulfur compound-grown fungal cells were prepared and subjected to protein extraction. For the heterotrophic growth, the fungus was cultured in a medium containing (g L^{-1}): maltose 1.0, KH_2PO_4 3.0, $(NH_4)_2SO_4$ 0.2, $MgSO_4 \cdot 7H_2O$ 0.5, $FeSO_4 \cdot 7H_2O$ 0.01, $CaCl_2 \cdot 2H_2O$ 0.25, $Na_2S_2O_3 \cdot 4H_2O$ 2.0, pH 5.0. Seven days after the inoculation of the spore suspension, fungal cells were collected by filtration as described in section 3-2-1-1. For the oligotrophic growth, the culture conditions were the same as those in section 3-2-1-1.

3-2-2-2 Protein extraction

The cells collected from maltose-containing or sulfur-containing medium were subjected to beads beating milling (Fig. 3-1). Twenty mM Tris-HCl buffer, pH 8.0 used for cell disruption contained 1 mM PMSF, and 1×cOmplete protease inhibitor (Roche, Mannheim, Germany). After centrifugation at 13,000×*g*, 4°C for10 min, the supernatant was collected and added with 4 volumes of ice-cold acetone for concentrating the proteins, and stand at -20 °C for two hours. After centrifugation at 13,000×*g*, 4°C for10 min, the supernatant was dumped off and the pellet was resuspended in 20 mM Tris-HCl buffer, pH 8.0 containing 1 mM PMSF and 1×cOmplete protease inhibitor. Then the obtained protein solution was subjected to centrifugation at 13,000×*g*, 4°C for 10 min. The soluble proteins (supernatant) was added with 3 volumes of 13.3 % (w/v) TCA in pure acetone containing 0.2% DTT. The mixture was statically kept at -20°C for 1.5 h. After centrifugation at 13,000×*g*, 4°C for10 min, the supernatant was descanted and the remaining pellet

was washed with iced pure acetone twice.

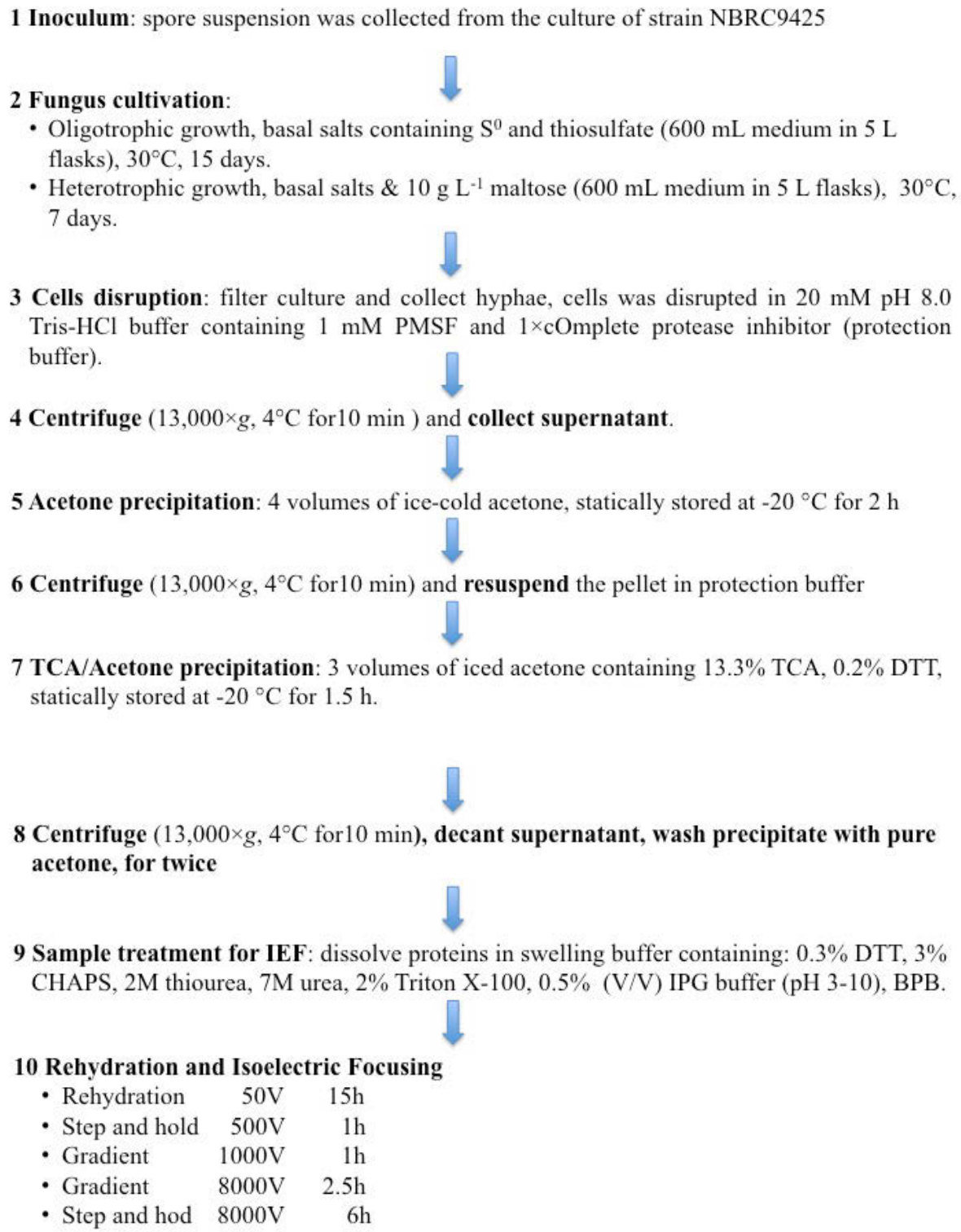

Fig. 3-1 Workflow of protein preparation and isoelectronic focusing.

3-2-2-3 Isoelectric focusing (IEF)

The obtained protein powder was dissolved in rehydration buffer which contained 0.3% (w/v) DTT, 3% (w/v) CHAPS, 2 M thiourea, 7 M urea, 2% (w/v) TritonX-100, 0.5% (v/v) immobilized pH gradient (IPG) buffer (GE Healthcare UK Ltd., Buckinghamshire, England), and 0.002% (w/v) BPB.

The first dimensional separation of the proteins was carried out using an Ettan IPGphor II isoelectric focusing system (Amersham Biosciences, Sweden). Two hundred μL protein solution was loaded onto a 13 cm strip holder. Immobiline DryStrip gels (13 cm, pH 3–10, GE Healthcare UK Ltd., Buckinghamshire, England) were covered on the protein solution. Five hundred μL cover fluid was loaded on the holder to prevent evaporation. After an active rehydration at 50V for 15h at 20°C, isoelectric focusing was performed under the following condition: 500V rapid for 1h, 1,000V linear for 1h, 8,000V for 2.5h, and 8,000V for 6h. Based on prior studies of 2-DE, the conditions of protein focusing are sample-specific. So a volt-hour optimization should be performed independently for each sample type.

3-2-2-4 SDS-PAGE

Focused IPG strips were equilibrated in equilibration solution (6 M urea, 30% glycerol [v/v], 75

mM Tris-HCl buffer [pH 8.8], 2% SDS [w/v], 0.002% [w/v] BPB) supplemented with 5% (w/v) DTT for 15 min in a first step and supplemented with 5% (w/v) iodoacetamide for 15 min in a second step.

SDS-PAGE was performed as described by Laemmli (1970) with a 12% polyacrylamide gel (Table 3-1, Table 3-2). Gently pushed the equilibrated Immobiline DryStrip gel down to the SDS-PAGE gel cassettes so that the entire lower edge of the Immobiline DryStrip gel was in contact with the top surface of the stacking gel. Ensured that no air bubbles were trapped between the Immobiline DryStrip gel and the stacking gel surface or between the gel backing and the glass plate. Agarose sealing solution was loaded onto the Immobiline DryStrip gel to prevent it from moving or floating in the electrophoresis buffer. After the separation of the proteins at 25 mA, the polyacrylamide gel was subjected to silver staining.

Table 3-1 Solutions for making a running gel of SDS-PAGE.

Solution	Volume (mL)
1.5 M Tris-HCl buffer (pH 8.8) containing 0.4% (w/v) SDS	5.3
30% (w/v) Acrylamide	10.5
1% BIS Acrylamide	4.5
Distilled water	0.5
10% (w/v) APS	0.1
TEMED	0.015

Table 3-2 Solutions for making a stacking gel of SDS-PAGE.

Solution	Volume (μL)
0.5 M Tris-HCl buffer (pH 6.8) containing 0.4% (w/v) SDS	450
30% (w/v) Acrylamide	300
1% BIS Acrylamide	234
Distilled water	816
10% (w/v) APS	9
TEMED	3.6

3-3 Results

3-3-1 *in vitro* activity of sulfur-oxidation of *F. solani* f.sp. *pisi* NBRC9425

3-3-1-1 Sulfur oxidation by strain NBRC9425 over a period of 15 days

Notably, dramatic decrease of culture pH was observed, which was consistent with the production of sulfate (Fig. 3-2). It was suggested that S^0 or thiosulfate was oxidized to sulfuric acid contributing to the decrease of culture pH. After 15 days cultivation on S^0, culture sample was collected to analyze sulfur oxidation.

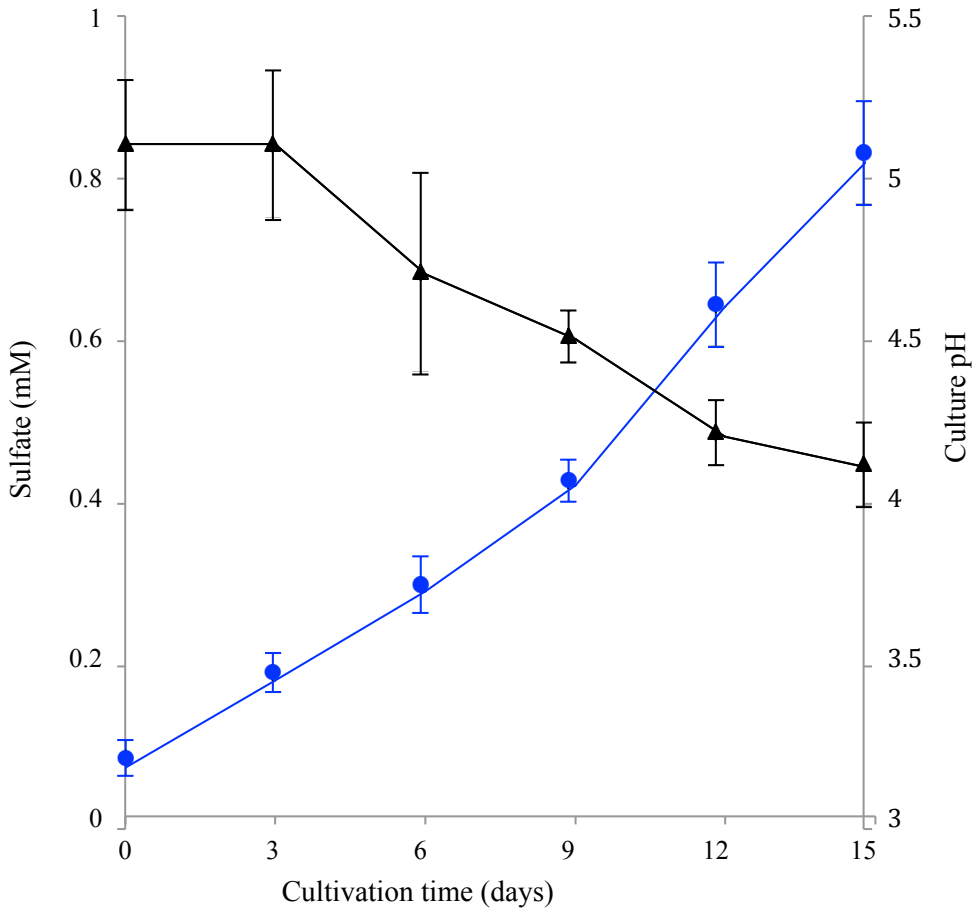

Fig. 3-2. Lithotrophic sulfur oxidation by *F. solani* f.sp. *pisi* NBRC9425 in 5 L flasks containing S^0 and thiosulfate incubated statically at 4°C for 15 days (three biological replicates). Symbols: ●, sulfate; ▲, culture pH.

3-3-1-2 Sulfur oxidation by secreted proteins of strain NBRC9425

The filtrate of culture was examined for the activity of sulfur-oxidizing enzymes. The filtrate of culture was extremely low in protein, and protein concentrations could not be determined by Lowry method. As shown in Table 3-3, culture filtrate exhibited no activity of sulfur-oxidation when GSSG/GS_nG, S^0, $S_2O_3^{2-}$, and SO_3^{2-} were used as substrates.

Culture filtrate was concentrated using 80% ammonium sulfate precipitation to a protein content of 2.3 μg mL^{-1} and tested for enzyme activities. However, no production of $S_2O_3^{2-}$, SO_3^{2-}, $S_4O_6^{2-}$, SO_4^{2-} was detected in the reaction mixture (Table 3-4). Centrifugal filtration was also used for concentrating culture filtrate with a final protein concentration of 3.14 μg mL^{-1}. However, no activity of sulfur-oxidizing enzymes was found (Table 3-5), either.

Table 3-3 Activity of sulfur-oxidizing enzymes of the culture filtrate of strain NBRC9425. One mL reaction mixture comprised 30 mM phosphate buffer (pH 6.0), enzymes solution, 1 mM of the substrates. After incubation at 30°C for 60 min, samples were collected and analyzed. Sulfur species yielding rate was calculated based on its production in the reaction mixture.

Substrate	Rate of yielding SO_3^{2-} (μmol min^{-1} mL^{-1})	Rate of yielding $S_2O_3^{2-}$ (μmol min^{-1} mL^{-1})	Rate of yielding $S_4O_6^{2-}$ (μmol min^{-1} mL^{-1})	Rate of yielding SO_4^{2-} (μmol min^{-1} mL^{-1})
GSSG/GS_nG	0[a]	0	0	0
S^0	0	o	0	0
$S_2O_3^{2-}$	0	-[b]	0	0
SO_3^{2-}	-	-	-	0

[a]: No activity.

[b]: Not determined.

Table 3-4 Activity of sulfur-oxidizing enzymes of the culture filtrate of strain NBRC9425 after concentrated by 80% ammonium sulfate precipitation. One mL reaction mixture comprises of 30 mM phosphate buffer (pH 6.0), enzyme solution (final concentration of protein: 0.2 μg mL^{-1}), 1 mM of the substrates. After incubation at 30°C for 60 min, samples were collected and analyzed. Sulfur species yielding rate was calculated based on its production in the reaction mixture.

Substrate	Rate of yielding SO_3^{2-} (μmol min^{-1} mL^{-1})	Rate of yielding $S_2O_3^{2-}$ (μmol min^{-1} mL^{-1})	Rate of yielding $S_4O_6^{2-}$ (μmol min^{-1} mL^{-1})	Rate of yielding SO_4^{2-} (μmol min^{-1} mL^{-1})
GSSG/GS_nG	0[a]	0	0	0
S^0	0	0	0	0
$S_2O_3^{2-}$	0	-[b]	0	0
SO_3^{2-}	-	-	-	0

[a]: No activity.

[b]: Not determined.

Table 3-5 Activity of sulfur-oxidizing enzymes of the culture filtrate of strain NBRC9425 concentrated by centrifugal filtration. One mL reaction mixture comprised of 30 mM phosphate buffer (pH 6.0), enzymes solution (final concentration of protein: 0.2 μg mL^{-1}), 1 mM of the substrates. After incubation at 30°C for 60 min, samples were collected and analyzed. Sulfur species yielding rate was calculated based on its production in the reaction mixture.

Substrate	Rate of yielding SO_3^{2-} (μmol min^{-1} mL^{-1})	Rate of yielding $S_2O_3^{2-}$ (μmol min^{-1} mL^{-1})	Rate of yielding $S_4O_6^{2-}$ (μmol min^{-1} mL^{-1})	Rate of yielding SO_4^{2-} (μmol min^{-1} mL^{-1})
GSSG/GS_nG	0[a]	0	0	0
S^0	0	0	0	0
$S_2O_3^{2-}$	0	-[b]	0	0
SO_3^{2-}	-	-	-	0

[a]: No activity.

[b]: Not determined.

3-3-1-3 Sulfur oxidation by cell-free extract of strain NBRC9425

Cell-free extract (0.51 mg mL^{-1} as protein concentration) was subjected to enzyme assays. Addition of various sulfur substrates for the test of enzyme activities showed no production of $S_2O_3^{2-}$, SO_3^{2-}, $S_4O_6^{2-}$, or SO_4^{2-} in the reaction mixture (Table 3-6).

Table 3-6 Activity of sulfur-oxidizing enzymes of cell-free extract (protein concentration: 0.51 mg mL^{-1}) of strain NBRC9425 concentrated by centrifugal filtration. One mL reaction mixture comprises of 30 mM phosphate buffer (pH 6.0), enzymes solution (final concentration of protein: 0.2 μg mL^{-1}), 1 mM of the substrates. After incubation at 30°C for 60 min, samples were collected and analyzed. Sulfur species yielding rate was calculated based on its production in the reaction mixture.

Substrate	Rate of yielding SO_3^{2-} (μmol min^{-1} mL^{-1})	Rate of yielding $S_2O_3^{2-}$ (μmol min^{-1} mL^{-1})	Rate of yielding $S_4O_6^{2-}$ (μmol min^{-1} mL^{-1})	Rate of yielding SO_4^{2-} (μmol min^{-1} mL^{-1})
$GSSG/GS_nG$	0	0	0	0
S^0	0	0	0	0
$S_2O_3^{2-}$	0[a]	-[b]	0	0
SO_3^{2-}	-	-	-	0

[a]: No activity.

[b]: Not determined.

3-3-1-4 Sulfur oxidation by sulfur-grown cells of strain NBRC9425

After cultivating strain NBRC9425 in sulfur-containing medium for 15 days, fungal cells were collected and washed. The cells were subjected to enzyme assays, but no production of $S_2O_3^{2-}$, SO_3^{2-}, $S_4O_6^{2-}$, or SO_4^{2-} was detected in the reaction mixture.

3-3-2 Two dimensional electrophoresis of the proteome of strain NBRC9425

3-3-2-1 Effects of volt-hour on protein focusing

Using the protocol shown in Fig. 3-1, the proteins extracted from maltose grown cells exhibited lots of horizontal streakings and vertical streakings (Condition 1 in Table 3-7). After extending the focusing time at 8,000V from 0.5h to 5h (Condition 2 in Table 3-7), some vertical streaking disappeared. But horizontal streaking was a still a problem. The horizontal streaking suggested the existence of non-protein impurities in the extracted proteins. As a result, optimization of both protein extraction and volt-hour was obligated.

Table 3-7 Effects of protein focusing time on IEF.

Changes in work flow (Fig. 3-1)	Condition 1 (maltose-grown cells)	Condition 2 (maltose-grown cells)
Step 10	Step and hold: 50v 15h	Step and hold: 50v 15h
	Step and hold: 500v 1h	Step and hold: 500v 1h
	Gradient: 1000v 1h	Gradient: 1000v 1h
	Gradient: 8000v 2.5h	Gradient: 8000v 2.5h
	Step and hold: 8000v 0.5h	Step and hold: 8000v 5h

3-3-2-2 Optimization of protein extraction and volt-hour of IEF

As shown in Table 3-8, protein extraction procedure and the IEF procedure was modified to remove the non-protein impurities and to optimize the volt-hour conditions. For condition 3 and 4, RNase was used to treat the extracted proteins to remove RNA. In addition, high speed

centrifugation was performed to remove polysaccharides. Compared to condition 2, condition 3 was better because less streakings were observed (Fig. 3-3A). It suggested that RNase treatment and high speed of centrifugation worked. An extension of protein focusing in condition 4 lead to a less streaking (Fig. 3-3B), suggesting an inadequate focusing in condition 3.

As a result, RNase and DNase were used to treat the protein sample and the volt-hour was increased (condition 5, 6 in Table 3-8). For the proteins extracted form maltose-grown cells, the modified procedure proved to be efficient in removing the non-protein impurities and protein separation. But in the case of proteins extracted form sulfur-grown cells, this modification did not work (Fig. 3-3C).

Table 3-8 Optimization of protein extraction and volt-hour in IEF.

Changes in work flow	Condition 3 (maltose-grown cells)	Condition 4 (maltose-grown cells)	Condition 5 (sulfur-grown cells)	Condition6 (maltose-grown cells)
After step 3	0.25 mg/mL RNase Treatment (1h 4 °C)	0.25 mg/mL RNase Treatment (1h 4 °C)	0.0125 mg/mL RNase 0.05 mg/mL DNase Treatment (1h 4 °C)	0.0125 mg/mL RNase 0.05 mg/mL DNase Treatment (1h 4 °C)
After step 6	High speed centrifugation (1h 4 °C 34780 ×*g*)	High speed centrifugation (1h 4 °C 34780 ×*g*)	High speed centrifugation (1h 4 °C 34780 ×*g*)	High speed centrifugation (1h 4 °C 34780 ×*g*)
Step 10	Step and hold：0v 15h Step and hold: 500v 1h Gradient: 1000v 1h Gradient: 8000v 2.5h Step and hold: 8000v 1.5h	Step and hold: 50v 15h Step and hold: 500v 1h Gradient: 1000v 1h Gradient: 8000v 2.5h Step and hold: 8000v 2h	Step and hold: 50v 15h Step and hold: 500v 1h Gradient: 1000v 1h Gradient: 8000v 2.5h Step and hold: 8000v 5h	Step and hold: 50v 15h Step and hold: 500v 1h Gradient: 1000v 1h Gradient: 8000v 2:30 Step and hold: 8000v 5h

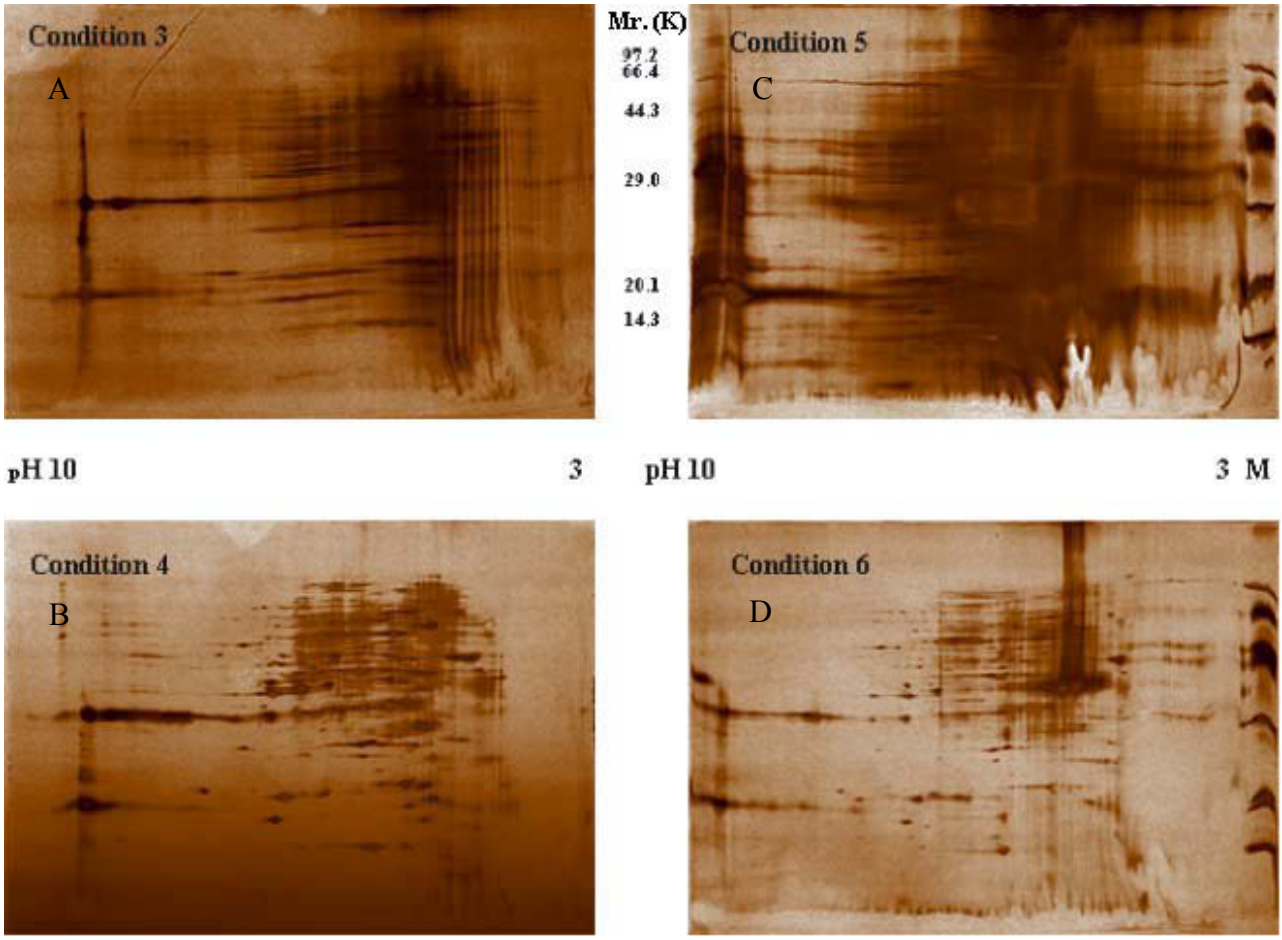

Fig. 3-3 Optimization of protein extraction and volt-hour in IEF. The protein extraction method and IEF conditions of condition 3, 4, 5and 6 are shown in Table 3-8.

3-3-2-3 Optimization of protein extraction of sulfur-grown cells.

Chloroform/methanol precipitation is efficient in removal of interfering agents such as lipids, nucleic acids from the protein samples, and it was performed to purify proteins extracted from sulfur-grown bacterial cells (Weissgerber et al., 2014). Thus, in this experiment chloroform/methanol precipitation was substituted for acetone precipitation. Fig. 3-4A shows that this chloroform/methanol precipitation was more efficient in removing impurities than acetone

precipitation. The soluble proteins extracted from sulfur-grown cells and maltose-grown cells exhibited significantly different patterns on polyacrylamide gel after two dimensional separation.

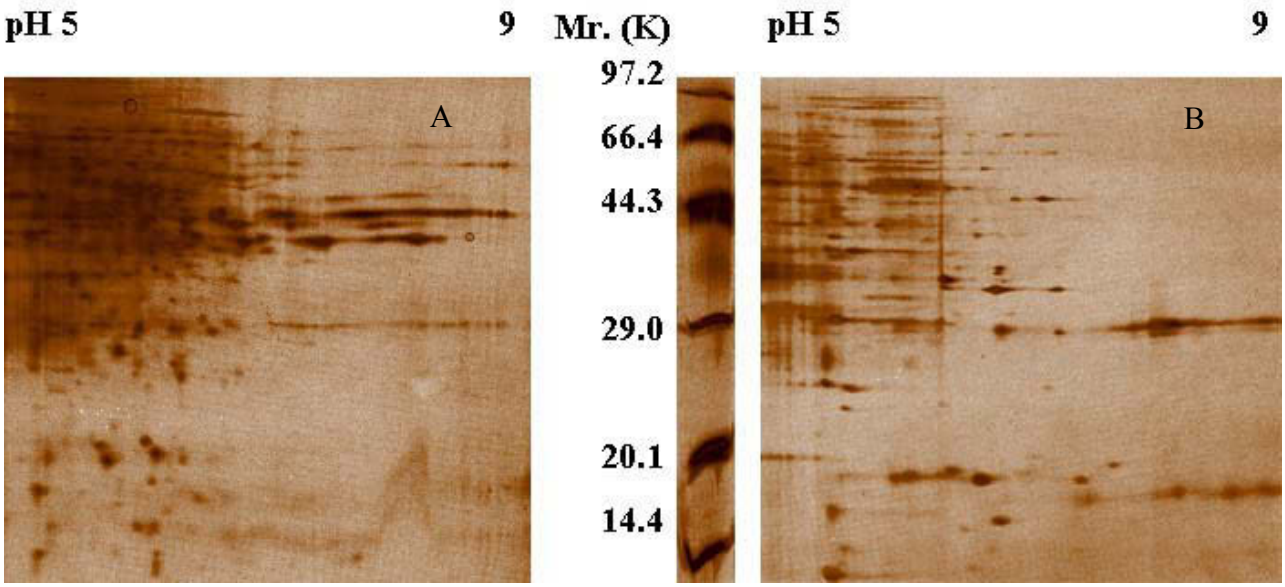

Fig. 3-4. 2-D display of soluble proteins of strain NBRC9425 grown on (A) sulfur (S^0 and thiosulfate), and (B) maltose.

3-4 Discussion

Strain NBRC9425 grew slowly in the organics-free sulfur-containing liquid medium. Sulfur oxidation was found as indicated by sulfate production and pH decrease. However, when the harvested fungal cells were incubated with GSSG/GS_nG, S^0, $S_2O_3^{2-}$, or SO_3^{2-} at 30°C, no $S_2O_3^{2-}$, SO_3^{2-}, $S_4O_6^{2-}$, SO_4^{2-} was produced after 60 min. It suggested that sulfur oxidation by the intact cells of strain NBRC9425 was relatively slow because of the relatively short reaction time compared to the culturing time of 15 days in the 5 L flasks. Theoretically, increasing the amount of fungal cells into test tubes of enzyme assays could increase the reaction products. However, it is not feasible because small amount of fungal filaments clung to larger volume of S^0 particles coherently. This

morphological property limited the amount of biomass loaded for enzyme assay.

When culture filtrate was tested for activities of sulfur-oxidizing enzymes, the negative results of enzyme assays might be due to the low protein content in the sample. As a result, samples were concentrated using ammonium sulfate precipitation. But the treatment rendered no positive results in enzyme assays. Because the process of ammonium sulfate precipitation can remove non-protein substances such as ions which might be essential for sulfur oxidation. Another disadvantage of ammonium sulfate precipitation is the denaturing of proteins. Centrifugal filtration causes little loss of biological activity, and the concentration of small molecules in the concentrated solution remains the same as that in the culture. Thus, centrifugal filtration could overcome the flaws caused by ammonium precipitation, and it was then used for concentration of the samples. However, the samples treated with centrifugal filtration still exhibited no activity. It seemed that the secreted proteins did not contain sulfur-oxidizing enzymes.

Sulfur-oxidation by microbes is usually an energy-yielding reaction. In prokaryotes, oxidation of sulfite and thiosulfate occur intracellularly. The proteins and enzymes in such reactions are membrane protein or intracellular proteins. As a result, cell-free extract of strain NBRC9425 was supposed to harbor activities of sulfur-oxidizing enzymes. However, none of the tested sulfur species were oxidized to a higher valence state. It is possible that sulfur oxidation in this fungus involves multiples proteins coordinating together located in higher structure in the fungal cell, and

the cells should maintain their integrate structure to exhibit sulfur-oxidation ability. As a result, cell-free extract obtained through disruption of fungal cells could not oxidize sulfur.

Most knowledge concerning sulfur oxidation in prokaryotes comes from enzyme assays and sequence analysis of specific gene clusters. DNA microarray technology has recently been applied to provide whole-genome transcriptional profile, which helps to provide a comprehensive and coherent picture of sulfur oxidation and the associated bioenergetics processes (Weissgerber et al., 2013). Whole-genome transcriptional profiling in response to sulfur and organic compounds does not always guarantee identification of proteins involved in sulfur oxidation because transcriptional change does not always result in protein composition change. As a result, comparative proteomic study provides solid evidence of proteins participating in sulfur oxidation by targeting the up-regulated proteins in response to sulfur.

In comparative proteomic studies, appropriate protein separation on 2-DE gel is clearly vital. Protein extraction is absolutely essential for good 2-DE results. Because of the diversity of protein sample types and origins, the optimal sample preparation procedure for any given sample must be determined empirically. The extracted proteins of maltose-grown cells showed lots of streakings in this study. It is thought that horizontal streakings usually result from incomplete focusing in the first dimension, protein overloading, poor protein solubilization, and non-protein impurities in the sample. Isoelectric focusing was increased in condition 2 (Table 3-7). But the results did not

change much while horizontal streakings, especially at acidic zone, still existed. Because nucleic acids can be stained with silver staining, the horizontal streakings seemed to be caused by nucleic acids. Some polysaccharides are negatively charged and can cause streakings. Some uncharged polysaccharides can block the pores of the gel, physically impeding focusing. After treated with DNase and RNase, followed by an ultracentrifugation, the majority of the streakings disappeared (Fig. 3-3).

After optimizing the precudures for protein preparation and the conditions of protein focusing, the proteomic profiles of maltose-grown and sulfur-grown cells of strain NBRC9425 were obtained (Fig. 3-4). The greatly distinctive pattern of the proteomic profiles in response to different energy substrates indicated significantly different metabolisms. The analysis of 2-DE gel and protein identification of the up-regulated proteins are yet to be performed.

Chapter 4. Chemolithotrophic growth of strain NBRC9425 on S^0

4-1 Introduction

As one of the most important reactions in sulfur cycling, lithotrophic sulfur oxidation has gained much importance during the last decades. Researchers have thoroughly investigated prokaryotic sulfur metabolism and the related physiology. However, prior studies have failed to characterize the physiological properties of lithotrophical sulfur oxidation by fungi. This current study aims to explore the physiological characteristics of strain NBRC9425 growing on sulfur-containing organics-free media. Previous studies concerning fungal growth on organics-free or carbon-deficient media have shown that contamination of exogenous organic compounds has to be addressed (Wainwright and Grayston, 1988; Parkinson et al., 1989; Jiang et al., 2017a).

Fungi have been observed in various oligotrophic environments in nature. Lechuguilla cave in New Mexico, USA, is an extreme environment for life. Given the depth of the cave, organic input is very limited. However, frequent fungal and bacterial colonization was found (Cunningham et al., 1995). It was thought that chemolithotrophic bacteria acted as primary producers in a unique subterranean microbial food chain (Cunningham et al., 1995). Recently, partly organically preserved and partly mineralized fungi were discovered in fractured crystalline rocks at a depth of 740 m at the Laxemar site in Sweden (Drake, et al., 2017). On the fungi found in this deep oligotrophic environment, the researcher proposed that the fungi needed to access to carbohydrates as energy source for their metabolism, and the methanotrophs and sulfate-reducing bacteria may

have acted as nutrients producer for the fungi and triggered the fungal colonization of the system.

On the other hand, only a small amount of fungi isolated from oligotrophic habitats were confirmed to be able to grow on organics-free media. In laboratories, fungal spores were found to germinate and produce hyphae in distilled water (Mirocha and Devay, 1971: Tribe and Mabedeje, 1972). Wainwright and Grayston (1988) found that a range of fungi belonging to the genera of *Aspergillus*, *Mucor*, and *Trichoderma* grew in a liquid medium lacking added carbon. But they thought that these fungi obtained energy by scavenging organic carbon from the growth medium or laboratory atmosphere.

However, *Fusarium solani* THIF01 could keep oxidizing S^0 as indicated by the decrease of culture pH and sulfate production (Li et al., 2010). It seemed that strain THIF01 could gain energy by oxidizing S^0 continuously not by scavenging organic compound contaminants.

In the Chapter 2 of this dissertation, lithotrophic sulfur oxidation by 7 fungal strains has been proved. In addition, it was found that the growth and sulfur-oxidation of fungi were distinctive when cultivated on agar-solidified medium and agarose-solidified medium. Dickinson and Bottomley (1980) thought that some fungi could conserve energy substances and then survive in carbon-deficient environments by utilizing such reserves. Taken together, in studies concerning lithotrophic sulfur oxidation by fungi, actions have to be taken to prevent contamination of organic

compounds in growth medium or laboratory atmosphere, and to exclude the effects of endogenous food reserves synthetized when growing heterotrophically. In this chapter, attempts were made to prevent the interference of organic compounds on the sulfur oxidation by strain NBRC9425.

4-2 Materials and Methods

4-2-1 Medium, inoculum preparation, and culture conditions

To ensure the absence of organic compounds, chlamydospores suspension was prepared and used as the inoculum instead of mycelial discs. After the fungus grew for 10 days on WS5-S agarose plates, its spores were harvested and rinsed with 2 mL sterilized saline poured onto a plate. One mL spore suspension was then inoculated into 200 mL Erlenmeyer flasks containing 40 mL WS5-S medium and incubated at 30°C without shaking. After 15 days, 20 μL culture was pipetted into the fresh medium, and incubated for another 15 days. This reinoculation procedure was performed 5 times to acclimate the fungus to oligotrophic conditions. The obtained culture was filtered (Filter Paper, LOT No. 70519102, 6-μm-pore size, ADVANTEC, Toyo Roshi Kaisha, Tokyo, Japan) to remove the S^0 particles and fungal hyphae, and the spores suspension (3.2×10^3 spores mL^{-1}) was stored at -80°C.

With the aim to avoid the carbon contaminants in the laboratory atmosphere and the autoclave chamber, 15 mL screw capped tubes (16.5 mm diameter × 130 mm length) containing 5 mL liquid WS5-S medium (3.0 g L^{-1} KH_2PO, 0.16 g L^{-1} NH_4Cl, 0.41 g L^{-1} $MgCl_2 \cdot 6H_2O$, 0.007 g L^{-1}

$FeCl_2 \cdot 4H_2O$, 0.25 g L^{-1} $CaCl_2 \cdot 2H_2O$, 10 g L^{-1} S^0, pH 5.0) were put in screw-capped glass bottles (101 mm diameter × 218 mm length) and were subjected to autoclave for 20 min at 120°C. In the experiments of fungal growth with thiosulfate, thiosulfate solution was subjected to filter sterilization (MILLEX® GV Filter Unit, LOT No. R7CA73006, 0.2-μm-pore size, Merck Millipore Ltd. Co. Cork, IRL) and added into the sterilized WS5-S medium to a final concentration of 2 g L^{-1}. Ten μL spores stock suspension was pipetted and transferred into the tubes (three biological replicates) and incubated at 30°C with reciprocal shaking at 110 rpm for 50 days in the dark.

4-2-2 Measurement of sulfur oxidation products and biomass

Lithotrophic sulfur oxidation by strain NBRC9425 was monitored over time interval. Produced sulfur species was measured as described in section 3-2-1-5. Ergosterol extracted from culture served as an indicator of living fungal biomass. The extraction and analysis of ergosterol were performed as described by Masaki et al. (2016) with minor modification. Fungal mycelia were added into 6 mL 1.4 M KOH solution (20 g KOH dissolved in 250 mL methanol and 50 mL ethanol). The mixture was heated at 75°C for 45 min. After the saponification, ergosterol was extracted with *n*-pentane. After cooling down to room temperature, the saponification mixture was added with 1.5 mL distilled water and 1.5 mL *n*-pentane. Upper phase containing ergosterol was collected after incubation for 5 min at room temperature. Then the extraction procedure was repeated for another twice. At last, *n*-pentane solution containing ergosterol was heated for 1 h in a

50°C water bath to evaporate the solvent. The pellet was dissolved in appropriate amount of methanol and stored at -20°C. The analysis of ergosterol should be performed within two days after the extraction. The measurement of ergosterol was performed with a High Performance Liquid Chromatography (HPLC, HP1100 Serires, Hewlet-Packard, Waldronn, Germany) with a Zorbax ODS column (DU PONT, Wilmington, DE, USA). Methanol was used as the mobile phase at a flow rate of 1 mL min^{-1}. Ergosterol was detected at 282 nm with UV detector.

4-2-3 Morphology of strain NBRC9425 growing on S^0

The fungal cells absorbing onto S^0 particles in WS5-S medium were stained with cotton blue and with 4,6-diamidino-2-phenylindole (DAPI). Microphotographs were taken using a BZ-8000 fluorescence microscope (KEYENCE, Osaka, Japan).

4-3 Results

4-3-1 Oxidation of S^0 and thiosulfate by strain NBRC9425 in organics-free medium

In the process of autoclave-sterilization, screw-capped tubes containing media were put in bigger screw-capped bottle to prevent organics contaminants in laboratory atmosphere and autoclave chamber. Chlamydospores of acclimated fungus growing in organics-free S^0-containing medium were used as inoculum. In this way, the effects of exogenous organics on lithotrophic growth of fungi were excluded. The value of culture pH stayed unaltered at 5.0 until the 5th day after inoculation and decreased to 4.8 on the 30th day. Fig. 4-1 shows the growth of strain NBRC9425

on organics-free medium and the oxidation of sulfur. When growing on S^0, the concentration of thiosulfate increased from 0 to 0.12 mM on the 20th day and decreased to 0.06 mM on the 50th day. At the same time, sulfate concentration kept increasing to 0.07 mM and 0.12 mM on the 20th and 50th day, respectively (Fig. 4-1A). The content of ergosterol increased to 13.81 mg L^{-1} by day 30 (Fig. 4-1B). These results strongly suggested that the fungus oxidized S^0 to thiosulfate, and to sulfate ultimately. During this period, no sulfur intermediates such as tetrathionate or sulfite, were detected in the culture.

When substituting thiosulfate for S^0 in WS5-S medium, production of sulfate was observed. However, it decreased after 5 days of cultivation (Fig. 4-1A). Although ergosterol content was too low to be quantified with HPLC, spore germination and hyphal growth could be observed with microscope. These findings suggest that thiosulfate could not be used as a sole energy source.

When the fungus was grown on both of S^0 and thiosulfate for 30 days, sulfate concentration and ergosterol content was 0.27 mM and 38.52 mg L^{-1}, respectively (Fig. 4-1). These values were much higher than those when the fungus was grown with S^0.

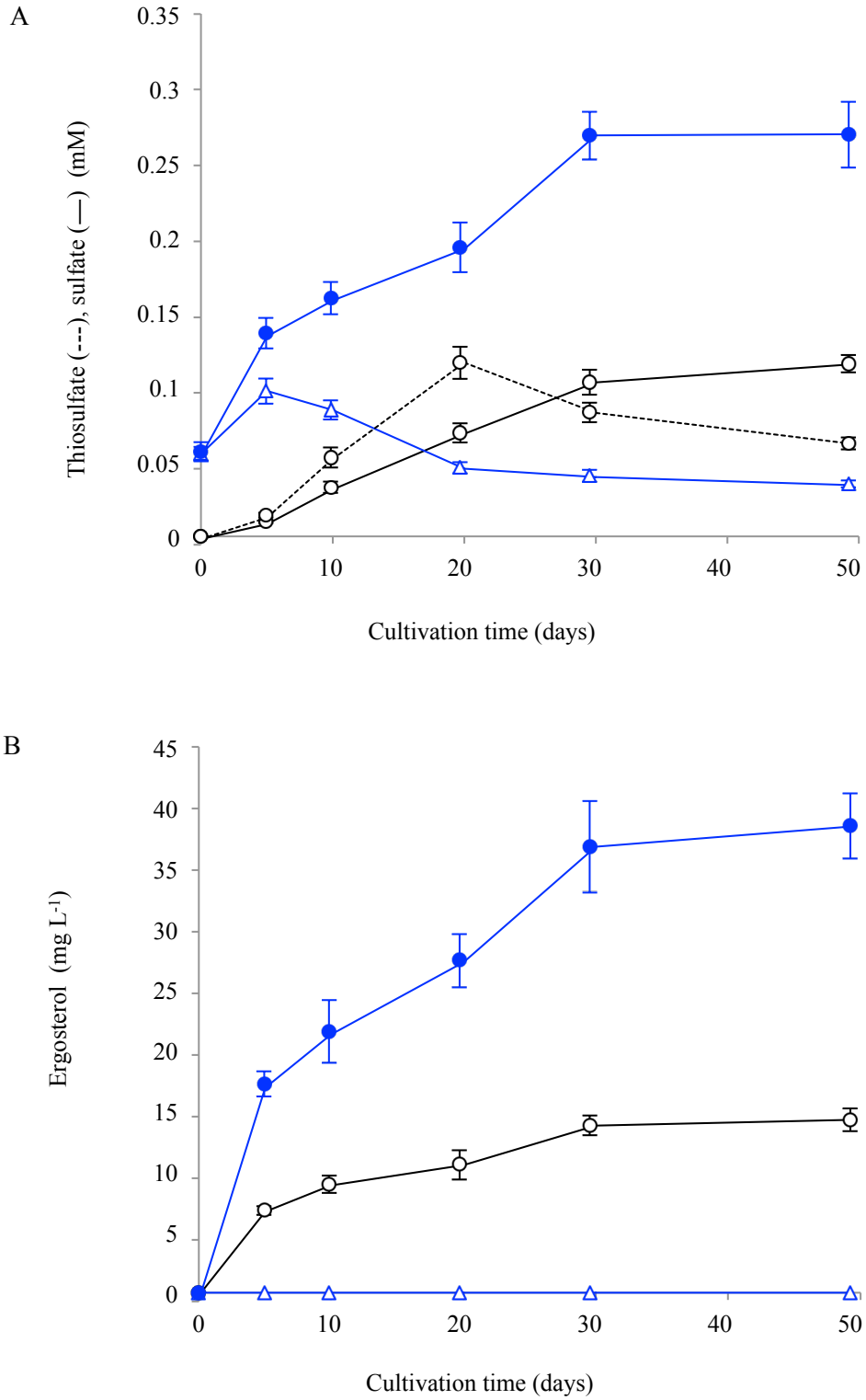

Fig. 4-1. Growth of *F. solani* f.sp. *pisi* NBRC9425 with S^0 or/and thiosulfate. Error bars indicate s.d., *n*=3. Symbols: ○, S^0 (10 g L^{-1}); △, thiosulfate (2 g L^{-1}); ●, S^0 plus thiosulfate.

4-3-2 Hyphae of strain NBRC9425 grown on S^0

When growing in WS5-S liquid medium, hypha of strain NBRC9425 did not disperse in the medium as they did in media containing exogenous organic compounds. Because of the high specific gravity, the particles of S^0 sunk to the bottom of medium in the screw-capped tubes. The fungus grew on the surface of S^0 particles, and the fungal hypha attached to S^0 particles coherently. The adsorption was too tight to be separated apart when centrifuged or vortexed. The aggregate comprising of S^0 and mycelia formed at the bottom of the tubes.

Fig. 4-2A and Fig 4-2C show that fungal hypha grew on the surface of the S^0 particles in WS5-S medium. The filaments intertwined together and formed a mycelial net which wrapped the S^0 particles. Unexpectedly, this fungus hardly grew when cultured in WS5-S medium in Erlenmeyer flasks incubated on a rotary shaker. As displayed in Fig. 4-2B &D, the fungus in circular shaking flasks was not likely to develop a fine mycelial sheet nor grew on the surface of S^0 particles.

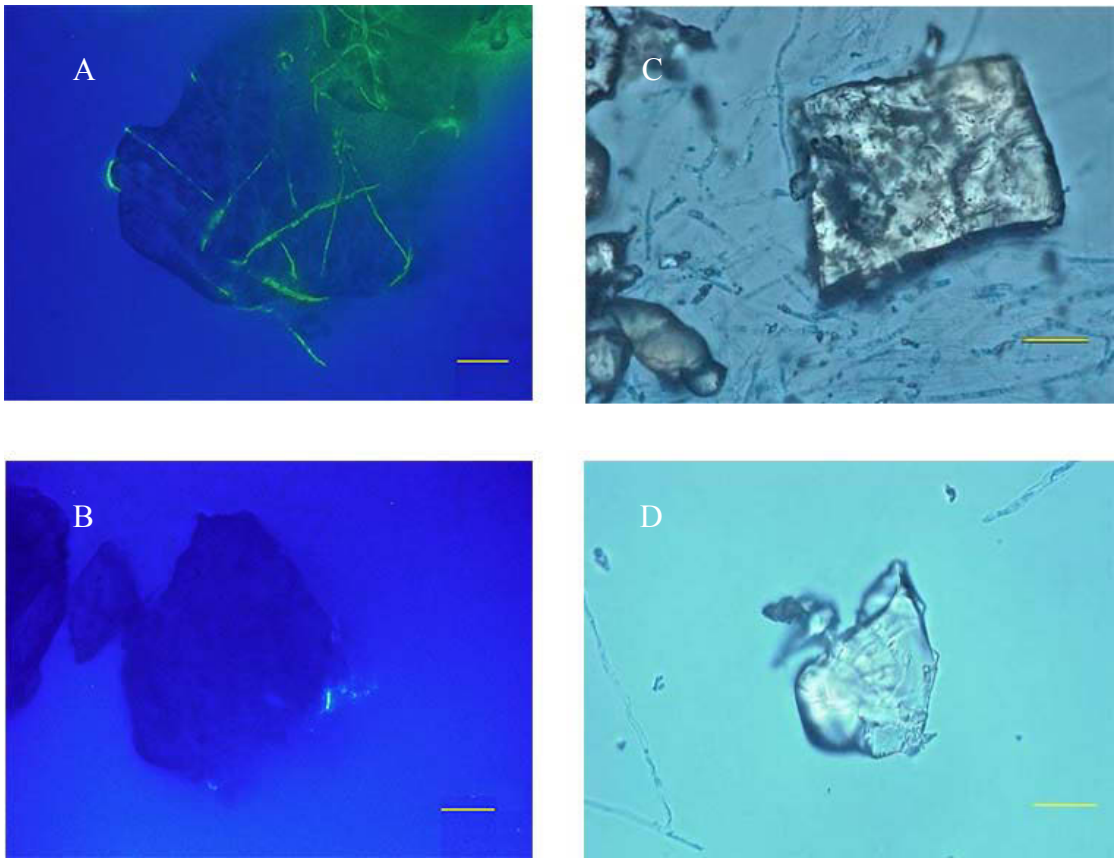

Fig. 4-2. Hyphae extension of *F. solani* f.sp. *pisi* NBRC9425 in S^0-containing WS5-S medium. Microphotographs of WS5-S liquid medium culture were taken five days after inoculation. (A) DAPI staining, reciprocal shaking tubes, (B) DAPI staining, circular shaking flasks, (C) Cotton blue staining, reciprocal shaking tubes, (D) Cotton blue staining, circular shaking flasks. The scale bars indicate 30.0 μm.

4-4 Discussion

Experimental results described in the Chapter 2 have demonstrated that trace amount of organic compounds in the agar medium affected sulfur oxidation greatly (Fig. 2-4A). In this chapter, it was further proved that the contaminants of organic compounds in laboratory atmosphere and the organic compounds reserved in fungal hyphae also could affect sulfur oxidation.

Nutrients are one of the main determinants for chlamydospore formation by some fungi. Chlamydospores of *Fusarium* species are the principal means of long-term survival during unfavorable periods in the soil (Couteaudier and Alabouvette, 1990; Lin and Heitman, 2005). *F. solani* THIF01 formed chlamydospores and mcroconidia on WS5 agar medium and on CMA medium, respectively (Li et al., 2010). In this study, the formation of chlamydospores and microconidia of strain NBRC9425 was also observed on WS5-S medium and PDA medium, respectively. PDA medium is abundant in nutrients and more preferable for the proliferation of this fungus. In order to minimize involvement of the trace organic contaminants in the medium or pre-formed mycelium, inoculum was prepared using chlamydospore suspension instead of mycelial disks that were cut from the agar plate. Mycelial discs were not ideal inocula for studies of fungal growth in organics-free medium, because some fungi have been found greatly efficient at conserving endogenous organic compounds, and the carbon stock in hyphae allows extensive hyphal growth under oligocarbotrophic conditions (Dickinson and Bottomley, 1980).

Obviously, contamination of organic compounds in laboratory atmosphere and chamber of autoclave pot is a problem during the sterilization of organics-free media. In this study, after the media was dispensed in screw-capped tubes, bigger screw-capped bottles were used to contain the tubes during autoclave. In this way, contaminants in laboratory atmosphere and chamber of autoclave pot got no access to the media.

The results proved the attempts were successful in minimizing the involvement of organic compounds. Culture pH value dropped from the initial 5.0 to 4.1 in 5 days after inoculating mycelial disks into WS5-S medium. When the inoculum was provided as chlamydospores suspension, no pH decrease was detected on the 5th day. The difference in pH decrease indicated that the trace amount of organic carbon in agar or the endogenous carbon reserves could enhance the oxidation of S^0. Although the culture pH did not change, production of sulfate and yield of biomass were recorded.

Wainwright and his teammates found that several fungi could grow in organics-free mineral-salts medium (Wainwright, 1993; Parkinson et al., 1989; Parkinson et al., 1990; Wainwright and Al-Talhi, 1999). As there was no added energy source available in the medium, the authors thought that some of the fungi obtained energy source by scavenging organic contaminants from the medium and laboratory (Wainwright and Grayston, 1988). Recently, Jiang's group found that a fungal species isolated from limestone, and 81 other fungal species isolated from a carbonate cave could grow in organics-free medium (Jiang et al., 2017a; 2017b). They prepared inoculum from fungi-growing PDA plates, and found that the fungi formed hyphae networks on organics-free medium (Jiang et al., 2017a). These findings manifest that fungi can reserve organics efficiently in nutritional environments and utilized this stock in oligotrophic environments. Collectively, in order to prove the ability of lithotrophic sulfur oxidation by fungi, organics contaminants should be removed and fungal cells collected from organics-free media should be used as inoculum.

Spores of strain NBRC9425 germinated and the hypha grew chemolithotrophically in screw-capped tubes with reciprocal shaking or in flasks without shaking. When grown in circularly shaken flasks, the spores were also able to germinate. However, the fungus was unable to develop mycelial sheet, and fungal hypha tended to fragment to pieces because of vigorous shaking. It seems that the fragile fungal hyphae grown on carbon-deficient media cannot extend well in vigorously shaking flasks. These results suggest that the experiments of chemolithotrophic sulfur oxidation of fungi cannot be performed with shaking flasks. On the contrary, culturing in shaking flasks is a universal way to cultivate fungi in liquid media, which might be one of the reasons why the ability of lithotrophic sulfur oxidation of fungi had been disapproved in the previous studies.

Strain NBRC9425 could use S^0 as an energy source, but it barely grew in a medium containing thiosulfate and mineral salts. Although thiosulfate was also oxidized to sulfate, the concentration of sulfate decreased 5 days after inoculation. From these results, it could be concluded that S^0 could be used as a sole energy source but thiosulfate could not. For the first 5 days of growth on thiosulfate, the fungus seemed to utilize thiosulfate as sulfur source for biosynthesis, and the endogenous carbon reserves as a carbon source. It appeared that the decrease of sulfate concentration 10 days after inoculation was due to sulfate assimilation by the fungus. The addition of thiosulfate into S^0-containing mineral-salts medium, sulfate production and biomass yield were greatly increased. This phenomenon suggested that thiosulfate served as a faster sulfur source than

S^0. Most microorganisms assimilate sulfur in the form of sulfate and use it for the synthesis of sulfur-containing amino acids. In some microorganisms, thiosulfate was a more favorable sulfur source than sulfate for the synthesis of cysteine (Funahashi et al., 2015; Kawano et al., 2017).

Tetrathionate or other polythionate was not detected in the culture, suggesting that this fungus did not follow "Tetrathionate Intermediate (S_4I) pathway" which was found in some beta- and gammaproteobaceria, but not in alphaproteobacteria (Trudinger, 1964; Ghosh and Dam, 2009). Although oxidation of thiosulfate seems not to be an energy-generating process in strain NBRC9425, this sulfur compound could enhance the production of sulfate and the yield of biomass in WS5-S medium remarkably (Fig. 4-1A). It appears that since the media used in this study did not contain sulfate, generally regarded as the major sulfur source used for the biosynthesis of sulfur-containing molecules such as cysteine and methionine by fungi, thiosulfate might serve as a faster sulfur source than S^0 and subsequently lead to the stimulation of transformation. In *Escherichia coli*, thiosulfate was found to be more advantageous as a sulfur source (Kawano et al., 2017). From these results, strain NBRC9425 can be concluded to be able to grow chemolithotrophically using S^0 as a sole energy source.

Chapter 5. Effects of organic compounds on sulfur oxidation by strain NBRC9425

5-1 Introduction

Microbial metabolism of lithotrophic sulfur oxidation can be affected by simultaneous presence of organic substance. Sulfur metabolism features of different groups of sulfur-oxidizers are quite distinct. Sulfur-oxidizing anoxygenic photolithotrophic bacteria are not able to use water as electron donor. They possess a light-dependent energy transfer process when reduced sulfur compounds serve as electron donor. This group of sulfur-oxidizer mainly comprises of green sulfur bacteria (GSB), purple sulfur bacteria (PSB), purple nonsulfur bacteria (PNSB), aerobic anoxygenic bacteriochlorophyll-containing bacteria, Heliobacteria, and filamentous aerobic/anoxygenic phototrophs belonging to *Chloroglexaceae* (Table 5-1). These photolithotrophic bacteria clearly share similarities in terms of nutritional requirements. However, GSB are not capable of chemotrophical growth with oxygen as the terminal electron acceptor or assimilating various organic compounds as PSB do (Table 5-1). Generally, only simple organic compounds can be assimilated by some members of anoxygenic photolithotrophic bacteria. For some lithoheterotrophs in this group, reduced inorganic sulfur compounds can enhance the utilization of organic carbon (Sorokin et al., 2000).

Table 5-1 Physiological characteristics of sulfur metabolism of anoxygenic phototrophic bacteria (adapted from Ghosh and Dam, 2009).

Taxonomic affiliation	Nutrition type	Electron donor	Capability of assimilating organic compounds	Representative species
GSB				
Chlorobi	Obligately phototrophic; potentially mixotrophic	S^{2-}, $S_2O_3^{2-}$	The mixotrophs can photoassimilate a few simple organic substances	*Chlorobaculum tepidum*, *Chlorobaculum thiosulfatiphilum*
PSB				
Gammaproteobacteria, *Chromatiaceae*	Obligately phototrophic; facultatively phototrophic	Sulfide, thiosulfate (only used by the facultative phototrophs)	The facultative phtotrophs can assimilate some organic compounds	*Allochromatium warmingii*, *Isochromatium buderi*, *Thiospirillum jenense*, *Allochromatium vinosum*
Gammaproteobacteria, Some members of *Ectothiorhodospiraceae*	Facultative phototrophs [also grow chemo (litho-/organo)(auto-/hetero) trophically]; photoorganoheterrotroph	S^{2-}, $S_2O_3^{2-}$ and SO_3^{2-} (by the facultative phototrophs)	The facultative phototrophs can assimilate a variety of organic substances	*Allochromatium vinosum, Ectothiorhodospira shaposhnikovii, Ectothiorhodospira vacuolata, Halorhodospira halophile, Ectothiorhodosinus mongolidum*
PNSB				
Alphaproteobacteria,	Photoheterotroph; photolithoautotroph	S^{2-}, $S_2O_3^{2-}$	The photoheterotrophs can utilize organic substances	*Rhodopseudomonas palustris, Rhodovulum sulfidophilum, Rhodocyclus prupureus*
Betaproteobacteria	Photoheterotroph; photolithoautotroph	S^{2-}, $S_2O_3^{2-}$	The photoheterotrophs can utilize organic substances	*Rhodocyclus prupureus*
Aerobic anoxygenic bacteriochlorophyll-containing bacteria				
All *Alphaproteobacteria*, except for *Roseotales depolymerans*	Photolithoheterotroph	S^{2-}, S^0, SO_3^{2-}, $S_2O_3^{2-}$	Various simple organic substances	*Roseinatronobacter thiooxidans*

Heliobacteria				
The family *Heliobacteria*	Photoheterotroph, chemoheterotroph	S^{2-}	Few organic carbon sources	*Heliobacterium sulfidophilum, Heliobacterium undosum*
Filamentous anoxygenic phototrophs				
Chloroflexaceae	Photoheterotoph, phtoautotroph	S^{2-}	The phtoheterotrophs can assimilate organic substances	*Chloroflexus aurantiacus, Oscillochloris trichoides*

As another important group of sulfur-oxidizers, sulfur-chemotrophic prokaryotes are highly taxonomically and ecologically multiple. The physiology, biochemistry, molecular mechanism of sulfur oxidation by them are also of diversity (Ghosh and Dam, 2009). Sulfur-chemotrophic bacteria include facultative chemolithoautotrophs, obiligate chemolithoautotrophs, chemolithoheterotrophs or mixotrophs (Table 5-2). Organic compounds do not solely support the growth of the facultatively chemolithoautotrophs, such as *Thiomonas intermedia* (formerly *Thiobacillus intermedius*) and *Thiomonas delicatus* (formerly *Thiobacillus delicatus*), but can enhance the lithotrophic growth on reduced inorganic sulfur compounds (Katayama et al., 1984). On the other hand, chemolithoheterotrophs, such as *Silicibacter pomeroyi*, can not oxidize inorganic sulfur compounds in the absence of organic compounds (González et al., 2003). Most sulfur-oxidizing archaea belong to the order of the Sulfolobales which utilize S^{2-}, S^0, H_2, and simple and complex organic substances as energy sources (Dahl et al., 2008).

Table 5-2 Physiological characteristics of sulfur metabolism of chemotrophic bacteria (adapted from Ghosh and Dam, 2009).

Nutrition type	Taxonomic affilation	Electron donor	Capability of assimilating organic compounds	Representative species
Facultatively chemolithotrophic bacteria				
	Alphaproteobacteria	$S_4O_6^{2-}$, $S_2O_3^{2-}$, SO_3^{2-},S^{2-}, S^0	Simple organic substrates	*Paracoccus* spp.
	Betaproteobacteria	$S_4O_6^{2-}$, $S_2O_3^{2-}$	Simple organic substrates	*Tetrachiobacter kashmirensis*
	Gammaproteobacteria	$S_4O_6^{2-}$, $S_2O_3^{2-}$, SO_3^{2-},S^{2-}, S^0	Simple organic substrates	*Acidithiobacillus caldus*
Obligately chemolithotrophic bacteria				
	Betaproteobacteria	$S_nO_6^{2-}$, $S_4O_6^{2-}$, $S_2O_3^{2-}$, S^{2-}, S^0	No	*Thiobacillus denitrificans*
	Deltaproteobacteria	$S_2O_3^{2-}$, SO_3^{2-}	No	*Desulfocapsa sulfoexigens*
	Epsilonproteobacteria	$S_2O_3^{2-}$	No	*Sulfurimonas denitrificans*
	Gammaproteobacteria	$S_nO_6^{2-}$, $S_4O_6^{2-}$, $S_2O_3^{2-}$, S^{2-}, S^0	No	*Thermithiobacillus tepidarius*
	Aquificales	Both $S_2O_3^{2-}$ and H_2	No	*Aquifex aeolicus*

Prior studies about sulfur oxidation by fungi pointed that sulfur oxidation by fungi was a chemoorganiotrophic process. Grayston and Wainwrigh (1987) found that the added thiosulfate could enhance the heterotrophic growth of *Aspergillus niger* and *Trichoderma harzianum* on

sucrose, and they thought that these fungi were capable of chemolithoherotrophic growth on sucrose and thiosulfate. Chapter 1 and chapter 4 have manifested that organic compounds, even trace amount of them, could greatly affect the sulfur oxidation of strain NBRC9425. A question raised concerning the nutritional requirement by strain NBRC9425.

5-2 Materials and Methods

5-2-1 Effect of yeast extract on sulfur oxidation by strain NBRC9425

Five mL WS5-S medium was added with 0 to 200 mg L^{-1} yeast extract (BD Bacto™, Becton, Dickinson and Company, Sparks, MD, USA). The medium was dispersed in 15 mL screw-capped tubes in five biological replicates. The tubes were then autoclaved as described in section 4-2-1. Ten μL spores stock suspension was pipetted and transferred into the tubes and incubated at 30°C with reciprocal shaking at 110 rpm for 5 days in the dark.

Thiosulfate, sulfate, and ergosterol were estimated as described above. Morphology of the fungus growing in the medium was analyzed as described above.

5-2-2 Effect of different organic compounds on sulfur oxidation by strain NBRC9425

Sixty mL WS5-S medium was supplemented with different organic compounds: 2 g L^{-1} yeast extract; 2 g L^{-1} Potato Dextrose Broth; 1 g L^{-1} Potato Dextrose Broth + 1 g L^{-1} yeast extract; 2 g L^{-1} maltose; 1 g L^{-1} maltose + 1 g L^{-1} yeast extract; 2 g L^{-1} glucose; 1 g L^{-1} glucose + 1 g L^{-1} yeast

extract. The medium was dispensed in 300 mL flasks in three biological replicates. One mL spore suspension was inoculated into the autoclaved medium and incubated at 30 °C for 7 days with a rotary shaking speed of 120 rpm. pH and thiosulfate concentration were examined as described above to investigate the sulfur oxidation ability.

Effects of different concentrations of carbon sources on sulfur oxidation were then tested. Strain NBRC9425 was grown in submerged WS-5 medium supplemented with different concentrations of carbon source (2 g L^{-1} maltose, 2 g L^{-1} yeast extract, 5 g L^{-1} maltose, 5 g L^{-1} yeast extract, 10 g L^{-1} maltose, 10 g L^{-1} yeast extract, 1 g L^{-1} maltose + 1 g L^{-1} yeast extract, 2.5 g L^{-1} maltose + g L^{-1} g/l yeast extract, 5 g L^{-1} maltose + 5 g L^{-1} yeast extract)

5-3 Results

5-3-1 Effect of yeast extract on sulfur oxidation by strain NBRC9425

Fig. 5-1A shows that sulfate production increased with the supplemented yeast extract. In accordance with the discoveries in the previous Chapters of this dissertation, even little amount of organic compounds can affect sulfur oxidation by the fungus. When growing on 10 mg L^{-1} yeast extract, 0.051 mM thiosulfate and 0.032 mM sulfate were produced. These values are much higher than those when the fungus was grown without organic compounds (0.014 mM thiosulfate and 0.01 mM sulfate, respectively). Fig. 5-1B shows that the biomass yield was also greatly enhanced with the added yeast extract.

Higher concentration of yeast extract supplemented in to WS5-S medium lead to more produced thiosulfate and sulfate, which did not necessarily mean that a better ability of sulfur oxidation by the fungus was rendered because higher biomass was achieved at the same time. The values of produced sulfur compounds were divided by the ergosterol contents, optimal values of the quotient were obtained at 15 mg L^{-1} yeast extract (Fig. 5-1B). It suggested that when the fungus was grown on 15 mg L^{-1} yeast extract, the most oxidized sulfur compounds were produced on a single fungal cell basis for comparison.

5-3-2 Morphology of strain NBRC9425 grown on yeast extract

Fig. 5-2 shows that when strain NBRC9425 was grown on 200 mg L^{-1} yeast extract, the fungal hypha did not attach to the S^0 particles as they did in organics-free medium. Fig. 5-3 shows that fungal biomass increased with the added yeast extract. At low concentration of yeast extract, such as 0-40 mg L^{-1}, the fungus attached to S^0 particles and they formed aggregate on the bottom of the media. At higher concentration of yeast extract (200 mg L^{-1}), the mycelia seemed hydrophilic and suspended in the medium (Fig. 5-3I). In addition, the diameter of fungal hyphae extending in nutritional media was much bigger than that in organics-free medium (Fig. 5-2; Fig. 4-2).

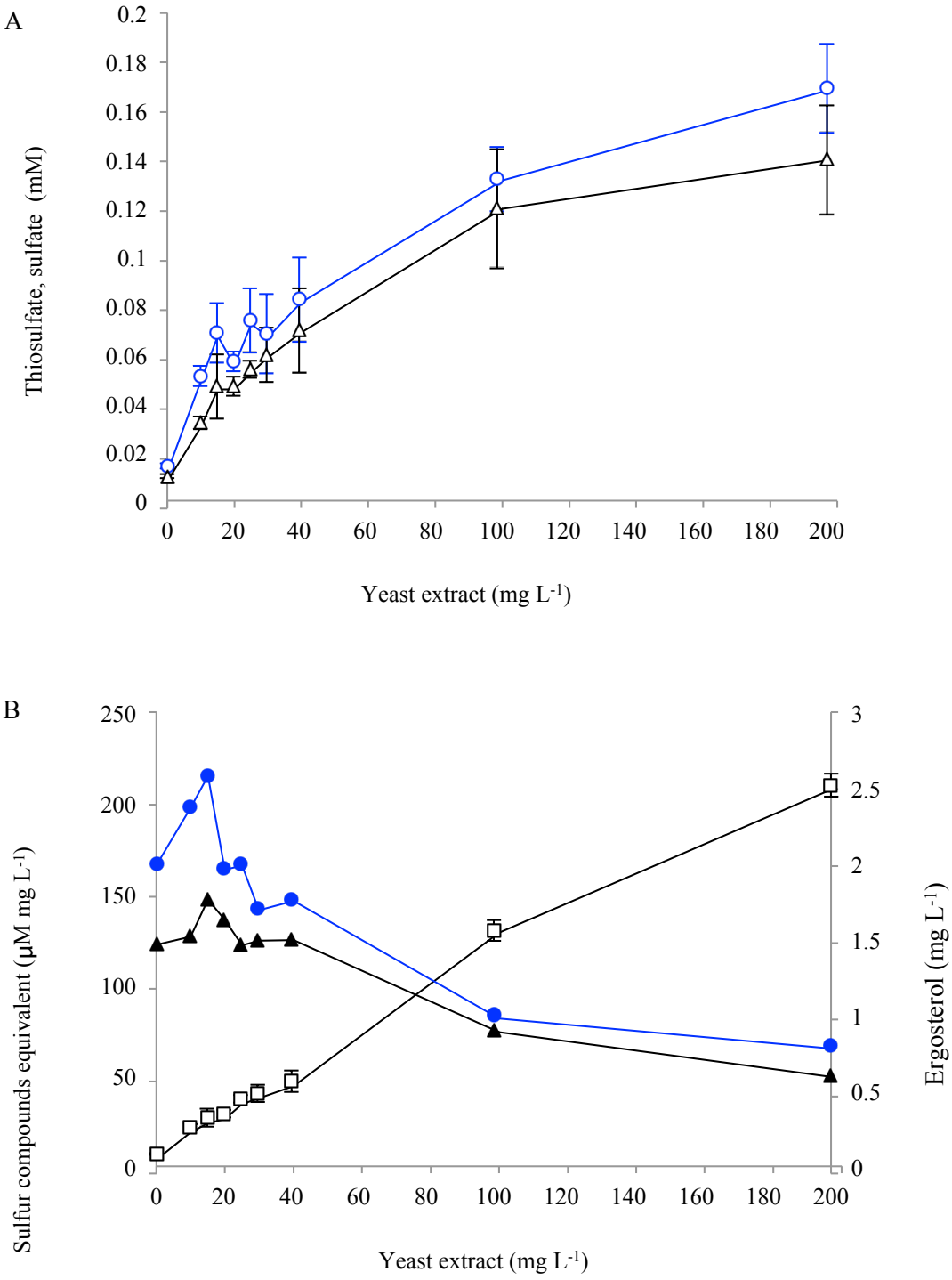

Fig. 5-1. Production of thiosulfate and sulfate from S^0 (A) and the growth (B) of *F. solani* f.sp. *pisi* NBRC9425 after 5 days cultivation in WS5-S medium supplemented with different concentrations of yeas extract. Error bars indicate s.d., *n*=5. Symbols: ○, thiosulfate; △, sulfate; □, ergosterol; ●, thiosulfate/ergosterol content; ▲, sulfate/ergosterol content.

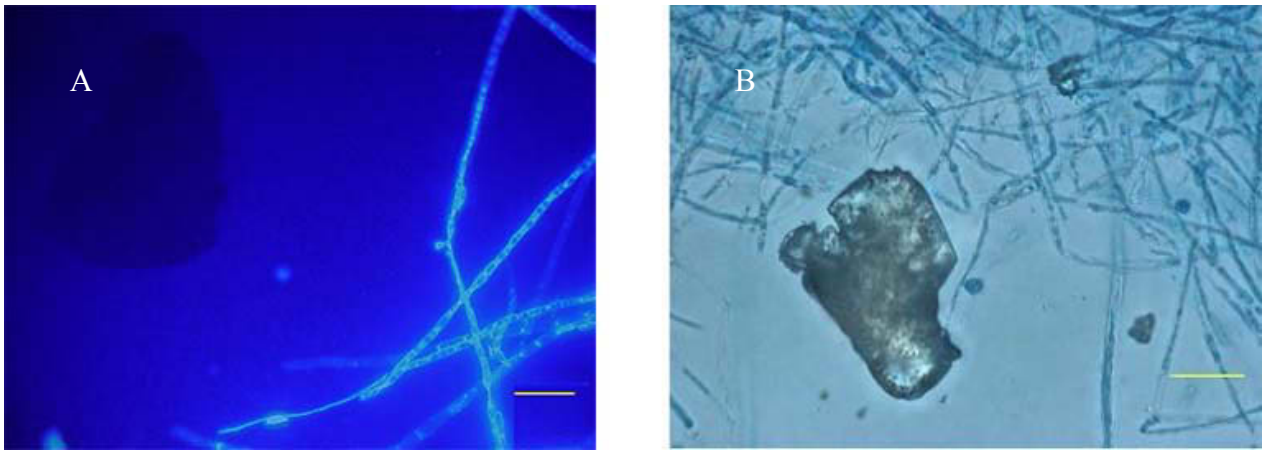

Fig. 5-2. *F. solani* f.sp. *pisi* NBRC9425 grew in S^0-containing WS5-S medium supplemented with 200 mg L^{-1} yeast extract. Microphotographs of WS5-S liquid medium culture were taken five days after inoculation: A, DAPI staining; B, cotton blue staining. The scale bars indicate 30.0 μm.

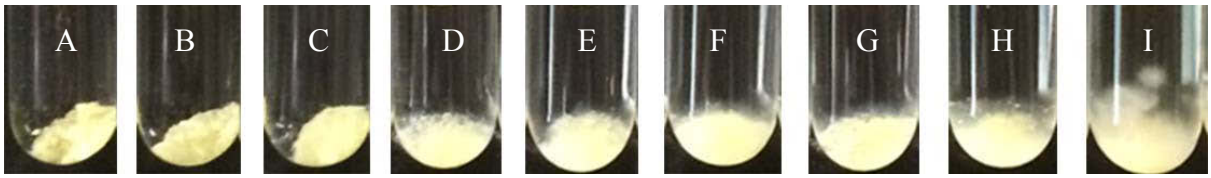

Fig. 5-3. *F. solani* f.sp. *pisi* NBRC9425 grew in S^0-containing WS5-S medium supplemented with different concentrations of yeast extract (mg L^{-1}): A, 0; B, 10; C, 15; D, 20; E, 25; F, 30; G, 40; H, 100; I, 200.

5-3-3 Effects of different organic compounds on sulfur oxidation by strain NBRC9425

S^0 was mainly oxidized to sulfate when strain NBRC9245 utilized carbohydrates (potato extract, maltose, and glucose) as carbon source. When grown on WS5-S medium supplemented with yeast extract, S^0, on the other hand, was mainly oxidized to thiosulfate. Yeast extract contains sulfate which was utilized efficiently for sulfur assimilation by strain NBRC9425. The negative number of sulfate production (-0.075 mM) in Fig. 5-4 indicates that the sulfate assimilated in yeast extract supplemented medium outnumbered the sulfate as a product of S^0 oxidation.

The most thiosulfate production was achieved when the fungus was grown on yeast extract, and the most sulfate production was obtained when grown on maltose (Fig. 5-4). Then the effects of different concentrations of yeast extract and/or maltose were examined. It is noteworthy that when the supplemented yeast extract increased to 0.5% and 1%, sulfate production was greatly enhanced (Fig. 5-5). The most production of thiosulfate and sulfate were achieved at 0.5% yeast extract and 0.5% maltose, respectively.

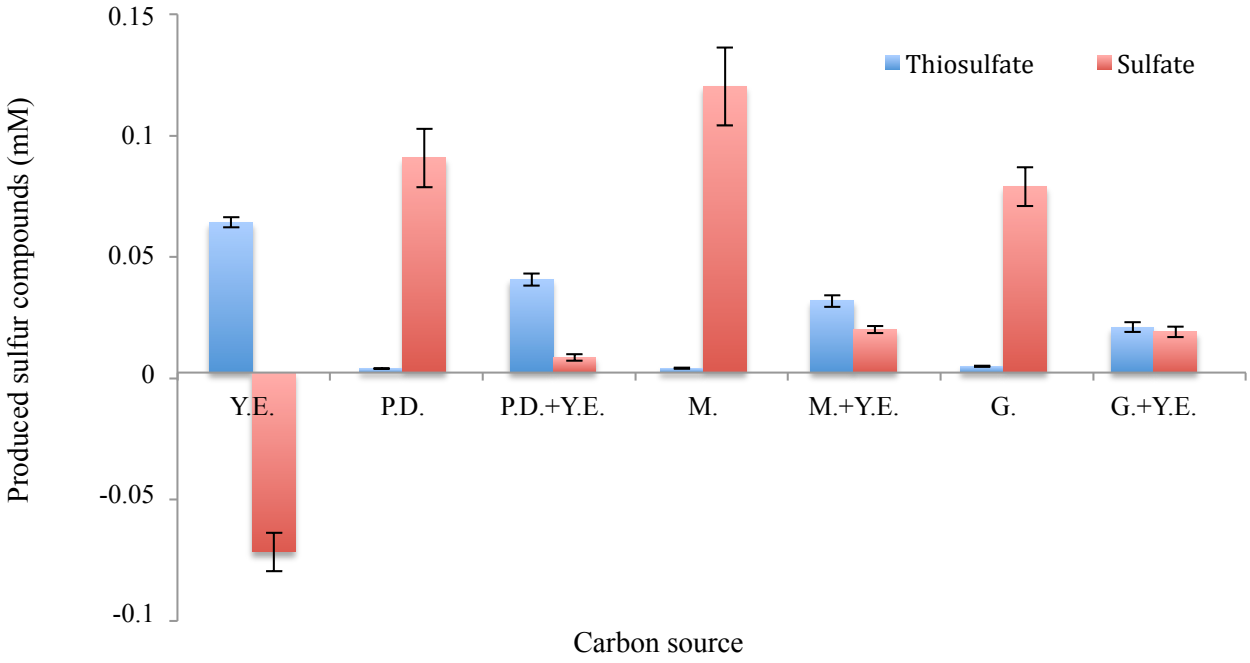

Fig. 5-4. Effects of different carbon sources on sulfur oxidation by strain NBRC9425. The fungus was cultivated in WS5-S medium supplemented with 0.2% different organic compounds (triplicates): yeast extract (Y.E.), potato dextrose extract (P.D.), P.D. + Y.E., maltose (M.), M. + Y.E., glucose (G.), G. + Y.E. After 5 days growth, thiosulfate and sulfate were determined by ion chromatography. Error bars correspond to s.d.

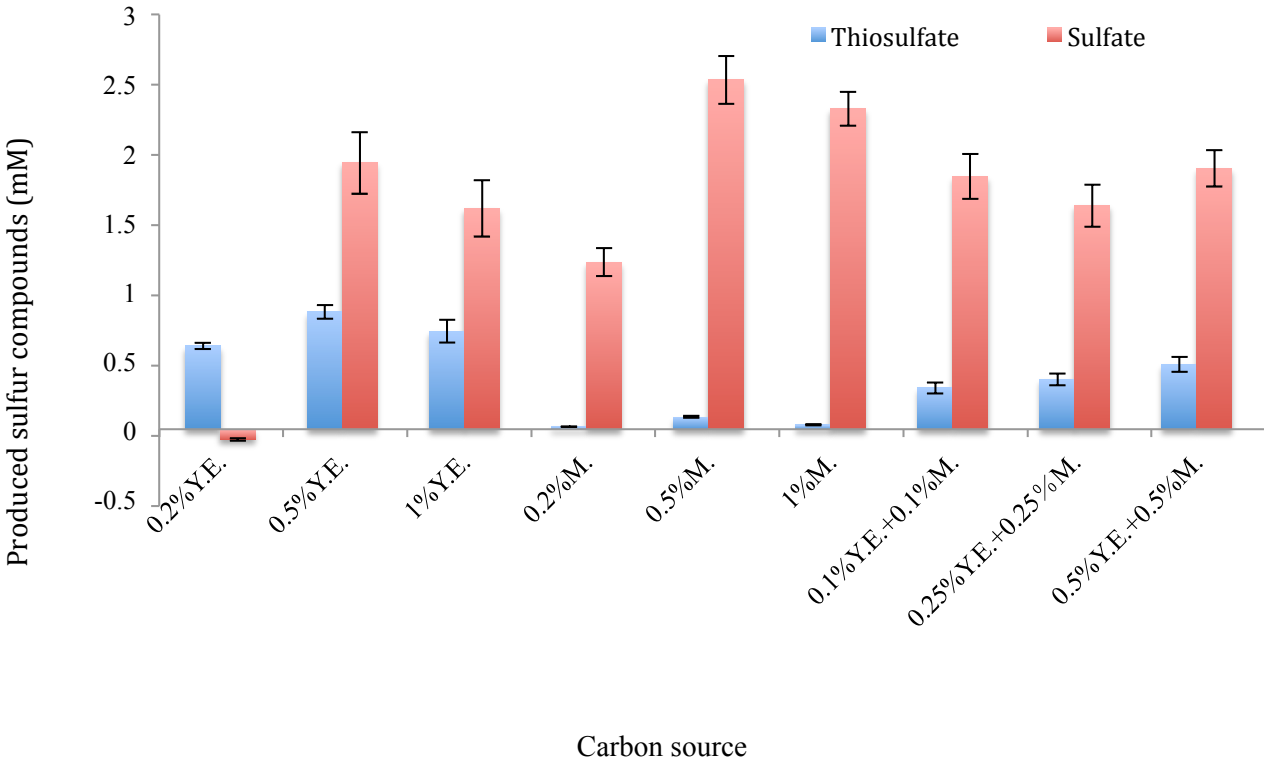

Fig. 5-5. Effects of different concentrations of carbon sources on sulfur oxidation by strain NBRC9425. The fungus was cultivated in S^0-containing WS5-S medium supplemented with different concentrations of organic compounds (triplicates): 0.2% (w/v) yeast extract (Y.E.); 0.5% (w/v) Y.E.; 1% (w/v) Y.E.; 0.2% (w/v) maltose (M.); 0.5% (w/v) M.; 1% (w/v) M.; 0.1% (w/v) Y.E. + 0.1% (w/v) M.; 0.25% (w/v) Y.E. + 0.25% (w/v) M.; 0.5% (w/v) Y.E. + 0.5% (w/v) M. After 5 days growth, production of thiosulfate and sulfate was determined by ion chromatography. Error bars correspond to s.d.

5-4 Discussion

The features of nutritional requirement by strain NBRC9425 are different from those of previously reported sulfur-metabolizing microbes. The fungus NBRC9425 could utilize either S^0 or organic compounds as a sole energy source, and organic compounds, both simple and complex substances, could enhance S^0-oxidation and fungal growth. However, the addition of organic compounds to WS5-S medium did not shift the metabolism of strain NBRC9425 from chemolitoautotroph to chemoheteroorganotroph. In this study, the quotient obtained by dividing the produced thiosulfate and sulfate by the ergosterol content served as a good indicator of sulfur oxidation ability (referred as thiosulfate equivalent and sulfate equivalent, respectively). It was intriguing to find that, when grown with different concentrations of yeast extract, both of the optimal thiosulfate equivalent and sulfate equivalent were observed at 15 mg L^{-1} yeast extract. It indicates that when the fungus was cultivated with less than 15 mg L^{-1} yeast extract, S^0 served as the substance and the generated energy was used for biosynthesis. However, in the presence of more yeast extract, the efficiency of S^0 utilization decreased because yeast extract was used as the energy source.

In the absence of organic compounds, fungal hyphae of strain NBRC9425 attached to the surface of S^0. High concentrations of yeast extract (200 mg L^{-1}) made hyphae more hydrophilic and disperse in the medium. Smits et al. (2003) reported that the growth condition affected the surface hydrophobicity of filamentous fungi. Distinct fungal contact angles were found when *Laccaria*

trichodermophora grew on potato dextrose agar and Modified Melin Norkrans (MMN) media, which the author proposed was attributed to be the depletion of carbon source in MMN medium as compared to PDA medium (Chau et al., 2009). *Acidithiobacillus ferrooxidans* cells were found to adhere onto hydrophobic surfaces when growing on S^0 and attached exclusively to negatively charged compounds when growing on ferric iron (Gehrke et al., 1998). An alteration in outer membrane proteins and extracellular polymeric substances composition was observed when *A. ferrooxidans* utilized S^0 and ferric iron (Kucera et al., 2012). Similar phenomena were observed for a *Mycobacterium* isolate which grew on the surface of S^0 particles (Kusumi et al., 2011).

Chapter 6. General discussion

6-1 Characteristics of lithotrophic sulfur oxidation by fungi

6-1-1 Abundance of lithotrophic sulfur oxidization by fungi

Sulfur oxidation by microorganisms plays a fundamental role in the global sulfur cycling. Widely accepted, it is prokaryotic sulfur oxidation that mainly drives the global cycling. Moreover, prokaryotic sulfur oxidation has critically influenced academic dialogue on taxonomy, ecology, physiology, biochemistry and molecular biology of microbial sulfur metabolism. To date, much information has been obtained about the natural occurrence of sulfur-oxidizing bacteria and archaea. The biotechnological application of sulfur-oxidizing prokaryotes in agriculture and industry has gained much importance in recent years. Enzymes and pathways have propelled to the forefront in investigations of microbial sulfur metabolism.

The wide distribution, ecological, and taxonomical diversity of sulfur-oxidizing fungi have long been recognized (Czaban and Kobus, 2000; Wainwright, 1989). However, this recognition is confined to chemoorganotrophic or chemolithoheterotrophic sulfur oxidation, and it is thought that fungi are not capable of dissimilatory sulfur oxidation. Previous studies failed to discover lithotrophic sulfur oxidation by fungi, except the one about the chemolithotrophic growth of *Fusarium solani* THIF01, isolated from deteriorated sandstone in Angkor monuments, Cambodia, on S^0 (Li et al., 2010). This fungus harbored an endobacterium *Bradyrhizobium* sp., and the endosymbiont might be the actual player of sulfur oxidation because *Bradyrhizobium* can oxidize

sulfur chemolithotrophically (Masuda et al., 2010; Xia et al., 2014).

It is reasonable to hypothesize that fungi are also able to oxidize reduced inorganic sulfur compounds lithotrophically. Firstly, the oligocarbotrophic growth capability of fungi in laboratory has been highlighted in previous studies (Wainwright and Gryston 1988). Carbon dioxide fixation by fungi has been found (Parkinson et al., 1991). These findings prove that fungi are able to grow lithotrophically in laboratory. Secondly, some bacteria and archaea can use inorganic reduced sulfur as energy source under oligotrophic conditions. Given that lithotrophic sulfur oxidation is an ancient microbial metabolic process and occurs on a global abundance basis (Friedrich et al., 2005), it is quite possible that fungi have inherited the ability of assimilatory sulfur oxidation.

It is also quite possible that lithotrophic sulfur oxidation by fungi occurs widely in nature. Fungi have been frequently observed in carbon-limiting environments (Cunningham et al., 1995; Drake et al., 2017). Colonies of a wide range of microbes, including archaea, bacteria and fungi, are found in Angkor monuments at the UNESCO World Cultural Heritage site of Cambodia (Lan et al., 2010). Hu et al. (2013) considered that fungi might play a very important role in the dynamics of microbial biofilms on the sandstone in Angkor monuments because of their ubiquity. Sulfur-oxidizing fungi found in nutrients-limiting deteriorated sandstone are important members among the observed fungi at Angkor sites. Chemolithotrophic sulfur-oxidizing bacteria oxidize reduced inorganic sulfur compounds to sulfuric acid which causes biodeterioration of sandstones in

Angkor Wat, Bayon, and Phnom Krom temples (Kusumi, et al, 2011). Nineteen sulfur-oxidizing fungal strains were isolated from the deteriorated sandstones at Angkor sites (Li et al., 2010). In this dissertation, 7 fungal strains belonging to fungal genera of *Fusarium*, *Gllocladium*, *Trichoderma*, *Aspergillus*, were found to be able to oxidize S^0 on organics-free medium. As a result, it is likely that on the nutrient-limiting sandstones in Angkor monuments, fungi, like some prokaryotic sulfur oxidizers, are capable of chemolithotrophic growth on oxidize inorganic reduced sulfur and act as one of the main contributors to the deterioration of sandstones in Angkor temples.

The findings in this dissertation about lithotrophic sulfur-oxidation by fungi may reshape current knowledge about biodeterioration. Besides sulfur-oxidizing fungi in Angkor temples, lithotrophic sulfur oxidation by fungi may play a vital role in industrial processes.

Fungi cause biodeterioration on various inorganic materials of natural or anthropogenic: cave rocks, minerals, antique marbles, and concrete by means of mycelia invasion and organic acids excreting (Gorbushina et al. 1993; Gómez-Alarcón et al. 1994; Gu et al. 1998; Burford et al. 2003; Bastian and Alabouvette 2009). Previous studies about biodeterioration on these materials concluded that autotrophic prokaryotes formed a chemosynthetic base through sequestration of carbon dioxide to support fungi under these nutrition-limiting environments. However, the discovery about lithotrophic sulfur oxidation by fungi suggests that fungi can act as a precursor in nutrient-limiting materials and provide organics for other forms of life. As well as mycelial invasion and organic

acids excreting, sulfuric acid as a product of lithotrophic sulfur oxidation by fungi is an agent leading to deterioration.

Sulfur-oxidizing bacteria have been applied in wastewater treatment, and related studies have interested the academic community. Few efforts have been made to apply fungi to removing sulfur compounds in wastewater. Little is known about the biochemical, ecological, and physiological characteristics of sulfur-oxidizing fungi in wastewater. Now that the ability of lithotrophic sulfur oxidation by fungi has been manifested, fungi would prove their value in transforming toxic sulfur species, such as sulfide and thiosulfate, into sulfate in wastewater lacking of organic compounds.

It is well accepted that acid-tolerant autotrophs are the main contributors of biomining and the eukaryotic sulfur-oxidizers feed on the nutrients synthetized by the prokaryotes (Rawlings and Johnson, 2007; Rawlings, 2005). Acid-tolerant sulfur-oxidizing bacteria have been applied in biomining process, and little attention has been paid to sulfur-oxidizing fungi. According to the theory established in this dissertation, sulfur oxidation by fungi in biomining has been underrated. The fungi that feed on metal sulfides may have potential in rendering efficient, low-cost, and environmentally friendly process of metal recovery.

6-1-2 Lithotrophic sulfur oxidation by fungi

From the 7 named fungal strains capable of oxidizing sulfur lithotrophiclly, *F. solani* f.sp. *pisi*

NBRC9425 was chosen because it harbors no endobacteria and it exhibited higher activity of sulfur oxidation indicated with bigger clearing zone formed on S^0-containing agarose medium and lower pH of liquid mineral salts medium containing S^0. Physiological features of sulfur oxidation by strain NBRC9425 were then investigated.

Like in studies of autotrophically growing bacteria, efforts should be made to ensure no contaminants from laboratory atmosphere or the autoclave chamber when making media for culturing lithotrophic fungi. What is more, inoculum for culturing fungi lithotrophiclly should also be prepared carefully in terms of contamination of organic compounds. Mycelial discs cut from agar medium are not appropriate inocula in this study because agar contains trace organic compounds. Seed culture collected from organics-free medium can not be used as inoculum because the organic compounds reserved in the fungal cells provide endogenous carbon for hyphae extension. Chlamydospores were formed when *F. solani* THIF01 grew on WS5 agar medium and macroconidia on CMA medium (Li et al., 2010). In the current study, NBRC9425 formed chlamydospores and microconidia on WS5-S medium and PDA medium, respectively. It has been found that *Fusarium* fungi survive from unfavorable environments by means of chlamydospores formation. PDA is rich in organic compounds which serve as more favorable energy sources for strain NBRC9425.

In addition to prevention from contamination, fungal acclimation to lithotrophic growth conditions

should be performed. In a study of chemolithotrophic sulfur oxidation by *Acidithiobacillus ferrooxidans*, the authors used the bacterial cells grown on S^0 for more than 5 years as the inoculum (Kucera et al., 2012). In this study, strain NBRC9425 grew slowly in WS5-S liquid medium (Flask 1 in Fig. 6-1) when condia collected from fungus-growing PDA medium were used as an inoculum. When chlamydospores collected from WS5-S agarose medium were inoculated into WS5-S liquid medium, the fungal growth was also slight (Flask 2-A in Fig. 6-1). After the fungus was reinoculated into fresh medium for four times, biomass yield increased (data not shown). It was likely that the reinoculation acclimated the fungus to the organics-free medium and S^0 as a sole energy source. Culture in Flask 2-E (Fig. 6-1) was filtered to remove the hypha and S^0 particles, and the filtrate containing chlamydospores served as inoculum in this dissertation.

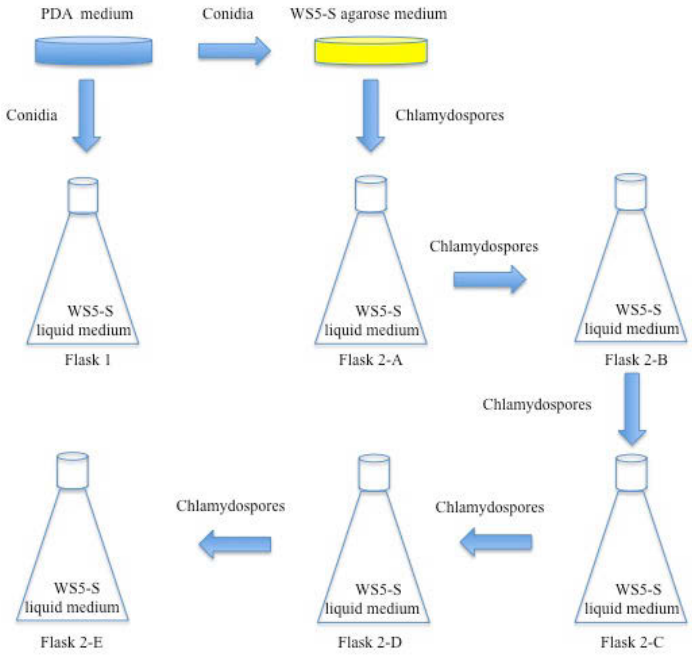

Fig. 6-1 Acclimation of strain NBRC9425 to lithotrophic growth on S^0.

The absorption of fungal hyphae to S^0 in organics-free medium is a rare finding of mycological physiology. Under certain circumstances, fungi incline to attach to the surface of insoluble carbonaceous materials which could subsequently be solubilized to nutrient source available to fungi (Wainwright and Grayston, 1988). Wainwright et al., (1986) found that a range of fungi could absorb insoluble particulates in Czpek Dox liquid medium. *Thichoderma harzianum* adsorbed activated charcoal on an agar medium containing 50 μg mL^{-1} sucrose (Wainwright and Grayston, 1988). In the present study, organics-free medium containing S^0 particles was used to cultivate the fungus. The observed adsorption of S^0 particles by fungal hyphae indicates that the fungus is capable of preying on insoluble energy substances, no matter carbonaceous or mineral.

Thiosulfate is an important energy source utilized by various sulfur-oxidizing prokaryotes. However, strain NBRC9425 could not associate oxidation of thiosulfate to biosynthesis as indicated by biomass yield (Fig. 4-1B). Addition of thiosulfate to WS5-S medium containing S^0 led to increases of biomass yield and sulfate production (Fig. 4-1A), suggesting that thiosulfate may serve as a more favorable sulfur source than S^0. Fig. 4-1A shows that when the fungus was growing in a medium containing S^0 as the sole sulfur source, S^0 was oxidized to sulfate while thiosulfate was produced as an intermediate. It indicates that sulfate was needed for fungal growth and served as a more favorable sulfur source than thiosulfate.

Now that lithotrophic S^0 oxidation by fungi has been proved, it seemed that the frequently observed fungi in Angkor monuments feed on S^0. It raises questions about the origin of S^0 and the process in which S^0 becomes available to the fungal sulfur-oxidizers in the deteriorated sandstone. Hosono et al. (2006) found that the content of sulfur in the freshly quarried sandstones at the Angkor site was 0.01-0.15% (wt.), mainly in the form of sulfate salts. The sulfate-reducing microorganisms may reduce sulfate to reduced inorganic sulfur compounds, which are then available for sulfur-oxidizing fungi. In this scenario, the question is answered why deterioration at the inner part of the buildings in Angkor sites is much severer: the rain washes the nutrients off the exterior wall, so biodeterioration rarely occurs on the exterior wall; the interior pillar and walls in Angkor monuments provide sulfur for microbes and suffer from serious biodeterioration. On the other hand, many bats can be seen inhabiting at Angkor site. The sulfur compounds in the bat droppings might be utilized as a sulfur source. As a result, chemolithotrophic and chemoorganotrophic sulfur-oxidizers can utilize such compounds and grow in these temples.

The distinctive physiological characteristics of lithoautorophic sulfur oxidation by strain NBRC9425 point a possibility that enzymes and pathways of fungal sulfur oxidation are different from the established prokaryotic ones. In the present study, enzyme assays failed to detect *in vitro* activities of sulfur-oxidizing enzymes. The results of enzyme assays suggested that sulfur oxidation occur inside of the fungal cells and that the integrated structure of fungal cell is indispensable to maintain the activity of sulfur oxidation by strain NBRC9425. Thus, enzyme

assays are not appropriate methods to identify sulfur-oxidizing enzymes in strain NBRC9425. Comparative proteomic studies have proved to be efficient in identifying proteins involved in a specific metabolism. In this dissertation, optimizing conditions of protein extraction and 2-DE were performed. It was found that the proteomic profiles of maltose-grown fungal cells and sulfur-grown fungal cells were greatly different. Clearly, the identification of the up-regulated proteins in sulfur-grown fungal cells is promising to find the sulfur-oxidizing enzymes.

6-1-3 Availability of organic compounds on sulfur oxidation

Strain NBRC9425 was able to grow chemolithotrophically on S^0. Both complex and simple organic compounds had positive effects on fungal growth and sulfur oxidation. These physiological properties were different from those of neither facultatively chemolithoautotrophic prokaryotes nor chemolithoheterotrophic prokaryotes. In addition, its nutritional requirement was similar to a microaerobic, thermotolerant, sulfur-oxidizing chemolithomixotrophic bacterium *Thiomicrospira thermophile* sp. nov. (Takai et al., 2004).

Morphological characteristics of strain NBRC9425 were very different when cultured in organics-containing medium and organics-free medium. The diameter of organics-grown fungal hyphae was bigger than that of sulfur-grown fungal hyphae (Fig. 4-2; Fig. 5-2). Sulfur-oxidation by fungi is generally thought to be an oxygen-dependent process, so studies of sulfur-oxidation by fungi were performed with shaking flasks in previous reports. When cultured in media containing

organic compounds (and S^0), fungal hypha of strain NBRC9425 extended well in vigorously shaking flasks. However, the smaller fungal cells tended to fragment in shaking flasks. The morphological change was overlooked in previous studies about fungal growth on organics and S^0. It was found in these study that even very trace amount of organic compounds could affect the fungal sulfur oxidation. When the WS5-S medium was supplemented with 10 mg L^{-1} yeast extract, the produced thiosulfate and sulfate increased to 3.6 and 3.2 folds, respectively. At the same time, the living fungal biomass indicated by ergosterol content also increased to 3.2 folds. The dramatic enhancement in sulfur oxidation by the trace amount of organic compounds explained the frequently observed fungi-like microorganisms in positive MPN cultures of deteriorated sandstone samples from Angkor monuments (Li et al. 2010): firstly, the ability of chemolithotrophic sulfur oxidation makes the fungi obtain energy and survive on the nutrients-poor sandstone. Secondly, these "precursor" provides a chemosynthetic base for the sulfur-oxidizers to thrive.

6-2 Conclusions

As prior studies about lithotrophic sulfur oxidation by microorganisms have focused on prokaryotes, this dissertation aims to explore the chemolithotrophic growth of fungi on reduced inorganic sulfur compounds. Named fungal strains were screened to select a model strain. Activities of sulfur oxidizing enzymes in fungi were examined. Physiological characteristics of sulfur oxidation by fungi on organics-free and carbon-added media were also investigated.

Seven named strains belonging to genera *Gliocladium, Fusarium*, *Trichoderma*, and *Aspergillus* formed clearing zone on agarose-solidified WS5-S medium, and they oxidized S^0 to thiosulfate and sulfate in WS5-S liquid medium. No 16S rRNA genes were amplified from the genomic DNA of the fungal strains, indicating that these fungi did not harbor endobacteria. These results indicated that the wide distribution of lithotrophic sulfur oxidation by fungi. *F. solani* f.sp. *pisi* NBRC9425 formed bigger clearing zone and produced more sulfuric acid. As a result, strain NBRC9425 was selected as the model strain in this dissertation.

The culture filtrate of strain NBRC9425 growing in organics-free, S^0-containing submerged medium did not oxidize GSSG/GS_nG, S^0, $S_2O_3^{2-}$, or SO_3^{2-} in test tubes. The concentrated culture filtrate also did not exhibit oxidation activity. It meant the extracellular proteins did not contain sulfur-oxidizing enzymes. Cell-free extract did not show any activity, indicating that fungus cells

should be maintained with integrated structure to oxidize sulfur. Extensive work was performed to optimize the conditions of protein extraction and isoelectric focusing. 2D display of soluble proteins of strain NBRC9425 grown on the different energy sources suggested greatly distinct metabolism. This study paves way for comparative proteomic study for identifying proteins participating in sulfur oxidation.

In physiological study of lithotrophic sulfur oxidation by strain NBRC9425, WS5-S medium containing mineral salts and S^0 was used. Chlamydospores suspension was used as an inoculum. Strain NBRC9425 grew chemolithotrophically on WS5-S medium. S^0 could be oxidized to sulfate, and thiosulfate was produced as an intermediate. Culture pH did not change after 5 days cultivation. When mycelial discs cut from PDA plates served as inoculum, culture pH decreased, indicating more produced sulfate. The differences in culture pH suggest that organic compounds in mycelial discs affected the fungal sulfur oxidation. Supplemented thiosulfate dramatically stimulated the biomass yield and the lithotrophic sulfur oxidation of the fungus in WS5-S medium. The fungus did not grow on the medium containing mineral salts and thiosulfate. It can be concluded that S^0 could be utilized as a sole energy source by strain NBRC9425 and thiosulfate could not. As a sulfur source, sulfate was more favorable than thiosulfate.

When strain NBRC9425 was cultured in WS5-S medium supplemented with different concentrations of yeast extract (0–200 mg L^{-1}), both produced thiosulfate and sulfate increase with

yeast extract. The living fungal biomass indicated by ergosterol content also increased when the fungus was grown on more yeast extract. The highest values of sulfur compounds equivalent, the quotient of sulfate/thiosulfate divided by ergosterol, were achieved at 15 mg L^{-1} of yeast extract. These finding indicated that organic compounds could enhance the sulfur oxidation by strain NBRC9425 and that the fungal metabolism shifted from chemolithotroph to chemoorganotroph when cultured with more than 15 mg L^{-1} of yeast extract.

In this study, fungal hypha attached to and extended on the surface of S^0 particles in WS5-S medium. Prior studies found that fungi were able to attach to insoluble carbonaceous materials and failed to characterize such morphological properties in organics-free media. Strain NBRC9425 barely grew in organics-free medium dispensed in circular-shaking flasks. It indicated that the fragile fungal hypha (with a smaller diameter than that grown in carbon-containing medium) could not extend in vigorously shaking flasks. Notably, fungal hypha did not attach to S^0 when WS5-S medium was supplemented with 200 mg L^{-1} yeast extract. It can be concluded that yeast extract served as a more favorable energy source than S^0. In the presence of 2 g L^{-1} yeast extract, S^0 was mainly oxidized to thiosulfate and little sulfate was produced. However, when WS5-S was added with 2 g L^{-1} carbohydrates, such as potato dextrose broth, maltose, and glucose, S^0 was oxidized to sulfate and little thiosulfate was accumulated. It indicated that strain NBRC9425 did no need to further oxidize thiosulfate to sulfate in the presence of yeast extract because yeast extract contains sulfate. These finding also further indicated that sulfate might serve as a favorable sulfur source

than thiosulfate.

Currently, only a few studies were conducted on lithotrophic sulfur oxidation by fungi. Since CO_2 fixation by fungi has been observed (Parkinson et al. 1991), this dissertation paves the way to further investigations of the biochemistry and molecular biology of chemolithotrophic sulfur oxidation in fungi.

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