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ISOLATION OF BACTERIA IN EFFECTIVE MICROORGANISMS (EM) SUITABLE FOR DEGRADATION OF THIOSULFATE

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ABSTRACT

A total of 60 potential sulfur-oxidizing bacteria isolated from the Effective Microorganisms (EM) sample were screened for sulfide oxidase producer. Out of 60 isolates, 11 strains were able to produce sulfide oxidase and the most superior sulfide oxidase producer was known as strain SO02. Strain SO02 produced 6.83 U/ml sulfide oxidase with specific activity of 2.32 U/mg. Strain SO02 was later identified as *Serratia* sp. via biochemical test and 16S rRNA gene sequence analysis. The optimum condition of sulfide oxidase production was obtained at pH 5.0, 30˚C in the presence 0.2% (w/v) peptone and 0.5 mM thiosulfate. Under optimized condition, sulfide oxidase activity was 3.848 U/ml with specific activity of 5.460 U/mg. There were 60.2% and 90.7% improvements in sulfide oxidase production and specific activity after the optimization studies, respectively. Development of oxygen control strategy towards sulfide oxidase production showed that the most favorable condition for the production of sulfide oxidase was without dissolved oxygen control strategy. Sulfide oxidase activity and the specific activity without oxygen control strategy were obtained up to 4.843 U/ml and 6.764 U/mg, respectively. However, sulfide oxidase production under oxygen control condition was 13% lower as compared to the condition without oxygen control with sulfide oxidase activity of 4.216 U/ml. This might occurred due to low oxygen concentration in the fermenter where oxygen was required in the sulfur oxidation. The specific oxygen uptake rate using strategy of without dissolved oxygen control was 0.499 mg O_2/ g cell/ min.

ABSTRAK

Sejumlah 60 bakteria berpotensi mengoksidakan sulfur telah dipencilkan daripada sampel Mikroorganisma Efektif (EM) dan telah disaring untuk penghasil sulfid oksida. Daripada 60 isolat, 11 strain menunjukkan keupayaan menghasilkan sulfid oksida dan penghasil terbaik sulfid oksida dikenali sebagai strain SO02.

Strain SO02 menghasilkan 6.83 U/ml sulfid oksida dengan aktiviti spesifik sebanyak 2.32 U/mg. Strain SO02 kemudiannya dikenalpasti sebagai *Serratia* sp. melalui ujian biokimia dan analisis jujukan gen 16 S rRNA. Keadaan optimum bagi penghasilan sulfid oksida diperolehi pada pH 5.0, 30˚C dengan kehadiran 0.2 % (b/i) pepton dan 0.5 mM thiosulfat. Dibawah keadaan optimum, aktiviti sulfid oksida telah dicapai pada 3.848 U/ml dengan aktiviti spesifik sebanyak 5.460 U/mg. Terdapat 60.2% dan 90.7% peningkatan dalam penghasilan sulfid oksida dan aktiviti spesifik selepas kajian pengoptimuman dilakukan, masing-masing. Pembangunan strategi kawalan oksigen terhadap penghasilan sulfid oksida menunjukkan keadaan yang sesuai bagi penghasilan sulfid oksida adalah tanpa strategi kawalan oksigen terlarut. Aktiviti sulfid oksida dan aktiviti spesifik menggunakan strategi tanpa kawalan oksigen diperolehi sebanyak 4.843 U/ml dan 6.764 U/mg, masing-masing. Walaubagaimanapun, penghasilan sulfid oksida dibawah keadaan kawalan oksigen adalah 13% lebih rendah jika dibandingkan dengan keadaan tanpa kawalan oksigen dengan aktiviti sulfid oksida sebanyak 4.216 U/ml. Ini mungkin berlaku disebabkan oleh pengurangan kepekatan oksigen di dalam fermenter dimana oksigen diperlukan dalam pengoksidaan sulfur. Kadar penggunaan oksigen spesifik dengan menggunakan strategi tanpa kawalan oksigen adalah 0.499 mg O_2/ g cell/ min.

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Sulfur is one of the most important elements for sustaining life on earth. It was divided into organic and inorganic sulfur compounds (SC). The organic sulfur occurs as volatile compounds for example dimethyl sulfide (DMS), methanethiol (MT) and dimethyl disulfide (DMDS) which can be found in wastewater and night soil treatment plants (Cho *et al*., 1992). On the other hand, inorganic sulfur compounds include hydrogen sulfide, sulfite, elemental sulfur, tetrathionate and thiosulfate. The biochemical significance of sulfur is tremendous. Sulfur is required because of its structural role in the amino acids cysteine and methionine, and because it is present in number of vitamins, such as thiamine, biotin and lipoic acid, as well as in coenzyme A (Madigan *et al*., 2000).

There are many sources of inorganic sulfur compounds be presented on earth. These include geological, biological and anthropogenic sources of sulfur. Volcanoes, hot springs and hydrothermal vents are the major natural sources of reduced sulfur compounds (Madigan *et al*., 2000). Besides that, industrial activity including petroleum and natural gas extraction and refining, pulp and paper manufacturing, rayon textile production, chemical manufacturing, agricultural and waste disposal add inorganic sulfur compounds to the environment (Park *et al*., 2002).

Sulfur undergoes number of biological transformation in nature carried out exclusively by microorganisms. The transformations of inorganic sulfur compounds in nature have been formalized in the so-called sulfur cycle. The microorganisms that participate in sulfur cycle are physiologically diverse and comprise both heterotrophic and autotrophic organisms (Peck, 1961). Microorganisms that are capable of oxidizing reduced inorganic sulfur compounds with sulfate as the end product are known as sulfur-oxidizing bacteria (SOB). Most of the known sulfur-oxidizing bacteria belonging to genera *Thiobacillus, Thiothrix, Beggiatoa*, *Thiomicrospira* and *Achromatium* (Das *et al*., 1996). The sulfur-oxidizing bacteria can be isolated from acid, neutral or alkaline environments, form cold, moderate or hot habitats, as well as from low to highly saline waters and soils.

In sulfur cycle, nature balances the inorganic sulfur oxidations through the biological reduction of sulfate to sulfide (Das *et al*., 1996). The process of sulfate reduction is synonym to hydrogen sulfide (H_2S) production or sulfidogenesis, as a result of sulfate-reducing bacteria (SRB) metabolism (Ruby *et al*., 1981). Sulfides ion or hydrogen sulfide gases are highly undesirable in the environment because of acute neuro-toxicity towards human and animals and are corrosive to metallic infrastructure.

Many literatures have been enlightened regarding microbial capacity to enzymatically oxidize sulfide. Sulfide oxidase is the key enzyme responsible for the catalysis of sulfide ions oxidation (Mohapatra *et al*., 2006). The aim of this work is to study the production of sulfide oxidase that was responsible in oxidation of sulfide to sulfate.

1.2 Objective of the Study

The main objectives of this study are:

- To isolate and screen potential sulfur-oxidizing bacteria from Effective Microorganisms (EM) sample that have a capability to produce sulfide oxidase.
- To identify and characterize the highest sulfide oxidase producer using biochemical test and 16S rRNA gene analysis.
- To optimize sulfide oxidase production using batch culture culture.

1.3 Scope of the Study

The potential sulfur-oxidizer was isolated from Effective Microorganisms (EM) sample. The morphological, physiological and biochemical characterization along with 16S rRNA gene analysis of the culture were carried out with the purpose of identifying the microorganism.

Optimization of sulfide oxidase production by SO02 strain was performed in this study using the shake flask culture system. The parameters involved are:

- pH
- Temperature
- Various nitrogen sources
- Various nitrogen source concentrations
- Various thiosulfate concentrations
- Various reduced inorganic sulfur compounds

The optimized cultural condition in shake flask will be applied in the fermenter in order to study the oxygen requirement of the potential selected strain.

CHAPTER 2

LITERATURE REVIEW

2.1 Problem Background

Pulping site, petroleum refinery plant, sewage treatment facility and livestock rising farm emit very offensive odours and most of them contain sulfur compounds (Park *et al*., 2002). Among many sulfur-containing compounds, hydrogen sulfide is notorious compound which is very toxic and corrosive. Hydrogen sulfide production which gives rotten egg smell can cause community problems and create unpleasant condition of working area. Besides that, the discharged of untreated industrial waste containing sulfur compounds into receiving stream will contaminate the habitat and give rise to toxic impact to the aquatic community. Therefore, removal of sulfur compounds especially in the waste is necessary.

Traditionally, the removal of sulfide was achieved by physiochemical methods such as air stripping, chemical precipitation and oxidation which use strong oxidizing agents such as hydrogen peroxide, ozone and hypochlorite. However, processes based

upon such agents are risky due to potential hazards associated with handling of these chemicals (Mohapatra *et al*., 2006).

Currently, microbiological bioprocessing is believed to be an efficient and environmental friendly method for sulfide removal owing to its cost effectiveness, low energy demand for the process operation and reduced consumptions of hazardous chemicals (Oprime *et al*., 2001). Microbiological treatment was carried out through the enzymatic oxidation of sulfide which catalyzes by an enzyme known as sulfide oxidase (Mohapatra *et al*., 2007).

2.2 Sulfur and the Importance of Sulfur

Sulfur is a major inorganic element that is essential for the entire biological kingdoms because of its incorporation into amino acids, proteins, enzymes, vitamins, lipids, carbohydrates and other biomolecules (Madigan *et al*., 2000). In plants, humans and animals, sulfur occurs in various biological structures. Sulfur is present in body tissue as part of the amino acids, methionine, cysteine and taurine. Besides that, the occurrence of sulfur in the living biological organisms can be found as coenzyme A, glutathione, chondroitin sulfate, heparin, fibrinogen, estrogen, ferredoxin and many more (Komarnisky *et al*., 2003).

According to Komarnisky *et al*. (2003), inorganic sulfur compounds can be found in the form of sulfate, sulfide, sulfite, thiosulfate, elemental sulfur and polythionates. Sulfate appears to be the most stable and abundant form of sulfur available for use by living organism in the biosphere. Sulfur amino acids and other

organic compounds in living cells contain sulfur in the lowest oxidation state (-2). This is the same oxidation state that is found in hydrogen sulfide (H_2S). It is evident that sulfate or other forms of sulfur in the oxidation state greater than -2 must be reduced to sulfide before it can be used for the synthesis of amino acids. Unlike plants and certain microorganism, monogastric animals and human are unable to synthesize sulfur-containing amino acid such as methionine and cysteine from inorganic sulfur compounds. Therefore, sulfur has to be supplied through food; and plants are important sources of sulfur for humans and animals. Sulfur plays many important roles in industries. Sulfur dioxide and its salts are commonly used as inhibitors of enzymatic and non-enzymatic browning, broad spectrum antimicrobial agents, dough conditioners, bleaching agents and antioxidants. Six sulfating agents, approved by the Food and Drug Administration are used as food preservatives: sodium sulfite, sulfur dioxide, sodium bisulfite, potassium bisulfite, sodium metabisulfite and potassium metabisulfite. The antimicrobial properties of sulfur dioxide and sulfite are used to control the growth of spoilage microorganisms in dried fruits, fruit juices and wine.

Thiosulfate is used in a variety of industrial processes where it serves as a fixing agent for the complexation of silver in photoprocessing solutions. Thiosulfate also has been used as a dechlorinating agent for municipal wastewaters, prior to discharge into receiving stream. In addition, thiosulfate salts are commonly used to control scaling in scrubbers used for desulfurization units, while in mining operations they enhance the leaching of precious metals such as gold from their ores (Schreiber and Pavlostathis, 1998).

In many parts of the world, concentration of sulfur element in soil is too low, resulting in reduce sulfur content of plant foodstuff used by humans and animals (Komarnisky *et al*., 2003). For that reason, in agricultural industries, sulfur fertilizers have been widely used to increase the soil sulfur level. Sulfur fertilizers are available mainly in three chemical forms: sulfate (SO_4^{2-}), elemental sulfur (S^0) and thiosulfate

($S_2O_3^{2-}$). Production of sulfate from either elemental sulfur or thiosulfate in soils is believed to be mainly a microbially-mediated process carried out under aerobic conditions by many different microorganism and fungi (Barbosa-Jefferson *et al*., 1998).

2.3 Sulfur Cycle

Sulfur, in addition to carbon, nitrogen, oxygen, hydrogen and phosphorus is one of the major metabolic nutrients in the ecosphere. Like other elements, it is cycled and metabolized through the lithosphere, hydrosphere and atmosphere in the biogeochemical cycle (Moss, 1978).

The sulfur cycle comprises an interrelated set of oxidation-reduction reactions of inorganic and organic sulfur compounds with a change in the reduction state of the sulfur atoms from -2 (sulfide) to +6 (sulfate) through several common intermediates, such as polysulfide, elemental sulfur, thiosulfate, polythionates and sulfite (Sorokin, 2003). The major available forms of sulfur in nature are sulfate and sulfide in water or soil, and sulfur dioxide in the atmosphere, whilst thiosulfate, polythionate, sulfoxides and elemental sulfur play a smaller but significant role. The sulfur chemistry is complicated by many oxidation states sulfur can assume. The oxidation states of sulfur is summarize in Table 2.1.

The global transport cycle of sulfur is given Figure 2.1. Sulfide originates from anaerobic sulfate reduction by sulfate-reducing bacteria and also from the decay of organic matter (Kuenen, 1975). Besides that, sulfide was also produced by the emission of geochemical sources in sulfide springs or volcanoes (Madigan *et al*., 2000). From a microbiological point of view, the production of sulfide was balanced by the processes

that involved variety of bacteria found in soil or water and able to oxidize sulfide (IPCS, 1981). However, under aerobic conditions sulfide was oxidized both chemically and biologically to sulfate. In nature, variety of reduced inorganic sulfur compounds such as sulfur, thiosulfate and sulfite occur as intermediates for sulfide oxidation to sulfate. As these compounds are slowly oxidized by a chemical reaction with oxygen, it is obvious that biological oxidation play an important role in the recycling of reduced inorganic sulfur compounds under aerobic condition (Kuenen, 1975). Microorganism which are specialized in the oxidation of sulfur compounds are known as sulfur-oxidizing bacteria that have the ability to oxidize reduced sulfur compounds and gain energy from these processes. The end product of oxidative activity is the production of sulfate which is extremely stable towards further chemical activity in nature (IPCS, 1981).

Table 2.1: Oxidation state of sulfur in common compounds (Komarnisky *et al*., 2003).

Oxidation state	Compounds
-2	Sulfide, HS^-; thiocyanate, SCN^-
-1	Disulfide, S_2^{2-}
0	Elemental sulfur, S_8; polysulfide
+1	Dichlorodisulfane, S_2Cl_2
+2	Sulfoxylate, SO_2^{2-}
+3	Dithionite, $S_2O_4^{2-}$
+4	Sulfite, SO_3^{2-}; sulfur dioxide, SO_2
+5	Dithionate, $S_2O_6^{2-}$
+6	Sulfate, SO_4^{2-}

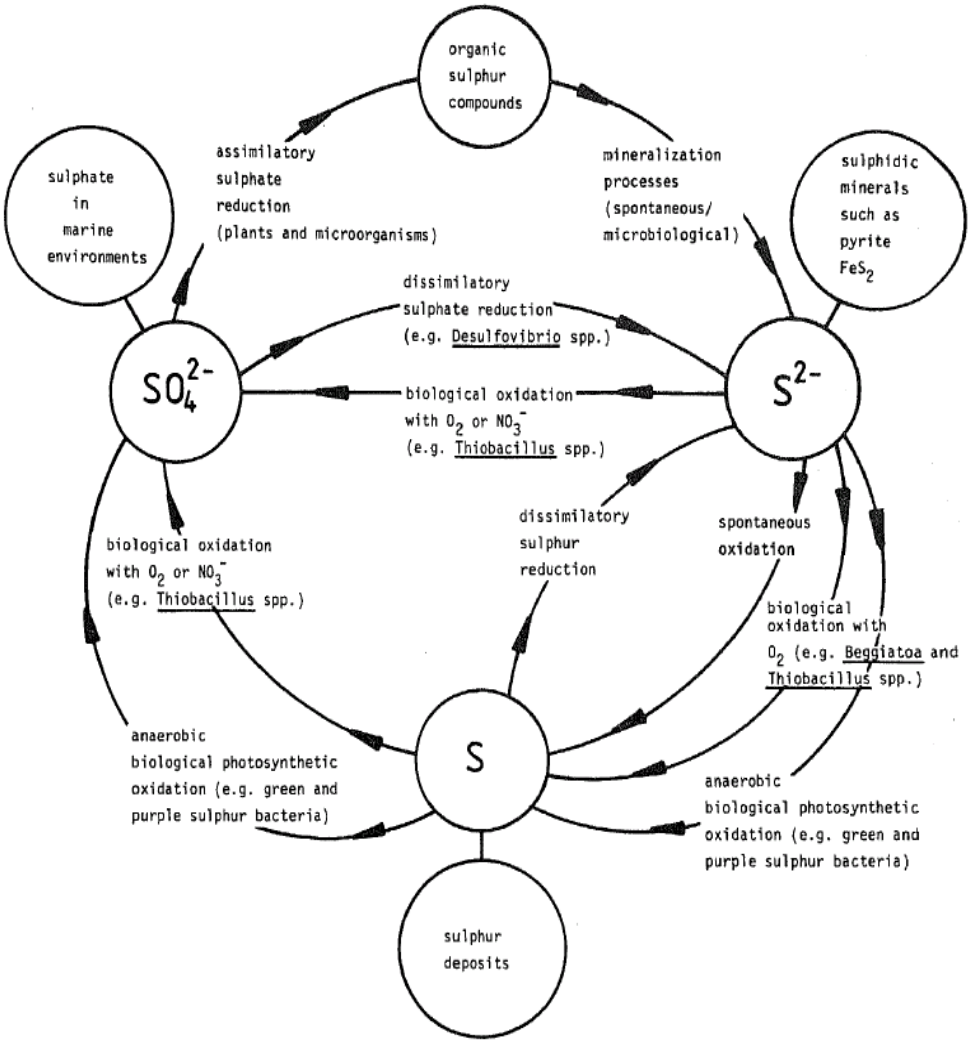

Figure 2.1: The global sulfur cycle (Bos and Kuenen, 1983)

2.4 Sources of Sulfur Compounds

Release of sulfide to variety of environment media (air, surface water, groundwater, soil and sediment) occur both naturally and as a result of human activity. In the natural cycle of sulfur, sulfide and sulfate appears to be the principal sulfur components in the environment.

Natural sources such as swamps, bogs and volcanoes account for about 90% of the total amount of H_2S in the atmosphere (ATDSR, 2004). Swamps, bogs and shallow lakes are the favourable environment for H_2S evolution where there is a highly organic materials presence, oxygen deficiency and the development of sulfur bacteria (Moss, 1978). Sulfides are also found in petroleum and natural gas as a result of sulfate-reducing bacteria activity or other sulfidogenic bacteria (Gevertz *et al*., 2000).

A number of bacteria, fungi and actinomycetes (a fungus-like bacteria) are known to release H_2S during the decomposition of sulfur-containing proteins or by the direct reduction of sulfate. The heterotroph *Proteus vulgaris* is an example of a common bacteria that produces H_2S when grown in the presence of protein. The reduction of sulfate to H_2S can be accomplished by anaerobic sulfate-reducing bacteria such as *Desulfovibrio* and *Desulfotomaculum* (IPCS, 1981). Hence, H_2S production can be expected in conditions under depletion of oxygen with the availability of organic material and sulfate.

H_2S was also emitted by some higher plant species as a byproduct of sulfite metabolism. The H_2S emission was studied in detail in squash and pumpkin plant (*Cucurbita pepo* L.) by Wilson *et al*. (1978). They found out that the emission was completely light-dependent and increased with light intensity in squash and pumpkin

plant. Rennerberg (1989) has stated that H_2S emission by higher plants seems to be a significant contribution to the biogeochemical cycle of sulfur.

In addition, H_2S was also produce by living organisms, including human being through the digestion and metabolism of sulfur-containing materials. Sulfide was produces as a result of bacterial reduction of inorganic sulfate and sulfite, and also by fermentation of sulfur-containing amino acids cysteine and methionine.
H_2S was also produced in human large intestine by natural bacteria present in both intestinal tract and mouth (CICAD, 2003). In addition, sulfide production in human mouth is a component that causes the bad breath (halitosis) (ATDSR, 2004).

The anthropogenic (man-made) sources of sulfur as a result of human activities include combustion of fossil fuels, refining petroleum, coking of coal, electric power generation and other industrial processes (ATDSR, 2004) have a large impact on the atmospheric sulfur balance. Combustion of sulfur-containing compounds in fossil fuels emits sulfur oxides, SO_2 which produced at the lower atmosphere (Soleimani *et al*., 2007). Moreover, the SO_2 can penetrate soils directly from the atmosphere and becomes oxidized to sulfate, and soils are available as sources of sulfur compounds. More commonly, sulfur enters soil system through addition of sulfur-containing fertilizers.

H_2S is also frequently found in industrial settings where it is either used as a reactant or produces as a by-product of manufacturing or industrial processes. Examples of these processes are tanneries, wastewater treatment facilities, manure and sewage facilities, rayon manufacturing plants, Kraft paper mills, drug manufacturing and food processing plants (IPCS, 1981). H_2S may also enter the environment through accidental release, from leakage during manufacture or utilization, or as a result of improper disposal of industrial waste (ATDSR, 2004). Discharge liquid waste from these industries can release H_2S to receiving waters. Since H_2S is a natural component of

petroleum, it may also be released into the environment during extraction, transport and refining of these sources.

The anthropogenic emissions of gaseous sulfur compounds are approximately two or three times as large as those from natural sources (Aneja and Cooper, 1978). The natural and anthropogenic sources of sulfur are summarized in Table 2.2.

Table 2.2: Main sources of sulfur in environment.

Natural Sources	Anthropogenic Sources
- Environment • Hydrothermal vents • Volcanoes • Petroleum reservoir • Soil • Swamps, bogs - Bacterial activity • SOB and SRB activity - Biogenic emissions • From vegetations (higher plants, wetlands, lands and ocean - Human metabolism • In large intestine, mouth, muscles - Sulfur storage • Gypsum, pyrite	Coke oven plants Coking of coal Combustion of fossil fuels Drug manufacturing Electric power generation Fertilizers Food processing Iron smelters Kraft paper mills Livestock raising farm Rayon manufacturing plants Refining petroleum, Tanneries Tar and asphalt manufacturing plants Wastewater treatment facilities

2.5 The Detrimental Effects of Reduced Sulfur Compounds

Odour from dairy farm, sanitary station and petrochemical plants can contaminate nearby environments and cause severe complaints. Significant components present in the odour are sulfur compounds and among these, H_2S is often the most abundant (Oh *et al*., 1998). H_2S is a colourless and flammable gas with a characteristic odour of rotten eggs (Wang, 2002). It is produced naturally as well as a result of human activity. H_2S which is very toxic and corrosive gas are detrimental in the environment due to many terrible effects towards humans, living organisms, environment and industrial facilities (Mohapatra *et al*., 2006).

Primary exposure of general population to H_2S is most likely occurs through inhalation of ambient air, and the gas is rapidly absorbed through the lungs in human (CICAD, 2003). As H_2S is part of the natural environment, the general population will have some exposure to H_2S. Populations living in areas of geothermal activity or near industrial sites may be more likely to be exposed to higher levels of H_2S. Besides that, workers employed at facilities where H_2S is produce as a byproduct, such as farms with manure storage pits, petroleum or natural gas drilling operations, landfills and waste water treatment plants may also be exposed to high level (ATDSR, 2004).

Study by WHO (2000), showed that H_2S poisoning could give rise to respiratory irritation, pulmonary oedema, and strong simulation in the central nervous system which leading to convulsions, unconsciousness and death. In fatal human intoxication cases, brain oedema, degeneration and necrosis of the cerebral cortex and the basal ganglia have been observed. They also pointed out that workers, who were exposed to H_2S daily, often complained of such symptoms as fatigue, somnolence, headache, irritability, poor memory, anxiety, dizziness and eye irritation. CICAD (2003) has reported that

there is also some evidence that exposure to H_2S may be associated with an increased rate of spontaneous abortion.

Furthermore, sulfide has hazardous effects on industrial facilities where it brings damage to plant equipments, concrete and steel structures caused by sulfide-initiated corrosion. Sulfides are also undesirable during oil production operations because of their corrosiveness and ability to form insoluble metal sulfides that can lead to a loss of reservoir permeability (Gevertz *et al*., 2000).

Thiosulfate is likely to become part of the industrial waste-stream and later discharged into a receiving stream. A number of microorganisms residing in the stream can use thiosulfate as an energy source, whereby thiosulfate is oxidized and oxygen being consumed. Therefore, thiosulfate contributes to the biochemical oxygen demand (BOD) of the stream and potentially impact the community (Schreiber and Pavlostathis, 1998). Thiosulfate is also toxic by causing mortality to the aquatic organisms. Therefore, treatment of thiosulfate-containing wastewaters is often desirable prior to final disposal.

2.6 Control of Sulfides and Reduced Sulfur Compound

Study by Oprime *et al*. (2001)shown that, among various reduce inorganic sulfur compounds exists in environment, H_2S or sulfides are the most common compounds that associated with human health risk and destructive effects on building structure. The removal of H_2S from biogas, natural or industrial gases is an important concern of environmental technology since this gas is extremely hazardous and corrosive.

Conventional physiochemical processes of H_2S removal are mainly based in its oxidation by air in the presence of several catalysts such as potassium permanganate ($KMnO_4$), ferric ion (Fe^{3+}) and active coal. Besides that, the detrimental activities of sulfide-producing bacteria can be controlled by the effective use of oxidizing biocides such as chlorine (Cl_2), hydrogen peroxide (H_2O_2) and NaClO (Oprime *et al*., 2001). The use of these biocides is the most successful method in controlling unwanted activities in surface facilities. However, processes based upon such agents are expensive due to the high cost involved in the facility installation, as well as the operational cost due to high energy demand and toxic chemical usage (Sorokin, 1994).

On the other hand, the biological treatment of sulfides using enzymatic oxidation of sulfide oxidase has drawn attention since this method is more efficient and environmental friendly. The bioconversion of sulfide using sulfide oxidase is believed to be cost effective, higher sulfide removal efficiency, ability to operate at mild conditions and reduced production of secondary pollution (Mohapatra *et al*., 2007). The application of sulfide oxidase to remove harmful sulfide may potentially utilize for the industrial processes and wastewater treatment. Sulfide oxidase has been produce by number of autotrophic and heterotrophic bacteria, known as sulfur-oxidizing bacteria.

2.7 Sulfur-Oxidizing Bacteria (SOB)

Reduced inorganic sulfur compounds (RISCs) are exclusively oxidized by prokaryotes, with sulfate as a major oxidation product (Friedrich *et al*., 2001). Sulfur-oxidizing bacteria represent an important functional group in biogeochemical cycling of sulfur and the species are distributed over the α-, β- and γ-*Proteobacteria* (Dam *et al*., 2007).

Sulfur-oxidizing bacteria are physiologically diverse and comprise both autotrophic and heterotrophic microorganisms (Peck, 1961). Sulfur-oxidizing bacteria are characterized by their ability to catalyze the oxidation of reduced sulfur compounds (H_2S, thiosulfate, sulfite, polythionate or elemental sulfur) with oxygen as terminal electron acceptor (Graff and Stubner, 2003). The ability of sulfur-oxidizing bacteria to oxidize the sulfur compounds under alkaline, neutral and acidic condition has been reviewed previously (Friedrich *et al*., 2001). The representatives of sulfur-oxidizing bacteria are displayed in Table 2.3.

The role of autotrophic sulfur bacteria is widely recognized in oxidation of reduced sulfur compounds in natural and industrial habitats, as well as their physiology, biochemistry, phylogeny and genetics are well established. Autotrophic sulfur-oxidizing bacteria are capable of growing chemolithoautotrophically with sulfur compounds as energy source and carbon dioxide as sole carbon source (Graff and Stubner, 2003). The autotrophic sulfur-oxidizing bacteria were believed to be able to derive metabolically useful energy from the oxidation of reduced inorganic sulfur compounds (Kuenen, 1975).

Most of the well known autotrophic sulfur-oxidizing bacteria belongs to the families of the *Thiobacteriaceae, Beggiatoaceae* and *Achromatiaceae* and commonly called as colourless sulfur bacteria (Kuenen, 1975). A lot of previous research has mainly focused on the genus *Thiobacillus*. The thiobacilli are a small group of microorganisms whose energy metabolism is uniquely adapted to obtain all the energy required for growth from oxidation of inorganic sulfur compounds to sulfate, and utilize carbon dioxide for the synthesis of cellular materials (Peck, 1961).

Examples of autotrophic sulfur-oxidizing bacteria that has been isolated and studies are *Halothiobacillus neapolitanus* (Wood *et al*., 2005), *Halothiobacillus kellyi* (Sievert *et al*., 2000), *Thiobacillus caldus* (Hallberg *et al*., 1996), *Thiobacillus thioparus* (Cho *et al*., 1991a), *Thiobacillus thiooxidans* (Hirano *et al*., 1996). Besides that, numerous studies of sulfur oxidation have been performed on *Thiobacillus denitrificans* and *Thiobacillus ferooxidans* which utilize nitrate and ferric ions in place of oxygen as electron acceptor, respectively (Peck, 1961).

However, oxidation of sulfur compounds is not restricted to the true sulfur bacteria; this process also occurs in heterotrophic bacteria isolated from soil and marine environment. Most of the heterotrophic bacteria that have such activities belong to genera *Arthrobacter* (Mohapatra *et al*., 2006), *Halomonas* (Sorokin, 2003), *Xanthobacter*, *Escherichia coli* (Das *et al*., 1996), *Klebsiella* (Mason and Kelly, 1988) and *Pseudomonas* (Schook and Berk, 1978).

On the other hand, only few of these heterotrophs have the ability to generate biologically useful energy from the oxidation of reduced inorganic sulfur compounds. Few representatives of this type of heterotrophic sulfur-oxidizing bacteria are *Limnobacter thiooxidans* (Spring *et al*., 2001), *Bosea thiooxidans* (Das *et al*., 1996), *Thiobacillus* Q (Gommers and Kuenen, 1988), *Ottowia thiooxidans* and *Roseinatronobacter thiooxidans*. These heterotrophs are increasing their growth yield in presence of thiosulfate or sulfide, which is indicative of a lithoheterotrophic metabolism (Sorokin *et al*., 2005a).

Teske *et al*. (2000) and Ruby *et al*. (1981) stated that two groups of heterotrophic sulfur-oxidizing bacteria with different pH responses can be distinguished, the acid-producing and the base-producing thiosulfate oxidizers. These heterotrophic bacteria can oxidize reduced sulfur compounds, but do no depend on this reaction for growth

(Teske *et al*., 2000). An increase in pH during growth of heterotrophic bacteria during thiosulfate oxidation was due to tetrathionate production (Sorokin, 2003). Oxidation of thiosulfate to tetrathionate probably does not supply energy for the growth of cells capable of catalyzing this oxidation (Peck, 1961).

Ruby *et al*. (1981) have described locally isolated sulfur-oxidizing bacteria from hydrothermal vents that comprised the heterotrophic base-producing, aerobic or facultatively anaerobic, rod-shaped bacteria that oxidize thiosulfate to tetrathionate without measurable enhancement of growth. Amongst the pure cultures of base-producing sulfur-oxidizing bacteria described in Sorokin's publication, the organisms are the members of γ-*Proteobacteria*, which include *Pseudoalteromonas*, *Halomonas* and *Pseudomonas stutzeri* (Sorokin, 2003).

Table 2.3: Sulfur-oxidizing bacteria.

Categories	Sulfur-Oxidizing Bacteria	Total Activity (U)	References
Autotrophs	*Halothiobacillus neapolitanus*	n.d	Wood *et al*., 2005
	Halothiobacillus kellyi	n.d	Sievert *et al*., 2000
	Thiobacillus thiooxidans	n.d	Hirano *et al*., 1996
	Thiobacillus sp. W5	15.8	Visser *et al*., 1997
Facultative	*Tetrathiobacter kashmirensis*	n.d	Ghosh *et al*., 2005
Heterotrophs	*Arthrobacter* sp.	2.56	Mohapatra *et al*., 2006
	Bosea thiooxidans *Xanthobacter* *Escherichia coli*	n.d	Das *et al*., 1996
	Citreicella thiooxidans *Ottowia thiooxidans* *Roseinatronobacter thiooxidans*	n.d	Sorokin *et al*., 2005a
	Klebsiella aerogenes	n.d	Mason and Kelly, 1988
	Pseudomonas aeruginosa	n.d	Schook and Berk, 1978
	Thiobacillus strain AL3	0.64	Sorokin *et al*., 1998
	Bacillus sp.	11.7	Nakada and Ohta, 1999

* n.d – not determined

To our knowledge, the involvement of *Serratia* in the oxidation of sulfur compounds has never been reported. However, there are reports about *Serratia* regarding sulfate formation from the hydrolysis of organic sulfate by enzymes termed sulfatase. *Serratia* was reported to synthesize arylsulfatase in the presence of methionine or taurine as the sulfur source (Murooka *et al*., 1980; Yamada *et al*., 1978). Arylsulfatase are considered to be important for sulfate ester mineralization in soils that contain high levels of sulfur in the form of sulfate ester (Murooka *et al*., 1980). This statement showed the ability of *Serratia* to utilize sulfur compounds in the organic form and produce sulfate from the process.

The ability of sulfur-oxidizing bacteria to oxidize sulfide and reduced inorganic sulfur compounds are attributed to an enzyme system present in the cell (Kurosawa *et al*., 1997), by which sulfide or the reduced inorganic sulfur compounds are biologically oxidize to sulfate (Das *et al*., 2006). Although in general sulfate is the major oxidation product of reduced inorganic sulfur compounds oxidation, other end-products or intermediates may be formed depending on the metabolic pathway used by the particular organisms (Schreiber and Pavlostathis, 1997). These intermediates compounds include polythionates, sulfite or elemental sulfur. The mechanism of inorganic sulfur oxidation appeared to differ radically among different sulfur bacteria (Kelly *et al*., 1997).

2.8 Sulfide Oxidase

There are a few reports on the biological role of the sulfide oxidase in heterotrophs. Sulfur metabolism in microorganisms are complicated due to involve of several enzymes and intermediates during the oxidation of sulfur compounds. Sulfide oxidase which catalyzes the oxidation of sulfide has been characterized from

Arthrobacter sp. and *Bacillus* sp. BN53-1 and was concentrated in this study (Mohapatra *et al*., 2006; Nakada and Ohta, 1999).

According to the Mohapatra *et al*. (2006) report, the purified sulfide oxidase was showed to be monomer with a molecular weight of 43 kDa. This molecular weight was found to be higher compared to the purified enzyme from the *Bacillus* sp. BN53-1 which is 37 kDa (Nakada and Ohta, 1999). The sulfide oxidase isolated from *Arthrobacter* sp. was cell-bound and had broad pH activities which are potentially useful in application of the wastewater treatment process (Mohapatra *et al*., 2006).

2.9 Oxidation of Inorganic Sulfur Compounds Pathway

According to Kelly (1999), through work has been carried out in order to elucidate the pathways of sulfur oxidation to sulfate. Most of the studies in sulfur oxidation pathways were conducted in chemolithotrophic bacteria with the best example in the genera of thiobacilli. During the studies, their finding showed that two or more oxidation pathway exists with the organisms capable of doing these activities being distributed through diverse genera of the proteobacteria. The oxidation pathway for the chemolithotrophic bacteria were differs among the organism. The sulfur oxidation pathway scheme illustrated in Figure 2.2 showed the general mechanism of reduced inorganic sulfur compounds oxidation by sulfur-oxidizing bacteria involving various sulfur enzymes.

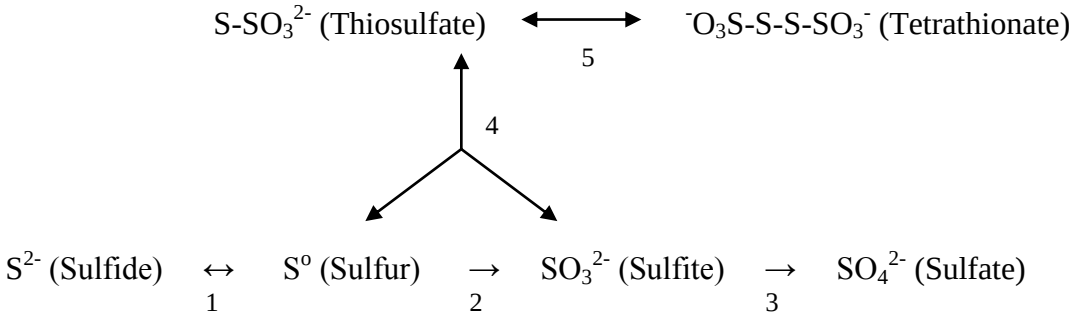

Figure 2.2: Oxidation of inorganic sulfur compounds pathway. The enzymes responsible for the reactions were identified as follows: reaction 1- sulfide oxidase; reaction 2 - sulfur-oxidizing enzyme (sulfur oxygenase); reaction 3 – sulfite oxidase; reaction 4 – rhodanese (thiosulfate – cleaving enzymes, sulfur transferase); and reaction 5 – thiosulfate-oxidizing enzyme (Suzuki, 1994)

2.10 Application of Sulfide Oxidase

It has been reported that a number of microorganisms are able to enzymatically oxidize sulfides. Sulfide oxidase can be useful in deodorizing farm animal feces (Yun and Ohta, 1997), mitigation of sulfides from effluent streams (Visser *et al*., 1997), landfills, wastewater facilities and also oil-field brine (Gevertz *et al*., 2000). The application of sulfide oxidase is summarized in Table 2.4.

Table 2.4: Application of sulfide oxidase

Microorganisms	Applications	References
Thiobacillus thiooxidans	Biological deodorization	Hirano *et al*., 1996
Thiomicrospira VO *Arcobacter* FWKO B	Oil field brine	Gevertz *et al*., 2000
Thiobacillus sp. W5	Wastewater	Visser *et al*., 1997
Bacillus sp. *Streptomyces* sp.	Compost piles	Nakada and Ohta, 1999
Thiobacillus sp.	Biological deodorization	Cho *et al*., 1991b

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 Sulfur-Oxidizer Medium Preparation

Selective medium containing sodium thiosulfate was used for isolation and enrichment of microorganism (Asku *et al*., 1991). Thiosulfate was supplemented in this medium as a sulfur source to the microorganism. It has been used as a preferred sulfur compound for sulfur-oxidizing bacteria laboratory studies, due to its stability at neutral pH and metabolized by all sulfur-oxidizing bacteria (Smith and Strohl, 1991). The medium was prepared by dissolving 10 g Bacto peptone (Becton-Dickinson), 1.5 g di-potassium hydrogen phosphate, 0.75 g ferric ammonium sulfate, 1.0 g sodium thiosulfate and 15.0 g agar in 1000 mL distilled water. The pH value of medium was adjusted to 7.0 using 1 M HCl. The medium was sterilized in autoclave at 121˚C and 101.3 kPa for 20 minutes and subsequently allowed to cool to about 45˚C, a temperature at which the agar remains molten. The molten agar medium was then poured into sterile Petri dishes and allowed the agar to solidify at room temperature. The liquid sulfur-oxidizer medium contains the same composition with sulfur-oxidizer agar except the 1.5 % (w/v) agar.

3.2 Culture Maintenance and Preservation

The sulfur-oxidizing bacteria was inoculated into fresh liquid sulfur-oxidizer medium and grown until the culture reached optical density of 0.5-0.8 at 600 nm. Cells were then harvested by centrifuging the culture at 4000 rpm for 20 minutes using refrigerated centrifuge. The cell-free supernatant was discarded and the resulting pellets were suspended with sulfur-oxidizer medium and mixed homogenously by vortex. Afterward, the pure culture of sulfur-oxidizing bacteria was transferred into cryovial containing cryopreservation beads stock and preserved at -20˚C for future experimental use.

3.3 Experimental Design

The experimental design of this study was divided into three stages which include isolation, characterization and identification of selected sulfur-oxidizer, optimization of sulfide oxidase in shake flasks and lastly the production of sulfide oxidase in bioreactor (Figure 3.1). This study also assesses the capability of newly isolated bacteria to break down thiosulfate into sulfate. Besides that, oxygen requirement of the bacteria during sulfide oxidase production in the bioreactor was also studied.

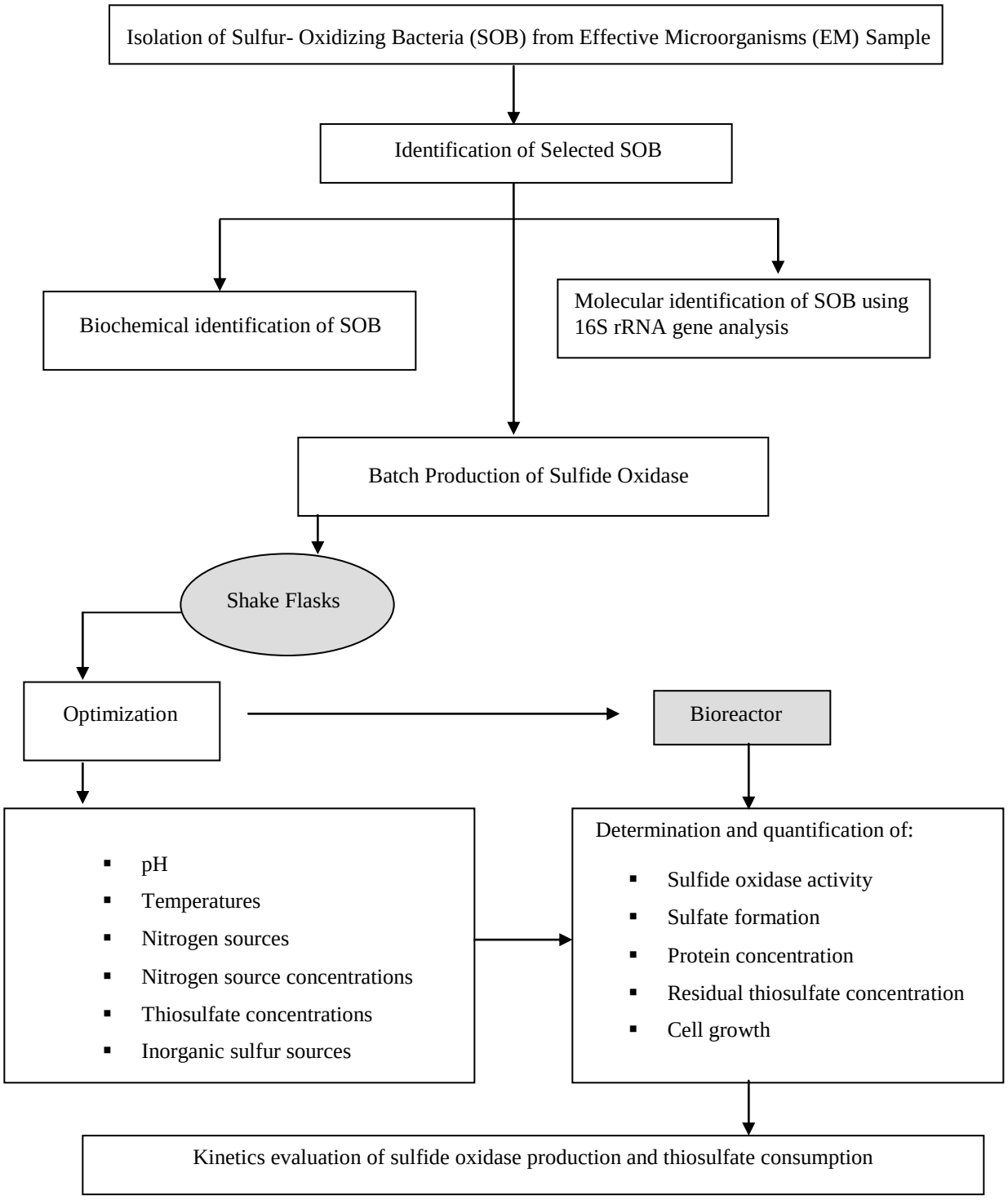

Figure 3.1: Experimental design chart

3.4 Inoculum Preparation

An inoculum was used as a starter culture in all fermentation process carried out during the optimization studies (section 5.3.3). Cells of sulfur-oxidizing bacteria were inoculated into 150 mL of sulfur-oxidizer liquid medium in 500 mL flask and incubated at 30˚C with 200 rpm agitation. The microbial growth was monitored using the turbidity measurement at 600 nm wavelength with a spectrophotometer. Once the initial OD reaches 0.5-0.8, the culture was ready to use as inoculum. Inoculation was carried out by transferring the inoculum into another sulfur-oxidizer broth medium for the optimization experiments.

3.5 Analytical Procedures

3.5.1 Determination of Cell Mass Concentration

Cell dry weight measurement was used to quantify the cell mass concentration in samples. About 5 mL aliquots were centrifuged for 20 minutes at 4000 rpm at 4˚C. The clear supernatant was carefully discarded and the remaining pellet was suspended using the medium broth. The mixture was mixed using vortex and poured into holding reservoir fitted on the pre-weighted dried 0.22 µm cellulose acetate filter membrane. The cell paste was dried in oven until a constant membrane weight was obtained. The differences in weight between membrane (empty) and membrane with cell paste was used to determine the dry weight of cell mass as g/L.

$$\text{Cell dry weight}(\text{g/L}) = \frac{\text{Weight of dried membrane and cell paste}(\text{g}) - \text{weight of membrane}(\text{g})}{\text{Volume of sample}(\text{L})} \quad \ldots(3.1)$$

3.5.2 Determination of Specific Growth Rate and Doubling Time

In microbiology, the word growth is defined as an increase in the number of microbial cells in a population, which can also be measured as an increase in microbial mass (Madigan *et al*., 2000). Growth rate (μ) is the change in cell number or cell mass per unit time, while doubling time (t_d) is the interval time where the number of cells doubles during each unit time period.

The specific growth rate was measured from the slope of cell mass curve (dry weight) by delineating points between the log growth phase, as represents by the equation below:

$$\mu = \frac{(\ln X_t - \ln X_0)}{t} \qquad \ldots(3.2)$$

Where;

X_0 = cell mass concentration at time 0 (g/L)

X_t = cell mass concentration at time t (g/L)

t = elapsed time between measurement (h)

μ = specific growth rate (h^{-1})

The relation between specific growth rate and doubling time of the cell mass is obtained from equation below:

$$t_d = \frac{\ln 2}{\mu} = \frac{0.693}{\mu} \qquad \ldots(3.3)$$

Where;

μ = specific growth rate (h^{-1})

t_d = doubling time (h)

3.5.3 Determination of Kinetic Parameters

Yield coefficient can be used to determine the growth kinetics. A measure of the overall efficiency of the conversion of substrate to cell mass or specific product is given by a yield coefficient (Y) which can be stated as $Y_{x/s}$, $Y_{p/s}$ and $Y_{p/x}$. The growth and product yield coefficient, $Y_{x/s}$ and $Y_{p/s}$, are very important parameters since they represent the efficiency of conversion of the substrate into cell mass or product.

Growth yield coefficient ($Y_{x/s}$) can be defined as dry cell weight obtained per amount of substrate consumed. The growth yield coefficient is an important as a mean of expressing the quantitative nutrient requirement of microorganism.

$$Y_{x/s} = \frac{\Delta X}{\Delta S} \qquad \ldots(3.4)$$

Where;

$Y_{x/s}$ = Growth yield coefficient (g dry cell weight /mg substrate consumed)

ΔX = Concentration of cell mass (g/L)

ΔS = Concentration of substrate consumed (mg/L)

Product yield coefficient ($Y_{p/s}$) can be defined as:

$$Y_{p/s} = \frac{\Delta P}{\Delta S} \quad \ldots(3.5)$$

Where;

$Y_{P/s}$ = Product yield coefficient (g product /mg substrate consumed)

ΔP = Concentration of product formed (g/L)

ΔS = Concentration of substrate consumed (mg/L)

Product yield coefficient ($Y_{p/x}$) that relates product formation with cell mass production is state as below:

$$Y_{p/x} = \frac{\Delta P}{\Delta X} \quad \ldots(3.6)$$

Where;

$Y_{p/x}$ = Product yield coefficient (g product /g cell mass)

ΔP = Concentration of product formed (g/L)

ΔX = Concentration of cell mass (g/L)

The productivity of a culture system maybe described as the output of cell mass per unit time of the fermentation. Thus, the productivity of a batch culture maybe represented as:

$$R_{batch} = \frac{X_{max} - X_0}{t_{final} - t_{initial}} \quad \ldots(3.7)$$

Where;

R_{batch} = cell productivity (g/L/h)

X_0 = initial cell concentration at inoculation (g/L)

X_{max} = maximum cell concentration (g/L)

$t_{initial}$ = initial time (h)

t_{final} = time at maximum cell concentration (h)

Sulfate productivity, $R_{sulfate}$ can be defined as:

$$R_{sulfate} = \frac{P_{max} - P_0}{t_{final} - t_{initial}} \quad \ldots(3.8)$$

Where;

$R_{sulfate}$ = sulfate productivity (g/L/h)

P_0 = initial sulfate production (g/L)

P_{max} = maximum sulfate production (g/L)

$t_{initial}$ = initial time (h)

t_{final} = time at maximum cell concentration (h)

The rate of growth associated products formation in a fermentation process is directly related to cells growth. The patterns of cells growth and product accumulation can be described by measuring the specific product formation rate (q_p). It shows that when product formation is growth associated, the specific rate of product formation (q_p) increase with specific growth rate.

$$q_{sulfate} = Y_{p/x} \, x \, \mu \quad \ldots(3.9)$$

Where;

$q_{sulfate}$ = specific rate of sulfate formation (g/g/h)

$Y_{p/x}$ = product yield coefficient base on cell concentration (g product /g cell mass)

μ = specific growth rate (h^{-1})

3.5.4 Sulfate Ion Determination

The amount of sulfate ion (SO_4^{2-}) produced during growth of sulfur-oxidizing bacteria on thiosulfate was determined spectrophotometrically. Sulfate was measured by adding 1:1 barium chloride solution (10% w/v) with supernatant followed by mixing the suspensions vigorously (Cha *et al*., 1999). A resulting white turbidity due to barium sulfate formation was measured at 450 nm with a Jenway 6300 spectrophotometer, U.K.

3.5.4.1 Sulfate Calibration Curve

Potassium sulfate (K_2SO_4) was used as standard to construct a sulfate calibration curve according to Kolmert *et al*. (2000). Standard sulfate solutions were made by dissolving K_2SO_4 in deionized water to known concentrations in the range 0 to 3 mM. The amount of turbidity formed is proportional to the sulfate concentration. The standard curve for sulfate measurement is presented in Appendix A1.

3.5.5 Sulfide Oxidase Assay

The sulfide oxidase activity was determined by measuring the product of enzymatic reaction, sulfate (SO_4^{2-}) in the reaction mixture. The enzyme assay protocol was originated from Hirano *et al*. (1996) method and modified in this study to fit with the sulfate measurement.

The reaction was initiated by addition of 0.5 mL of sodium sulfide (Na_2S) solution into the reaction mixture that contain 4.5 mL of 0.1 M sodium acetate buffer (pH 5.6) and 1 mL supernatant. The Na_2S solution was prepared by dissolving 0.06 g Na_2S in an alkaline solution consisting of 0.16 g NaOH, 0.02 g EDTA $Na_{2.}$ $2H_2O$ (sodium ethylenediaminetetraacetic acid), 2 mL glycerol and 40 mL distilled water. The Na_2S solution was freshly prepared prior to use. The mixture was incubated for 30 minutes at 30˚C and the reaction was subsequently terminated by the addition of 1.5 mL NaOH (1.0 M) followed by thorough mixing.

Concentration of sulfate ion formed during sulfide oxidase assay was detected by the reaction of equal volume of barium chloride solution (10% w/v) and reactant and the absorbance was measured at 450 nm using spectrophotometer. The measurement of sulfate ion in the sample was based upon the formation of barium sulfate after addition of barium chloride which leads to the white turbidity. The amount of turbidity formed is proportional to the sulfate concentration in the sample. One unit of sulfide oxidase activity was defined as amount of enzyme required to produce 1 µmol sulfate per hour per mL (U/mL).

3.5.6 Protein Concentration Determination

Protein concentration in the supernatant sample was colorimetrically determined according to Lowry method (Lowry, 1951). In this assay, when protein is placed in an alkaline system containing copper ion (Cu^{2+}), a colored complex forms between the peptide bonds of the protein and the copper ions. The Lowry protein assay applied the Folin-Ciocalteau phenol reagent to enhance the color response resulting a blue color complex. The absorbance of the complex was measured spectrophotometrically at wavelength of 750 nm. The concentration of protein was calculated from a standard curve generated using the Bovine Serum Albumin (BSA) as standard. Reagent preparation and the standard curve (0-1 mg/mL) for protein quantification is presented in Appendix A2.

3.5.7 Thiosulfate Determination

Thiosulfate analysis was conducted by adding 0.5 mL sodium thiocynate or supernatant into test tube containing 1 mL phosphate buffer. The mixture was added up with 1 mL deionized water to make the volume up to 2.5 mL followed by mixing well. Potassium cyanide solution (1.25 mL) was then added to the mixture, mix well and leave for 10 minutes. After that, 0.375 mL copper sulfate solution was placed to the mixture, vortex and leave again. After 20 minutes of incubation at room temperature, 0.75 mL ferric nitrate solution and 1.375 mL deionized water was added with mixing after each addition. The absorbance of the mixture was read at 460 nm using spectrophotometer. For thiosulfate analysis, thiocyanate was used as standard to quantify thiosulfate concentration in the supernatant. The standard curve for thiosulfate measurement in concentration range from 0 to 1 mM is presented in Appendix A3.

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CHAPTER 4

ISOLATION, SCREENING AND IDENTIFICATION OF POTENTIAL SULFUR-OXIDIZER FROM EFFECTIVE MICROORGANISMS (EM) SAMPLE

4.1 Introduction

Sulfide and thiosulfate are the most abundant reduced inorganic sulfur species in the environment and converted to sulfate primarily by bacterial action in the sulfur cycle. Sulfides are detrimental in the environment because of the toxicity to human health and corrosive effect on concrete and metallic infrastructure, besides it strong unpleasant smell (Oprime *et al*., 2001). While thiosulfate is one of the intermediate product of sulfide oxidation process in sulfur cycle and considered as important in nature.

The use of microorganisms able to remove reduced inorganic sulfur compounds in nature has been considered as a potential alternative. Oxidation of reduced inorganic sulfur compounds especially sulfide and thiosulfate was exclusively carried out by sulfur-oxidizing bacteria. The ability of sulfur-oxidizing bacteria to oxidize sulfide was

contributed to an enzyme system present in the cell known as sulfide oxidase and sulfate is the expected end product of reduced inorganic sulfur compounds oxidation by the sulfur-oxidizing bacteria (Visser *et al*., 1997).

The potential for oxidation of inorganic sulfur compounds, such as sulfide and thiosulfate is widely spread among the bacteria. Most of the known sulfur-oxidizing bacteria are members of the so-called 'colourless sulfur bacteria' belong to genera *Thiobacillus, Thiothrix, Thiomicrospira, Beggiatoa* and *Achromatium* (Gommers and Kuenen, 1988). However, there are many heterotrophic bacteria especially those belonging to the γ-subdivision of the *Proteobacteria* that are capable of oxidizing thiosulfate and sulfide (Sorokin *et al*., 2005b). Most of the example of heterotrophic bacteria belongs to the genera *Pseudomonas, Xanthomonas, Halomonas, Escherichia coli* and *Klebsiella* (Gommers and Kuenen, 1988).

This study describes the isolation work of potential sulfur-oxidizing bacteria from Effective Microorganisms (EM) sample. The isolates were then screened for sulfide oxidase production. The superior sulfur-oxidizing bacteria was identified using biochemical tests and 16S rRNA gene analysis.

4.2 Experimental Design

The flowchart of the experimental design carried out in this study is summarized in Figure 4.1.

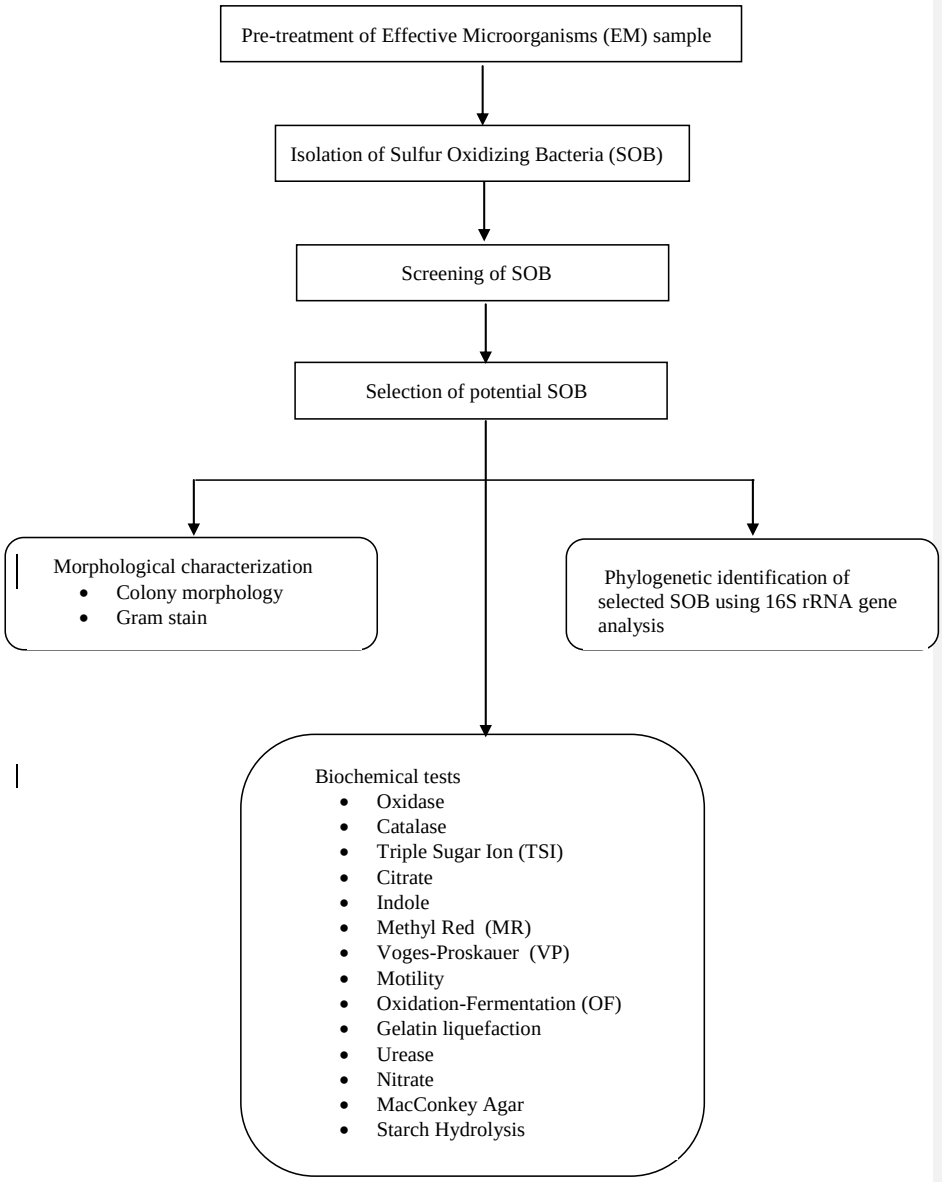

Figure 4.1: Experimental design chart

4.3 Materials and Methods

4.3.1 Media Preparation

The composition and preparation of sulfur-oxidizer medium (SOM) used throughout this study was listed in section 3.1.

4.3.2 Pre-treatment of Effective Microorganisms (EM) Sample

The EM sample that has been used as a source for isolation of potential sulfur-oxidizing bacteria was kindly donated from Associate Professor Dr. Zainoha Zakaria from Chemistry Department, UTM. In order to ascertain the recovery of viable microorganisms in the sample, the EM was pre-treated prior to cultivation. Pre-treatment was done by mixing 1 mL of EM with 1 mL molasses and 18 mL of sterile distilled water (method from previous work). The treated sample was then incubated at 30˚C overnight to enhance the population of microorganisms in the EM sample.

4.3.3 Isolation of Sulfur-Oxidizing Bacteria

Isolation of sulfur-oxidizing bacteria was performed by using direct plating method. The treated EM sample (0.1 mL) was poured onto the sulfur-oxidizer medium

agar. The sample was spread evenly on the sulfur-oxidizer medium plate using the sterile glass spreader. The plates were incubated at 30˚C for 24 h. The well defined isolated colonies appeared on the plate were picked up by wire loop and restreak on the other sulfur-oxidizer medium agar plate for purity conformation.

4.3.4 Screening of Sulfur-Oxidizing Bacteria

Screening of potential sulfur-oxidizing bacteria was performed by inoculating 10 % (v/v) inoculum into sulfur-oxidizer medium broth formulated by Asku *et al*. (1991). The cultures were then incubated at 30˚C and agitated at 200 rpm until the optical density of the cultures reached 0.6 – 0.8. The cultures were centrifuged at 4000 rpm, 4˚C for 30 minutes. The supernatant was analyzed for sulfide oxidase activity and protein concentration (section 3.5.4 - 3.5.6). Culture that gives the highest sulfide oxidase activity was selected and used throughout the studies.

4.3.5 Analytical Procedures

Quantification of sulfide oxidase activity and protein concentration that was determined in this works was described in detail in section 3.5.

4.3.6 Morphological Characterization

4.3.6.1 Colony Morphology

Morphological characteristics of strain SO02 that include configuration, margin, elevation and colour of the bacteria were examined using 24 h culture that was previously cultivated on sulfur-oxidizer medium agar. The morphology of the culture colony was observed using a stereomicroscope.

4.3.6.2 Cellular Morphology

The overnight-grown culture of strain SO02 was used for the observation of gram stained under 1000x immersion oil objective of bright-field microscope (Olympus C-35 AD-4). The procedures for gram staining can be reviewed in Appendix A7.

4.3.7 Biochemical Tests

Biochemical characterizations test of strain SO02 were performed using 24 h culture. The tests were include the carbohydrate metabolism test (starch hydrolysis, Methyl Red, Vogues Proskauer, Simmons citrate and triple sugar iron), nitrogen metabolism test (indole, urease, nitrate, motility and gelatin), specific enzymatic test

(catalase and oxidase) and growth on differential medium (MacConkey agar). The biochemical tests were performed as described by MacFaddin (1980). The procedure of biochemical tests is presented Appendix A7. The biochemical test results along with morphological and staining results are the key for the bacterial identification process. Bergey's Manual of Determinative Bacteriology has been used as reference for classification and identification of the SO02 bacteria (Holt *et al*., 1994).

4.3.8 Identification of Potential Microbe using 16S rRNA Gene Analysis

Strain SO02 showing the superior sulfide oxidase activity was identified at 16S rRNA gene level according to the steps shown in Figure 4.2.

4.3.8.1 DNA Extraction

The potential sulfur-oxidizing bacteria which known as SO02 strain in this work was grow on sulfur-oxidizer medium agar. The cell suspension for DNA extraction was prepared from 24 h culture incubated at 30˚C with agitation of 200 rpm. The 16S rRNA gene analysis began with DNA extraction of SO02 bacteria. The solutions needed in 16S rRNA gene analysis were provided in the Wizard® Genomic DNA Purification Kit (Promega Corp., Madison, Wis.).

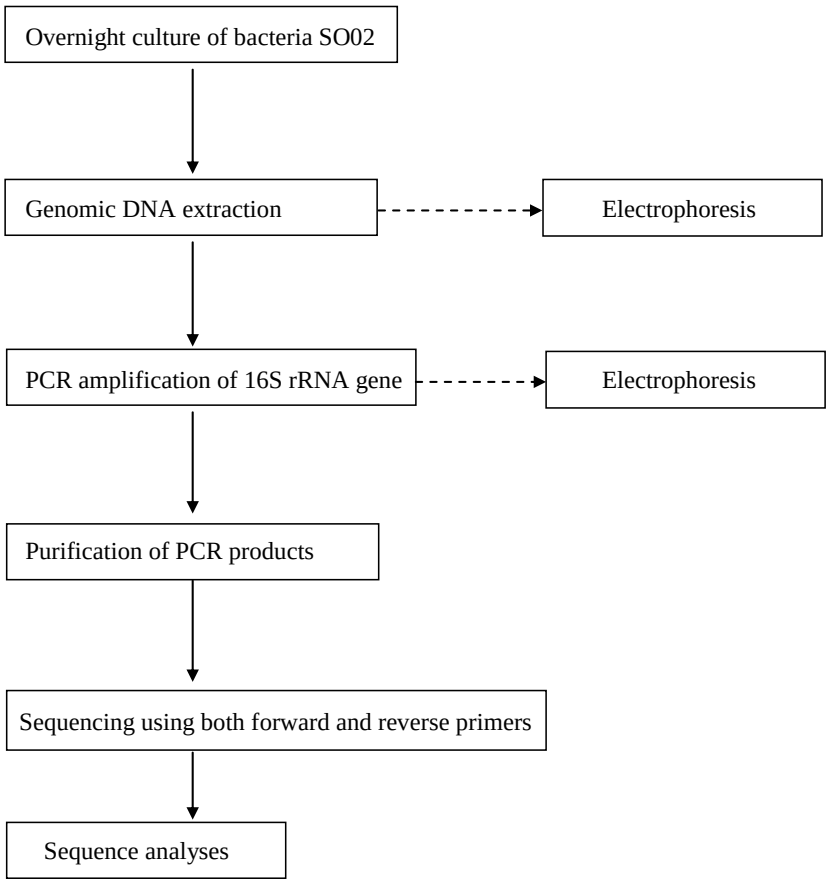

Figure 4.2: Outline of bacterial 16S rRNA gene identification

The overnight-grown culture with optical density, OD_{600nm} of 0.5 - 0.8 was transferred into microcentrifuge tube and centrifuged at 14,000 rpm for 5 min. Approximately 600 µl Nuclei Lysis Solution was added to the pellet and resuspended gently. The mixture was incubated at 80˚C for 5 min, and allowed to cool at room temperature. Afterward, 3 µL RNase solution was added to the cell lysate, mixed by inverted the tube 2-5 times and incubated at 37˚C for 30 min. After the sample cool at room temperature, 200 µL of Protein Precipitation Solution was added to the RNase-treated cell lysate. The sample was vortex vigorously at high speed for 20 seconds to mix the Protein Precipitation Solution with the cell lysate and incubated on ice for 5 min. After that, the sample was centrifuged at 14 000 rpm for 5 min.

The next step on DNA extraction was known as DNA precipitation and rehydration. The supernatant containing DNA obtained from the previous spin was transferred into a new clean microcentrifuge tube containing 600 µL of room temperature isopropanol. The sample was gently mix by inversion until the thread-like strands of DNA form a visible mass and centrifuged at 14,000 rpm for 5 min. The supernatant was carefully poured off and the tube was drain on a clean absorbent paper. The pellet was added with 600 µL of room temperature 70% (v/v) ethanol and gently inverts several times to wash the DNA pellet. The sample was centrifuged for 5 min and supernatant containing ethanol was discarded from the tube. The tube was drain on absorbent paper and the pellet was allowed to air-dry for 15 min. Approximately 100 µL Rehydration Solution was added and pellet containing DNA was rehydrate by incubating at 65˚C for 1 h. The isolated genomic DNA was stores at -20˚C until further uses.

4.3.8.2 Analyzing Genomic DNA

The extracted genomic DNA was analyzed via agarose gel electrophoresis to prove the presence of DNA after the extraction procedures. The agarose gel was prepared by dissolving 1.0 % (w/v) agarose in 1X Tris-Acetate-EDTA (TAE) buffer by heating the agarose solution in a microwave oven. TAE buffer was used because it provides faster electrophoretic migration of linear DNA and better resolution of supercoiled DNA. Ethidium bromide (10 mg/mL) was added to the molten agarose and poured slowly into a casting tray that was previously inserted with comb to form wells. The gel was left to cool and solidify for 20-40 min before removing the comb from the solidified gel. The tray was then placed onto the electrophoresis tank with sample wells are near to the cathode (black). DNA sample will migrate toward the anode (red) during the electrophoresis process. The electrophoresis tank was filled up with 1X TAE buffer until the gel was submerged under the buffer. Sample of genomic DNA (5 µL) was prepared by mixing with 2 µL loading dye before loading the mixture into respective wells on the agarose gel. The agarose gel electrophoresis was performed at 70 volts for 1 h or until the blue dye has migrated about two-third the length of the gel. The gel was then removed from the tray and place on the Gene-Flash to visualize and photographed the observed DNA band.

4.3.8.3 Polymerase Chain Reaction (PCR)

The PCR amplification for the nearly full-length 16S rRNA gene of SO02 bacteria was conducted using the universal primers, pA and pH' (Edwards *et al*., 1989). The sequences for the primers are displayed in Table 4.1.

Table 4.1: Sequences of universal primers (pA and pH') (Edwards *et al*., 1989)

Primers	Sequences
pA (Forward)	5'-AGA GTT TGA TCC TGG CTC AG-3'
pH' (Reverse)	5'-AAG GAG GTG ATC CAG CC-3'

The PCR reaction mixture was prepared by adding 10 µL of template DNA into tube containing 2.0 µL of each forward and reverse primer (10 pmol of each primer), 25 µL of 2X PCR master mix and topped up with 11 µL distilled water. Amplification was performed in a GeneAmp PCR system 9700 thermal cycler (Perkin-Elmer Corp., Emeryville, Calif.), under the following parameters: an initial denaturation at 94˚C for 3 min; 25 cycles, denaturation at 94˚C for 1 min, annealing at 50˚C for 1 min, and extension at 72˚C for 2 min; with 8 min final extension at 72˚C. The expected PCR products (1.5 kb) were cooled at 4˚C until removed and can be stored at -20˚C until further uses. The amplified 16S rRNA gene was now continued with purification procedures before sequencing can be done.

4.3.8.4 Analyzing PCR Products

The presence of PCR products can be distinguished using agarose gel electrophoresis (section 4.3.8.2). The PCR products were electrophoresed through 1.0 % (w/v) agarose gels containing ethidium bromide in 1X TAE buffer as the running buffer. A GeneRuler™ 1kb DNA Ladder Plus (Fermentas) was used as marker for sizing and approximate quantification of PCR products. Five microlitres of PCR products or molecular weight marker was mixed with 2 µL of blue loading dye. The

first lane of agarose gel was loaded with marker followed by loading the other wells with PCR products. The electrophoresis was then performed at 70 volts for 30 min. The gel was observed and photographed using the Gene-Flash.

4.3.8.5 Purifying PCR Products

Purification of PCR products was performed using the QIAquick® PCR Purification Kit (Qiagen). For PCR purification protocol, all the centrifugation steps were performed at 14 000 rpm using tabletop microcentrifuge (Eppendorf AG) at room temperature.

For PCR purification, 200 µL of Buffer PB was added to 40 µL of PCR sample and mixed up by inverted the tube for several times. A QIAquick spin column was placed in a provided 2 mL collection tube and sample was applied to the QIAquick column followed by centrifugation for 60 seconds. The flow-through liquid in the collection tube was discarded and the QIAquick column was placed back in the tube. The DNA was wash by adding 0.75 mL Buffer PE to the QIAquick column and centrifuge for 60 seconds. The flow-through was discarded and the QIAquick column was put back in the tube. The empty QIAquick column was centrifuge for an additional 1 min and continued by placing the QIAquick column in a clean 1.5 mL microcentrifuge tube. For complete elution of bound DNA from the column membrane, 50 µL of Buffer EB was directly dispensed to the center of the QIAquick membrane and centrifuge for 1 min. The average ~~eluate~~elute volume of purified PCR product was about 48 µL.

4.3.8.6 Sequence Analyses of 16S rRNA Gene

The PCR products were sent to First Base Laboratories Sdn. Bhd. for sequencing. Multiple sequence alignment against sequences from database was performed using BLASTn (Basic Local Alignment Search Tool), an online service provided by NCBI (National Center for Biotechnology Information) (http://www.ncbi.nlm.nih.gov/BLAST). The BLASTn program was used to identify the bacteria strain SO02 up to the genus and species level.

4.4 Results and Discussion

4.4.1 Isolation and Screening of Sulfur-Oxidizing Bacteria

Isolation of potential sulfur-oxidizing bacteria was initiated from Effective Microorganisms (EM) sample. EM has been used to manage odors and also as deodorizer for barns, waste treatment areas, home and also industrial (Sawada, 2006). Treatment of palm oil mill effluent (POME) ponds in Kota Tinggi, Johor with addition of EM shows that EM is able to treat the foul odor problem (Khamis, 2006). This indicates the existence of sulfur-oxidizing activity in ponds after addition of EM and increase the possibility to isolate sulfur-oxidizing bacteria from the EM sample.

Prior to isolation, the EM was pre-treated with molasses to sustain viable cells for the isolation purpose. Molasses is a byproduct of sugar production and contains carbohydrate, nitrogenous substances, vitamins and trace minerals (Yeon, 1988). Addition of molasses to the EM may increases the possibility to isolate the heterotrophic

microorganisms instead of autotrophic organisms due to the presence of organic compounds. The heterotrophic microorganisms will use the organic chemicals instead of inorganic as source of energy and electron donor (Madigan *et al*., 2000).

Isolation of potential sulfur-oxidizing bacteria was done in medium containing thiosulfate as a selective agent and incubated under aerobic condition. The sulfur-oxidizer medium contains sodium thiosulfate and without the addition of particular defined carbon sources to stimulate the consumption of thiosulfate by the bacteria (ref).

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The bacterial isolation was initiated by direct plating of EM-treated sample onto solid sulfur-oxidizer media using the spread plate technique and incubated aerobically at 30˚C. After 24 h, total of 60 colonies of potential sulfur-oxidizing bacteria was observed on sulfur-oxidizer plate medium. At this point, the colonies were considered as positive strains of sulfur-oxidizing bacteria due to the ability of microorganisms to grow on sulfur-oxidizer agar in the presence of thiosulfate (ref). The isolates were picked and restreak onto the new sulfur-oxidizer medium. Repeated picking and streaking of a well-isolated colony will ensure of obtaining a pure culture. The single colony obtained on the agar was considered as a pure isolate containing only one type of microorganism. The assumption is made based on that each viable cell can yield one colony (Madigan *et al*., 2000).

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The 60 colonies were subjected to undergo a screening process in order to choose potential superior extracellular sulfide oxidase producer. The screening of sulfide oxidase producers was done by measuring sulfide oxidase production during the bacterial growth. Out of 60 isolates, 11 isolates were found to have capability to convert sulfide into sulfate during sulfide oxidase assay (section 3.5.3) (Table 4.2). The highest sulfide oxidase producer was SO02 bacteria which produced sulfide oxidase activity up to 6.38 U/mL and 2.32 U/mg of specific activity.

Table 4.2: Screening results for sulfur-oxidizing bacteria

Strain No.	Sulfide Oxidase Activity (U/mL)	Protein concentration (mg/mL)	Specific Activity (U/mg)
01	2.65	2.76	0.96
02	**6.38**	**2.75**	**2.32**
03	2.26	2.79	0.81
04	4.03	1.98	2.04
05	6.03	2.75	2.19
06	2.60	2.77	0.94
07	0.56	2.55	0.22
08	1.00	2.70	0.37
09	2.99	2.82	1.06
10	0.35	2.92	0.12
11	3.51	2.93	1.20

The selected sulfur-oxidizing bacteria, SO02 was subjected to the biochemical tests and 16S rRNA gene analysis to identify the biochemical characteristics of the ~~culture~~ bacteria and also to determine the phylogenetic status of the bacteria.

4.4.2 Characterization and Identification of Potential Sulfur-Oxidizing Bacteria

4.4.2.1 Morphological and Cultural Characteristics

The SO02 bacteria which was isolated from EM and grown on sulfur-oxidizer medium agar produced circular, smooth, mucoid, round, opaque and creamy morphology. Single colony appeared less than 24 h at 30˚C. The SO02 bacteria was gram negative with rod shapes (Figure 4.3). The sulfur-oxidizing bacteria colonies grown on the sulfur-oxidizer medium agar were displayed in the Figure 4.4.

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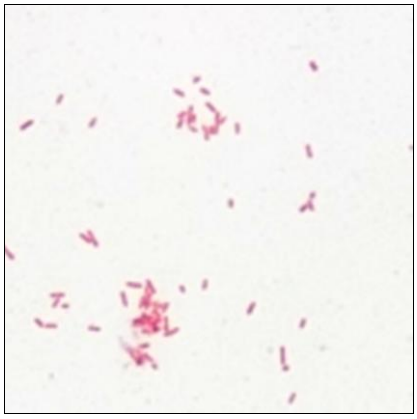

Figure 4.3: Gram morphology of strain SO02 under light microscope (1000x magnification)

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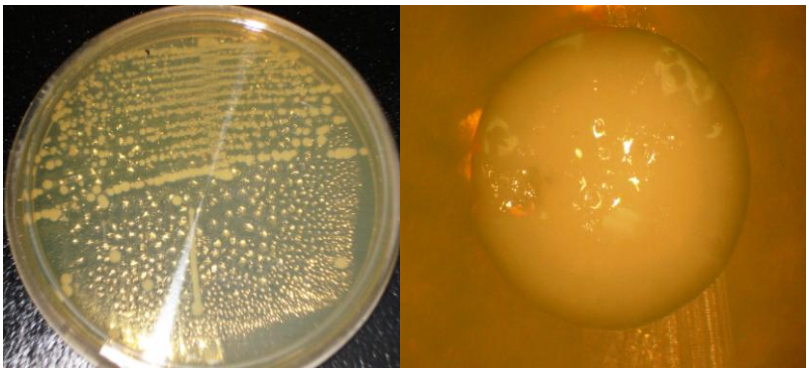

Figure 4.4: Strain SO02 on sulfur-oxidizer medium agar and colony morphology of the bacteria

4.4.2.2 Biochemical Features of strain SO02

Biochemical characteristics of microorganism were studied to identify the genus and species of unknown bacteria. Microorganisms are extremely versatile and their range of metabolic capabilities is very large (Norrell and Messley, 2003). These characteristics can be used to demonstrate the exceptional metabolic diversity of prokaryotic organisms and aid in differentiation between closely related genera or species (MacFaddin, 1980). Therefore, the strain SO02 was subjected to the biochemical tests in order to identify the bacteria.

Biochemical characteristics of strain SO02 is presented in Table 4.3. The strain SO02 showed an ability to grow on MacConkey agar with colourless or slightly whitish

colonies indicating that strain SO02 is gram negative enteric bacteria and non-lactose fermenter. MacConkey agar is a solid differential medium mainly used in identification of lactose fermenting, gram negative enteric pathogens and inhibits the growth of gram positive microorganism.

Strain SO02 was also tested for the ability ~~of microorganism~~ to hydrolyze starch by enzymatic action of amylase. The isolate showed negative result with starch hydrolysis test demonstrating that strain SO02 was unable to utilize starch due to lack of amylase production by the strain itself.

Besides amylase, the isolate was also ~~checked~~tested with ~~specific enzymatic tests including~~for catalase and ~~oxidase. The SO02 culture was tested~~and ~~positive~~gave positive results for both ~~enzyme~~ enzyme~~which showed the presence of catalase and oxidase in the cells~~. Catalase is an enzyme produce by many organisms which converts hydrogen peroxides into water and oxygen and cause foaming due to release of oxygen (Norrell and Messley, 2003). While, oxidase is an enzyme involved in oxidation reaction of microorganisms due to the presence of cytochrome oxidase system in aerobic and facultative anaerobic bacteria (Norrell and Messley, 2003; MacFaddin, 1980). The presence of oxidase in the cells suggesting that the strain SO02 was capable to carry out the oxidation process required in the sulfur compounds oxidation.

Carbohydrate metabolism of isolate was assessed by conducting tests involving Methyl Red (MR), Vogues Proskauer (VP), Simmons citrate, Oxidation - Fermentation (OF) and triple sugar iron (TSI). The MR-VP tests are useful in the differentiating between members of the *Enterobacteriaceae*. MR is a test that was used to identify bacteria that produced stable acid end products by means of mixed acid fermentation of glucose. Whereas, the VP test was used to determine the ability of organism to produce a neutral end product, acetoin from glucose fermentation (MacFaddin, 1980). The strain

SO02 was tested negative for MR and positive for VP. The combination of MR-VP test agreeds that strain SO02 produced acetoin during the glucose fermentation instead of acids, which increase the pH of media above 5.0.

Table 4.3: Biochemical characteristics of strain SO02

Tests	Results
Gram stain	Gram negative
MacConkey	+ ve
Oxidase	+ ve
Catalase	+ ve
Starch Hydrolysis	- ve
Methyl Red	- ve
Voges - Proskauer	+ ve
Simmons Citrate	+ ve
Oxidation - Fermentation	+ ve / + ve
Triple Sugar Ion	Acid/Acid No gas/No H_2S
Indole	- ve
Gelatin	+ ve
Urease	- ve
Nitrate	+ ve
Motility	- ve

* + ve = positive result; -ve result = negative result

Citrate agar was used to determine the capability of strain SO02 to utilize citrate as a sole carbon source for metabolism with resulting alkalinity (MacFaddin, 1980). The ~~citrate agar grown with strain SO02 was observed to turns~~ the green ~~colour~~color of agar into blue indicating that citrate has been utilized by the SO02 culture.

Oxidation – Fermentation (OF) test determines whether an organism metabolizes glucose by oxidation or fermentation. Some bacteria are capable of metabolizing glucose that is exhibit by acid production only under aerobic conditions, while others produce acid both aerobically and anaerobically (MacFaddin, 1980). The SO02 culture was tested positive for both tests suggesting that the bacteria was glucose fermenters and facultative anaerobes bacteria.

Triple sugar iron (TSI) agar is a medium used in the identification of gram negative enteric rods. This medium measures ability of bacteria to utilize three sugars, glucose, lactose and sucrose and also production of hydrogen sulfide (H_2S). The SO02 culture turns the slant and butt of the medium from red to yellow indicating acid production due to sugar fermentation and showed the ability of bacteria to ferment glucose and also sucrose or lactose. The test also showed that no H_2S and other gases was produced by the bacteria which could be visualized by lacking of blackening of the medium and the cracks or air gap at the bottom of agar during growth on TSI agar, respectively.

Nitrogen metabolism of strain SO02 was assessed by growing the culture in media without the addition of carbohydrate. This condition forces the organism to utilize any available proteins and amino acids presence in the media (Norrell and Messley, 2003). The nitrogen metabolism tests include indole, gelatin liquefaction, urease, nitrate reduction and motility. Strain SO02 was tested negative for the indole test by the absence of red colour in the surface of the medium after addition of Kovacs'

reagent. The negative result showed that SO02 culture was unable to produce indole as a result of amino acid tryptophan breakdown due to lack of tryptophanase in the cell of SO02.

The gelatin liquefaction test was used to determine the ability of a microbe to produce gelatinases that digest and liquefy gelatin (MacFaddin, 1980). Strain SO02 gave positive result towards this test by producing gelatinases which hydrolyze the gelatin into soluble peptides and amino acid.

Urea is a by-product of protein and nucleic acid decomposition. This test was conducted in order to investigate the ability of isolate to detoxify urea and hydrolyze it to ammonia and carbon dioxide which raise the pH of media (Norrell and Messley, 2003). The urease test is helpful to differentiate members of the *Enterobacteriaceae*. The strain SO02 was found to be urease-negative and this result indicated the non existence of urease in the SO02 cell culture.

Nitrate reduction is a characteristic of several organisms resulting in the formation of nitrite or some other nitrogenous compound such a nitrogen gas. The SO02 culture gave positive result for this test, which showed the ability of isolate to reduce nitrate into nitrite.

The aim of motility test is to determine whether the organism has a capability to motile or non-motile. Bacteria have flagella which facilitate the movement of the bacteria through the motility soft media which produced cloudy appearance. The strain SO02 has been tested negative due to growth of bacteria that only occurred along the stab line instead of spreading into the medium. From this result, it can be concluded that the strain SO02 was non-motile organism due to lack of flagella.

~~From the results of biochemical tests (Table 4.3), the biochemical characteristics of strain SO02 has pointed out to the characteristic of *Serratia* sp (Holt *et al*., 1994).~~ The bacteria strain SO02 identification was proceeded with 16S rRNA analysis to confirm the result of the biochemical tests since the bacterial identification by standard biochemical tests has a drawback due to possibility of strain to exhibit biochemical profiles that do not fit the patterns (Lau *et al*., 2002). Therefore, molecular approach of identification by 16S ribosomal RNA (rRNA) gene sequencing was studied to confirm the identity of strain SO02.

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4.4.2.3 Identification of strain SO02 using 16S rRNA Gene Analysis

The analysis of nucleic acid sequences (DNA) coding for 16 small ribosomal RNA (16S rRNA) gene is particular useful for phylogenetic analysis and characterization of an organism. The comparison of the gene sequences of bacterial species has shown that the 16S rRNA gene is highly conserved within a species and among species of the genus and can be used as a standard for bacterial identification (Lau *et al*., 2002). Due to the difficulty of obtaining intact RNA from bacteria, the 16S rDNA fragment which consist for almost the entire 16S rRNA gene will be targeted and amplify during the Polymerase Chain Reaction (PCR) ~~amplification (Edwards *et al*., 1989)~~. DNA is more suitable for PCR and much easier to obtain and even minimal amounts of DNA are sufficient for the amplification reaction (Edwards *et al*., 1989).

In this study, the genomic DNA of strain SO02 was successfully extracted. This was visibly shown by the clear band obtained by agarose gel electrophoresis. The extracted DNA was then ~~carried on~~ subjected to ~~with~~ PCR amplification using the universal primers pA and pH' as stated in section 4.3.8.3. The primers were design in

corresponds to the conserved regions of *E. coli* ~~(Edwards *et al*., 1989)~~. The forward primer, pA corresponds to the nucleotide positions 8 to 27 of *E. coli* 16S rRNA. While the reverse primer, pH'corresponding to *E. coli* 16S rRNA position 1542-1522 (Edwards *et al*., 1989). As ~~can be seen~~shown in Figure 4.5, the primers directed the synthesis of 1.5 kb DNA fragment indicating that the DNA fragment responds to the 16S rRNA gene was successfully amplified.

The pure PCR product was sent for sequencing to the First Base Laboratories Sdn. Bhd and the partial 16S rDNA sequence obtained was analyzed using the BLASTn online service provided by NCBI. Based on the BLASTn search, the partial sequence of 16S rDNA of strain SO02 shared 99% similarity with *Serratia* sp. From this result, together with the biochemical tests result ~~the~~ SO02 culture isolated from the EM could be confirmed ~~was identified~~ as *Serratia* sp. Therefore, ~~T~~the isolate was designated ~~known as~~as *Serratia* sp. strain SO02 ~~in this study~~.

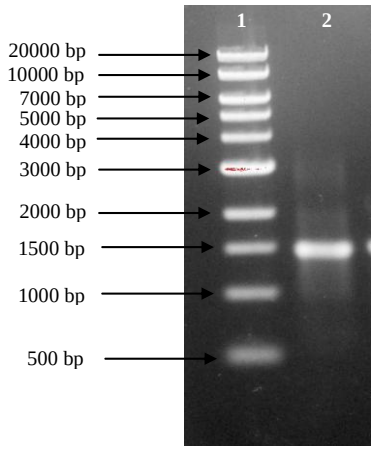

Figure 4.5: Agarose gel electrophoresis

Lane 1: GeneRuler, DNA ladder marker

Lane 2: PCR product of strain SO02

As determined by the 16S rRNA gene sequence analysis, the genus *Serratia* belongs to the γ-subclass of the *Proteobacteria* and under the family of *Enterobactericeae*. The member of the genus can be isolated from oil, water, plant surfaces and other environmental sites (Holt *et al*., 1994). Besides *Serratia*, the other genus of known sulfur-oxidizing bacteria situated under the family of *Enterobactericea* is *Escherichia* and *Klebsiella* (Mason and Kelly, 1988). While member of genus of sulfur-oxidizing bacteria that share the same subclass of the γ-*Proteobacteria* are *Xanthomonas* (Cho *et al*., 1992), *Pseudomonas* (Schook and Berk, 1978) and *Beggiatoa* (Das *et al*., 1996).

4.5 Conclusions

Total of 60 cultures of potential sulfur-oxidizing bacteria was isolated from EM sample. Out of 60 cultures, 11 strains were observed to produce sulfide oxidase. The highest sulfide oxidase producer was known as strain SO02 with sulfide oxidase activity of 6.38 U/mL and 2.32 U/mg specific sulfide oxidase activity. The physiological properties and phylogeny analysis of the newly isolated bacteria indicate that strain SO02 shared~~ing a~~ sequence similarity of 99% with the *Serratia* sp. The new isolate was then designated ~~known~~ as *Serratia* sp. strain SO02 throughout the study.

CHAPTER 5

OPTIMIZATION OF SULFIDE OXIDASE PRODUCTION BY *Serratia* sp. strain SO02

5.1 Introduction

Sulfur-oxidizing bacteria utilize reduced inorganic sulfur compounds for the biosynthesis of cellular material or transform these compounds as a respiratory energy generating process. For heterotrophic microorganisms, oxidation of thiosulfate or reduced inorganic sulfur compounds does not supply energy for the growth of cells (Das *et al*., 1996). Thiosulfate is the common oxidizable substrate suitable for growth of sulfur-oxidizing bacteria and has been widely used in the laboratory. The non-toxic characteristic of thiosulfate allows easier manipulation than hydrogen sulfide when growing the sulfur-oxidizing bacteria (Velasco *et al*., 2004).

There are few reports on the biological role of sulfide-oxidizing enzyme in heterotrophic microorganisms (Cho *et al*., 1992; Ohta *et al*., 1997; Nakada and Ohta,

1999). Sulfide oxidase is responsible in oxidation of sulfide with sulfate as a result of the process (Mohapatra et al., 2006).

In this study, a locally isolated sulfide oxidase producer, *Serratia* sp. strain SO02 was optimized for the maximum production of sulfide oxidase in a batch culture.

5.2 Experimental Design

The flowchart of the experimental design carried out in this study is summarized in Figure 5.1

5.3 Materials and Methods

5.3.1 Media Preparation

The sulfur-oxidizer medium (SOM) used in this optimization experiments were prepared as described in section 3.1. The original composition of sulfur-oxidizer medium was modified for the optimization of sulfide oxidase production.

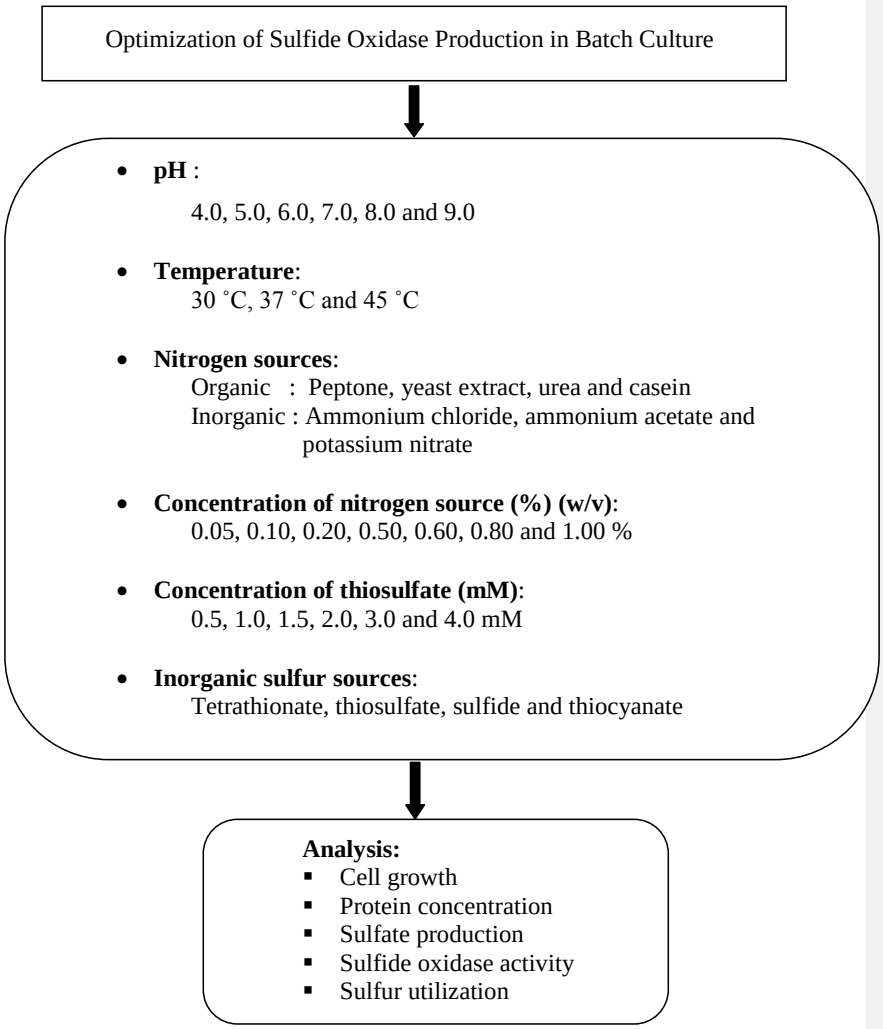

Figure 5.1: Experimental design for optimization of sulfide oxidase production by *Serratia* sp. strain SO02

5.3.2 Analytical Procedures

Determination of cell concentration, sulfide oxidase activity, sulfate production, protein concentration and thiosulfate utilization were described in detail in section 3.5.1 to section 3.5.7.

5.3.2.1 Tetrathionate, Thiocyanate and Sulfide Determination

Tetrathionate, thiocyanate and sulfide utilization by *Serratia* sp. strain SO02 were determined according to the modified method of Kelly *et al*. (1969). Tetrathionate reaction was conducted at room temperature but without the addition of Cu^{2+} as a catalyst. Reagents for the tetrathionate assay were prepared as similar to thiosulfate assay procedures (section 3.5.7) but without copper sulfate solution. This assay procedure was also applied for thiocyanate and sulfide determination as described by Sorokin *et al*. (2001) and Gommers and Kuenen (1988), respectively. Thiocyanate was used as standard to estimate the tetrathionate, thiocyanate and sulfide concentration in the supernatant fraction. The procedures and standard curve for tetrathionate, thiocyanate and sulfide estimation was presented in Appendix A5. The standard curve was constructed with thiocyanate concentration in the range of 0-1.0 mM.

5.3.3 Optimization of Sulfide Oxidase Production in Shake-Flask Culture

Six parameters were studied for the optimization of sulfide oxidase production by conventional methods. The parameters include effects of various initial pH (pH 4.0-9.0), incubation temperature (30˚C - 45˚C), nitrogen sources (yeast extract, urea, casein, ammonium chloride, ammonium acetate and potassium nitrate), concentration of nitrogen source (0.05 to 1.00% w/v), concentration of thiosulfate (0.5 – 4.0 mM) and various inorganic sulfur sources (potassium tetrathionate, sodium sulfide and sodium thiocyanate). Fermentation was carried out in a batch process using shake-flask culture technique.

5.3.3.1 Effect of Various Initial pH

The initial pH of the culture media was adjusted within 4.0 to 9.0 using 1 M HCl or 1 M NaOH. This pH range was chosen in order to study effect of pH on the bacterial growth and sulfide oxidase production at a wide range of pH and as referred to Mohapatra *et al*. (2006) study. pH adjustment was done before autoclaving process. After autoclaving and cooling, 10% (v/v) inoculum with optical density of 0.5-0.8 was added to each flask. The cultures were incubated at 30˚C with 200 rpm agitation speed and samples were withdrawn at every 3 hours interval to analyze as described in section 3.5.1 to section 3.5.7.

The initial pH of culture media was adjusted within 4.0 to 9.0 using 1 M HCl or 1 M NaOH. pH adjustment was done before autoclaving process. After autoclaving and cooling, 10% (v/v) inoculum with optical density of 0.5-0.8 was added to each flask.

The cultures were incubated at 30˚C with 200 rpm shaking speed and samples were withdrawn at 3 hours interval to analyze as described in section 3.5.1 to section 3.5.7.

5.3.3.2 Effect of Various Incubation Temperatures

The influence of incubation temperature was studied at various temperatures of 30˚C, 37˚C and 45˚C, keeping the optimum initial pH at 5.0. The culture was agitated at 200 rpm for 24 hours. Samples were withdrawn for every 3 hours interval for the analysis purpose.

5.3.3.3 Effect of Various Nitrogen Sources

Sulfur-oxidizer liquid medium was amended with varying nitrogen sources. Nitrogen sources was chosen based on Mohapatra *et al*. (2006) and Saxena *et al*. (2007) studies. Peptone in the original formulation was substituted with other organic nitrogen sources, particularly yeast extract, urea and casein; inorganic sources, particularly ammonium chloride, ammonium acetate and potassium nitrate. The concentration of nitrogen sources was remained the same as original composition, at 1% (w/v). Each medium was adjusted to pH 5.0 and the cultures were incubated at 30˚C. Samples were withdrawn at regular interval and kept frozen until further analysis was conducted.

5.3.3.4 Effect of Various Peptone Concentration

The sulfur-oxidizer medium was prepared by varying the concentration of peptone from 0.05 to 1.00% (w/v). Each flask of medium was adjusted to pH 5.0 and supplemented with peptone at varying concentration. Fermentation was conducted at 30˚C with agitation speed of 200 rpm. Samples were eluted at appropriate time intervals and analysis was conducted as illustrated in section 3.5.1 to section 3.5.7.

5.3.3.5 Effect of Various Thiosulfate Concentration

To study the effect of thiosulfate concentration on sulfide oxidase production and thiosulfate utilization by *Serratia* sp. strain SO02, sulfur-oxidizer medium was modified by varying the concentration of thiosulfate in each flask. Concentration of thiosulfate in this experiment was in range of 0.5 – 4.0 mM and supplied with peptone as nitrogen source at concentration of 0.2% (w/v) with initial pH of 5.0. Culture media were incubated at 30˚C for 24 hours and analysis was performed at 3 hours intervals to determine the favorable concentration of thiosulfate to achieve maximum sulfide oxidase activity.

5.3.3.6 Effect of Various Reduced Inorganic Sulfur Sources

The original composition of sulfur-oxidizer medium uses thiosulfate as a sulfur source. The medium was amended by replacing sodium thiosulfate with a variety of inorganic sulfur sources such as potassium tetrathionate, sodium sulfide and sodium thiocyanate at concentration of 0.5 mM. The choice of sulfur sources that were used in this study was referred to Mohapatra *et al*. (2006) study. Incubation was conducted at 30˚C with agitation speed of 200 rpm. Samples were taken out at regular period and analyze as stated in section 3.5.1 to section 3.5.6 and section 5.3.2.

5.4 Results and Discussion

5.4.1 Optimization of Sulfide Oxidase Production

5.4.1.1 Effect of Initial pH on Sulfide Oxidase Production

Sulfide oxidase synthesis and bacterial growth of *Serratia* sp. strain SO02 was observed in sulfur-oxidizer medium over a wide range of pH, between pH 4.0 to 9.0. Effect of initial culture pH on production of sulfide oxidase by *Serratia* sp. strain SO02 is summarized in Table 5.1 and Figure 5.2. The initial pH culture was observed to have a pronounced effect on sulfide oxidase production. Studies of pH effects towards enzyme production demonstrated that sulfide oxidase synthesis was repressed when the initial pH medium was above pH 7.0 (Figure 5.2). The results suggested that there was stimulation of sulfide oxidase synthesis at pH 5.0 and higher enzyme production at this pH was concluded as a result of increased cell growth (UI Qader *et al*., 2006). A drastic decrease in enzyme activity was observed when *Serratia* sp. strain SO02 was grown in

pH 9.0 (0.357 U/mL). At this pH, the activity of sulfide oxidase was decreased about 7.8 fold compared to the highest activity that was attained at pH 5.0 (2.929 U/mL).

In this study, pH 5.0 was observed to be the required pH for the maximal production of sulfide oxidase in sulfur-oxidizer medium by *Serratia* sp. strain SO02 culture. Hirano *et al*. (1996) also used the same initial culture pH in his work for cultivation of *Thiobacillus thiooxidans* for removal of hydrogen sulfide and trimethylamine. Besides that, another *T. thiooxidans* had been grown at pH 5.0 on thiosulfate by Jefferey *et al*. (2001) to study the mechanism of inorganic sulfur compound oxidation by the bacteria. However, Mohapatra *et al*. (2006) had reported in their work that the optimum pH for sulfide oxidase production by *Arthrobacter* sp. strain FR-3 in GY medium was pH 7.5.

As stated in Table 5.1, thiosulfate utilization by the bacteria was observed to increase as initial pH of the medium increase but decreased when pH of the medium above 6.0 achieving maximum utilization at this pH. This result suggested that thiosulfate utilization under alkaline condition was low because of the reduction in cell mass as compared to the maximum cell mass achieved at pH 6.0. The decrease of *Serratia* sp. strain SO02 cell mass concentration at alkaline condition of pH 8.0 and 9.0 was probably due to the alteration in three-dimensional protein structure of the bacterial enzyme systems (Hogg, 2005). The same pH (pH 6.0) was discovered by Sorokin *et al*. (2005b) for the optimal thiosulfate oxidation with haloarchaea strain HG 1.

Table 5.1: Effect of various initial culture pH towards batch fermentation of *Serratia* sp. strain SO02 in shake-flask culture

Kinetic Parameters/ pH	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9
Specific growth rate, μ (h^{-1})	0.147	**0.150**	0.164	0.168	0.170	0.183
Doubling time, t_d (h)	4.730	**4.620**	4.220	4.140	4.080	3.780
X_{max} (g/L)	5.898 ± 0.397	5.693 ± 0.383	6.053 ± 0.408	5.569 ± 0.375	5.470 ± 0.368	5.402 ± 0.364
Cell mass productivity, R_{batch} (g/L/h)	0.194	**0.205**	0.213	0.195	0.216	0.193
Max sulfide oxidase activity (U/mL)	1.170 ± 0.079	2.929 ± 0.197	2.191 ± 0.148	1.533 ± 0.103	0.807 ± 0.054	0.375 ± 0.025
Protein concentration (mg/mL)	2.888 ± 0.388	**2.693** ± 0.362	2.376 ± 0.319	3.034 ± 0.408	3.071 ± 0.413	3.113 ± 0.418
Specific activity (U/mg)	0.407 ± 0.027	**1.092** ± 0.074	0.926 ± 0.062	0.508 ± 0.034	0.264 ± 0.018	0.121 ± 0.008
Concentration of thiosulfate consumed (mM)	0.308	**0.410**	0.431	0.277	0.225	0.205
Thiosulfate utilization (%)	7.692	**10.256**	10.769	6.921	5.632	5.130
Max sulfate product (g/L)	0.375	**0.404**	0.320	0.184	0.087	0.060
Sulfate productivity, $R_{sulfate}$ (g/L/h)	0.016	**0.0156**	0.013	0.008	0.004	0.003
Specific rate of sulfate formation, q_p (g/g/h)	0.012	**0.011**	0.010	0.007	0.002	0.003
[1]$Y_{x/s}$ (g cell / mg thiosulfate)	0.135	**0.107**	0.106	0.151	0.198	0.201
[2]$Y_{p/s}$ (g sulfate / mg thiosulfate)	0.011	**0.008**	0.006	0.006	0.004	0.003
[3]$Y_{p/x}$ (g sulfate / g cell)	0.080	**0.076**	0.061	0.039	0.014	0.012

[1] $Y_{x/s}$ = Growth yield coefficient (g dry cell weight /mg substrate consumed)
[2] $Y_{P/s}$ = Product yield coefficient (g product /mg substrate consumed)
[3] $Y_{p/x}$ = Product yield coefficient (g product /g cell mass)

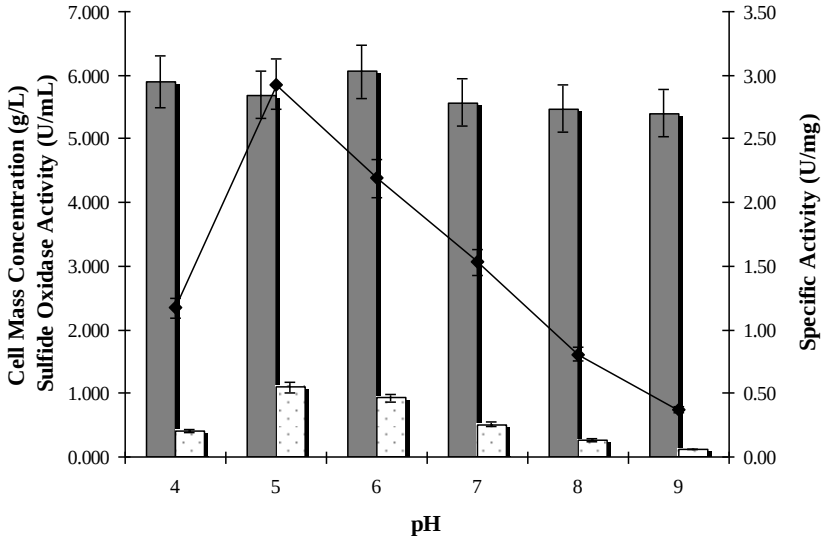

Figure 5.2: Effect of initial pH culture towards sulfide oxidase activity (-♦-), specific activity (-□-) and *Serratia* sp. strain SO02 growth (-■-)

Results showed that initial culture pH of 5.0 and 6.0 were required for ensuring the excellent growth of *Serratia* sp. strain SO02 bacteria in the presence of thiosulfate. However, optimal pH for the growth of *Serratia* sp. strain SO02 and sulfide oxidase production were found to be different, as maximal cell growth was observed at pH 6.0 (6.053 g/L) while the production of sulfide oxidase was optimum at pH 5.0. Besides that, sulfate production by *Serratia* sp. strain SO02 was also observed to be maximum at pH 5.0 (0.404 g/L). This situation suggested that the bacteria prefer slightly acidic conditions with pH 5.0 to produce maximal sulfide oxidase and sulfate rather than pH 6.0.

5.4.1.2 Effect of Temperature on Sulfide Oxidase Production

The influence of temperature on the performance of sulfide oxidase production and microbial growth is presented in Table 5.2. Optimum temperature for the production of sulfide oxidase occurred at 30˚C which is proportional to high cell density of *Serratia* sp. strain SO02 (5.936 g/L). Both sulfide oxidase production and bacterial growth decreased with the increment of temperature beyond 30˚C. As the temperature rise to 45˚C, the activity of sulfide oxidase reduced by 3.68 fold as compared to the optimal temperature (30˚C), proving that temperature plays a major role in sulfide oxidase production by *Serratia* sp. strain SO02. The reduction of sulfide oxidase activity beyond 30˚C corresponded to a decrease in specific growth rate (μ) of the culture. The μ value of *Serratia* sp. strain SO02 decreased by 1.60 fold when the temperature rised from 30˚C to 45˚C. According to Hogg (2005), the reduction of enzyme activity may be due to the enzyme denaturation that leads to the loss of catalytic properties.

The *Serratia* sp. strain SO02 was able to survive at temperature within 30˚C and 37˚C, whereas at 45˚C the bacterial growth was sharply declined. These results indicated that *Serratia* sp. strain SO02 is a mesophiles microorganism, where the optimal bacterial growth achieved at a midrange temperature. At optimal temperature, chemical and enzymatic reactions in the cell proceeded at rapid rates and the bacterial growth becomes faster (Madigan *et al*., 2000). Beyond the optimal temperature, the growth starts to decrease due to the denaturation of the bacterial enzymes and caused the rate of growth to fall sharply (Hogg, 2005).

Table 5.2: Effect of various temperatures towards batch fermentation of *Serratia* sp. strain SO02 in shake-flask culture

Kinetic Parameters/ Temperature	30˚C	37˚C	45˚C
Specific growth rate, μ (h^{-1})	**0.181**	0.148	0.116
Doubling time, t_d (h)	**3.834**	4.687	6.001
Max cell mass, X_{max} (g/L)	**5.936 ± 0.400**	5.012 ± 0.338	1.694 ± 0.114
Cell mass productivity, R_{batch} (g/L/h)	**0.244**	0.196	0.352
Max sulfide oxidase activity (U/mL)	**2.917** ± 0.196	1.533 ± 0.103	0.761 ± 0.051
Protein concentration (mg/mL)	**3.436** ± 0.119	3.528 ± 0.122	3.524 ± 0.122
Specific activity (U/mg)	**1.011** ± 0.159	0.524 ± 0.082	0.226 ± 0.042
Concentration of thiosulfate utilized (mM)	**0.441**	0.338	0.133
Thiosulfate utilization (%)	**11.026**	8.462	3.337
Max sulfate product (g/L)	**0.385**	0.192	0.061
Sulfate productivity, $R_{sulfate}$ (g/L/h)	**0.016**	0.011	0.003
Specific rate of sulfate formation, $q_{sulfate}$ (g/g/h)	**0.139**	0.008	0.023
[1]$Y_{x/s}$ (g cell / mg thiosulfate)	**0.084**	0.078	0.307
[2]$Y_{p/s}$ (g sulfate / mg thiosulfate)	**0.008**	0.002	0.001
[3]$Y_{p/x}$ (g sulfate / g cell)	**0.077**	0.054	0.199

Serratia sp. strain SO02 was shown to achieve optimal growth at temperature of 30˚C and produced 5.936 g/L cell mass concentration at the end of incubation. At this point of temperature, *Serratia* sp. strain SO02 achieved the highest specific growth rate with value of 0.1808 h^{-1}, compared to incubation at 37˚C and 45˚C. The value of doubling time, 3.83 h indicated that *Serratia* sp. strain SO02 populations required less time to generate cells during the bacterial growth. The cell mass productivity of *Serratia* sp. strain SO02 at 30˚C was attained at 0.244 g/L/h. On the other hand, drastic

[1] $Y_{x/s}$ = Growth yield coefficient (g dry cell weight /mg substrate consumed)
[2] $Y_{P/s}$ = Product yield coefficient (g product /mg substrate consumed)
[3] $Y_{p/x}$ = Product yield coefficient (g product /g cell mass)

decreased of cell growth can be observed at 45˚C incubation with concentration of cell mass produced at 1.694 g/L and the lowest value of μ (0.116 h^{-1}).

As well as sulfide oxidase production, temperature also affected sulfate formation by *Serratia* sp. strain SO02. It appeared that the maximum production of sulfate occurred at 30˚C and sulfate concentration start to decrease beyond that temperature. *Serratia* sp. strain SO02 produced sulfate at 30˚C with concentration of 0.385 g/L and decreased to 0.061 g/L when the bacteria was grown at 45˚C. This is reasonable because apparently at 45˚C bacterial growth was repressed and thiosulfate utilization was reduced, thus leads to least amount of sulfate produced. Wang *et al*. (1979) suggested that product formation can be similarly related to nutrient consumption, and product formation cannot be occurred without the presence of cells. Thus it was expected that growth and product formation were closely related to nutrient utilization, and that product formation will be coupled to cell growth (Wang *et al*., 1979).

Thiosulfate utilization capability of *Serratia* sp. strain SO02 was observed to be optimum at 30˚C (11.0 %). A shift to higher temperature of 45˚C appears to affect thiosulfate utilization capability of *Serratia* sp. strain SO02 by 3.31 % lower as compared to the optimal temperature (30˚C). *Serratia* sp. strain SO02 is a mesophile microorganism and thus mesophilic temperature was suitable for thiosulfate utilization by the bacteria. Furthermore, many researchers used 30˚C to investigate thiosulfate utilization by sulfur-oxidizing bacteria. Ito *et al*. (2004) and Sorokin *et al*. (2005b) reported that 30˚C is the most suitable temperature for cultivation and utilization of thiosulfate by sulfur-oxidizing bacteria. However, selection of temperature depends on the species and natural habitat of the bacteria itself. Sulfur-oxidizing of *Thermus scotoductus* for example, showed an ability to grow and consumed thiosulfate at high temperature of 65˚C because the bacteria was originally isolated from a hot spring in Iceland (Skirnisdottir *et al*., 2001b).

Results suggested that *Serratia* sp. strain SO02 required temperature at 30˚C for cell growth, synthesis of sulfide oxidase and also thiosulfate utilization. Therefore, 30˚C was chosen as the optimal temperature for *Serratia* sp. strain SO02 fermentation. Similar finding has been reported on *Arthrobacter* sp. where growth and production of sulfide oxidase was found to be maximal at 30˚C fermentation by Mohapatra *et al*. (2006).

5.4.1.3 Effect of Nitrogen Sources on Sulfide Oxidase Production

Effect of nitrogen source towards sulfide oxidase synthesis and cell growth was studied by amended sulfur-oxidizer medium with varying nitrogen sources. Peptone in the original formulation was substituted with other organic nitrogen sources, particularly yeast extract, urea and casein; inorganic nitrogen sources, particularly ammonium chloride, ammonium acetate and potassium nitrate. The concentration of nitrogen source in the sulfur-oxidizer medium was maintained as original composition at 1% (w/v). The influence of organic and inorganic nitrogen sources on cell growth and enzyme yield was depicted in Table 5.3.

Table 5.3: Effect of 1 % (w/v) organic and inorganic nitrogen sources towards batch fermentation of *Serratia* sp. strain SO02 in shake flask culture

Kinetic Parameters/ Nitrogen Sources	Ammonium Acetate	Ammonium Chloride	Potassium Nitrate	Peptone	Yeast Extract	Casein Hydrolysate	Urea
Specific growth rate, μ (h^{-1})	0.042	0.128	0.102	**0.181**	0.232	0.294	0.057
Doubling time, t_d (h)	16.348	5.436	6.816	**3.834**	2.983	2.361	12.203
Max cell mass, X_{max} (g/L)	0.571 ± 0.038	2.853 ± 0.192	2.382 ± 0.160	5.935 ± 0.400	9.700 ± 0.653	14.748 ± 0.993	0.676 ± 0.046
Cell mass productivity, R_{batch} (g/L/h)	0.008	0.360	0.272	**0.084**	0.447	0.745	0.018
Max sulfide oxidase activity (U/mL)	1.226 ± 0.038	1.022 ± 0.069	1.703 ± 0.115	**2.917** ± 0.196	1.941 ± 0.131	0.863 ± 0.058	1.249 ± 0.084
Protein concentration (mg/mL)	1.028 ± 0.200	0.735 ± 0.143	0.840 ± 0.164	**2.988** ± 0.583	2.274 ± 0.444	2.921 ± 0.570	0.797 ± 0.155
Max specific activity (U/mg)	1.209 ± 0.155	1.408 ± 0.181	2.054 ± 0.264	**0.989** ± 0.127	0.864 ± 0.111	0.299 ± 0.038	1.588± 0.204
Concentration of thiosulfate consumed (mM)	0.185	0.246	0.277	**0.441**	0.410	0.205	0.144
Thiosulfate utilization (%)	4.615	6.154	6.923	**11.026**	10.256	5.128	3.590
Max sulfate product (g/L)	0.023	0.118	0.126	**0.244**	0.355	0.045	0.029
Sulfate productivity, $R_{sulfate}$ (g/L/h)	0.001	0.008	0.007	**0.016**	0.039	0.002	0.001
Specific rate of sulfate formation, $q_{sulfate}$ (g/g/h)	0.009	0.009	0.008	**0.139**	0.011	0.001	0.005
[1]$Y_{x/s}$ (g cell / mg thiosulfate)	0.005	0.078	0.053	**0.385**	0.175	0.583	0.020
[2]$Y_{p/s}$ (g sulfate / mg thiosulfate)	0.003	0.013	0.003	**0.008**	0.008	0.002	0.002
[3]$Y_{p/x}$ (g sulfate / g cell)	0.209	0.071	0.074	**0.077**	0.048	0.003	0.083

[1] $Y_{x/s}$ = Growth yield coefficient (g dry cell weight /mg substrate consumed)
[2] $Y_{p/s}$ = Product yield coefficient (g product /mg substrate consumed)
[3] $Y_{p/x}$ = Product yield coefficient (g product /g cell mass)

The influence of organic and inorganic nitrogen sources on sulfide oxidase production is demonstrated in Table 5.3 and **Figure 5.5**. The supplementation of culture medium with peptone obviously stimulated bacterial growth and enhanced the sulfide oxidase production (2.917 U/mL). In this study, sulfide oxidase production was also enhanced by the presence of yeast extract and potassium nitrate with sulfide oxidase activity of 1.941 U/mL and 1.703 U/mL, respectively. Asgher *et al*. (2007) stated that organic sources like yeast extract and peptone usually have stimulating effect on α-amylase synthesis. The result of this study is in good agreement with Asgher *et al*. (2007) where stimulation of sulfide oxidase activity can be seen when peptone and yeast extract was supplied in the medium. Addition of potassium nitrate into the medium was unable to enhance the microbial growth to the level where peptone and yeast extract was used, but nitrate still induce sulfide oxidase production with activity of 1.703 U/mL. The positive effect of nitrate to induce sulfide oxidase synthesis might be contributed by the biochemical characteristic of *Serratia* sp. strain SO02 itself that able to use nitrate in the medium (refer to section 4.4.2.2).

Comment [kobenz1]: Why

Reduction of sulfide oxidase activity by 3.40 fold was observed in the presence of casein hydrolysate in the medium as compared to the use of peptone. Even though supplementation with casein improved cell growth to the highest level (14.748 g/L) as compared to peptone and yeast extract, evidently it decreased sulfide oxidase yield. Addition of casein only yields sulfide oxidase activity at 0.863 U/mL, the lowest amongst the tested nitrogen source. This result proves that supplementation of *Serratia* sp. strain SO02 with casein only encourage cells to grow rapidly at the highest specific growth rate (0.294 h^{-1}) instead of producing sulfide oxidase which is shown in the lowest sulfide oxidase yield (0.863 U/mL).

In urea-containing medium, repression of sulfide oxidase production and microbial growth was demonstrated by *Serratia* sp. strain SO02. This was differed from

Taylor and Hoare (1969) who reported that *Thiobacillus novellus* was able to grow aerobically in thiosulfate-containing medium with the supplementation of urea. Presence of urea in the medium failed to stimulate cell growth and sulfide oxidase production by *Serratia* sp. strain SO02. This could be attributed by the inability of *Serratia* sp. strain SO02 to use urea as a nitrogen source due to lack of urease. Biochemical characteristic of *Serratia* sp. strain SO02 has proved that the bacteria was unable to degrade urea (refer to section 4.4.2.2).

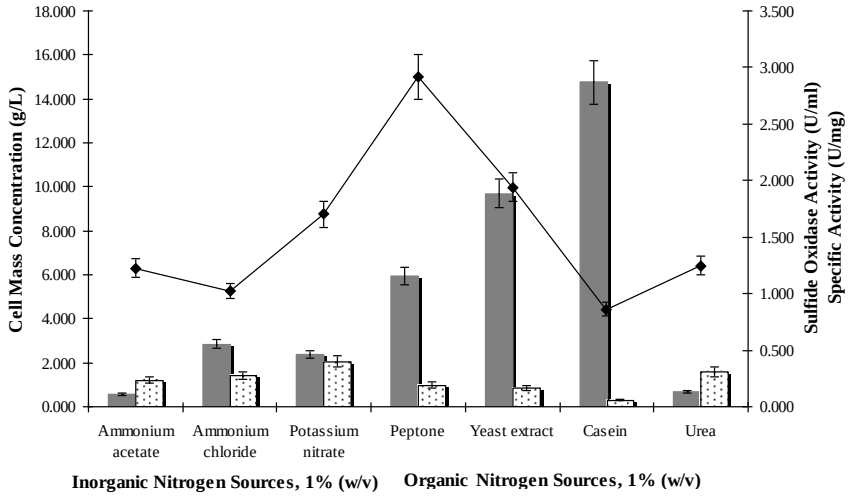

Figure 5.3: Influence of various nitrogen sources at concentration of 1% (w/v) towards sulfide oxidase activity (-♦-) and *Serratia* sp. strain SO02 growth (-■-).

Most enzymes including amylase, protease and nitrate reductase are repressed by ammonium salts (Saxena *et al*., 2007; Sanchez and Demain, 2002). In this study, ammonium salts also showed an inhibitory effect towards bacterial growth and sulfide oxidase production considerably. Fermentation with ammonium acetate and ammonium

chloride as a nitrogen source yielded sulfide oxidase activity of 1.226 U/mL and 1.022 U/mL, respectively. Mohapatra *et al*. (2006) also observed that ammonium salts repressed the production of sulfide oxidase by the *Arthrobacter* sp.

Highest reduction for the specific sulfide oxidase activity occurred when medium was added with organic nitrogen sources except for urea. A reduction over 1.69 fold and 3.18 fold in the specific activity was observed when yeast extract and casein hydrolysate were used in the medium as compared to peptone, respectively. This indicated the presence of high concentration of unwanted protein inside the culture came from yeast extract and casein hydrolysate. Meanwhile, medium with inorganic nitrogen sources minimized the presence of foreign protein and caused the specific activity to increase. Supplement of medium with potassium nitrate enhanced the specific activity by 2.08 fold as compared to peptone due to low concentration of protein was produced by the bacteria during the fermentation process.

Referring to Table 5.3, the results showed that the addition of organic nitrogen source in medium was favorable for the enhancement of cell mass production, whereas inorganic nitrogen materials failed to improve growth of *Serratia* sp. strain SO02. The highest cell mass concentration of *Serratia* sp. strain SO02 was attained at 14.748 g/L when casein hydrolysate was supplied as a nitrogen source in medium. During growth with casein, *Serratia* sp. strain SO02 achieved maximal specific growth rate among other tested nitrogen source at 0.294 h^{-1} and fastest time for cell doubling at 2.361 h. However, among organic nitrogen sources tested, only urea displayed an inhibitory effect on *Serratia* sp. strain SO02 cell mass production. The presence of urea in the medium inhibits cell mass production with X_{max} obtained at 0.676 g/L and longer doubling time at 12.203 h. Supplementation of inorganic nitrogen materials in medium however, suppressed the growth of *Serratia* sp. strain SO02. Cultivation of *Serratia* sp. strain SO02 with ammonium acetate as nitrogen supply generates 0.571 g/L cell mass which corresponds to specific growth rate of 0.042 h^{-1}. This outcome showed that

inorganic nitrogen sources exhibit inhibitory effect on microbial growth, and therefore *Serratia* sp. strain SO02 required organic nitrogen supply in sulfur-oxidizer medium for favorable growth. ~~This is probably due to~~

Comment [kobenz2]: why

Effect of varying nitrogen sources towards thiosulfate utilization and sulfate production was studied and summarized in Table 5.3. The end results displayed that maximal utilization of thiosulfate was achieved when *Serratia* sp. strain SO02 bacteria was grown in medium containing 1 % (w/v) peptone, followed by yeast extract. Repression of thiosulfate utilization as well as sulfate production by *Serratia* sp. strain SO02 bacteria was observed when casein, urea and ammonium salts were employed. The presence of casein hydrolysate in media however gave rise to bacterial growth but failed to increase the thiosulfate utilization. This might be related to the composition of casein that is rich with amino acids and protein (Yeon *et al*., 1988), which encouraged cells to consume casein rather than using the thiosulfate to support the bacterial growth. Addition of urea and ammonium salts in medium however, inhibits bacterial growth and also the capability of the bacteria to degrade thiosulfate, leads to poor utilization of thiosulfate and eventually the synthesis of sulfate. Maximal utilization of thiosulfate at 11 % achieved in medium supplemented with 1 % (w/v) peptone which give yield to sulfate production of 0.385 g/L, while the lowest thiosulfate utilization (3.6 %) was observed when 1 % (w/v) urea was supplied in the medium with sulfate formation of 0.029 g/L.

Kinetic parameters on influence of various nitrogen sources were analyzed to study the importance of suitable nitrogen substrate towards thiosulfate utilization by *Serratia* sp. strain SO02 bacteria. Table 5.3 shows that medium supplemented with peptone was a favorable medium for the production of sulfide oxidase, even though yeast extract was observed to enhance more cell production. The cell mass yield on thiosulfate consumed ($Y_{x/s}$) were higher than sulfate yield ($Y_{p/s}$) proved that the metabolic pathway of the microbes was directed to the production of cell mass rather

than sulfate itself. The highest cell mass yield was obtained with organic nitrogen sources (casein, yeast extract and peptone) followed by ammonium chloride, potassium nitrate, ammonium acetate and urea. Meanwhile, the highest sulfate yield was attained when ammonium chloride is used as a nitrogen source followed by peptone, yeast extract, potassium nitrate, ammonium acetate, urea and casein.

For this, we can conclude that inorganic nitrogen sources including ammonium salts and organic nitrogen sources namely casein hydrolysate and urea are qualify as poor nitrogen sources for the sulfide oxidase synthesis. The addition of peptone enhanced the production sulfide oxidase as compared to other nitrogen sources. The order of sulfide oxidase production under the influence of various nitrogen sources was peptone > yeast extract > potassium nitrate > urea > ammonium salts > casein hydrolysate. Peptone was found as the best nitrogen source for sulfide oxidase production and was selected for the subsequent studies.

5.4.1.4 Effect of Peptone Concentration on Sulfide Oxidase Production

The effect of peptone concentration on microbial growth, sulfide oxidase production and thiosulfate utilization were studied in batch culture. The purpose of this study was to determine the optimum concentration of peptone required for the enhancement of sulfide oxidase production by *Serratia* sp. strain SO02 bacteria. The nitrogen source in the original medium was modified by varying peptone concentration within 0.05 to 1.0 % (w/v). The results from this study were analyzed and summarized in Table 5.4. Table 5.4 showed the influence of peptone concentration on the performance of *Serratia* sp. strain SO02 bacteria fermentation.

Table 5.4: Effect of various peptone concentrations towards batch fermentation of *Serratia* sp. strain SO02 bacteria in shake flask culture

Kinetic Parameters/ Peptone Concentrations	**0.05%**	**0.1%**	**0.2%**	**0.5%**	**0.6%**	**0.8%**	**1.0%**
Specific growth rate, μ (h^{-1})	0.101	0.111	**0.135**	0.134	0.170	0.166	0.181
Doubling time, t_d (h)	6.843	6.239	**5.119**	5.184	4.070	4.188	3.834
Max cell mass, X_{max} (g/L)	2.518 ± 0.170	3.194 ± 0.215	3.622 ± 0.244	3.876 ± 0.261	6.097 ± 0.411	6.382 ± 0.430	5.935 ± 0.400
Cell mass productivity, R_{batch} (g/L/h)	0.099	0.151	**0.187**	0.118	0.256	0.233	0.244
Max sulfide oxidase activity (U/mL)	1.873 ± 0.126	2.423 ± 0.163	3.632 ± 0.245	3.008 ± 0.203	3.008 ± 0.203	2.952 ± 0.199	2.917 ± 0.196
Protein concentration (mg/mL)	0.995 ± 0.194	1.196 ± 0.233	1.355 ± 0.264	2.216 ± 0.432	2.257 ± 0.440	2.415 ± 0.471	2.987 ± 0.583
Specific activity (U/mg)	1.907 ± 0.245	2.052 ± 0.264	2.714 ± 0.349	1.375 ± 0.177	1.350 ± 0.174	1.238 ± 0.159	0.989 ± 0.127
Concentration of thiosulfate utilized (mM)	0.461	0.574	**0.713**	0.482	0.462	0.461	0.441
Thiosulfate utilization (%)	11.538	14.359	**17.820**	12.052	11.538	11.538	11.026
Max sulfate product (g/L)	0.175	0.185	**0.201**	0.224	0.348	0.379	0.385
Sulfate productivity, $R_{sulfate}$ (g/L/h)	0.003	0.007	**0.004**	0.005	0.009	0.014	0.016
Specific rate of sulfate formation, $q_{sulfate}$ (g/g/h)	0.004	0.005	**0.005**	0.006	0.007	0.009	0.139
[1]$Y_{x/s}$ (g cell /mg thiosulfate)	0.030	0.051	**0.053**	0.059	0.111	0.109	0.084
[2]$Y_{p/s}$ (g sulfate /mg thiosulfate)	0.002	0.003	**0.001**	0.002	0.004	0.007	0.008
[3]$Y_{p/x}$ (g sulfate /g cell)	0.044	0.046	**0.036**	0.047	0.040	0.055	0.077

[1] $Y_{x/s}$ = Growth yield coefficient (g dry cell weight /mg substrate consumed)
[2] $Y_{P/s}$ = Product yield coefficient (g product /mg substrate consumed)
[3] $Y_{p/x}$ = Product yield coefficient (g product /g cell mass)

The influence of peptone concentration on sulfide oxidase synthesis by *Serratia* sp. strain SO02 was investigated and delineated in Table 5.4. Production of sulfide oxidase increased for 1.9 fold when 0.2 % (w/v) peptone was added into the medium. However, the sulfide oxidase activity dropped by 1.2 fold when the concentration of peptone exceeded 0.2 % (w/v). The addition of 0.2 % (w/v) peptone into the medium increased the sulfide oxidase activity up to 3.632 U/mL. As shown in Figure 5.4, increasing the concentration of peptone up to a level of 1.0 % (w/v) evidently did not enhance the enzyme production. Peptone at concentrations of 0.5-1.0 % (w/v) did not shown any significant effect on sulfide oxidase production. This result shows the inverse relationship between sulfide oxidase and peptone concentration. In other words, increasing the peptone concentration was unfavorable for the production sulfide oxidase which leads to the reduction of sulfide oxidase activity. This condition suggested that higher concentration of peptone had inhibitory effect on the synthesis of sulfide oxidase. This could be due to the complex nature of peptone and some of its constituents at higher concentration that might have a toxic effect on enzyme production (Singh *et al*., 2007).

The original composition of sulfur-oxidizer medium employed 1.0 % (w/v) peptone as a nitrogen source. An improvement of up to 20 % was observed in sulfide oxidase activity when 0.2 % (w/v) peptone was used in the medium. As a result, peptone with concentration of 0.2 % (w/v) was selected as a concentration for the subsequent studies of sulfide oxidase production. This finding was in contrast from Mohapatra *et al*. (2006) which used 1.0 % (w/v) yeast extract to produce sulfide oxidase from *Arthrobacter* sp.

The increased concentration of peptone beyond 0.2 % (w/v) reduced the specific sulfide oxidase activity. A reduction of specific activity in relation to high protein content showed that the addition of high peptone concentration into a culture induced the production of protein. The reduction of specific sulfide oxidase activity by 2.7 fold at

1.0 % (w/v) peptone concentration as compared to 0.2 % (w/v) peptone revealed that the undesirable protein production was greatly induced instead of the sulfide oxidase production. The presence of readily available protein in the peptone also affected the value of specific sulfide oxidase activity.

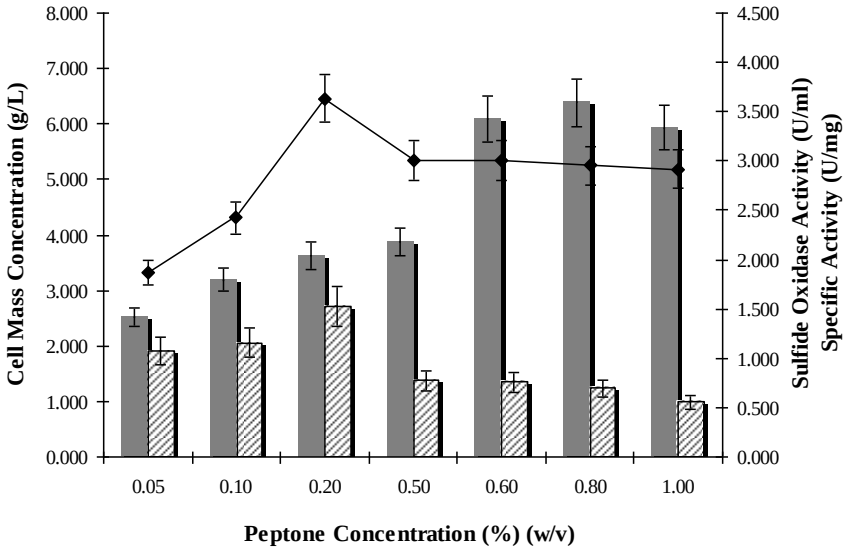

Figure 5.4: Impact of various peptone concentrations towards *Serratia* sp. strain SO02 growth (-▪-), sulfide oxidase activity (-♦-) and specific activity (-▨-)

The presence of peptone in sulfur-oxidizer medium was suspected to affect the thiosulfate utilization by *Serratia* sp. strain SO02. Referring to Table 5.4, thiosulfate consumption by *Serratia* sp. strain SO02 was increased proportionally to the increment of peptone up to 0.2% (w/v). However, the thiosulfate consumption was reduced when the concentration of peptone increased beyond this value. This leads to suggestion that higher peptone concentration resulted in loss of thiosulfate oxidizing capability. The

presence of high peptone in the medium stimulates the bacteria to consume peptone more than thiosulfate to support the bacterial life, and consequently slow down the thiosulfate utilization by bacteria in medium containing high peptone concentration. This condition leads to the incomplete utilization of thiosulfate in the culture.

To avoid the repression of thiosulfate utilization by *Serratia* sp. strain SO02, the level of peptone supplemented in the culture medium should be limited. Therefore, only a small amount of nitrogen is required to maintain the cell viability. Low concentration of peptone acted as a growth factor to support the microbial growth (Das *et al*., 1996). In this study, 0.2% (w/v) peptone was selected as the optimal concentration since the maximal percentage for thiosulfate utilization (18%) was obtained at this concentration. An increment of 38% for the thiosulfate utilization was achieved when 0.2% (w/v) peptone was used in the medium compared to the previous concentration of peptone (1.0%).

Table 5.4 illustrated the effect of peptone concentrations on microbial growth and specific growth rate of *Serratia* sp. strain SO02 bacteria. Microbial growth was monitored and it showed that the cell mass concentration was increased corresponding to the increment in peptone concentration. This report suggests that higher concentration of peptone enhanced the growth of *Serratia* sp. strain SO02. The relationship between cell growth and specific growth rate (μ) can also be seen in Table 5.4. The μ value seems to increase proportionally to the peptone concentration. The specific growth rate (μ) was increased for 44% when the peptone concentration varies from 0.05 to 1.0 % (w/v). The specific growth rate (μ) which was faster in medium containing high concentration of peptone shows the presence of high nitrogen compounds in the medium leads to the increase of specific growth rate. Furthermore, the decrease of doubling time (t_d) about 1.8 fold at 1.0 % concentration as compared to 0.05 % (w/v) peptone had clearly shown the rapid production of cell in medium with high quantity of peptone (1.0 % w/v).

Table 5.4 summarized the influence of peptone concentration on sulfate production. Sulfate formation by *Serratia* sp. strain SO02 was seems to increase in parallel to peptone concentration. In contrast to sulfide oxidase production, the sulfate formation increase proportionally with the increment in the peptone concentration. This might be happen due to high cell mass production with the increase of peptone concentration in the medium which leads to the rise of sulfate production. Maximum sulfate production (0.385 g/L) was attained in the presence of 1.0 % (w/v) peptone in the media.

5.4.1.5 Effect of Thiosulfate Concentration on Sulfide Oxidase Production

In this work, the effect of thiosulfate concentration towards cells growth, sulfide oxidase synthesis and thiosulfate utilization were investigated. The idea of this work was to discover the suitable concentration of thiosulfate for *Serratia* sp. strain SO02 in order to enhance the enzyme production and thiosulfate consumption. Concentration of thiosulfate in sulfur-oxidizer medium was varied in a range of 0.1 - 4.0 mM keeping the optimized condition as obtained previously. Results in this study are illustrated in Table 5.5. Table 5.5 shows the influence of thiosulfate concentration on the performance of *Serratia* sp. strain SO02 fermentation process.

As shown in Table 5.5 and Figure 5.5, sulfide oxidase activity did not significantly affected by the increased in thiosulfate concentration up to 4.0 mM. As the concentration increased to 1.5 mM, sulfide oxidase activity was slightly increased and decline beyond the concentration. The maximal sulfide oxidase activity of 3.905 U/mL was observed when 1.5 mM thiosulfate was incorporated in the medium. Mohapatra *et al*. (2006) uses 1 mM thiosulfate to investigate effect of the sulfur compound towards sulfide oxidase production.

Comment [kobenz3]: why

Table 5.5: Effect of various thiosulfate concentrations towards batch fermentation of *Serratia* sp. strain SO02 bacteria in shake flask culture

Kinetic Parameters/ Thiosulfate Concentration	**0.1 mM**	**0.5 mM**	**1.0 mM**	**1.5 mM**	**2.0 mM**	**3.0 mM**	**4.0 mM**
Specific growth rate, μ (h^{-1})	0.130	**0.142**	0.141	0.136	0.122	0.120	0.127
Doubling time, t_d (h)	5.34	**4.87**	4.91	5.09	5.67	5.78	5.46
Max cell mass, X_{max} (g/L)	3.498 ± **0.236**	**3.461** ± **0.233**	3.361 ± **0.266**	3.312 ± **0.223**	3.312 ± **0.223**	3.343 ± **0.225**	3.338 ± **0.225**
Cell mass productivity, R_{batch} (g/L/h)	0.107	**0.109**	0.120	0.115	0.120	0.117	0.117
Max sulfide oxidase activity (U/mL)	3.258 ± **0.219**	**3.848** ± **0.259**	3.825 ± **0.258**	3.905 ± **0.263**	3.065 ± **0.206**	3.088 ± **0.208**	3.644 ± **0.245**
Protein concentration (mg/mL)	0.524 ± **0.070**	**0.692** ± **0.093**	0.779 ± **0.105**	0.757 ± **0.102**	0.865 ± **0.116**	0.952 ± **0.128**	0.959 ± **0.129**
Specific activity (U/mg)	6.241 ± **0.420**	**5.590** ± **0.376**	4.932 ± **0.332**	5.184 ± **0.349**	3.558 ± **0.240**	3.260 ± **0.220**	3.817 ± **0.257**
Concentration of thiosulfate utilized (mM)	0.301	**0.399**	0.404	0.441	0.511	0.588	0.651
Thiosulfate utilization (%)	46.24	**51.220**	31.08	27.63	27.24	22.22	18.58
Max sulfate product (g/L)	0.190	**0.270**	0.271	0.258	0.234	0.264	0.215
Sulfate productivity, $R_{sulfate}$ (g/L/h)	0.011	**0.018**	0.011	0.021	0.016	0.018	0.010
Specific rate of sulfate formation, $q_{sulfate}$ (g/g/h)	0.012	**0.018**	0.018	0.018	0.013	0.016	0.014
[1]$Y_{x/s}$ (g cell /mg thiosulfate)	0.073	**0.061**	0.071	0.060	0.047	0.032	0.042
[2]$Y_{p/s}$ (g sulfate /mg thiosulfate)	0.006	**0.011**	0.006	0.005	0.006	0.004	0.003
[3]$Y_{p/x}$ (g sulfate /g cell)	0.092	**0.123**	0.126	0.131	0.109	0.133	0.105

[1] $Y_{x/s}$ = Growth yield coefficient (g dry cell weight /mg substrate consumed)
[2] $Y_{P/s}$ = Product yield coefficient (g product /mg substrate consumed)
[3] $Y_{p/x}$ = Product yield coefficient (g product /g cell mass)

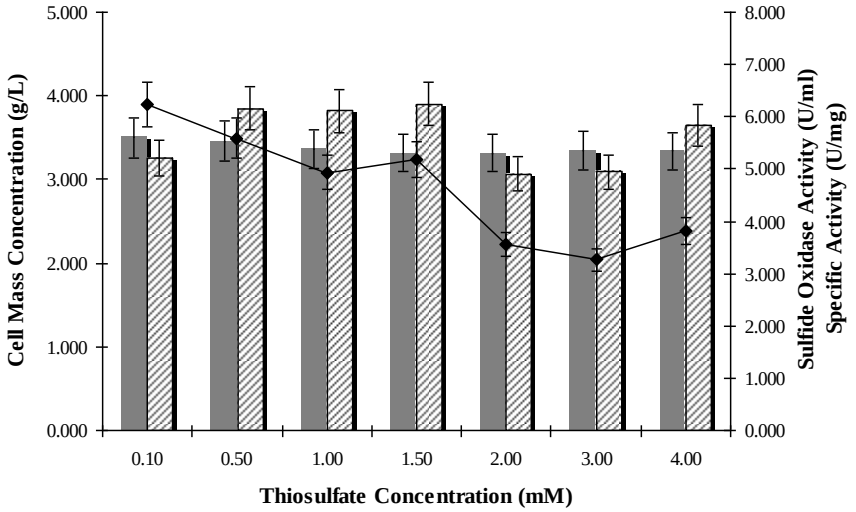

Figure 5.5: Impact of various thiosulfate concentrations towards *Serratia* sp. strain SO02 growth (-■-), sulfide oxidase activity (-▨-) and specific activity (-♦-)

As the concentration of thiosulfate rise, the protein production by the bacteria seems to increase as well. The protein production by *Serratia* sp. strain SO02 increase about 1.7 fold as the thiosulfate concentration raise from 0.1 mM to 4.0mM. The specific activity of sulfide oxidase declined by 1.6 fold with the decrease in cell mass production when the thiosulfate concentration increases to 4.0 mM. The reduction of specific activity revealed that more undesirable protein was produced when the thiosulfate concentration increased.

Referring to Table 5.5, the increased thiosulfate concentration did not result in the significant increment of cell mass production. The amount of cell mass produced at thiosulfate concentration ranging from 0.1- 4.0 mM was 3.312 - 3.498 g/L. This condition suggested that the growth of *Serratia* sp. strain SO02 was not influenced by the amount of added thiosulfate in the medium. The same condition occurred to Schook

and Berk (1978) who found out that the increasing amount of thiosulfate in the growth medium does not stimulate bacterial growth. Hence, oxidation of thiosulfate probably does not supply energy to enhance the cells growth which capable of catalyzing the thiosulfate oxidation (Peck, 1961). This finding is in contrast with Spring *et al*. (2001) which observed the increase in growth yield of *Limnobacter thiooxidans* with the increment of thiosulfate in the medium.

According to our experience when *Serratia* sp. strain SO02 was grown in the thiosulfate-containing medium, an increase in culture pH from 5 to neutral condition was observed. Based on the referred literature, the increase in pH during thiosulfate oxidation might be due to tetrathionate production during growth of *Serratia* sp. strain SO02 (Sorokin, 2003). Sorokin (2003) has reported that *Halomonas* increased the pH of medium during thiosulfate oxidation from 7 to 9 due to the tetrathionate production. Phylogenetic analysis of *Halomonas* revealed that this bacteria was the member of the γ-*Proteobacteria* which is the same phylum with the isolated *Serratia* sp. strain SO02 (Sorokin, 2003). Besides *Halomonas*, other heterotrophic sulfur-oxidizing bacteria that phylogenetically affiliated to the γ- *Proteobacteria* are *Pseudomonas*, *Escherichia coli*, *Xanthomonas* and *Beggiatoa* (Gommerz and Kuenen, 1988; Das *et al*., 1996).

The effect of differ thiosulfate concentration towards thiosulfate utilization was illustrated in Table 5.5. As previously discussed, thiosulfate concentration in range of 0.1 – 4.0 mM did not significantly affect cell mass and sulfide oxidase production. On the other hand, it greatly influenced the *Serratia* sp. strain SO02 ability to convert thiosulfate into sulfate. The thiosulfate utilization increased slowly with the increasing in thiosulfate concentration up to a maximum level observed at 0.5 mM (51.2%). This was followed by inhibition of thiosulfate utilization at high concentration which leads to less consumption of thiosulfate by this bacteria. According to Spring *et al*. (2001), concentration of thiosulfate above the optimum inhibits the bacterial growth and thiosulfate utilization by the bacteria.

Comment [kobenz4]: why

As shown in Table 5.5, thiosulfate utilization was decreased by 63.7% when 4.0 mM thiosulfate was used in the medium as compared to 0.5 mM thiosulfate. The maximal consumption was achieved at low concentration of thiosulfate, 0.5 mM with percentage of utilization at 51.2 %. With the intention of improving the thiosulfate utilization percentage, 0.5 mM was chosen as the concentration of thiosulfate required by *Serratia* sp. strain SO02 for the subsequent study. At this point of concentration, more than 50% of thiosulfate was consumed by *Serratia* sp. strain SO02 within 24 hours. As described by Eccleston and Kelly (1978), we can conclude that very low thiosulfate concentration allowed very high oxidation rate. Higher thiosulfate concentration only resulted in loss of thiosulfate oxidizing capacity and decrease in cell mass production. This might be due to the thiosulfate toxicity at high concentration that can repressed cell growth and also thiosulfate oxidizing capability of the bacteria (Gommers and Kuenen, 1988).

Table 5.5 shows the effect of various thiosulfate concentrations in the sulfur-oxidizer medium towards sulfate formation by *Serratia* sp. strain SO02. The sulfate production was raised to the maximum amount of 0.271 g/L at 1.0 mM thiosulfate and fall off to 0.215 g/L when the thiosulfate concentration increased to 4.0 mM. This condition indicates that high concentration of thiosulfate was not suitable for the production of sulfate by *Serratia* sp. strain SO02. The same situation happened to Skirnisdottir *et al*. (2001a) which observed the conversion of thiosulfate to sulfate by *Hydrogenobacter* sp. was reduced with the increase thiosulfate concentration.

Comment [kobenz5]: why

5.4.1.6 Effect of Sulfur Sources on Sulfide Oxidase Production

In this work, studies were done to determine which inorganic sulfur compounds could be utilized as sources of sulfur for *Serratia* sp. strain SO02 and effect of different sulfur sources towards the production of sulfide oxidase. The concentration of sulfur sources were kept low as obtained in section 5.4.1.5 to avoid the substrate inhibition due to high concentration of substrate. Various inorganic sulfur sources were use to replace thiosulfate in the sulfur-oxidizer medium. The sulfur compounds tested were sodium or potassium salts of tetrathionate, sulfide, thiocyanate and thiosulfate.

The influence of varying sulfur sources towards sulfide oxidase production is illustrated in Table 5.6 and Figure 5.6. The ability of *Serratia* sp. strain SO02 to oxidize sulfide when grown on individual sulfur compounds was determined by measuring sulfate production during the sulfide oxidase assay. The *Serratia* sp. strain SO02 was induced to grow on each of the sulfur sources to test the capability of the bacteria to produce sulfide oxidase in the presence of different sulfur substrate. Table 5.6 shows that the crude enzyme of *Serratia* sp. strain SO02 was able to produce sulfate during sulfide oxidase reaction when grown on each sulfur substrate but the lowest activity showed when using thiocyanate.

Cells of *Serratia* sp. strain SO02 grown in thiocyanate exhibited the lowest sulfide oxidase activity (1.960 U/mL) amongst the tested sulfur. Thus, suggesting that incorporation of thiocyanate in medium failed to stimulate sulfide oxidase activity at the high level. Based on the results, thiocyanate is not a suitable sulfur compound to induce the production of sulfide oxidase by *Serratia* sp. strain SO02. This could be due to the toxicity of the thiocyanate itself. Thiocyanate ($N{\equiv}C{-}S$) is a C_1 sulfur species and degradation of thiocyanate molecule release sulfide, ammonia and carbon dioxide that can serve as energy, carbon, nitrogen or sulfur source.

Table 5.6: Effect of various inorganic sulfur compounds at 0.5 mM concentration towards batch fermentation of *Serratia* sp. strain SO02 in shake flask culture

Parameters/ Sulfur Sources	None	Sulfide	Tetrathionate	Thiosulfate	Thiocyanate
Oxidation state of sulfur	-	-2		**+2**	-2
Specific growth rate, μ (h^{-1})	0.139	0.127	0.133	**0.142**	0.129
Doubling time,t_d (h)	4.99	5.39	5.22	**4.87**	5.36
Max cell mass, X_{max} (g/L)	3.382 ± 0.228	2.798 ± 0.118	3.931 ± 0.265	**3.461 ± 0.233**	3.432 ± 0.231
Cell mass productivity, R_{batch} (g/L/h)	0.183	0.101	0.212	**0.109**	0.225
Max sulfide oxidase activity (U/mL)	1.102 ± 0.142	3.294 ± 0.222	4.901 ± 0.234	**3.848 ± 0.259**	1.960 ± 0.021
Protein concentration (mg/mL)	0.827 ± 0.211	0.659 ± 0.089	0.733 ± 0.050	**0.708 ± 0.095**	0.558 ± 0.075
Specific activity (U/mg)	1.355 ± 0.174	5.019 ± 0.338	6.738 ± 0.367	**5.460 ± 0.368**	3.676 ± 0.248
Concentration of sulfur utilized (mM)	-	0.192	0.236	**0.399**	0.057
Sulfur utilization (%)	-	47.368	30.474	**51.220**	7.547
Max sulfate product (g/L)	0.132	0.235	0.234	**0.270**	0.133
Sulfate productivity, $R_{sulfate}$ (g/L/h)	0.011	0.020	0.013	**0.018**	0.011
Specific rate of sulfate formation, $q_{sulfate}$ (g/g/h)	0.006	0.015	0.010	**0.018**	0.006
[1]$Y_{x/s}$ (g cell / mg sulfur)	-	0.345	0.081	**0.061**	0.766
[2]$Y_{p/s}$ (g sulfate / mg sulfur)	-	0.103	0.005	**0.011**	0.038
[3]$Y_{p/x}$ (g sulfate / g cell)	0.048	0.115	0.0745	**0.123**	0.049

[1] $Y_{x/s}$ = Growth yield coefficient (g dry cell weight /mg substrate consumed)
[2] $Y_{P/s}$ = Product yield coefficient (g product /mg substrate consumed)
[3] $Y_{p/x}$ = Product yield coefficient (g product /g cell mass)

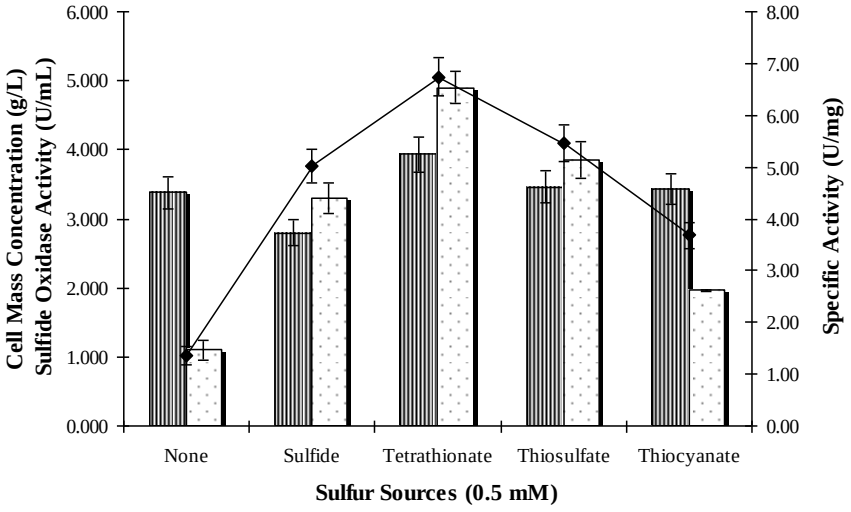

Figure 5.6: Impact of various sulfur sources at 0.5 mM concentration towards sulfide oxidase production (-□-), specific activity (-♦-) and *Serratia* sp. strain SO02 growth (-▮-)

However, release of ammonia shows toxicity effect on growth and activity of the bacteria. This has been stated by Sorokin *et al*. (2001) which discovered that sulfur-oxidizing bacteria strain ARh 2 lost its thiosulfate-oxidizing activity completely in culture grown with thiocyanate. In this study, cell mass production of 3.432 g/L by thiocyanate-grown cells was observed to be comparable with cells that were grown in thiosulfate (Table 5.6). The ability to grow in thiocyanate-containing medium and still retained sulfide oxidase activity indicates that *Serratia* sp. strain SO02 was tolerance to the thiocyanate at initial concentration of 0.5 mM.

Growth of *Serratia* sp. strain SO02 in thiocyanate-containing medium inhibits the utilization of thiocyanate. Only 7.6 % of thiocyanate was consumed by *Serratia* sp. strain SO02 in 24 h. According to Sorokin *et al*. (2001) report, the inability of *Serratia* sp. strain SO02 to consume thiocyanate may possibly occur due to lack of enzymes that responsible to break down the thiocyanate molecule, or released of ammonia in the medium which inhibit the thiocyanate utilization. Sorokin *et al*. (2001) stated that ammonia prevented thiocyanate utilization completely without influencing the growth yield. Even though *Serratia* sp. strain SO02 was unable to utilize thiocyanate, the growth of microorganism was unaffected and as good as growth in the thiosulfate-containing medium. The use of low thiocyanate concentration in the medium probably allowed the microorganism to achieve the stable growth (Sorokin *et al*., 2001).

The supplementation of sulfur-oxidizer medium with tetrathionate and thiosulfate obviously enhanced the production of sulfide oxidase by *Serratia* sp. strain SO02. These results showed that besides thiosulfate, tetrathionate is a possible good source of reduced inorganic sulfur compounds to enhance the production of sulfide oxidase by *Serratia* sp. strain SO02. It appeared that sulfide oxidase activity was induced when *Serratia* sp. strain SO02 was grown using sulfur sources that had low oxidative states excluding sulfide and thiocyanate. These assumptions were made referring to Schook and Berk (1978) report. Schook and Berk (1978) have found out that thiosulfate oxidase activity was induced when *P. aeruginosa* was grown in medium containing sulfur sources that had low oxidative states (tetrathionate, thiosulfate, sulfide), while low or no thiosulfate oxidase activity was observed with higher oxidative states of sulfur compounds.

In this study, it appeared that incorporation of sulfide in sulfur-oxidizer medium does not succeed to stimulate sulfide oxidase activity to the optimum level compared to the use of thiosulfate or tetrathionate as a sulfur substrate. Referring to the Schook and Berk (1978), they found out that sulfide-grown cells were able to stimulate high activity

of tetrathionate oxidase and metabisulfite oxidase. However, sulfide oxidase activity was not tested by Schook and Berk (1978) on that time. They also discovered that thiosulfate and tetrathionate-grown cells produced high level of metabisulfite oxidase activity compared to the other oxidase activity of *P. aeruginosa* (Schook and Berk, 1978). This trend showed that there are possibilities of using different sulfur compounds such as thiosulfate and tetrathionate to induce the activity of sulfide oxidase.

The *Serratia* sp. strain SO02 was observed to produce sulfide oxidase with activity of 1.102 U/mL in the absence of reduced sulfur compound. There was an increment of 3.7 and 3.1 fold of sulfide oxidase activity when thiosulfate or sulfide was supplied in the sulfur-oxidizer medium, respectively. Mohapatra *et al*. (2006) also found an increment in sulfide oxidase production when thiosulfate and sulfide was supplied in the GY medium. Production of sulfide oxidase by *Arthrobacter* sp. FR-3 increased by 1.2 and 1.2 fold in the presence of thiosulfate and sulfide in the medium, respectively (Mohapatra *et al*., 2006). According to Mohapatra *et al*. (2006), the production of extracellular sulfide oxidase in the absence of thiosulfate and sulfide suggests that the enzyme was constitutively expressed in small amount by the bacteria. The sulfide oxidase synthesis of *Xanthomonas* sp. strain DY44 isolated from peat was also constitutive, but this enzyme that was responsible for H_2S degradation is intracellular (Cho *et al*., 1992).

Referring to Table 5.6 and Figure 5.6, it was observed that *Serratia* sp. strain SO02 cell mass production in medium containing aforementioned compounds has no major differences as opposed to the cell growth in sulfur-free medium. In previous experiments, thiosulfate was used as a source of sulfur compound in the sulfur-oxidizer medium. Obviously in this experiment, the growth yield was not enhanced by the addition of thiosulfate in the medium, where the cell mass was produced at a level obtained without the presence of sulfur compounds. This result suggested that thiosulfate has no metabolic role in *Serratia* sp. strain SO02 metabolisms (London and

Rittenberg, 1966). In other words, thiosulfate did not serve as an energy source to enhance the growth of *Serratia* sp. strain SO02. However, addition of 0.5 mM tetrathionate seems to have small stimulatory effect on *Serratia* sp. strain SO02 cell mass yield. This finding was supported by Schook and Berk (1978), which found that tetrathionate appeared to affect growth of *P. aeruginosa*. On the contrary, Mason and Kelly (1988) showed that tetrathionate have no effect on growth of *P. aeruginosa* strain KF.

The addition of sulfide into the medium appeared to slightly repress the growth of *Serratia* sp. strain SO02. The cell mass production with the presence of sulfide in the medium was slightly declined as opposed to the medium with no added sulfur compounds. This condition could be happened due to the toxic properties of the sulfur compounds itself and it might slightly inhibit the growth of *Serratia* sp. strain SO02. Sulfide was known to have toxic effect on microorganisms (Lee et *al*., 2006).

Comment [kobenz6]: why

The capability of *Serratia* sp. strain SO02 to consume variety of sulfur compounds were studied and illustrated in Table 5.6. The results showed that at initial concentration of 0.5 mM, more than 45% of the tested sulfur compounds were consumed by the *Serratia* sp. strain SO02. Maximal utilization of sulfur compounds were observed when the culture was grown with thiosulfate and sulfide. Low utilization of sulfur substrate was discovered with the presence of thiocyanate in the medium.

Oxidation of thiosulfate in the medium with oxygen as an electron acceptor has been demonstrated by the *Serratia* sp. strain SO02. Thiosulfate oxidation occurred throughout the growth period with 51.2% thiosulfate being used within 24 hours. Whereas the consumption of tetrathionate by *Serratia* sp. strain SO02 occurred only at 30%, even though the maximum cell mass was produced while growing in the presence of tetrathionate.

Comment [kobenz7]: why

Throughout the growth period, 47.4% sulfide was used by *Serratia* sp. strain SO02 within 24 h incubation. The oxidation results with sulfide as substrate was slightly low compared to the thiosulfate, the usual sulfur substrate that was used during the whole optimization experiments. This might be happened due to the toxic effect of sulfide towards *Serratia* sp. strain SO02, that slightly slow down the sulfide consumption. Sulfide was known as compounds that were toxic to microorganisms (Lee *et al*., 2006), and the influence of sulfide toxicity was observed to affect the microbial growth of *Serratia* sp. strain SO02.

5.4.1.7 Time Course of Sulfide Oxidase Production by *Serratia* sp. strain SO02

In the investigation of optimized culture conditions, six parameters have been evaluated. The parameters include pH, temperature, nitrogen sources, concentration of peptone and thiosulfate and also sulfur sources. At the end of the optimization period, the optimized culture condition was achieved by considering two aspects which are sulfide oxidase production and thiosulfate utilization.

The optimized culture condition for *Serratia* sp. strain SO02 fermentation was achieved in sulfur-oxidizer medium at initial pH of 5.0, supplemented with 0.2% (w/v) peptone and 0.5 mM sodium thiosulfate with incubation temperature of 30˚C. The results on the time course studies of sulfide oxidase production and the cell growth of *Serratia* sp. strain SO02 grown in the optimized sulfur-oxidizer medium is display in Figure 5.7.

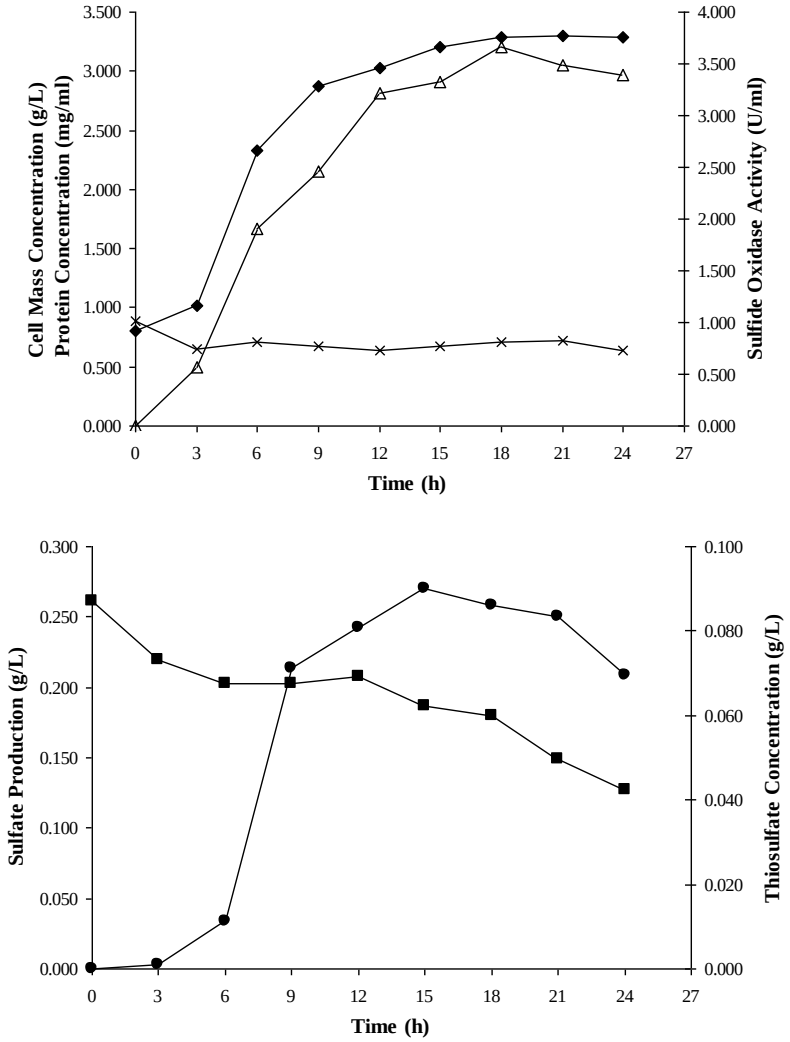

Figure 5.7: Time course of *Serratia* sp. strain SO02 batch fermentation during growth at optimized condition [pH 5.0, 30˚C, 0.2% (w/v) peptone and 0.5 mM thiosulfate]. Symbols represent; cell mass concentration (-♦-), sulfide oxidase activity (-Δ), protein concentration (-x-), thiosulfate utilization (-■-), sulfate production (-●-)

The time course profile of *Serratia* sp. strain SO02 in the optimized medium demonstrated that the production of cell mass was rapidly increased over 12 h incubation with specific growth rate, μ of 0.142 h^{-1}. This corresponds to a doubling time of 4.87 h with maximum cell mass of 3.461 g/L achieved at 21 h of incubation. *Serratia* sp. strain SO02 was discovered to enter the exponential phase less than 3 hours incubation without reaching the lag period. This phenomenon occurs when the inoculum that was used in the experiment was taken from the exponentially growing culture in the same medium with the same cultivation conditions. As stated by Madigan *et al*. (2000), cells in the exponential growth phase are usually in their healthiest state, thus help *Serratia* sp. strain SO02 to begin the exponential growth immediately in a new culture medium.

The production of extracellular sulfide oxidase increased significantly during the exponential growth phase of the culture with maximum activity of 3.848 U/mL at 18 h incubation time. After this point, sulfide oxidase activity was slightly decreased until the end of incubation. The trend of sulfide oxidase production linked with the cell growth suggested that the production of extracellular enzyme involved in sulfide oxidation was growth associated in *Serratia* sp. strain SO02 culture. Maximum activity of sulfide oxidase that was observed at the exponential growth phase suggests that sulfide oxidase of *Serratia* sp. strain SO02 was produced as a primary metabolite of the fermentation.

Thiosulfate was supplied at low concentration of 0.5 mM in order to minimize substrate inhibition due to the high concentration of substrate. Referring to Eccleston and Kelly (1978) report, low concentration of thiosulfate leads to the high oxidation rates, which was also observed with *Serratia* sp. strain SO02. During the growth, *Serratia* sp. strain SO02 used up to 51.2% thiosulfate within 24 h incubation time and produced 0.270 g/L sulfate as a result of thiosulfate utilization. There was an increased in sulfate production during the active growth of *Serratia* sp. strain SO02 with maximum sulfate achieved by 0.270 g/L at 15 h of incubation and was found to decrease

gradually until the end of incubation. The kinetic of sulfate synthesis under optimized condition was expressed in term of sulfate productivity ($R_{sulfate}$) and specific rate of sulfate formation ($q_{sulfate}$). The $R_{sulfate}$ and $q_{sulfate}$ of *Serratia* sp. strain SO02 was achieved with value of 0.018 g/L/h and 0.018 g/g/h, respectively. The $Y_{p/x}$ value of this fermentation was attained at 0.123 g/g. The yield coefficient of *Serratia* sp. strain SO02 fermentation against thiosulfate consumption was described by kinetic parameters of $Y_{x/s}$ and $Y_{p/s}$. The value of $Y_{x/s}$ and $Y_{p/s}$ obtained in the fermentation were 0.061 g/mg and 0.006 g/mg, respectively.

5.4.1.8 Comparative Study of Sulfide Oxidase Production before and after the Optimization Process

In order to see the improvement of sulfide oxidase production by *Serratia* sp. strain SO02, results obtained from the optimized conditions were compared with the results before the optimization studies were performed (Table 5.7).

Results presented in Table 5.7 showed an improvement in sulfide oxidase production, sulfate formation and thiosulfate utilization by *Serratia* sp. strain SO02 before and after the optimization studies. In optimized culture medium concentration of peptone was reduced from 1.0% (w/v) to 0.2% (w/v) in order to increase the production of sulfide oxidase and stimulate thiosulfate utilization by *Serratia* sp. strain SO02. However, the growth of *Serratia* sp. strain SO02 was decreased when concentration of peptone was reduced to 0.2% (w/v). Higher concentration of peptone improved the bacterial growth but eventually decrease sulfide oxidase yield. Hence, only a small amount of peptone was added to the sulfur-oxidizer medium to maintain the viability of cells.

Table 5.7: Comparative studies of sulfide oxidase production by *Serratia* sp. strain SO02 before and after the optimization studies

Kinetic Parameters	Before Optimization	After Optimization
Initial pH	7.0	5.0
Temperature (˚C)	30	30
Nitrogen source (1% w/v)	Peptone	Peptone
Peptone concentration (%)	1.0	0.2
Thiosulfate concentration (mM)	4.0	0.5
Specific growth rate, μ (h^{-1})	0.168	0.142
Doubling time, t_d (h)	4.14	4.87
Max cell mass, X_{max} (g/L)	5.569 ± 0.375	3.461 ± 0.233
Cell mass productivity, R_{batch} (g/L/h)	0.195	0.109
Max sulfide oxidase activity (U/mL)	1.533 ± 0.103	3.848 ± 0.259
Protein concentration (mg/mL)	3.034 ± 0.408	0.708 ± 0.095
Specific activity (U/mg)	0.508 ± 0.034	5.460 ± 0.368
Concentration of thiosulfate consumed (mM)	0.277	0.399
Thiosulfate utilization (%)	6.921	51.220
Max sulfate product (g/L)	0.184	0.270
Sulfate productivity, $R_{sulfate}$ (g/L/h)	0.008	0.018
Specific rate of sulfate formation, $q_{sulfate}$ (g/g/h)	0.007	0.018
[1]$Y_{x/s}$ (g cell /mg thiosulfate)	0.151	0.061
[2]$Y_{p/s}$ (g sulfate /mg thiosulfate)	0.006	0.006
[3]$Y_{p/x}$ (g sulfate /g cell)	0.039	0.123

[1] $Y_{x/s}$ = Growth yield coefficient (g dry cell weight /mg substrate consumed

[2] $Y_{p/s}$ = Product yield coefficient (g product /mg substrate consumed)

[3] $Y_{p/x}$ = Product yield coefficient (g product /g cell mass)

Under optimized fermentation conditions, improvement of sulfide oxidase synthesis (60.2%) and specific activity (90.7%) was observed as compared to the unoptimized conditions. Sulfide oxidase activity was increased from 1.533 U/mL to 3.848 U/mL after the optimization studies. While the specific sulfide oxidase activity increases from 0.508 U/mg to 5.460 U/mg once the optimization was performed. The decrease of added peptone into the medium evidently reduces the concentration of protein and enhanced the production of sulfide oxidase which can be seen in the increment of sulfide oxidase activity after the optimization.

The increment of 31.9% in sulfate production by *Serratia* sp. strain SO02 was attained under the optimized culture condition. Kinetic analysis of sulfate productivity, $R_{sulfate}$ (0.018 g/L/h) and specific rate of sulfate formation ($q_{sulfate}$) (0.018 g/g/h) was also increase by 2.3 and 2.7 fold with the increase in sulfate production as opposed to before the optimization process, respectively.

In optimized medium condition, peptone and thiosulfate concentration were cut down to minimize effect of high substrate inhibition. This greatly affects the percentage of thiosulfate utilization by *Serratia* sp. strain SO02. Before optimization study was conducted, the *Serratia* sp. strain SO02 consumes thiosulfate only about 7% from the initial concentration. However, once the optimized condition was applied more than 50% of thiosulfate at 0.5 mM concentration was consumed by the cells. Thus, suggests that *Serratia* sp. strain SO02 was able to consume thiosulfate only at low concentration.

Kinetic studies of yield coefficient with optimized conditions showed an increase of $Y_{p/s}$ and $Y_{p/x}$ value by 1.02 and 3.14 fold, respectively. The increase in $Y_{p/s}$ and $Y_{p/x}$ value was probably due to the improvement of sulfate production that was higher in the optimized condition. The yield of $Y_{x/s}$ however, decrease as a result of lower cell mass

production in medium containing reduced amount of peptone after the optimization was done.

5.5 Conclusion

Referring to the results obtained from the optimization studies, the best conditions for *Serratia* sp. strain SO02 fermentation was observed in medium containing 0.2% (w/v) peptone and 0.5 mM thiosulfate with initial pH of 5.0 and grown at 30˚C incubation temperature. Under the influence of optimized culture conditions, enhancement can be seen in sulfide oxidase production and sulfate yield along with the thiosulfate consumption by the cells. The optimized condition however, cause the decline in cell growth as a result of reduce peptone concentration. Nevertheless, the objective of this study was accomplished by improving the sulfide oxidase production after the optimization study was done.

CHAPTER 6

PRODUCTION OF SULFIDE OXIDASE IN BIOREACTOR BY *Serratia* sp. strain SO02 CULTURE

6.1 Introduction

Bioreactor or fermenter provides a controlled environment for the growth of microorganisms with the purpose to achieve the desired product of the fermentation processes. Environmental factors that are frequently monitored include temperature, dissolved oxygen tension (DOT) and pH of the culture. Thus, the development of bioreactor has greatly improved fermentation system and permits greater potential for monitoring and controlling pH and DOT levels. Applying bioreactor in fermentation process have provides culture with mixing efficiency and oxygen transfer capability (Betts and Baganz, 2006). High oxygen transfer rate supported growth of quickly respiring organisms, whereas effective mixing allowed the vessel to maintain homogenous conditions when dealing with viscous fermentation broth (Betts and Baganz, 2006). Bioreactors support rapidly metabolizing microbial cell cultivations and increase the amount of product that such bioprocess can yield (Betts and Baganz, 2006).

The dissolved oxygen concentration is an important operating parameter that influences the cellular metabolism and growth (Gawande *et al*., 2003). Therefore, oxygen demand of an industrial fermentation process is normally satisfied by aerating and agitating the fermentation broth. The agitation and aeration are important in order to supply oxygen for the microorganism in the fermentation process. The production of several enzymes by fermentation process such as inulinase and hemicellulase are influenced by the oxygen supply in a fermenter (Silva-Santisteban and Filho, 2005; Reddy *et al*., 2002). Therefore, oxygen requirements for the organisms are important to insure a successful biological conversion process.

In this study, the lab-scale fermenter was applied in order to study the aeration requirement for sulfide oxidase production by *Serratia* sp. strain SO02. Moreover, this study investigates development of aeration control strategy towards sulfide oxidase production by *Serratia* sp. strain SO02.

6.2 Materials and methods

6.2.1 Medium Composition

The medium composition for batch culture fermentation used in this study was presented in section 3.2. Composition of the medium and cultural condition was similar to the optimum condition previously obtained in shake flask culture. The prepared medium broth was transferred into the culture vessel and sterilized by autoclaved.

6.2.2 Fermenter Set Up

The schematic diagram of 2 L fermenter used for the enhancement of sulfide oxidase production is presented in Figure 6.1. The jacketed culture vessel consisted of borosilicate glass with an internal concave bottom section for optimum mixing rate even at low agitation speeds. The fermenter was equipped with control unit, peristaltic pump, motor, temperature sensor, air sparger, impeller, pH-electrode, pO_2-electrode, antifoam probe and level probe. A sterilizable polagraphic pO_2-electrode (Mettler Toledo AG, Switzerland) and glass pH electrode (Mettler Toledo) were used to measure the dissolved oxygen (DO) level and pH of the culture, respectively. The fermenter was fixed with two six-bladed Rushton impellers (diameter, d = 53 mm) on the stirrer shaft to provide agitations throughout the fermentations.

The prepared sulfur-oxidizer medium was transferred into the vessel and the top plate of the vessel was attached. Membrane filter and temperature electrode plug were covered with cotton and followed by wrapping with aluminium foil in order to protect them from the impact of steam during autoclaving. Tubings of the air inlet, sampling port and corrective solution supplies were clamped using the dovetail clamps attached to the culture vessel. The culture vessel was then sterilized by autoclaving at 121˚C for 20 min.

After autoclaving, the culture vessel was connected to air supply, thermostat system, cooling water supply, motor and the control unit. Before starting the fermentation, the pO2-electrode was polarized for at least six hours before calibration and measurement can be done. Polarization was done by connecting the electrode to the control unit and the system was switched on. The pO_2-electrode was calibrated in percentage of oxygen saturation, % pO_2. Calibration of pO_2-electrode was made in the culture vessel after autoclaving and under the conditions of fermentation. The pO_2-

electrode calibration includes zero and slope calibration. For zero calibration, the culture medium was sparged with nitrogen gas to displace any dissolved oxygen in the medium until the control system showed pO_2 value 0.00%. The calibration slope was performed by supplying air up to the saturation level that gives the electrode slope 100% pO_2. At this point, the fermenter was ready for inoculation and fermentation process.

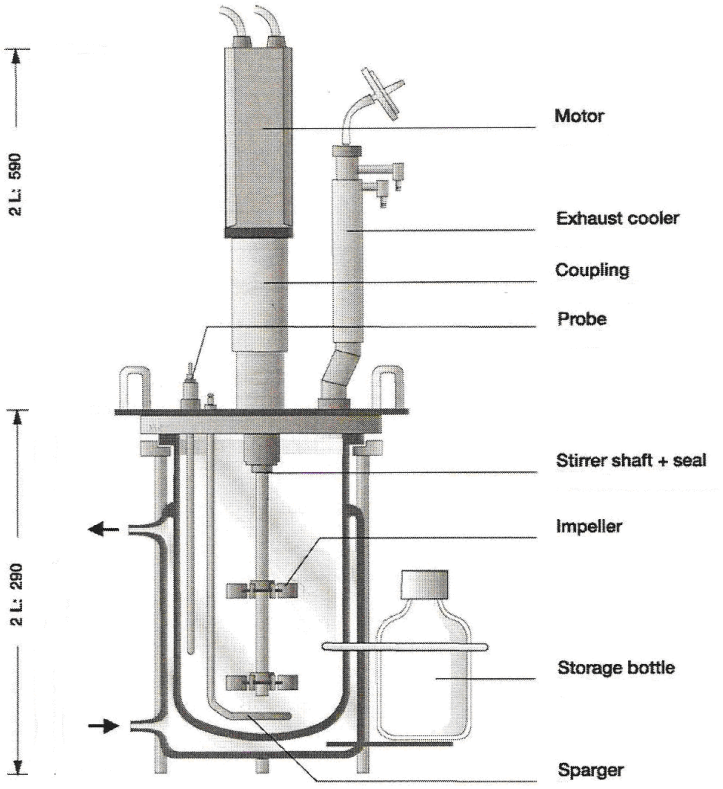

Figure 6.1: Setup and dimensions of the jacketed 2 L culture vessel (Biostat® B, Germany)

6.2.3 Production of Sulfide Oxidase in Batch Culture using 2L Fermenter

Batch fermentation was carried out using 2 L fermenter with starting volume of medium, 1.35 L. About 10% (v/v) of bacterial culture with optical density of 0.5 at 600 nm was used as inoculum. The fermentation was carried out at 30˚C with 200 rpm stirring rate and air flow rate at 1 l/min.

6.2.4 Analytical Procedures

Samples of the fermentation process were withdrawn from the culture vessel at regular time intervals. During the sampling period, the pO_2 value of the culture was note down. The sample was spin at 4000 rpm, 4˚C for 30 minutes and the supernatant was separated from the pellet by transferring the supernatant into new tube and kept frozen at -20˚C until further analysis. This supernatant was used for determination of sulfide oxidase activity, cell mass production, sulfate formation, protein concentration and thiosulfate utilization according to protocol described in section 3.5.

6.2.5 Determination of Oxygen Uptake Rate by Dynamic Gassing Out Technique

The specific oxygen uptake rate (qO_2) of *Serratia* sp. strain SO02 was determined using the dynamic gassing out method, where dissolved oxygen concentration in the fermentation broth was measured as a function of time using a polarographic oxygen electrode (Ozergin-Ulgen and Mavituna, 1998). The dissolved

oxygen concentration was determined by assuming the maximum concentration of oxygen in an air that sparged within the culture medium at 100% saturation to be 7.87 mg/L at 27°C (Rane and Sims, 1994). Protocol for determination of specific oxygen uptake rate (qO_2) and results of a typical dynamic gassing out study are illustrated in Appendix A6.

6.3 Results and Discussions

6.3.1 Sulfide Oxidase Production without Oxygen Control Strategy

Batch fermentation in a fermenter without oxygen control strategy was carried out in order to study the improvement of sulfide oxidase production by *Serratia* sp. strain SO02. Fermentation was conducted using the optimized conditions of shake flask culture with agitation speed and airflow rate fixed at 200 rpm and 1 l/min, respectively. The profiles of *Serratia* sp. strain SO02 fermentation is illustrated in Figure 6.2.

Sulfide oxidase started to accumulate at 0 h incubation after inoculum was introduced into the fermenter. It appeared that sulfide oxidase was already produced in a small amount during an inoculum stage. As a result, sulfide oxidase production was steadily increased as growth occurred. The extracellular sulfide oxidase was actively synthesized during the exponential growth phase of *Serratia* sp. strain SO02. Sulfide oxidase production peaked (4.843 U/ml) at 36 h of incubation when the culture was enter the stationary growth phase and was found to decline gradually until the end of fermentation process. The trend of sulfide oxidase synthesis was found to be more of the growth associated rather than the non-growth associated type. In addition, the specific sulfide oxidase activity of this fermentation was attained at 6.726 U/mg.

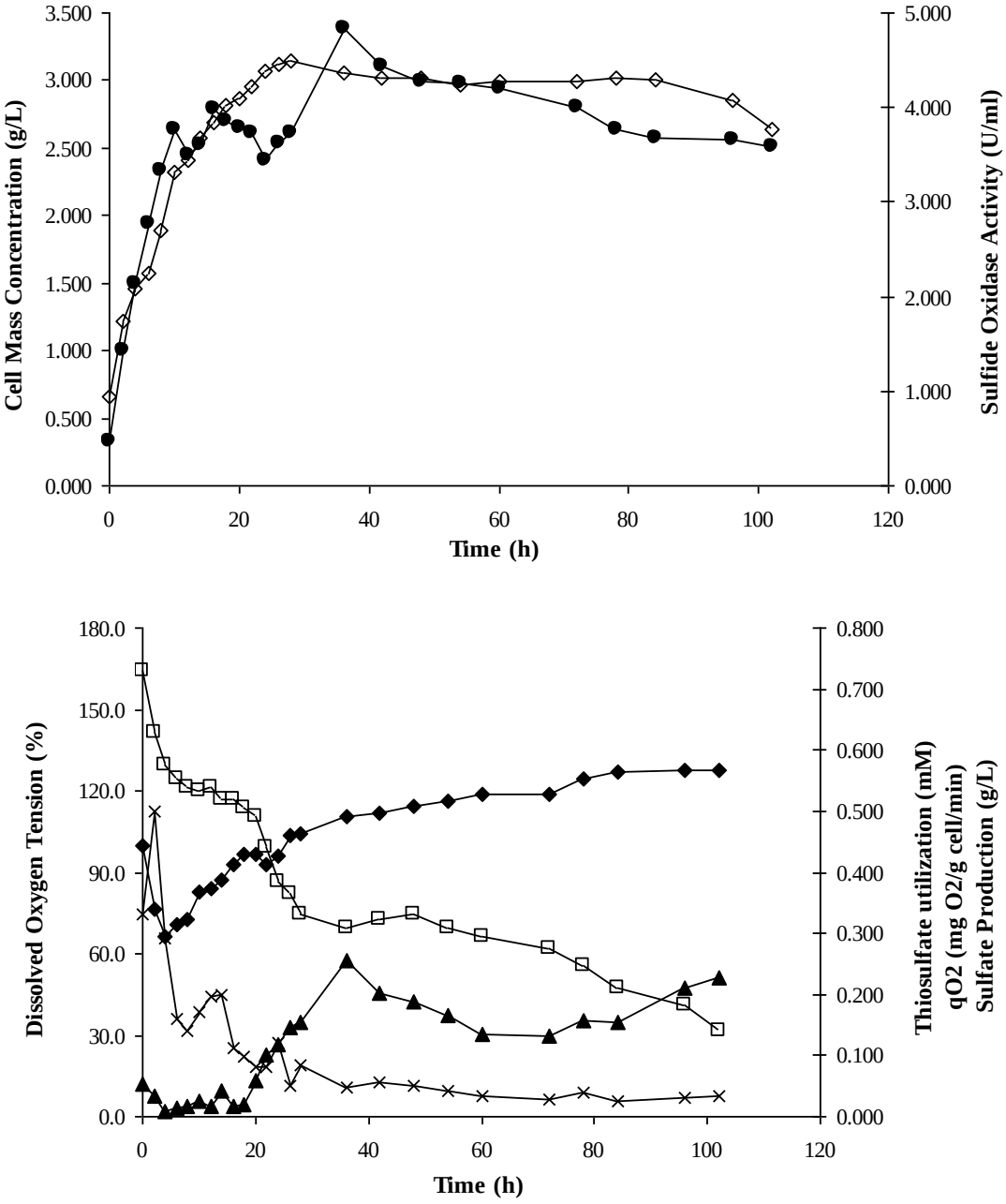

Figure 6.2: Time course of *Serratia* sp. strain SO02 fermentation in batch culture using without DOT control strategy. Symbols represent; cell mass concentration (-◊-), sulfide oxidase activity (-●-): thiosulfate utilization (-□-), sulfate production (-▲-), pO_2 (-♦-) and qO_2 (-×-)

Thiosulfate was actively consumed by *Serratia* sp. strain SO02 during the exponential growth phase of the bacteria up to 36 h of cultivation. Beyond this point, thiosulfate utilization by the bacteria was reduced as the culture reached the stationary growth phase. During the stationary phase, there is no net increase and decrease in cell number, and however cell functions still continue (Madigan *et al*., 2000). This indicates that thiosulfate utilization by the bacteria still occurred during the stationary phase but at the slower rate. Nevertheless, the incomplete utilization of thiosulfate was still observed in this fermentation. The slower rate of thiosulfate consumption during the stationary phase might be owing to deplete amount of essential nutrient in the culture medium that leads to the stationary cell growth (Wan Salwanis, 2005). As a result, thiosulfate utilization by the *Serratia* sp. strain SO02 was slower. During the fermentation process, about 80% of thiosulfate was utilized by *Serratia* sp. strain SO02 within 102 h of incubation time.

A typical growth curve with log and stationary phase was observed with *Serratia* sp. strain SO02 without reaching the lag phase. This happens when the inoculum was taken from culture that was exponentially grown in the same medium and condition with the working fermentation. The cell mass of *Serratia* sp. strain SO02 increased exponentially for 18 hours before reaching the maximum cell mass. Maximum cell mass concentration of 3.149 g/L was achieved at 28 h incubation when the cells growth enter the stationary phase. During the stationary phase, the cell growth was constant and decline slightly towards the end of fermentation process. The specific growth rate of *Serratia* sp. strain SO02 batch culture using fermenter was 0.1402 h^{-1}, corresponds to a doubling time of 4.94 h. Meanwhile, the productivity of cell growth (R_{batch}) was obtained at 0.08912 g/L/h.

The profile of dissolved oxygen tension (DOT) during the fermentation without DOT control was shown in Figure 6.2. The DOT value during the active cell growth of *Serratia* sp. strain SO02 decrease from 100% saturation prior to inoculation to

approximately 66% saturation within 4 to 6 h. The decreasing level of DOT occurred as a result of the increase in cell mass and specific oxygen uptake rate (qO_2) (Figure 6.2). The DOT increased to about 100% saturation towards the end of fermentation process. Increase of DOT in the culture medium was a result of slower cells growth and leads to the reduction of qO_2.

Figure 6.2 also showed the profile of qO_2 during the fermentation without DOT control. The qO_2 was evaluated based on dynamic gassing-out method which utilize an actively respiring culture to get a decrease in the slope of DOT versus time which represents the oxygen uptake rate (qO_2 X) (Rane and Simms, 1994; Ariff *et al*., 1996). The oxygen uptake during the fermentation is a dynamic process affected by cell growth and cell mass concentration. The qO_2 increased rapidly during the first 4 h of incubation due to an increase in cell growth and metabolic activities of *Serratia* sp. strain SO02. The maximum qO_2 was 0.498851 mg O_2/ g cell/ min, which occurred approximately 2 to 4 h after inoculation. At the end of incubation, the qO_2 decreased to approximately 0.034582 mg O_2/ g cell/ min.

Sulfate production by the *Serratia* sp. strain SO02 was gradually increased during the exponential growth phase of the culture. This leads to the suggestion that sulfate was produced as a primary metabolite of *Serratia* sp. strain SO02 during growth in the presence of thiosulfate. Primary metabolite was considered as equivalent to growth-linked product which is synthesized by growing cells (Stanbury and Whitaker, 1984). The maximum sulfate production (0.256 g/L) was attained at 36 h incubation during the stationary growth phase of the culture. Kinetic analysis of sulfate production in fermenter showed productivity ($R_{sulfate}$) and specific rate ($q_{sulfate}$) at value of 0.00561 g/L/h and 0.01180 g/g/h, respectively. In the meantime, the $Y_{p/x}$ value for this fermentation was obtained at 0.08420 g/g.

In this study, the yield coefficient of $Y_{x/s}$ and $Y_{p/s}$ was also investigated. The value of $Y_{x/s}$ and $Y_{p/s}$ obtained in this fermentation were 0.10270 g/mg and 0.00696 g/mg, respectively. According to this value, there are possibility that thiosulfate was utilized more for *Serratia* sp. strain SO02 growth rather than for sulfate production.

Throughout the fermentation, it was obvious that oxygen demand of *Serratia* sp. strain SO02 in this fermentation was lower than the oxygen supply of the fermenter. To study further the effect of control DOT on the production of sulfide oxidase, the subsequent fermentation will be conducted by maintaining the DOT at 30-50% saturation. The production of sulfide oxidase in this study was than compared to the fermentation without DOT control strategy.

6.3.2 Sulfide Oxidase Production with Oxygen Control Strategy

In previous fermentation, the dissolved oxygen tension (DOT) increase at the end of fermentation process indicating the cell growth has already reduced. The increase in oxygen demand occurred at the active period of cells growth and started to decrease beyond the requirement. This showed the oxygen demand for *Serratia* sp. strain SO02 fermentation was low, where the oxygen supply was greater than oxygen requirement. In this experiment, the DOT was controlled at 30 – 50 % saturation to investigate the effect of controlled DOT on growth, sulfide oxidase production and thiosulfate utilization.

Figure 6.3 presented the time course of sulfide oxidase production by Serratia sp. strain SO02. The maximum sulfide oxidase activity of 4.216 U/ml was attained after

36 h of fermentation with specific activity of 7.201 U/mg. The production of sulfide oxidase with DOT control was optimum at the same time (36 h) with sulfide oxidase production using strategy without DOT control (refer section 6.3.1). However, the enzyme activity obtained with DOT control strategy was 13% lower compared to experiment without DOT control. This might be occurred because of lower dissolved oxygen concentration in the fermenter where sulfur-oxidizing bacteria required oxygen for the oxidation process (Siegel, 1975). The control of DOT might have cause lower activity in sulfide oxidase production.

During the fermentation with controlled DOT, *Serratia* sp. strain SO02 utilized 68.4% of thiosulfate within 102 h incubation. The percentage of thiosulfate utilization obtained in this fermentation however, was 15% lowered as compared to previous experiment without DOT control (refer section 6.3.1). The decrease of 15% in thiosulfate utilization might due to oxygen limitation especially during the stationary growth phase of the bacteria (Figure 6.3). This situation can be correlated with theory where oxidizing process of sulfur compounds by bacteria needs oxygen molecule as an electron acceptor (Siegel, 1975).

The profile of *Serratia* sp. strain SO02 fermentation is presented in Figure 6.3. During fermentation process, the growth of microorganism increased rapidly within 16 to 18 h and reached the maximum cell mass at 3.191 g/L. The growth enters the stationary phase after 26 h of cultivation and remained constant towards the end of fermentation. The specific growth rate (μ) of this fermentation was attained by 0.1547 h^{-1} with doubling time of 4.48 h. The microorganism showed productivity in cell mass production (R_{batch}) within this fermentation process by 0.14955 g/L/h.

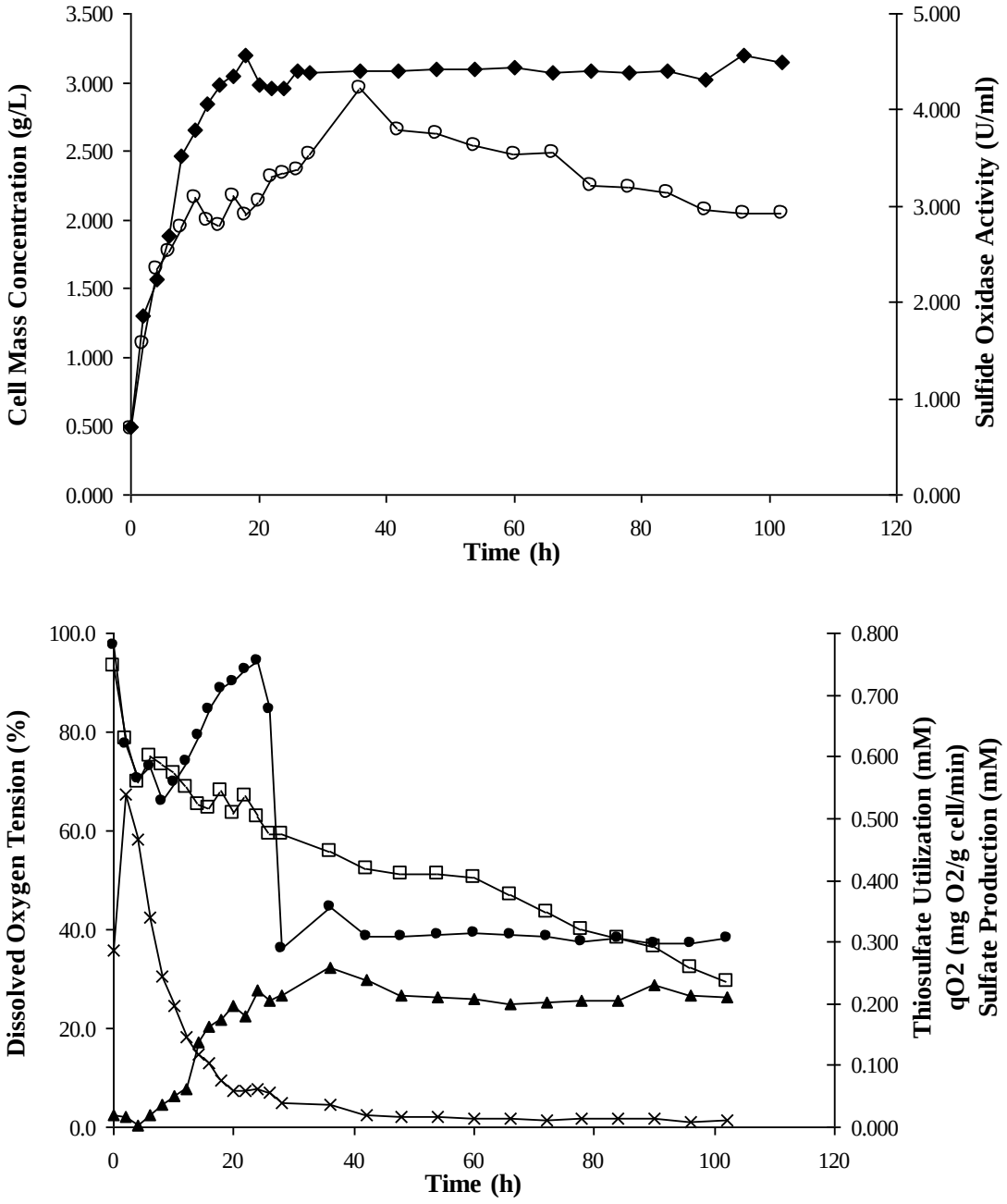

Figure 6.3: Time course of *Serratia* sp. strain SO02 fermentation in batch culture using DOT control strategy. Symbols represent; cell mass concentration (-♦-), sulfide oxidase activity (-○-): thiosulfate utilization (-□-), sulfate production (-▲-), pO_2 (-●-) and qO_2 (-×-)

During the active periods of cell growth, the DOT reduced from 98% saturation to 70% during the first 6 h after inoculation (Figure 6.3). The increased of oxygen demand by microorganism resulted in a decrease of DOT in the liquid phase of the fermenter (Rane and Simms, 1994). Decrease of DOT in the fermenter indicates the raise of specific oxygen uptake rate (qO_2) by microorganism where cells were actively use oxygen for cellular maintenance and metabolism of aerobic organism (Rane and Simms, 1994). Thus in this fermentation process, resulted in an increase in cell mass and sulfate concentration with time. For experiment in which DOT was controlled at 30 to 50% saturation, the maximum qO_2 was obtained with value of 0.538909 mg O_2/g cell/ min. Toward the end of fermentation process, the qO_2 value was decrease to 0.011789 mg O_2/ g cell/ min which indicate the decline of cell growth.

Maximum sulfate production was observed by 0.258 g/L after 36 h inoculation of medium with *Serratia* sp. strain SO02. The sulfate production in this fermentation gives the productivity value of 0.00234 g/L/h and specific rate of 0.001396 g/g/h. In addition to kinetic of sulfate production, the sulfate yield based on cell growth ($Y_{p/x}$) was obtained at 0.09022 g/g.

Throughout the fermentation, thiosulfate was serves as substrate for *Serratia* sp. strain SO02 growth with cell and sulfate production during the fermentation. The cell and product yield based on thiosulfate consumption for the period of this fermentation were 0.11843 g/g and 0.00430 g/g, respectively.

The control of DOT in fermenter obviously affected the production of sulfide oxidase and thiosulfate consumption by cells. Performance of *Serratia* sp. strain SO02 in the condition without DOT control is better as compared to the experiment with DOT control in term of sulfide oxidase activity and thiosulfate utilization. The presence of oxygen is believed to assist with the oxidation of reduced sulfur compounds. The

comparisons of this fermentation with shake flask culture and without DOT control culture are discussed in the next section to study the improvement of the fermentation performances.

6.3.3 Comparative Performances of *Serratia* sp. strain SO02 Fermentation between Shake Flask Culture, without DOT Control and DOT Control Strategy Culture

The improvement of sulfide oxidase production by *Serratia* sp. strain SO02 were observed by comparing the results obtained with shake-flask culture and fermenter (Table 6.1).

Results presented in Table 6.1, showed an improvement in sulfide oxidase synthesis from the fermentation with shake flask culture to the application in the fermenter. The use of fermenter provides effective mixing and aeration to the fermentation broth that assist with sulfide oxidase production by *Serratia* sp. strain SO02 (Betts and Baganz, 2006). In contrast with the fermenter, a major limitation of shake flask is their reliance on surface aeration, leading to the reduced oxygen transfer (Betts and Baganz, 2006). The oxygen limitation could alter the product formation rate and in this case the production of sulfide oxidase (Betts and Baganz, 2006).

The maximum synthesis of sulfide oxidase with activity of 4.843 U/ml was observed in the fermenter without DOT control. There was 32% improvement of sulfide oxidase synthesis in the fermenter as compared to the production in the shake flask culture. In the meantime, the DOT control caused the sulfide oxidase production to

slightly decline roughly about 13% in contrast to the fermentation without DOT control. In the fermenter, the production of sulfide oxidase exhibits the same peak, which is after 36 h inoculation and during the early stage of the stationary phase. The highest specific activity (7.201 U/mg) was attained in fermenter with DOT control due to the lowest protein production in this condition of fermentation.

Table 6.1: Comparative performances of *Serratia* sp. strain SO02 fermentation between shake-flask culture and fermenter

Parameters	**Fermenter**		
	Shake-Flask	**Without DOT Control**	**DOT Control**
Specific growth rate, μ (h^{-1})	0.1422	0.1402	0.1547
Doubling time, t_d (h)	4.87	4.94	4.48
X_{max} (g/L)	3.296	3.149	3.191
Cell mass productivity, R_{batch} (g/L/h)	0.1057	0.08912	0.14955
Max sulfide oxidase activity (U/ml)	3.665	4.843	4.216
Protein concentration (mg/ml)	0.89	0.72	0.59
Specific activity (U/mg)	5.200	6.764	7.201
Concentration of thiosulfate utilized (mM)	0.399	0.588	0.511
Thiosulfate utilization (%)	51.22	80.66	68.41
$Y_{x/s}$ (g cell / mg thiosulfate)	0.06060	0.10270	0.11843
$Y_{p/s}$ (g sulfate / mg thiosulfate)	0.01075	0.00696	0.00430
$Y_{p/x}$ (g sulfate / g cell)	0.12309	0.08420	0.09022
Max sulfate product (g/L)	0.270	0.256	0.258
Sulfate productivity, $R_{sulfate}$ (g/L/h)	0.01798	0.00561	0.00234
Specific rate of sulfate formation, $q_{sulfate}$ (g/g/h)	0.01750	0.01180	0.01396
Max specific oxygen uptake rate,q_{O2} (mg O_2/ g cell/ min)	-	0.498851	0.538909
DOT (%)	-		

Oxidation of thiosulfate by growing cells of *Serratia* sp. strain SO02 required oxygen that act as an electron acceptor (Pronk *et al*., 1990). The availability of dissolved oxygen in the fermenter might give effect on thiosulfate utilization by *Serratia* sp. strain SO02. This has been showed by a decrease in thiosulfate utilization when the DOT in the fermenter was controlled at 30-50% saturation. The decrease about 15% in thiosulfate consumption was observed in culture with DOT control as compared to the culture without control of DOT. This showed that oxygen might be importance for utilization of thiosulfate by *Serratia* sp. strain SO02. Fermentation in the fermenter along with prolonged incubation time was obviously enhanced the thiosulfate utilization with an increment of 36.5% as compared to the shake flask fermentation.

The cell mass production in fermenter without DOT control and with DOT control exhibits a slightly decline results by 1.04 and 1.03 fold as compared to the shake flask culture, respectively. Cell mass production was unpredictably higher in shake flask culture with maximum value of 3.296 g/L as compared to the fermentation in fermenter. On the other hand, the maximum specific growth rate (μ) of 0.1547 h^{-1} was obtained in culture with DOT control that corresponds to doubling time of 4.48 h. It also appears that maximum productivity of cell mass (R_{batch}) which is 0.14955 g/L/h was also observed in culture with DOT control.

The specific oxygen uptake rate (qO_2) values have been described by a model taking into account oxygen consumption by maintenance and growth. During periods of the active cell growth, the qO_2 by microorganism changes significantly. As described by Ozergin-Ulgen and Mavituna (1998), the peak in the qO_2 occurred during the initiation of the exponential phase and decrease gradually as time passed. Obviously, the maximum qO_2 in both cultures of the fermenter was peaked after 2 h incubation during the early stage of exponential phase. This maximum qO_2 indicates high demand of oxygen by microorganism due to thee increase in cell growth and metabolic activity at the period of time. Comparing the qO_2 values for both cultures, the maximum qO_2

(0.538909 mg O_2/ g cell/min) was observed in culture with DOT control and showed 7.43% increment compared to the culture without DOT control.

Sulfate was produced as a result of thiosulfate oxidation by *Serratia* sp. strain SO02 during growth. By comparing three conditions of fermentation, the maximum sulfate production (0.270 g/L) was observed in the shake flask culture system. This corresponds to the kinetic of sulfate productivity, $R_{sulfate}$ and specific rate of sulfate formation ($q_{sulfate}$) with value of 0.01798 g/L/h and 0.01750 g/g/h, respectively. As presented in Table 6.1, the highest $Y_{p/s}$ and $Y_{p/x}$ was observed in the shake flask culture due to high sulfate production with value of 0.01075 g/mg and 0.12309 g/g, respectively. Meanwhile the maximum $Y_{x/s}$ of 0.11843 g/mg was attained in fermenter culture with DOT control.

As a conclusion, by comparing the performances of sulfide oxidase production by *Serratia* sp. strain SO02, the maximal production of sulfide oxidase (4.843 U/ml) was observed in the fermenter under the condition of without DOT control. The improvement of sulfide oxidase production by 1.32 and 1.15 fold was achieved using the strategy of without DOT control as compared to the shake flask culture and culture with DOT control, respectively.

6.4 Conclusion

Sulfide oxidase production by *Serratia* sp. strain SO02 was successfully induced by changing the shake flask culture system to the fermenter. The aeration provided by the fermenter was observed to improve the production of sulfide oxidase and fulfill the objective of this study.

CHAPTER 7

CONCLUSION AND FUTURE RESEARCH

7.1 Conclusion

The optimization studies for sulfide oxidase production by locally isolated bacteria *Serratia* sp. strain SO02 has fulfill the main objective of this study. The findings are summarized as follows:

The 60 strains of potential sulfide oxidase producer were isolated from the Effective Microorganisms (EM) sample. Out of 60 strains, 11 able to produce sulfide oxidase and the most superior sulfide oxidase producer was recognized as strain SO02. The strain SO02 produced maximal sulfide oxidase amongst the screened cultures with sulfide oxides activity and specific activity value at 6.83 U/ml and 2.32 U/mg, respectively.

The isolate strain SO02 is a gram negative bacteria and grow on sulfur-oxidizer agar with circular, smooth, mucoid, opaque and creamy-coloured morphology. Based

on the BLASTn search of GenBank, the partial sequence of the 16S rDNA of strain SO02 shared 99% similarity with *Serratia* sp. The strain SO02 was known as *Serratia* sp. strain SO02 throughout the study.

The optimization of sulfide oxidase production was carried out using the shake flask culture. The optimum conditions for the production of sulfide oxidase were obtained at pH 5.0, 30˚C with the presence of peptone and thiosulfate as a nitrogen and sulfur source of the microbial growth. The optimum peptone and thiosulfate concentrations for the synthesis of sulfide oxidase production and thiosulfate utilization were achieved at 0.2 % (w/v) peptone and 0.5 mM thiosulfate. Under these optimized condition, the improvement of sulfide oxidase production and specific activity was attained up to 60.2% and 90.7%. Sulfide oxidase production by *Serratia* sp. strain SO02 after the optimization studies was observed by 3.848 U/ml with specific activity of 5.460 U/mg. The utilization of thiosulfate by *Serratia* sp. strain SO02 was also increase to 51.2% with after the optimization study.

Development of sulfide oxidase production in batch culture using a bioreactor was carried out in order to study the oxygen requirement of *Serratia* sp. strain SO02. Development of oxygen control strategy towards the production of sulfide oxidase showed that without dissolved oxygen tension (DOT) control strategy was the best condition for sulfide oxidase production. Using without DOT control strategy, the sulfide oxidase activity was attained at 4.843 U/ml with specific activity of 6.764 U/mg. Sulfide oxidase synthesis was evidently decreased by 13% under the condition of 30-50% oxygen control as compared to the without DOT control strategy. During the active growth of *Serratia* sp. strain SO02, the specific oxygen uptake rate (qO_2) increase rapidly due to the increase in cell mass concentration. The maximum qO_2 value of 0.498851 mg O_2/g cell/min and 0.538909 mg O_2/g cell/min was achieved during the fermentation process without the DOT control and DOT control strategy, respectively.

7.2 FUTURE RESEARCH

Several recommendations for future research of this study are listed below:

1. The production of sulfide oxidase in the bioreactor can be improved using several types of fermentation system such as fed batch or continuous system using free or immobilized cell culture.

2. Purification and characterization of sulfide oxidase from *Serratia* sp. strain SO02.

3. The pathway of reduced inorganic sulfur compound oxidation by *Serratia* sp. strain SO02 should be studied in detail.

5. The crude sulfide oxidase can be tested to the palm oil mill effluent (POME) pond in order to study the efficiency of sulfide oxidase to oxidize sulfide into sulfate.

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