CRANFIELD UNIVERSITY

Pakamas Prayuenyong

Biodesulphurisation of coal

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ABSTRACT

The emission of sulphur oxides during the combustion of coal is one of the causes of an environmental problem known as acid rain. Biodesulphurisation technology applied as a method to remove sulphur before coal combustion was investigated in this work. The desulphurisation abilities of three specific bacterial strains including *Rhodococcus erythropolis* IGTS8, *R. erythropolis* X309 and *Shewanella putrefaciens* strain NCIMB 8768 have been evaluated. *R. erythropolis* IGTS8 and X309 were found to be able to remove both inorganic and organic sulphur from model compounds and coal samples. Their abilities to remove sulphur from benzothiophene were observed for the first time. A novel desulphurising bacterium, *S. putrefaciens* was also found to be able to remove inorganic and organic sulphur from coal samples. The bacterium, however, lost its ability to remove organic sulphur from model compounds during the investigation. *R. erythropolis* IGTS8 presented the greatest desulphurisation efficiency among the three bacterial strains. Nevertheless, the desulphurisation activity of *R. erythropolis* IGTS8 was too low for an economical coal biodesulphurisation process as it removed only 32.0% of total sulphur in bituminous coal, and 21.1% of total sulphur in anthracite coal. Alternatively, coal biodesulphurisation can be carried out in inexpensive conditions by using the bacteria inherent in the coal itself. The type of coal has an important effect on desulphurisation efficiency since the sulphur reduction in bituminous coal, which is in a lower rank than anthracite, was greater than the sulphur reduction in anthracite coal. This work also developed and evaluated the analytical methods used in the field. A HPLC method was developed to detect the desulphurisation metabolites of model compounds. The techniques for measuring sulphur in coal were improved.

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SYMBOLS AND ABBREVIATION

ANOVA	Analysis of variance
ASTM	American Society for Testing and Materials
BCA	Bicinchoninic acid
BDS	Biodesulphurisation
BPSo	Biphenylene sultone
BSA	Bovine serum albumin
BSM	Basic salt medium
BT	Benzothiophene
CAAAs	Clean Air Act Amendments
CAPTO	Controlled-atmosphere programmed-temperature oxidation
CV	Coefficient of variation
DBT	Dibenzothiophene
DHBP	2,2'-dihydroxybiphenyl
DMSO	Dimethyl sulphoxide
DOE	The U.S. Department of Energy
DPDS	Diphenyl disulphide
EBC	Energy Biosystems Corporation
EDX	Energy-dispersive X-ray
EtOAc	Ethyl acetate
EPM	Electron probe microanalysis
FAD	Flavin adenine dinucleotide
FBC	Fluidised bed combustion
FGD	Flue gas desulphurisation
FMN	Flavin mononucleotide
FPD	Flame photometric detector
HBP	2-hydroxybiphenyl
HBPSi	2-hydroxybiphenyl 2'-sulphinate
HBPSo	2-hydroxybiphenyl 2'-sulphonate
HDS	Hydrodesulphurisation
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HEPPS	N-[2-Hydrocyethyl]piperazine-N'-[3-propanesulphonic acid]
HPEal	2-(2'-hydroxyphenyl)ethan-1-al
HPESi	(Z)-2-(2'-hydroxyphenyl)ethan-1-sulphinate
HPLC	High-pressure liquid chromatography
ICP	Inductively coupled plasma
ICP-AES	Inductively coupled plasma atomic emission spectrometry
IEA	International Energy Agency
IGT	Institute of Gas Technology
IGTS8	*Rhodococcus erythropolis* IGTS8
IPA	Iso-propanol
L.f.	*Leptospirillum ferrooxidans*
LSD	Least significant difference
LVHRMS	Low-voltage high-resolution mass spectrometry

MA	Micro BCA Reagent A
MB	Micro BCA Reagent B
MC	Micro BCA Reagent C
MITI	Ministry of International Trade and Industry
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NCIMB 8768	*Shewanella putrefaciens* NCIMB 8768
PASHs	Polyaromatic sulphur heterocycles
PEC	Petroleum Energy Center
SEM	Scanning electron microscopy
SPE	Solid-phase extraction
T2C	Thiophene-2-carboxylic acid
T.f.	*Thiobacillus ferrooxidans*
T.t	*Thiobacillus thiooxidans*
TPR	Temperature programmed reduction
VG	Vacuum generators
WR	Working reagent
X309	*Rhodococcus erythropolis* X309
XANES	X-ray absorption near edge spectroscopy
XPS	X-ray photoelectron spectroscopy

CHAPTER 1:

INTRODUCTION

INTRODUCTION

Coal has been accepted as a major source of energy for centuries. In addition, the International Energy Agency (IEA) has forecast a substantial increase in its use over the next few years, rising from 3.5 billion tonnes at present to over 5.3 billion tonnes per year (IEA, 1998). When coal is burnt its sulphur content combines with oxygen to form sulphur dioxide (SO_2), which contributes to both pollution and acid rain. Governments throughout the world have recognised the problems and moved to reduce the amount of SO_2 emission through legislation. For instance, the SO_2 emission limit for coal-fired utility boilers under Title IV of the 1990 Clean Air Act Amendments (CAAAs) has been set at 0.52 kg/10^6 kJ (Miller & Srivastava, 2000).

To meet this standard, flue gas desulphurisation (FGD) has been retrofitted to existing coal combustion plants in many countries (UK Clean Coal Technologies, 1998). In the FGD process, flue gas is sprayed with a slurry made up of water and an alkaline agent, usually lime or limestone (Figure 1.1). The SO_2 is converted into calcium sulphate (gypsum) and disposed of as a wet sludge. Fluidised bed combustion (FBC) has been used in another instance. This method cleans coal inside the furnace where the coal is actually burned (Figure 1.2). Coal is ground into small particles, mixed with limestone and injected with hot air into the boiler. This mixture, a bed of coal and limestone, is suspended on jets of air and resembles a boiling liquid. As the coal burns, the limestone acts as a sponge and captures the sulphur.

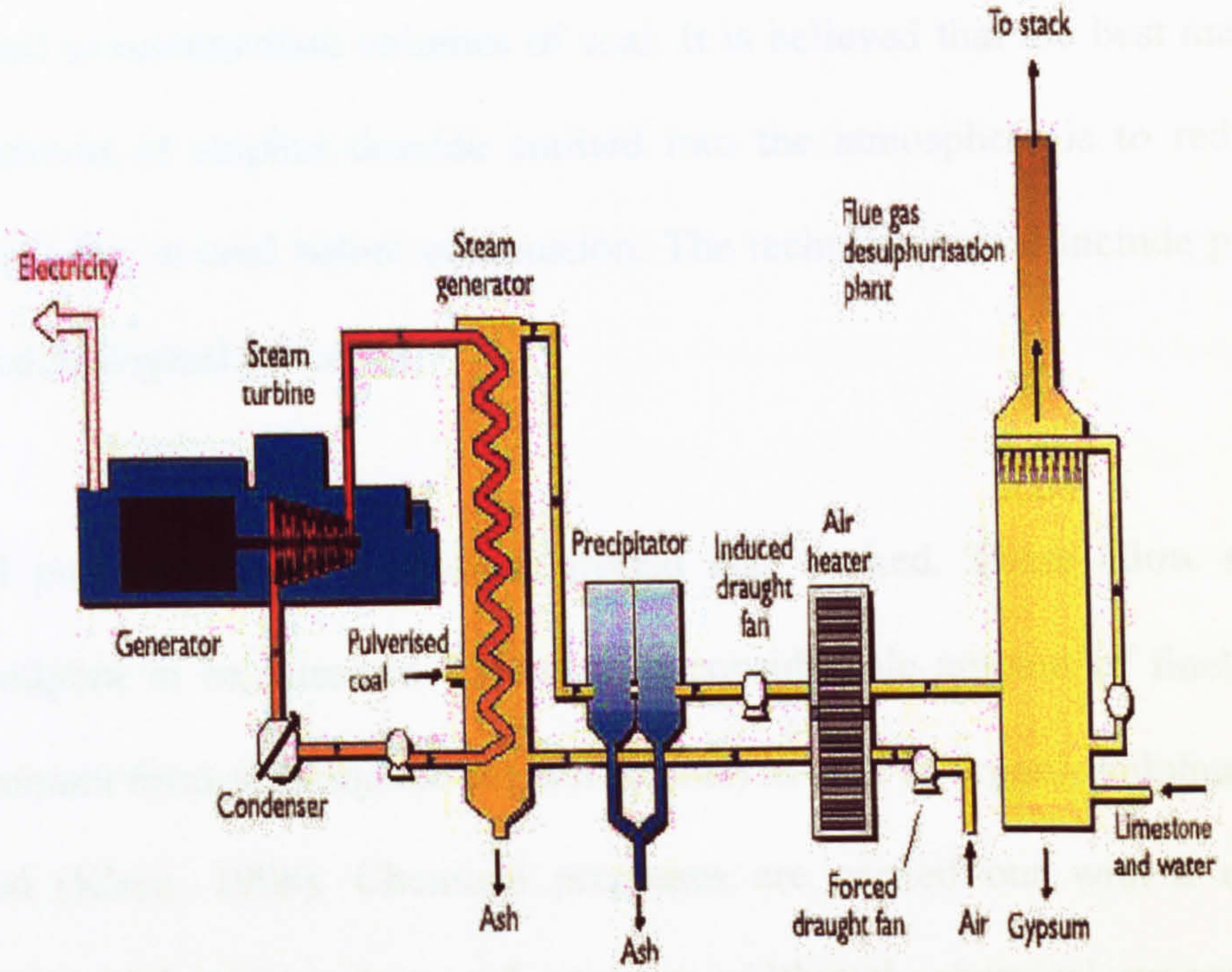


Figure 1.1 Typical coal fired power station with flue gas desulphurisation (UK Clean Coal Technologies, 1998).

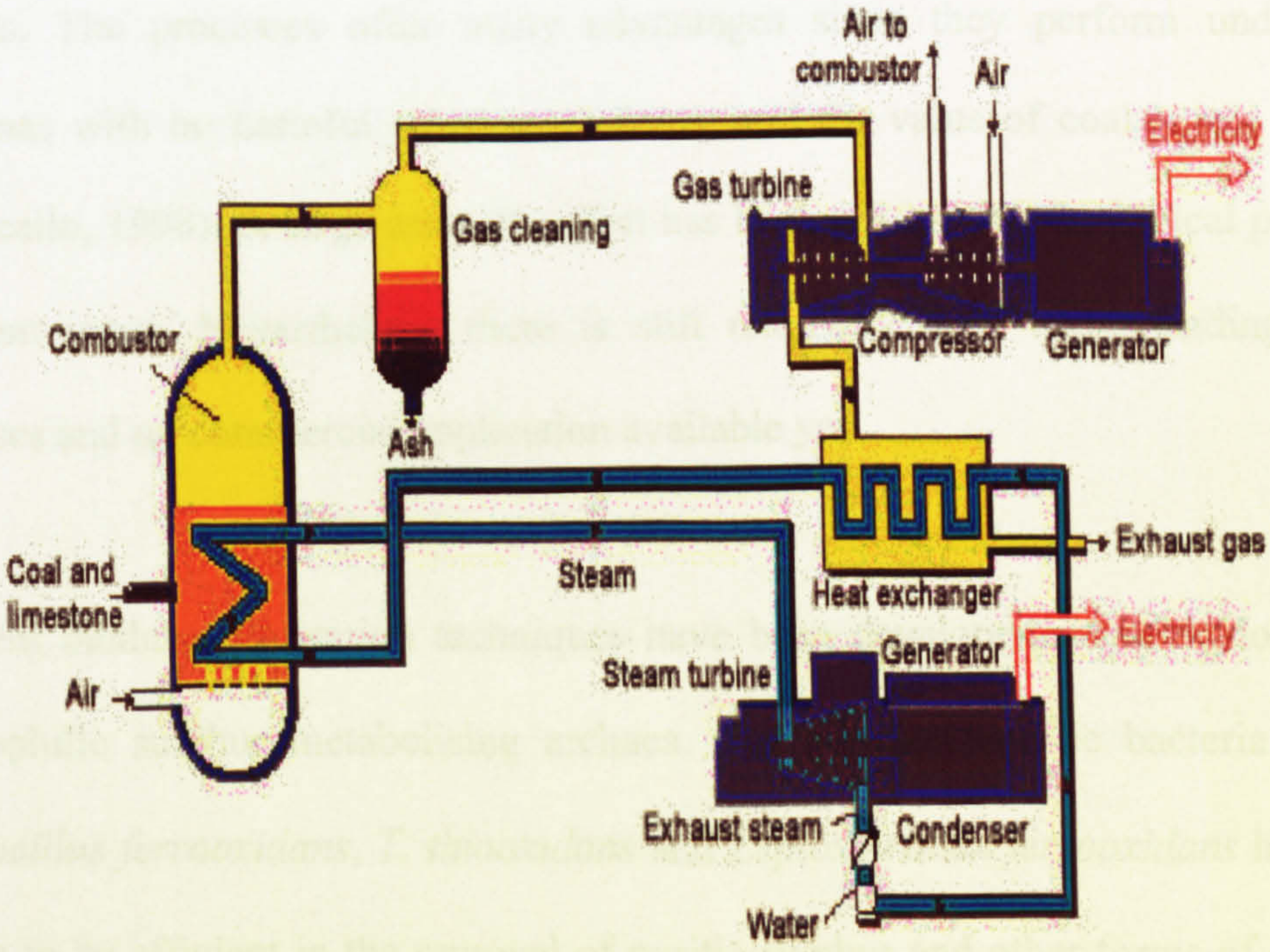


Figure 1.2 Pressurised fluidised bed combine cycle (UK Clean Coal Technologies, 1998).

Nevertheless, both FGD and FBC techniques are too expensive and impractical for users of small to intermediate volumes of coal. It is believed that the best method to limit the amount of sulphur dioxide emitted into the atmosphere is to reduce the amount of sulphur in coal before combustion. The techniques used include physical, chemical and biological processes.

In physical processes coal is crushed, ground and washed. These allow some of inorganic sulphur to be removed. However, a considerable amount of finely pyrite (the predominant form of inorganic sulphur in coal) as well as organic sulphur can not be removed (Klein, 1998). Chemical processes are carried out with a chemical catalyst under high temperature and pressure. Although chemical processes can remove both inorganic and organic sulphur, they are costly, producing hazardous products, and the structural integrity of the coal is affected. Biological processes based on degradation of sulphur compounds by microorganisms are the alternative methods. The processes offer many advantages since they perform under mild conditions with no harmful reaction products, and the value of coal is not affected (Monticello, 1998). A large research effort has been put into the biological processes in recent years. Nevertheless, there is still relatively little understanding in the processes and no commercial application available yet.

Different biodesulphurisation techniques have been developed using acidophilic or thermophilic sulphur metabolising archaea. The mesoacidophilic bacteria such as *Thiobacillus ferrooxidans*, *T. thiooxidans* and *Leptospirillum ferrooxidans* have been proven to be efficient in the removal of pyritic sulphur and other forms of inorganic sulphur, but inefficient in the removal of organic sulphur from coal (Juszczak *et al.*,

1995). The thermoacidophilic bacteria including *Sulfolobus acidocaldarius*, *Acidianus brierleyi*, *Metallosphaera sedula* and *Thiobacillus caldus* were reported to remove both inorganic and organic sulphur from coal. At the elevated temperature used for the thermophilic bacteria, the removal of inorganic sulphur is greater and faster. However, precipitation of iron sulphates also increases and counteracts the desulphurisation as the precipitates stick to the coal even after the washing step. Therefore, the mesoacidophilic bacteria seem to be more appropriate to apply for the removal of inorganic sulphur. In addition, the experiments using a sulphur model compound, dibenzothiophene (DBT) which is generally regarded as a model compound representative of the forms of organosulphur found in coal, have shown that the thermoacidophilic bacteria can only break the C-C bond, not the C-S bond of the compound. So that sulphur still remains in the compound and the caloric value of the compound is reduced (Konishi *et al.*, 1997). More preferable, desulphurising microorganisms should attack the sulphur without altering the carbon skeleton or affecting other valuable components in the coal structure. *Pseudomonas* strain Coal Bug One (CB1) isolated from coal has suggested that sulphur could be removed specifically from coal with little or no reduction of caloric value. Nevertheless, it is unfortunate that the desulphurisation ability of strain CB1 has been lost and the strain is no longer available for the research community.

Up to date, many researchers have carried out their work using *Rhodococcus* species. *R. erythropolis* IGTS8 has been the most extensively studied strain since it can selectively remove sulphur from DBT without reduction of caloric value (Kayser *et al.*, 1993). In practice, there are many DBT derivatives such as alkyl-substituted, and it is considered that the more complicated compounds are the more recalcitrant to

chemical desulphurisation (Ohshiro *et al.*, 1996). *R. erythropolis* IGTS8 has not been shown to desulphurise DBT bearing alkyl substitutions adjacent to the sulphur atom. *R. erythropolis* ECRD-1 which can selectively remove sulphur from these DBT derivatives was then isolated (Lee *et al.*, 1995). Interestingly, even with the obvious chemical similarity of DBT and benzothiophene (BT), the *Rhodococcus* species able to desulphurise DBT such as strain IGTS8 could not desulphurise BT (Gilbert *et al.*, 1998). *Gordonia* sp. strain 213E was later isolated for the desulphurisation of BT. Nevertheless, the strain 213E could not desulphurise DBT (Gilbert *et al.*, 1998). This absence of cross-reactivity is desirable. It is observable from the literature that all cultures proclaimed useful for removing sulphur are not versatile for a wide range of sulphur compounds. Furthermore, their desulphurisation abilities seem unstable and the reproducibility of results is poor. It is necessary to search for more stable and active microbial cultures with improved efficiency toward a wider variety of sulphur compounds in order to achieve a successful biodesulphurisation process.

Previous work at Cranfield Biotechnology Centre reported that when using *Shewanella putrefaciens* (NCIMB 8768) in clay desulphurisation, sulphur odour was reduced (Whittles, personal communication). A more detailed investigation was undertaken in the present study to examine the ability of this strain of *S. putrefaciens* in sulphur removal from the sulphur model compounds (both organic and inorganic forms). The effect of different parameters on the desulphurisation ability of this strain has been examined. The results achieved were compared to those obtained by the current desulphurising bacteria, *R. erythropolis* strain IGTS8 and strain X309.

Although there were many reports on the desulphurisation ability of *R. erythropolis* strain IGTS8, most of these investigations were carried out with sulphur model compounds which are recognised to behave differently to sulphur in coal (Davidson, 1994). Very few studies on biodesulphurisation of certain coal have been published. Therefore, experiments with coal samples performed in this study may provide data of greater relevance to coal biodesulphurisation than experiments which employ sulphur model compounds. Biodesulphurisation of two types of coal (bituminous and anthracite) by *R. erythropolis* IGTS8, *R. erythropolis* X309, *S. putrefaciens* and the bacteria inherent in the coal were investigated.

In addition, to gain more understanding of the coal biodesulphurisation processes, accurate analytical techniques for measuring sulphur in coal are required. The most common methods used are the standard methods of the American Society of Testing Materials (ASTM): a coal sample is analysed chemically to determine total sulphur and sulphate sulphur; pyritic sulphur is calculated from pyritic iron; and organic sulphur is obtained indirectly by subtracting the sulphate and pyritic sulphur contents from total sulphur content. The techniques are time consuming, cumbersome and not so consistent; many errors can be introduced in each stage of the analysis. Therefore, it is difficult to monitor the efficiency of the different bacterial desulphurisation processes with confidence. In this study, the alternative determination methods of sulphur forms in coal were proposed and evaluated.

CHAPTER 2:

LITERATURE REVIEW

2.1 INTRODUCTION

This Chapter is intended to cover the overview and progress achieved to date in coal desulphurisation. The focus is primarily on microbiological processes. At the end of this Chapter, the aims and objectives of this research project are detailed.

2.2 COAL FUEL

2.2.1 The origin of coal

Coal is a carbonaceous deposit derived from vegetation, which has been consolidated between other rock strata to form coal seams, and altered by the combined effects of microbial action, pressure and heat over a considerable period of time. Initially, the peat (the precursor of coal) was converted into lignite. Lignite was later changed into the range known as sub-bituminous. As the processes continued, the coal became harder and is classified as bituminous and then anthracite. The degree of metamorphism undergone by coal has an important bearing on its physical and chemical properties, and is referred to as the rank of the coal. Low rank coal, such as lignite and sub-bituminous coal, is typically softer, friable materials with a dull and earthy appearance. Higher rank coal is typically harder and stronger. Increasing rank is accompanied by a rise in the carbon and energy contents and a decrease in the moisture content of the coal as shown in Figure 2.1.

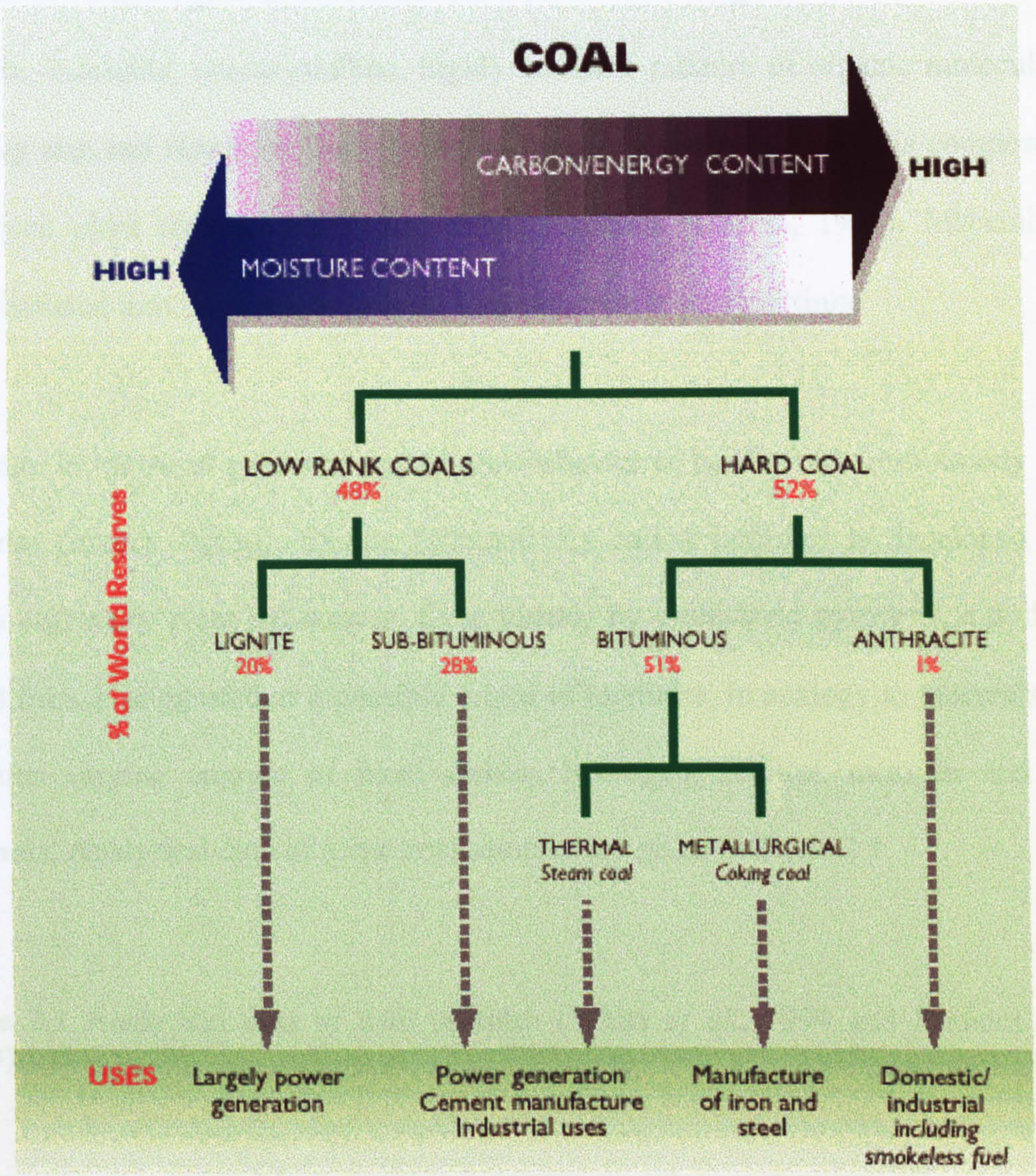


Figure 2.1 Rank of coal with world reserves and uses (World Coal Institute, 1999).

2.2.2 Coal structure

Coal is not a homogenous substance. As a result of its origin, coal is an almost non-volatile, insoluble, non-crystalline, highly complex mixture of organic molecules of varying size and structure. Coal, being an organic sedimentary rock, is composed of fossilised plant remains which are called macerals (Haenel, 1992). Macerals are differentiated into three major groups; vitrinite, exinite and inertinite.

Vitrinite is the most prevalent group and believed to be derived from woody plant material (mainly lignin). Exinite (alternatively called liptinite) is developed from lipids and waxy plant substances. Char formed by prehistoric pyrolysis, e.g. during wood fires, is suggested as a possible origin of inertinite. In analogy to macerals, coal contains varying amount of fixed carbon, hydrogen, sulphur, nitrogen and trace minerals. Analytical data of some coal samples are given in Table 2.1.

Table 2.1 Analytical data of coal samples (White *et al.*, 1994 and Rubiera *et al.*, 1999).

Analysis	Lignite	Sub-bituminous	Bituminous	Anthracite
Proximate analysis (wt%)				
Moisture	7.4	3.6	1.3	0.9
Volatile matter	40.1	33.7	34.8	7.1
Ash	20.0	26.1	8.4	33.0
Fixed carbon	32.5	36.6	55.5	59.0
Ultimate analysis (wt %)				
C	68.8	65.4	84.5	87.9
H	4.5	4.8	5.5	3.6
N	0.7	-	-	-
S	9.9	8.4	1.8	4.4
O	16.1	-	-	-
Form of sulphur (wt %)				
Pyritic	0.57	2.1	0.8	2.9
Sulphate	0.03	0.3	0	0.1
Organic	9.30	6.0	1.0	1.4

It is extremely difficult to detail the characteristic of coal structure. Many models of coal structure have been proposed. Figure 2.2 shown a model of hard coal was proposed by Wise (1981).

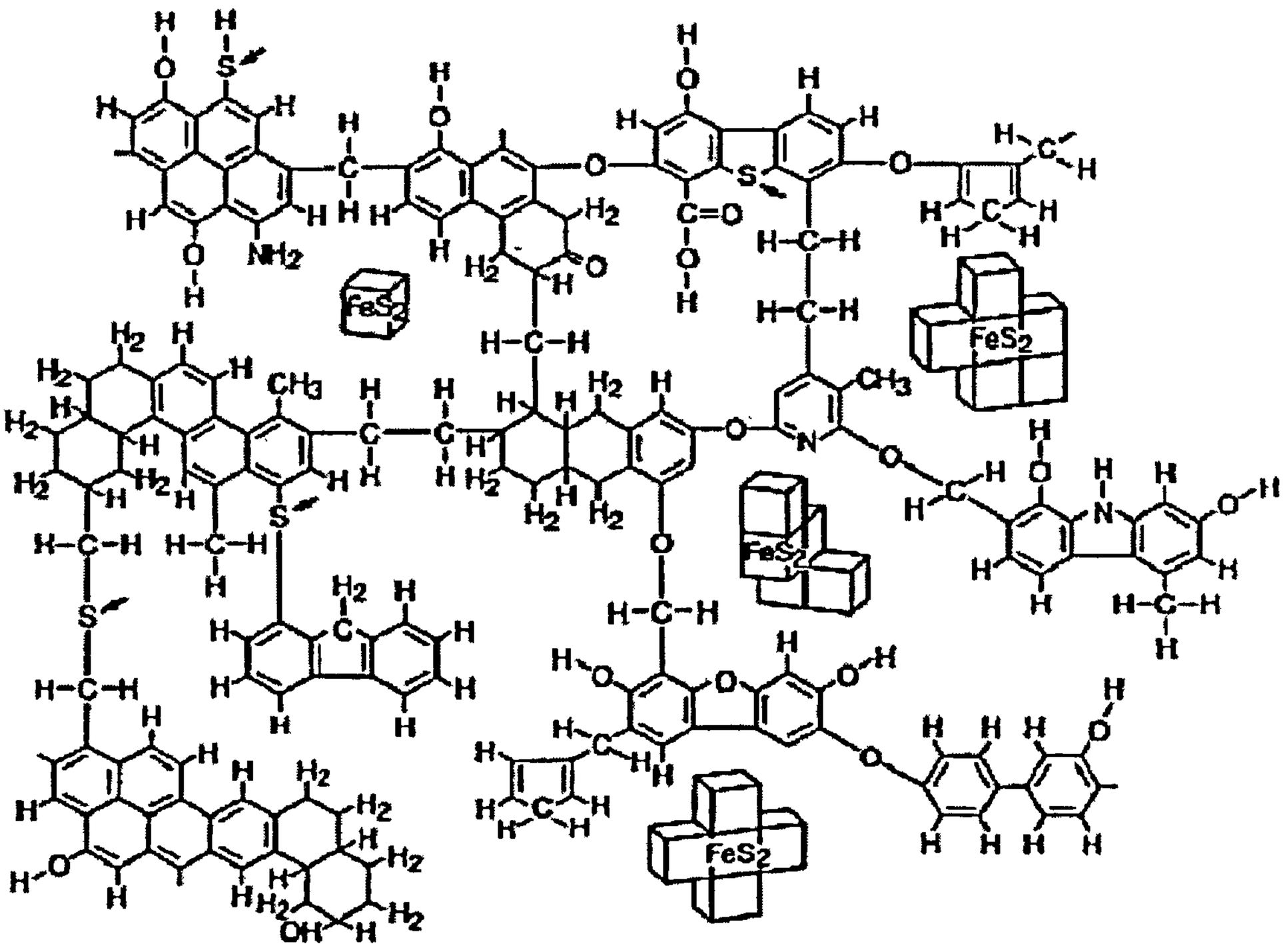


Figure 2.2 Structural model of hard coal (Wise 1981).

The model assumes that the aromatic and hydroaromatic structural units of three to five rings are cross-linked through short aliphatic and ether bridges to form macromolecular aggregates. Later, this model was adapted by Jones *et al.* (1999) to fit the ultimate analysis of the coal as shown in Figure 2.3.

As an alternative, a two-component system has been suggested for the vitrinite of bituminous coal (Haenel, 1992). This model largely abandons the concept of individual structures. A macromolecular, three-dimensional network of the coal substance forms the immobile phase in which is embedded a multitude of relatively small molecules of varying structures forming the mobile phase. The sketch of this model is shown in Figure 2.4. However, this model retains some of the previous structural concepts, for instance the identification of aromatic/hydroaromatic structures and aliphatic/ether bridges.

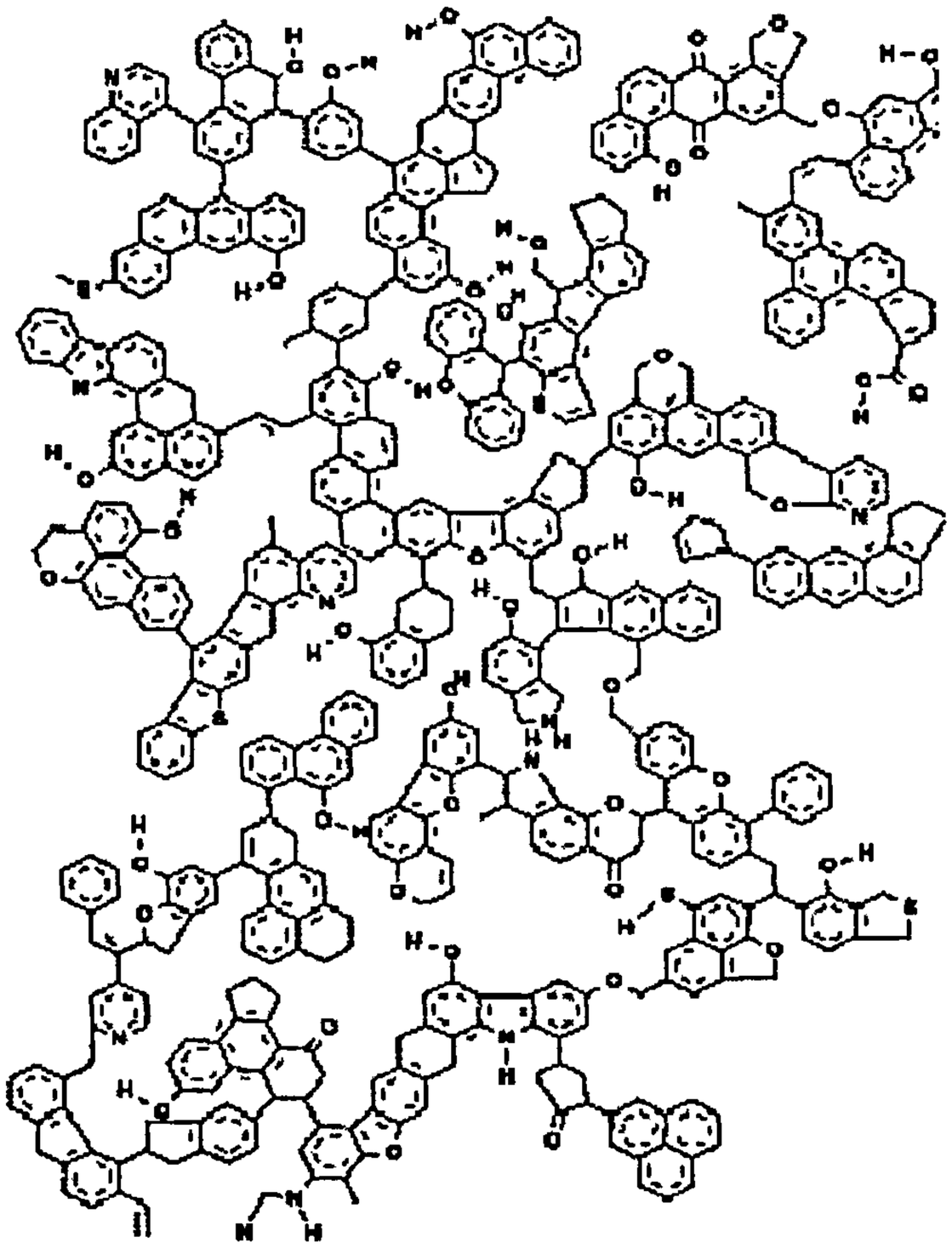


Figure 2.3 Structural model of bituminous coal (Jones *et al.*, 1999).

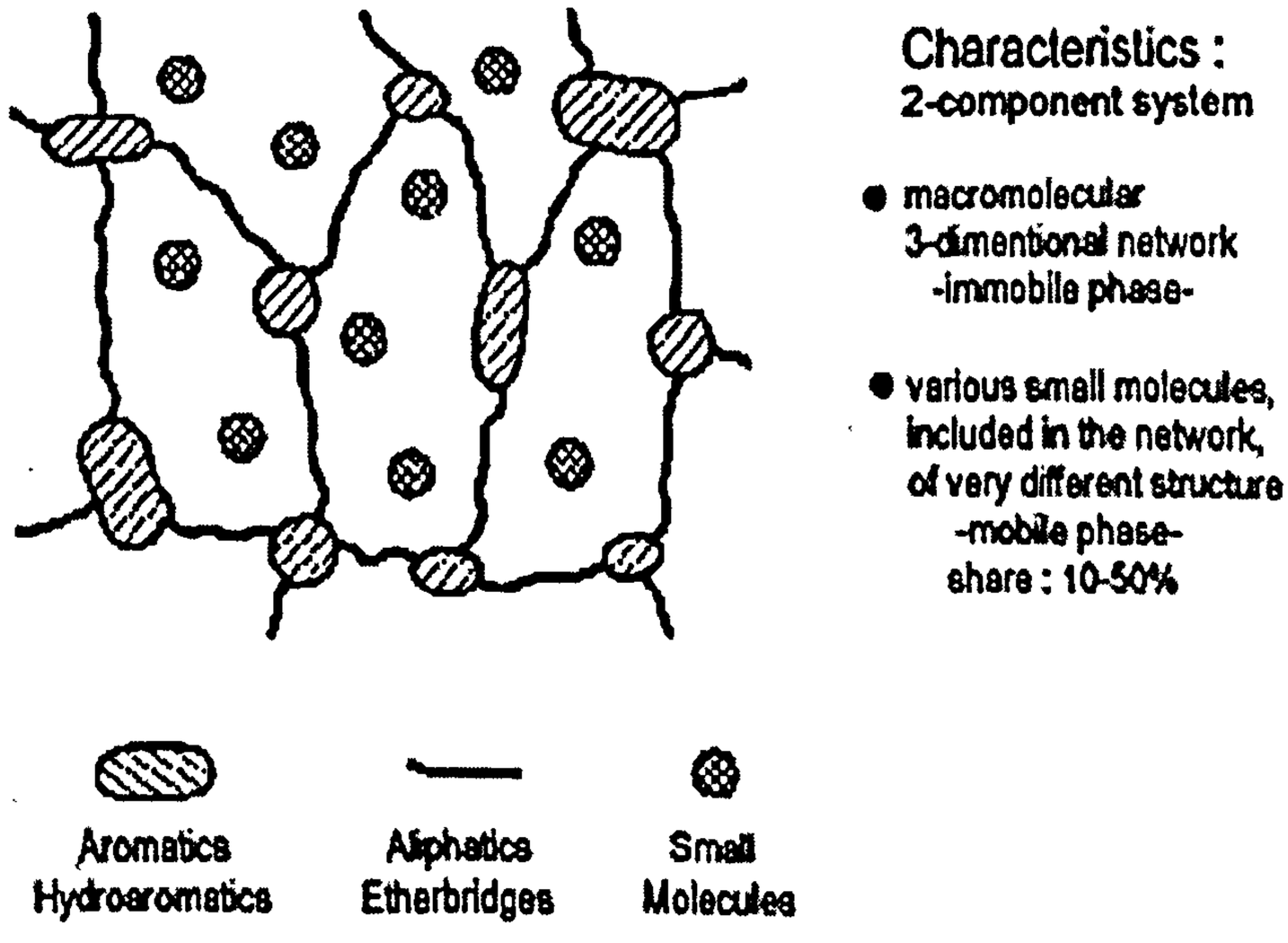


Figure 2.4 Conceptual coal model (Haenel, 1992).

2.3 SULPHUR COMPOUNDS

Living cells possess several organosulphur compounds, for example amino acids, vitamins, coenzymes, penicillins, biotin, sulphone, and chondroitin sulphate. Various amounts and selections of these compounds were transformed into different forms of sulphur in coal, depending upon the geobiology and geochemistry of sulphur cycling (Murty *et al.*, 1994).

2.3.1 Sulphur compounds in coal

Sulphur in coal is present in both inorganic and organic forms. The total sulphur contents vary considerably from <0.1 wt% to >7.0 wt%. The inorganic sulphur in coal consists predominantly of sulphides and sulphates. Sulphide minerals include pyrite (FeS_2), sphalerite (ZnS), galena (PbS), arsenopyrite (FeAsS) and others. The sulphate minerals include barite ($BaSO_4$), gypsum ($CaSO_4.2H_2O$), anhydrite ($CaSO_4$), and a number of iron sulphates and others (Calkins, 1994). The pyritic sulphur is generally the preponderant inorganic sulphur in coal. Particles of pyrite are randomly distributed as crystals throughout the coal but are not bound to it as shown in Figure 2.2 (Klein *et al.*, 1994). The pyrite is probably formed by reaction of the H_2S from bacterial reduction of sulphate with ferrous or ferric ions in the ground water (Calkins, 1994). The organic sulphur in coal is covalently bound into its large complex structure, so that it is more difficult to remove physically, chemically, or biologically in contrast to pyritic or inorganic sulphur (Constanti *et al.*, 1994). The organic sulphur in coal exists as both aliphatic and aromatic or heterocyclic forms, which can be classified into four groups (Klein *et al.*, 1994):

1) aliphatic or aromatic thiols (mercaptans, thiophenols);

2) aliphatic, aromatic, or mixed sulphides (thioethers);

3) aliphatic, aromatic, or mixed disulphides (dithioethers); and

4) heterocyclic compounds or the thiophene type (dibenzothiophenes).

Figure 2.5 represents the four types of organic sulphur compounds. There is considerable evidence that the low-rank coal contains more of the organic sulphur in aliphatic or labile form, whereas the higher-rank coal contains predominantly heterocyclic sulphur. The thiophenic sulphur compounds in high-sulphur coal can be grouped into families with up to six rings including thiophenes, benzothiophenes, dibenzothiophenes, naphthothiophenes, phenanthrathiophenes and anthrathiophenes. Many of these have structural isomers, and contain alkyl substituents up to C6 or longer. Some of these substituents are unsaturated (e.g. vinyl) or branched (Calkins, 1994). It is assumed that the thiophenic structures in coal originated through incorporation of inorganic sulphur into unsaturated compounds, followed by cyclisation and aromatisation of branched alkyl thiophenes.

Vetter *et al.* (1989) proposed that the organic sulphur in low-sulphur coal (<0.5 wt%) is obtained primarily from the sulphur components of the coal-forming plant such as cysteine and cystine (sulphur containing amino acids) whereas the sulphur in high-sulphur coal is obtained primarily from the bacterial reduction of sulphates from sea water or brackish water. Evidence for this came first from geology when it was noted that high-sulphur coal in the Illinois Basin were overlain by marine shale and limestone whereas lower-sulphur coal in the Appalachian Basin were not.

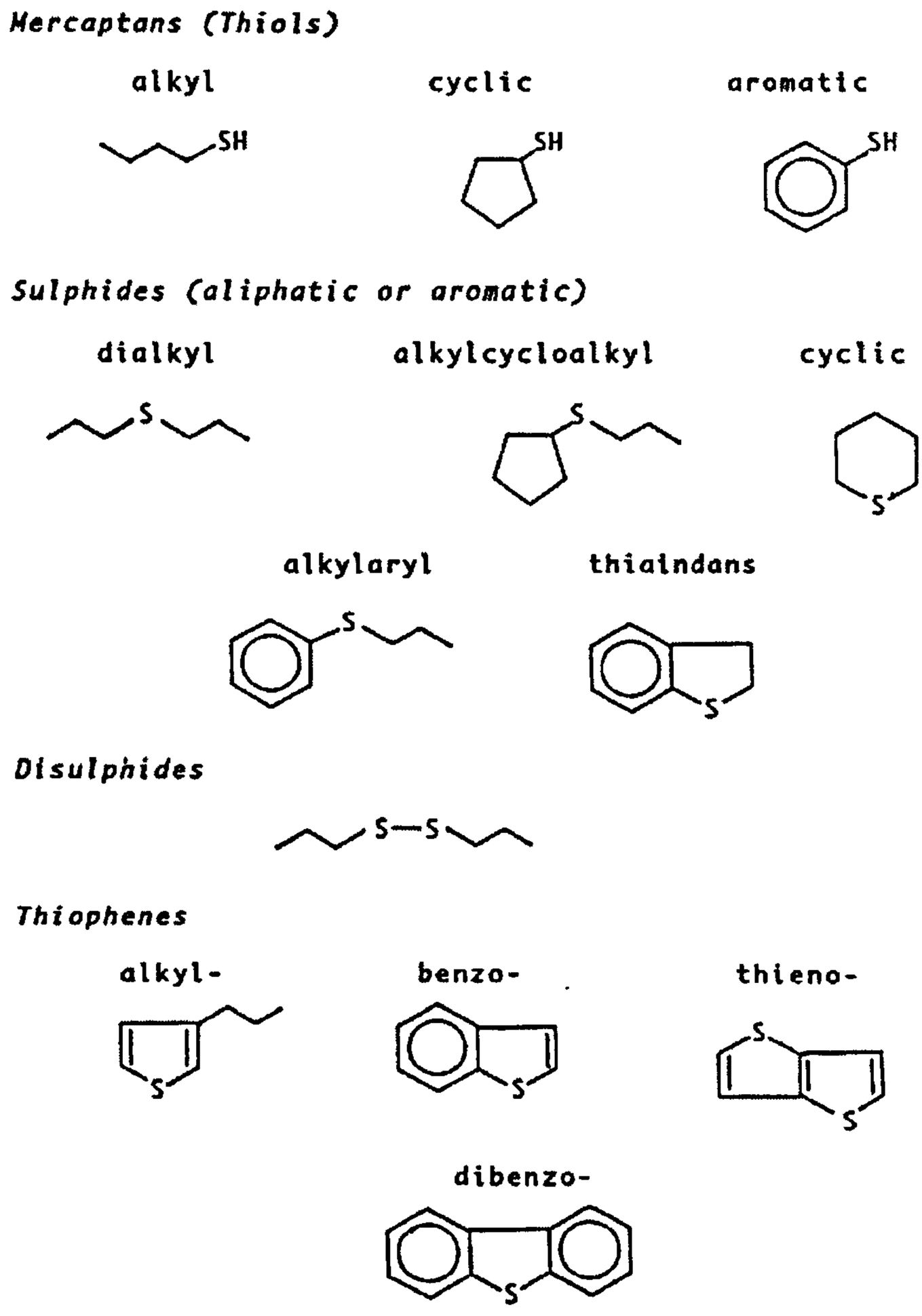


Figure 2.5 Types of sulphur-containing organic compounds identified in coal (Shennan, 1996).

2.3.2 Methods of analysing and identifying sulphur compounds

With the need for coal desulphurisation, techniques to quantify and identify sulphur compounds in coal are required. The customary methods used for the determination of total sulphur are combustion methods. The coal is oxidised in an oxygen bomb calorimeter or ignited in a mixture with magnesium oxide and sodium carbonate (Eschka mixture) and the resulting sulphate is determined gravimetrically as barium sulphate. For instance, in total sulphur analysis via ASTM D3177 coal is ignited with Eschka mixture and then dissolved in hot water to get barium sulphate ($BaSO_4$) as a precipitate which will be weighed for calculation (American Society for Testing and Materials, 1993). Alternatively, coal can be burned in a furnace in a stream of oxygen and the sulphur dioxide determined by infrared spectrometry (Leco method). This latter method requires calibration but is quicker and easier.

Because most of the sulphate minerals can be extracted with hydrochloric acid (HCl) whereas many of the sulphide minerals cannot, the sulphate sulphur are usually determined by extraction with HCl followed by titration of the acid extract or conversion to $BaSO_4$ for a gravimetric determination. In the ASTM D2492, sulphate sulphur is extracted using dilute HCl and then quantified using the gravimetric determination of $BaSO_4$ precipitate. The residue from sulphate analysis is further extracted for pyritic sulphur using dilute nitric acid (HNO_3). The pyritic sulphur is calculated following determination of iron concentration in the extract solutions by atomic absorption spectrometer by assuming a FeS_2 stoichiometry for pyrite (American Society for Testing and Materials, 1994). The organic sulphur is calculated by subtracting the sum of sulphate and pyritic sulphur from the total sulphur.

The calculation of organic sulphur is subject to many errors accumulated in the analyses of sulphate, pyritic and total sulphur. For example, the assumption that all of the iron sulphides extracted by nitric acid have the same stoichiometry as pyrite could cause inaccuracies in the calculation of pyritic sulphur. The sulphur attributable to sulphide minerals other than pyrite will also be unaccounted for in the calculation. Furthermore, there may be a contribution of iron from either the partial dissolution of iron silicate or iron oxide minerals leading to an over-estimation of pyritic sulphur (Kilbane, 1989). Therefore, more accurate analytical methods of sulphur forms in coal are required.

Recently, the sequential digestion method developed by Laban & Atkin (2000) has been reported for the direct determination of sulphate, pyritic and organic sulphur concentrations in coal. A three-stage extraction was developed, using acid digestion in a microwave oven. In the first stage, 5M HCl was used to dissolve sulphate phases in coal. Pyritic sulphur was then extracted using 2M HNO_3. The final stage, for the determination of organic sulphur, involved the use of concentrated HNO_3, HCl, hydrofluoric acid (HF) and boric acid for the complete decomposition of residue that remained following stage 2. The extract solutions from each stage were analysed for sulphur by inductively coupled plasma atomic emission spectrometry (ICP-AES). The sums of the three forms of sulphur have shown consistent agreement with certified total sulphur data for most of the coals studied. However, use of HF in the process poses a potential hazard which should be avoided. The technique is thereby not preferable.

Determining the actual forms of sulphur, other than organic or inorganic is considered to be more difficult. The flash pyrolysis technique was proposed. This technique applies heat to convert sulphur in coal to volatile compounds. The quantification of the organic sulphur forms was based on a study of organic sulphur model compounds which are recognise to behave differently to organic sulphur in coal (Davidson, 1994).

A reduction technique in which all the sulphur groups in coal could be reduced to H_2S by heating in the presence of reducing solvents was later proposed. Each reduction reaction has a given activation energy and frequency factor which is different for different sulphur groups. The problem was to determine these for each sulphur group, and ensure that all the sulphur in each group in the coal sample was reduced to H_2S. Additionally, the amount of H_2S produced needed to be measured accurately. Temperature programmed reduction (TPR) with an automated potentiometric method of determining H_2S has been later developed. This should improve the precision of detection, but it still suffers from limited quantitative reduction of the sulphur groups (Davidson, 1994).

Similarly, the controlled-atmosphere programmed-temperature oxidation (CAPTO), in which the sulphur compounds in coal were oxidised to SO_2, was invented. Lately, pyrolysis-gas chromatography, in combination with a flame photometric detector (FPD), a mass spectrometer or a low-voltage high-resolution mass spectrometry (LVHRMS) has been introduced. However, all of these methods still have their limitations.

All of the procedures described above are destructive methods. Non-destructive methods for sulphur determination are preferable. The instrumental techniques which have been predominant in sulphur determination in coal are based on electron microscopy. The general principle behind this is that a high energy electron beam interacts with atoms in the coal sample. The atoms which are struck by the electron beam are excited into a higher energy state. Characteristic X-rays are emitted as the atoms return to the stable ground state, and the intensity of the X-rays is measured. Sulphur is usually determined by measuring the area of its Kα peak (Davidson, 1994).

There have been some reports on the use of two closely related techniques; scanning electron microscopy (SEM) and electron probe microanalysis (EPM). Quantitative analysis by SEM and EPM is performed by comparing the X-ray intensity produced by the sample with that produced by a standard of known composition. The methods are time consuming as account is taken of the weight percentages of the various coal macerals. The sulphur content is not the same for every maceral in a sample.

Although infrared spectroscopy and nuclear magnetic resonance are routinely used for coal analysis, neither has proved satisfactory for sulphur. The instrumental techniques which have proved more successful are types of X-ray spectroscopy. X-ray photoelectron spectroscopy (XPS) is a technique which can provide information on the chemical state of elements in a solid state (Davidson, 1994). This solid is irradiated with monochromatic X-rays, which eject electrons from the core levels of the nuclei. The kinetic energy of the electrons, the binding energy, is characteristic of both the element and its state of chemical bonding. Sulphur at or near the surface of coal particles can be investigated by observing the S 2p peak, that is electrons ejected

from the 2p level. XPS analysis is limited by the escape depth of the electrons, usually about 2-4 nm. Therefore, XPS is primarily a surface analysis technique and, in untreated coal samples, there is a lack of resolution of the sulphur peaks. Deconvolution of the spectra and oxidation of the coal can improve matters, nevertheless the improvement is only partial.

X-ray absorption spectroscopy has been used in another instance. A coal sample is irradiated with an intense beam of X-rays until there is sufficient energy to eject an electron from an atom absorbing the X-rays. Then, the absorption rises rapidly to form the absorption edge (Davidson, 1994). The spectrum in the region of this edge is studied in X-ray absorption near edge spectroscopy (XANES). In general the K-edge XANES spectra do not show a great deal of resolution. Recently, L-edge XANES spectra have been obtained. XANES method suffers, as do other non-invasive methods, from inadequate resolution.

In fairness, there would seem to be no more reason to be sceptical of the non-invasive techniques than of the chemical techniques. They all use deconvolution of some sort. The non-invasive technique will hardly become a routine laboratory technique since it is a highly specialised technique and therefore not convenient for routine analysis. Nevertheless, the non-invasive techniques should provide a useful check on interpretation of data from the chemical techniques. Further studies and development of the analytical methods of sulphur are still required.

2.4 DESULPHURISATION PROCESSES

To decrease the sulphur content in coal before combustion, physical, chemical and biological processes have been applied. Physical methods involve grinding, screening and washing processes. The heavier pyritic sulphur particles can be removed by floating off the remaining coal or by magnetic attraction. However, a considerable amount of finely distributed pyritic and organic sulphur can remain in and attach to the coal particles.

The inability of physical methods to completely remove even the inorganic sulphur has led to the development of many chemical desulphurisation processes. These include carbonisation in different atmospheres, air oxidation, wet oxidation, Meyers process, chlorination and extraction with sodium hydroxide, copper chloride and ethanol solutions. Table 2.2 summarises the results of the chemical desulphurisation studies using Turkish lignite.

Hydrodesulphurisation (HDS), a physicochemical technique, has been applied as a conventional sulphur removal process worldwide. It is a high-pressure (150-250 psig) and high-temperature (200-425 °C) process (Monticello, 1998). Figure 2.6 presents a typical process layout of this technique.

Table 2.2 The results of chemical desulphurisation studies using Turkish lignite (Yaman *et al.*, 1995).

Method	**Sample**	**Temp. (°C)**	**Time (min)**	**Total sulphur removal (%)**
Carbonisation (Fischer retort)	From 11 regions	440	Until the gas release is complete	19.9-46.0
Steam Carbonisation	Beypazari	600	300	67.9
	Afsin	450	300	68.9
Carbonisation in CO_2	Cayirhan	800	60	64.9
Oxydesulphurisation	From 3 regions	125-175	60	20-23
Air oxidation	Cayirhan	550	15	41.92
Alkali extraction	Can	200	30	53.0
Ethanol-alkali extraction	Beypazari	243	60	86.6
Carbonisation in N_2	From 7 regions	900	30	31.2-79.1
Carbonisation in NH_3	From 7 regions	800	5	26.7-75.1
Air oxidation	From 7 regions	550	15	28.4-52.3
Alkali extraction	From 3 regions	70	480	61.1-75.0
Meyers	Cizre	40	720	10.76
Alkali extraction	Soma	27-187	30-150	5-33
Chlorination	Keles	74	300	25.36-58.99
	Agacli	74	300	23.58-71.07
Meyers	Keles	80	60	64.84
	Agacli	80	60	85.18
Oxidation by $CuCl_2$	Goynuk	150	210	52.95

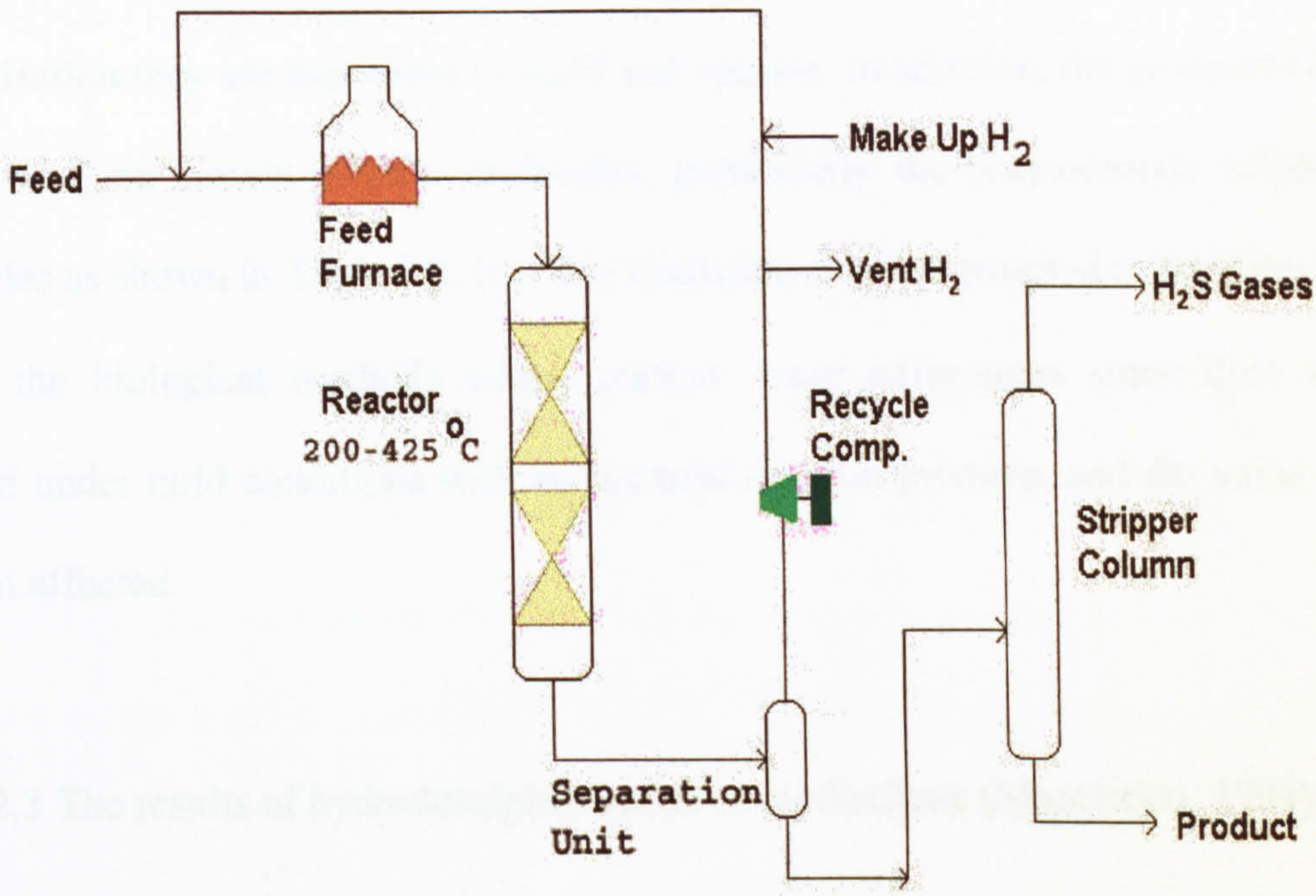
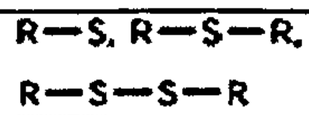
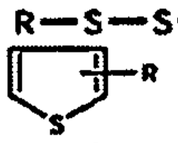
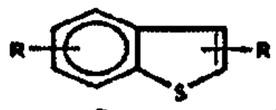
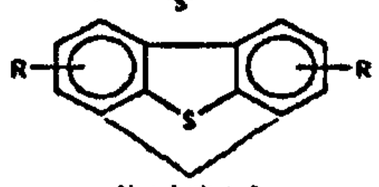
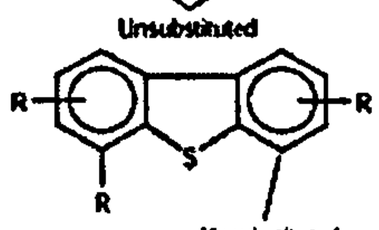
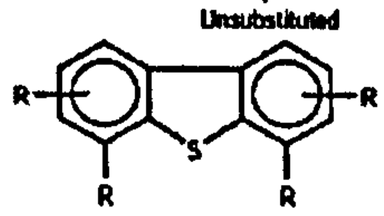


Figure 2.6 Typical hydrodesulphurisation process layout (Cyr, 2000).

In the HDS process, feed stock is mixed with hydrogen-rich make up gas and recycle gas. The mixture is heated in a reactor loaded with catalyst. The catalyst used is normally cobalt, molybdenum and nickel finely distributed on alumina. In the reactor sulphur is converted to hydrogen sulphide (H_2S). The reaction products leave the reactor and, after having been cooled to a low temperature (40-50 °C), enter a separation stage. The hydrogen-rich gas from the high-pressure separation is recycled to combine with the feed stock. The effluent is sent to a stripper column where H_2S is removed.

Although chemical and hydrodesulphurisation processes give a high rate of coal desulphurisation they are expensive to build and operate. In addition, the processes do not work well on certain sulphur molecules, particularly the polyaromatic sulphur heterocycles as shown in Table 2.3. It is this limitation that has tempted researchers to move to the biological methods which present many advantages since they are performed under mild conditions with no harmful reaction products and the value of coal is not affected.

Table 2.3 The results of hydrodesulphurisation of oil distillate (Monticello, 1998).

Sulphur compound classification	Structure	HDS difficulty	Compound's conc. (ppm)	
			Straight-run distillate	Cracked distillate
Non-thiophenic		Moderate	5000	300
Thiophenes		Very easy	0	0
Benzothiophenes		Very easy	1700	7300
Non-β-substituted DBTs		Easy	1000	1900
β-substituted DBTs		Moderate	1500	2300
Di--β-substituted DBTs		Difficult	600	900
Unassigned 3,4-ring S-containing molecules	Variable	Moderate	100	20
Unassingned 1,2-ring S-containing molecules	Variable	Moderate	5500	2800

2.5 BIODESULPHURISATION PROCESSES

2.5.1 State of the art

Since 1935, when an early account of microbial desulphurisation of crude oil was published, the ability of microorganisms to remove sulphur from coal has been of sporadic interest (Shennan, 1996). In the early 1950s, a series of U.S. patents were issued on microbial desulphurisation processes. However, these processes were found not to work. In the late 1970s and early 1980s the U.S. Department of Energy (DOE) and other organisations sponsored work around the country to crack this technology. The bacteria that had been isolated at that time were not appropriate for commercial desulphurisation technologies because they attacked the hydrocarbon portion of polyaromatic sulphur heterocycles (PASHs) and only coincidentally solubilised the sulphur molecules to water, thus removing them from oil.

Campbell's article in the late 1980s (Campbell, 1993) is the largest stimulation in development of biological desulphurisation processes. Bacteria that could liberate sulphur from the model PASH, dibenzothiophene, without attacking the hydrocarbon were identified. Some of the government, industry and academic groups encompassed in the field of biodesulphurisation are summarised in Table 2.4. Some of these have made significant contributions, e.g. Energy Biosystems Corporation (EBC) have constructed and operated a large-scale oil biodesulphurisation process (Figure 2.7) which presents many challenges.

Table 2.4 The organisation in biodesulphurisation technology (Monticello, 1998).

Organisation (In approximate order of capability)	Principal investigators	Sponsor	Collaborations, partnerships, or alliances
Energy Biosystems Corporation (EBC)	Monticello, Squires, Pienkos, Johnson, Childs, Gray Folsom, Pacheco, Mrachko	Public corporation	Total Raffinage, Texaco, Koch, Baker Petrolite, M.W. Kellogg, The U.S. Department of Energy (DOE), ATP
Petroleum Energy Center, Japan (PEC)	Yasui, Suzaki	Ministry of International Trade and Industry (MITI)	~12 Japanese companies
Tottori University Japan	Izumi	MITI	~12 Japanese companies
University of Tokyo, Japan	Omori, Kodama	MITI	~12 Japanese companies
Exxon Corporate Research	Grossman	Public corporation	Canadian National Research Council
University of Bologna, Italy	Setti	Enichem	Agip
Institute of Gas Technology (IGT)	Kilbane	DOE/EBC	EBC
Korean Institute of Science & Technology	Kim	Korean government	EBC, University of Calgary (Canada)
Oak Ridge National Laboratory	Kaufman	DOE	EBC, Texaco Exxon, Chevron Baker Petrolite
Brookaven National Laboratory	Premuzic	DOE	Unknown
University of Alberta, Canada	Fedorak	Canadian gevernment	EBC
Universitat Rovira I Virgili, Spain	Constanti	Catalan Spanish government	None
University of Notre Dame	Kulpa	EBC	EBC
Canadian National Research Council and University of Calgary, Canada	Voordouw (University of Calgary), Sankey (Imperial Oil)	Canadian government	Imperial Oil, Exxon

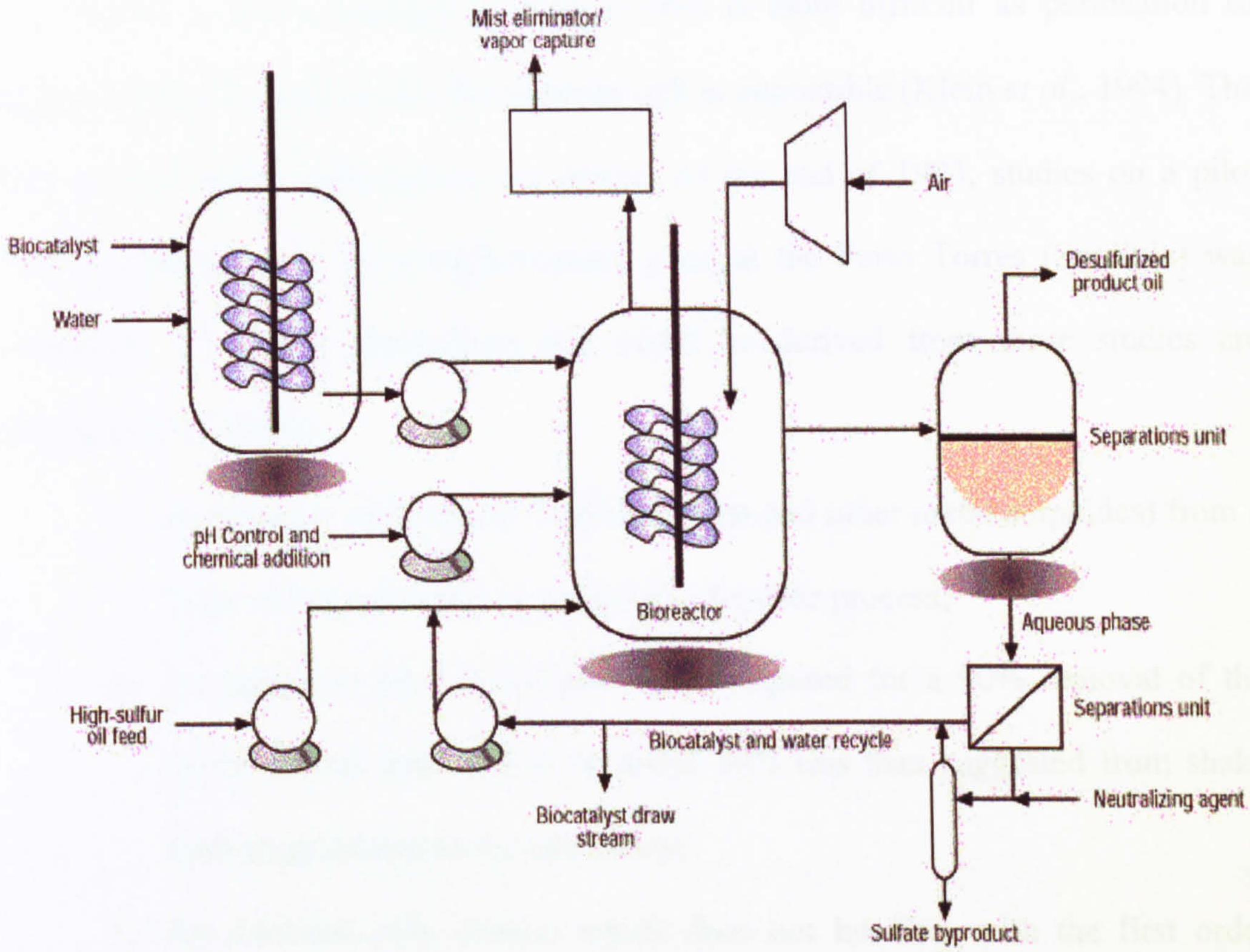


Figure 2.7 Schematic of oil biodesulphurisation process (Monticello, 1998).

In Figure 2.7, a biocatalyst is combined with water and transferred to the bioreactor. The biocatalyst slurry and high-sulphur petroleum feedstock are mixed with oxygen in a continuous stirred tank reactor. The desulphurised petroleum is separated from the aqueous/biocatalyst output stream. The aqueous phase is further treated to separate the biocatalyst and water. The sulphur by-product is removed from the process in the aqueous phase as sulphate, which can be disposed of as sodium sulphate (salt water) or ammonium sulphate, depending on local conditions. The biocatalyst/water mixture is recycled to the bioreactor after the spent biocatalyst is removed.

To compare with oil, desulphurisation of coal is more difficult as permeation of highly polymeric material into the bacterial cell is impossible (Klein *et al.*, 1994). The data on coal biodesulphurisation are scarce. At the end of 1993, studies on a pilot scale (1 ton/day) coal biodesulphurisation plant at the Porto Torres (Sardinia) was completed. The main conclusions that could be derived from these studies are (Camara *et al.*, 1997):

1) the removal of inorganic sulphur (pyrite and other metal sulphides) from a large variety of coals is a technically feasible process;
2) the residence time in the pilot plant required for a 90% removal of the pyrite present appeared to be about 50% less than suggested from shake flask experiments in the laboratory;
3) the maximal pulp density which does not interfere with the first order kinetics of pyritic sulphur removal appeared to be far higher than indicated in the literature. Almost 40% (w/v) pulp density does not interfere with the specific removal rate;
4) nevertheless, the economics of the technology are not yet favourable, mainly due to the high energy need of the reactor systems; and
5) *Thiobacillus ferrooxidans* does not play a key role in the process and thus is not an appropriate model organism. Mixed cultures containing *Leptospirillum ferrooxidans* seem to be far more important in bacterial leaching of sulphidic minerals.

In order to gain more understanding on coal biodesulphurisation process, mechanisms involved in the process are discussed next.

2.5.2 Biodesulphurisation of inorganic sulphur

Microbiological removal of inorganic sulphur from coal has been demonstrated in numerous laboratory studies over the past 30 years (Bos & Kuenen, 1990). Rossi (1993) found that pyrite bioleaching occurs in a three-phase system, the suspension of coal in an aqueous solution through which a stream of air + CO_2 is dispersed by suitable injectors. The presence of certain microbial strains, which can be mesophilic or thermophilic, in aqueous suspensions of finely ground pyrite in suitable inorganic salt solutions enhances the dissolution kinetics of the mineral.

Two mechanisms have been proposed for the biologically catalysed oxidation of pyrite by *Thiobacillus ferrooxidans*: a direct, and an indirect mechanism. In the direct mechanism, the pyrite is oxidised biologically and it requires physical contact between the bacterium and the pyrite particles as represented in Equation 1 (Larsson *et al.*, 1994).

$$4\,FeS_2 + 15\,O_2 + 2\,H_2O \xrightarrow{\textit{T. ferrooxidans}} 2\,Fe_2(SO_4)_3 + 2\,H_2SO_4 \qquad \text{(Equation 1)}$$

Several attempts have been made to demonstrate the direct attack of *T. ferrooxidans* on metal sulphides. It can be considered as a heterogeneous process in which the bacterial cell attaches itself to the sulphide crystal surface and the corrosion occurs in a thin film located in the interspace between the bacterial outer membrane and the sulphide surface. With certain coals, the direct mechanism for oxidation of pyrite may be limited because the microorganism is too large to enter most of the coal pores as shown in Figure 2.8.

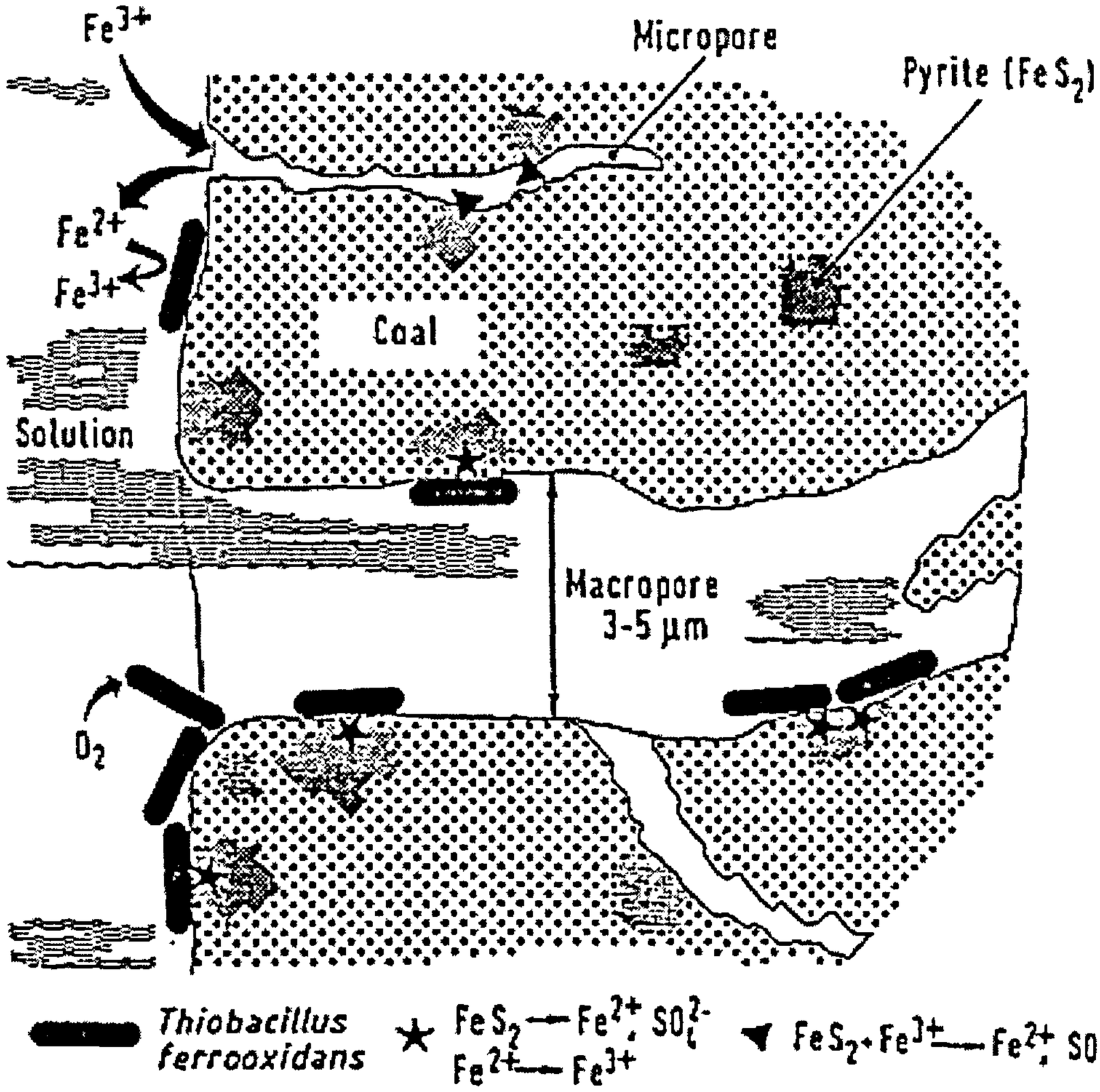


Figure 2.8 Bimodal pore structure of coal and pyrite oxidation (Hone *et al.*, 1987).

This suggests that pyrite oxidation in coal to a large extent must rely on the indirect mechanism. In the indirect mechanism, the bacterium oxidises ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}); the regenerated Fe^{3+} ions are then used for chemical oxidation of pyrite. Equation 2 and Equation 3 describe the indirect oxidation mediated by Fe^{3+} and *T. ferrooxidans* (Larsson *et al.*, 1994).

$$FeS_2 + 14\ Fe^{3+} + 8\ H_2O \xrightarrow{\textit{T. ferrooxidans}} 15\ Fe^{2+} + 16\ H^+ + 2\ SO_4^{2-} \qquad \text{(Equation 2)}$$

$$2\ Fe^{2+} + 2\ H^+ + 0.5\ O_2 \longrightarrow 2\ Fe^{3+} + H_2O \qquad \text{(Equation 3)}$$

The oxidation of ferrous iron in the absence of microorganisms is a slow process. It is considered to be the rate-limiting step for the oxidation of pyrite with ferric iron. Another option for the indirect mechanism is that the ferric iron oxidises the ferrous iron in the pyrite, leaving elemental sulphur behind as in Equation 4. The elemental sulphur is then oxidised to sulphate by the microorganisms, as shown in Equation 5.

$$FeS_2 + 2\ Fe^{3+} \xrightarrow{\textit{T. ferrooxidans}} 3\ Fe^{2+} + 2\ S^0 \qquad \text{(Equation 4)}$$

$$2\ S^0 + 3\ O_2 + 2\ H_2O \longrightarrow 2\ H_2SO_4 \qquad \text{(Equation 5)}$$

The rate of pyrite oxidation in coal might be mass-transport-limited rather than substrate-limited for the microorganism. Pyrite oxidation in coal seems to be a first-order reaction in pyrite concentration, whereas pyrite oxidation with pure pyrite is more complicated and depends on the ferric iron concentration as well as the pyrite concentration.

The formation of iron precipitates, mainly jarosites ($MFe_3(SO_4)_2(OH)_6$ where M stands for either hydronium, potassium, sodium or ammonium) is a problem in the oxidation of pyrite. At the elevated temperatures used for the thermophilic bacteria, the chemical reactions are faster, and the overall pyrite oxidation rate is greater than at temperatures applied for the mesophilic bacteria. However, elevated temperatures also increase the formation of jarosites which counteracts the desulphurisation as the precipitates stick to the coal even after the washing step. The concentration of soluble ferric iron also decreases. These conditions have a large impact on the chemical reactions involved in the indirect mechanism (Larsson *et al.*, 1994).

Recently, Schippers & Sand (1999) have proposed two indirect oxidation mechanisms for metal sulphides. Not only the oxidation of pyrite, the oxidation of other metal sulphides including molybdenite (MoS_2), tungstenite (WS_2), sphalerite (ZnS), chalcopyrite ($CuFeS_2$) and galena (PbS) were studied. The first mechanism is exclusively based on the oxidative attack of ferric iron on the acid-insoluble metal sulphides (FeS_2 , MoS_2 and WS_2). In this mechanism, the main sulphur intermediate is thiosulphate as shown in Equation 6 and Equation 7.

$$FeS_2 + 6\,Fe^{3+} + 3\,H_2O \text{ ----------> } S_2O_3^{2-} + 7\,Fe^{2+} + 6\,H^+ \qquad \text{(Equation 6)}$$

$$S_2O_3^{2-} + 8\,Fe^{3+} + 5\,H_2O \text{ ----------> } 2\,SO_4^{2-} + 8\,Fe^{2+} + 10\,H^+ \qquad \text{(Equation 7)}$$

The second mechanism allows for the dissolution of metal sulphides (ZnS, $CuFeS_2$ and PbS) by an attack of ferric iron and/or by protons. In this case, the main sulphur intermediate is polysulphide (and consequently elemental sulphur) as shown in Equations 8, 9 and 10.

$$MS + Fe^{3+} + H^{+} \text{ ----------> } M^{2+} + 0.5\ H_2S_n + Fe^{2+}\ (n \geq 2) \qquad \text{(Equation 8)}$$

$$0.5\ H_2S_n + Fe^{3+} \text{ ----------> } 0.125\ S_8 + Fe^{2+} + H^{+} \qquad \text{(Equation 9)}$$

$$0.125\ S_8 + 1.5\ O_2 + H_2O \text{ ----------> } SO_4^{2-} + 2\ H^{+} \qquad \text{(Equation 10)}$$

Consequently, the bacterial function is to generate sulphuric acid biologically to supply protons for hydrolysis attack and/or to keep the iron ions in an oxidised state (Fe^{3+}) for an oxidative attack (Figure 2.9). Three species of mesophilic bacteria: *T. ferrooxidans* (*T.f.*), *T. thiooxidans* (*T.t.*) and *Leptospirillum ferrooxidans* (*L.f.*) are mainly involved. *T. ferrooxidans* (a sulphur and iron oxidiser) and *L. ferrooxidans* (an iron oxidiser) are capable of oxidising pyrite when growing in pure culture. Whereas *T. thiooxidans* (a sulphur oxidiser) is not able to oxidise pyrite alone but grows on the sulphur released after the iron is oxidised (Rawlings *et al.*, 1999).

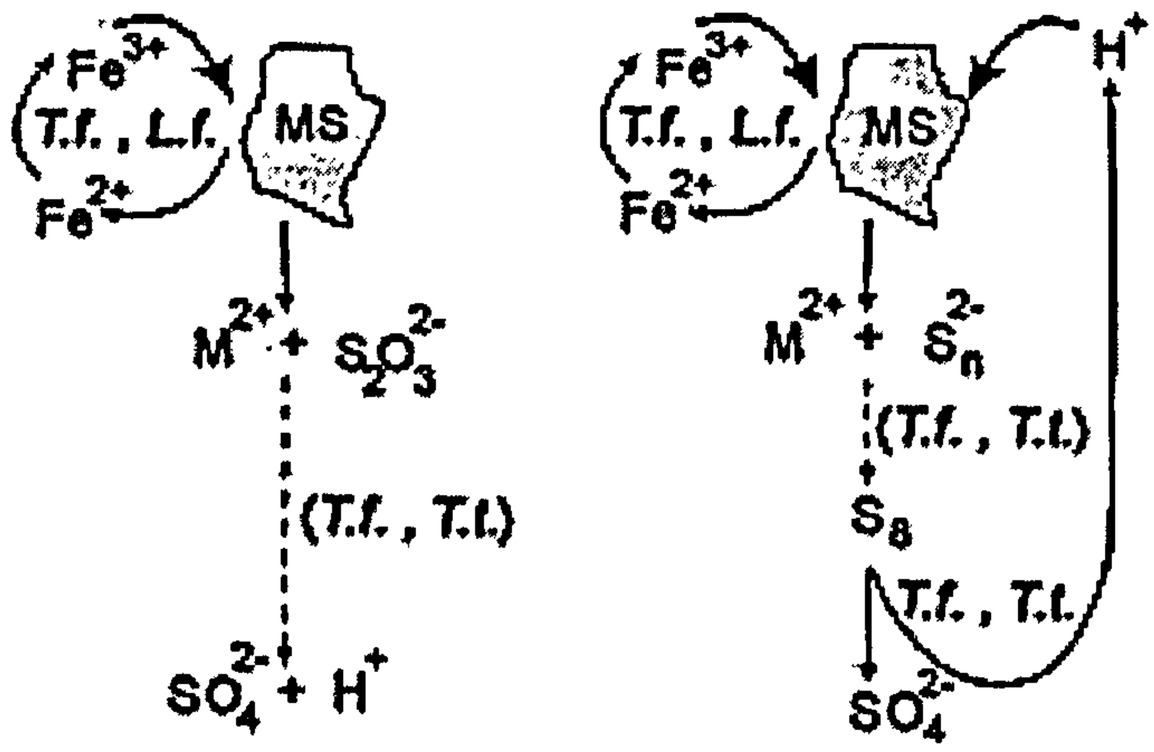


Figure 2.9 Bioleaching proceeds by two indirect mechanisms (Schippers & Sand, 1999).

The efficiency of microbial oxidation of pyrite depends on a number of parameters, for example the particle size of the pulverised coal, the pyrite content, nutrient media composition, pH, temperature, aeration and reactor design. Table 2.5 (Klein, 1998) summarises some major parameters with indications of the optimum conditions for high desulphurisation rates.

Different reactor systems for large-scale applications have been developed and proposed. A choice is generally available between heap (percolation) leaching (Beir, 1987) and slurry leaching (Beyer *et al.*, 1986); heap leaching is a less expensive approach than slurry leaching. However, reaction rates are faster in slurry leaching but these require fine grinding of coal and long residence times with aeration in large bioreactors. Surface area limits pyrite oxidation rates in heap leaching whereas biomass, up to a point, limits rates in slurry leaching (Olsson, 1994).

Alternatively, froth flotation methods can be used (Attia, 1990). The principal of this method is that the bacterium could selectively adhere to pyrite rather than to coal in coal-pyrite mixtures despite the fact that the total surface area of the pyrite was much less than that of the coal (Ohmura *et al.*, 1993). Its adhesion induced the suppression of pyrite floatability by changing the surface property of pyrite from hydrophobic to hydrophilic (Ohmura & Saiki, 1994). Because pyrite does not float with coal it can be collected as tailings from the bottom along with the ash minerals during the froth floatation (Raman *et al.*, 1995).

Table 2.5 Parameters on biodepyritisation of coal (Klein, 1998).

Process Parameter	Influence on	To obtain maximum pyrite oxidation rate
Bioreactor		
Type	Mixing Mass transfer O_2-, CO_2–supply Mechanical shear stress	Pachuca tank
Operation	Efficiency	Plug flow multi-stage
Coal		
Quality	Pyrite -concentration -distribution -crystal size	Pyrite crystal of small size but accessible for microorganisms
Pulp density	Substrate concentration Mixing Mechanical shear stress	20-30% (w/v)
Particle size	Pyrite accessibility Mixing Mechanical shear stress	Powder coal <0.5 mm
Microorganisms		
Concentration/ Species	Growth rate Pyrite oxidation rate	Mixed cultures, enriched from coal relevant
Reaction conditions		
Temperature	Bacterial activity Rate of chemical pyrite oxidation Oxygen/carbon dioxide solubility	Thiobacillus (30-35 °C) *Sulfolobus* (70-75 °C)
pH	Precipitation of jarosite Bacterial activity	pH 1.8
Nutrients	Bacterial activity Precipitation of jarosite	N-, P-alimentation
O_2-, CO_2-supply	Bacterial activity	>10% Saturation

Based on laboratory results, it was proposed to treat coal slurries in an industrial scale in large Pachuca tank reactors. These are 3-phase slurry reactors, cylindrical in cross-section with a conical bottom. The various units and the layout of an industrial-scale plant for the treatment of coal slurry are shown in Figure 2.10.

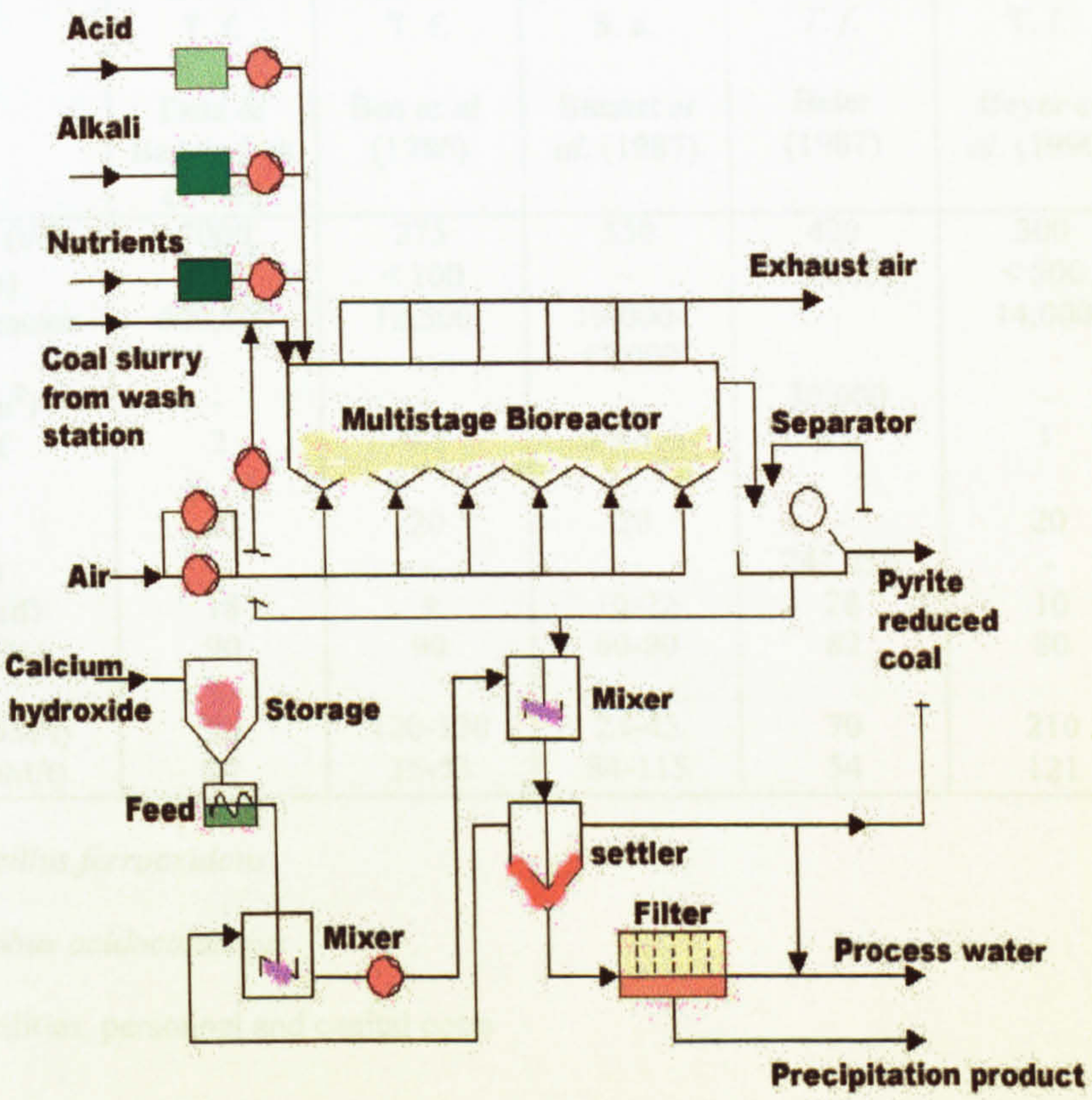


Figure 2.10 Process flow sheet of a plant for coal biodepyritisation (Klein, 1998).

An industrial-scale commercial operation of coal biodesulphurisation has not yet been performed. Consequently, published statements concerning cost-effectiveness are based on results from lab-scale and pilot-scale tests as shown in Table 2.6.

Table 2.6 Cost estimation for coal biodepyritisation at an industrial scale (Klein, 1998).

Process	Slurry 28 °C T. f.	Slurry 30 °C T. f.	Slurry 70 °C S. a.	Heap *T.f.*	Slurry 30 °C T. f.	Slurry 30 °C T. f.
Reference	Detz & Barvinshak (1979)	Bos *et al.* (1986)	Biesert *et al.* (1987)	Beier (1987)	Beyer *et al.* (1990)	Loi *et al.* (1994)
Coal throughput (t/d)	8000	275	550	420	300	300
Particle size (μm)	< 74	< 100	-	< 50,000	< 500	< 60
Required total reactor volume (m^3)	600,000	12,500	19,000-43,000	-	14,000	14,000
Required area (m^2)	-	-	-	30,000	-	-
Concentration of pyrite (%)	2	0.5	0.8-1.6	0.6	1	2
Coal (%w/v)	20	20	20	-	20	20-40
Trickling (m^3/d)	-	-	-	241.250	-	-
Residence time (d)	18	9	10-22	28	10	5
Pyrite removal (%)	90	90	60-90	82	80	90
Specific costs						
-Investment (DM/t)	38	100-130	24-45	70	210	210
-Operation* (DM/t)	27	35-53	84-115	54	121	80

T.f. = *Thiobacillus ferrooxidans*

S. a. = *Sulfolobus acidocaldarius*

* Including utilities, personnel and capital costs

It can be pointed out that coal biodepyritisation is a sufficiently well-known process, at least as far as its fundamentals are concerned, but some controversy still exists as to its technical and economic profitability, or at least its competitiveness with conventional desulphurisation methods. To date, there is no commercial biodepyritisation available since there are faster and less expensive physical and chemical methods for the removal of inorganic sulphur. Further basic research on biodepyritisation will certainly be useful, especially in regard to leaching rate enhancement and bioreactor design. In addition, the construction of a pilot plant is needed to fill in the missing technical and economic data essential for scale-up to industrial application. Therefore, future research should be directed towards well-defined objectives aimed at developing the reliable scale-up procedures and improving the commercial processes.

More importantly, it is necessary for biodesulphurisation process to remove not only inorganic sulphur but also organic sulphur, otherwise the process may not be commercial viable. The literature focused on bioremoval of organic sulphur is discussed in the next Section.

2.5.3 Biodesulphurisation of organic sulphur

Early attempts on biodesulphurisation of organic sulphur were considered failures because the bacteria that were isolated could not specifically remove sulphur, and moreover the fuel value was decreased. Initial attention has focused on bioremoval of sulphur from dibenzothiophene (DBT) since it represents a major proportion of thiophenic sulphur found in most fuels.

The first reported pathway for DBT biodegradation was called the Kodama pathway, as shown in Figure 2.11 (McFarland *et al.*, 1998). It involves initial oxidative attack and ring cleavage of one of DBT's aromatic rings, and in some cases the formation of a sulphoxide or sulphone (Kodama *et al.*, 1973). The destruction of C-C bonds and unacceptable loss of fuel value is found in this pathway. In addition, a specific sulphur release is not found since formyl benzothiophene remains as a dead end metabolite.

In the mid-1980's, researchers at the Atlantic Research Corporation proposed a strain Coal Bug One (CB1) of *Pseudomonas* that could convert dibenzothiophene to 2,2'-dihydroxybiphenyl (DHBP) and free inorganic sulphur (Campbell, 1993). This report suggested that sulphur could be removed specifically from DBT and possibly from fossil fuels with little or no reduction in the fuel's caloric value. It renewed interest in the potential use of bacteria for removal of organosulphur. However, the strain CB1 has lost its viability during a period of transfer to ARCTECH, Inc., a company formed to pursue the commercial development of this strain (McFarland *et al.*, 1998).

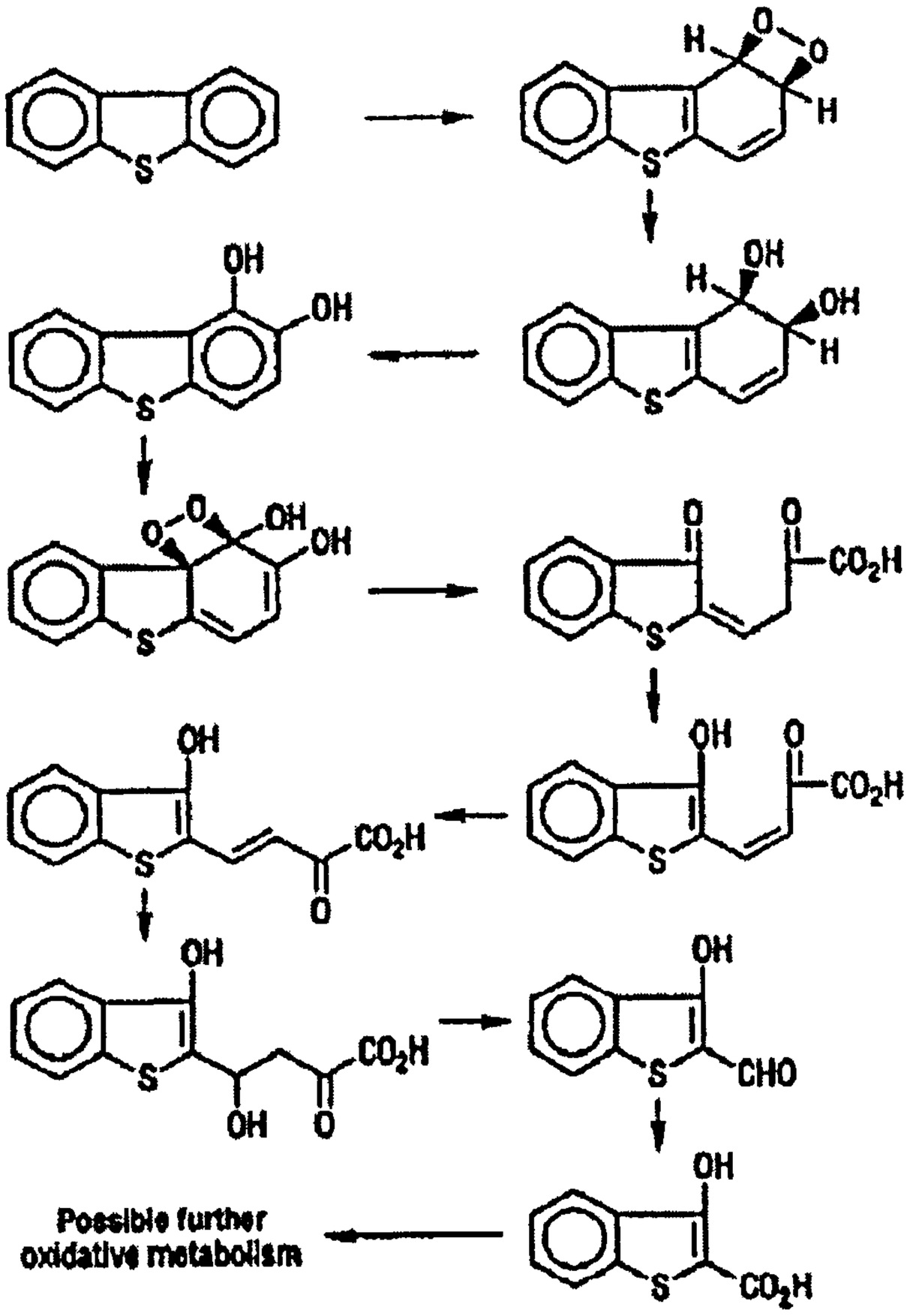


Figure 2.11 Kodama pathway of DBT degradation (McFarland *et al.*, 1998).

Later, a sulphur specific pathway, sometimes called the 4S pathway, was proposed by Kilbane (1990). It presents the sequential metabolism of DBT to DBT-sulphoxide, DBT-sulphone, DBT-sulphonate, 2-hydroxybiphenyl (HBP) and sulphate. According to the 4S pathway, bacteria selectively oxidise the sulphur atom in DBT with no cleavage of C-C bonds, thereby maintaining the caloric value of the fuel (Bressler *et al.*, 1998).

The isolation and characterisation of *Rhodococcus erythropolis* IGTS8 led to major advancements in the investigations of DBT-biodesulphurisation. Figure 2.12 shows biodegradation of DBT by this strain using the modified 4S pathway in which a sulphinate, rather than a sulphonate, was an intermediate (Bressler *et al.*, 1998).

The genes responsible for the DBT-desulphurisation phenotype in strain IGTS8 were organised into a single operon, variously referred to either as the sulphur oxidation (*sox*) operon or the desulphurisation (*Dsz*) operon (Oldfield *et al.*, 1998). The *DszC* gene encoded for a monooxygenase that, in a two-step process, oxidised DBT to DBT-sulphoxide and then to DBT-sulphone. DBT-sulphone was oxidised by another monooxygenase, which was the product of the *DszA* gene to yield 2-hydroxybiphenyl 2'-sulphinate (HBPSi). In the last step product of the *DszB* gene, which was a novel desulphinase converted HBPSi to HBP and released sulphite. The desulphinase was the slowest of the three enzymes; thus it controlled the rate of the desulphurisation. The metabolic pathway is shown in Figure 2.13 (Gray *et al.*, 1996).

Gray *et al.* (1996) also proposed that a fourth enzyme, a *DszD*, should be involved in the degradation pathway of DBT. The role of *DszD* in catalysis was to supply the two monooxygenases with reduced flavin, hence it should be a nicotinamide adenine dinucleotide, reduced form: flavin mononucleotide (NADH:FMN) oxidoreductase. The enzyme was specific for NADH and FMN, nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) and flavin adenine dinucleotide (FAD) could not act as substituents. It can be summarised that the DBT degradation pathway consists of two monooxygenases, which are not typical in the sense that they appear to utilise free flavin ($FMNH_2$) as a substrate, and a desulphinase.

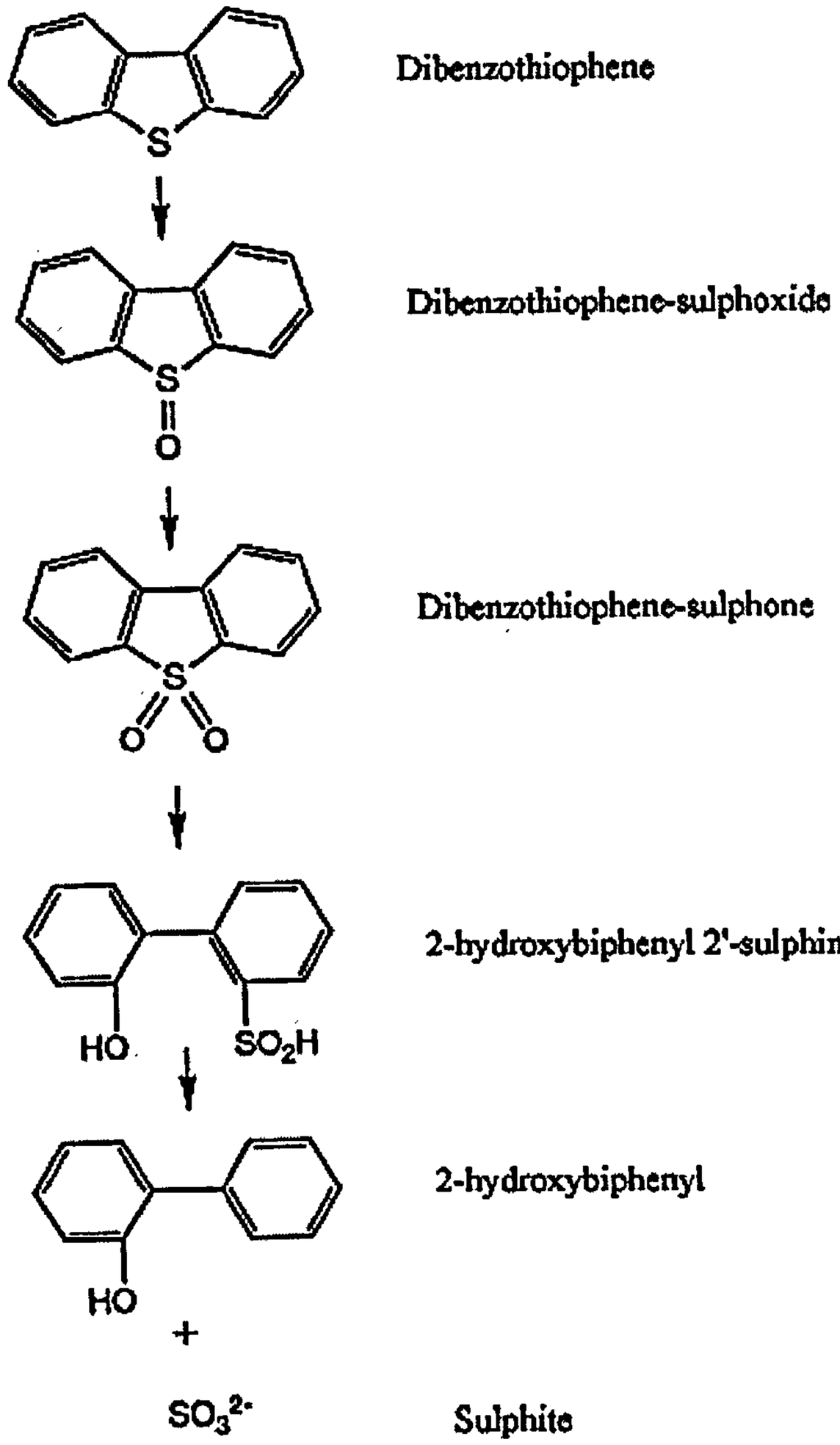


Figure 2.12 Modified 4S pathway of DBT degradation (Bressler *et al.*, 1998).

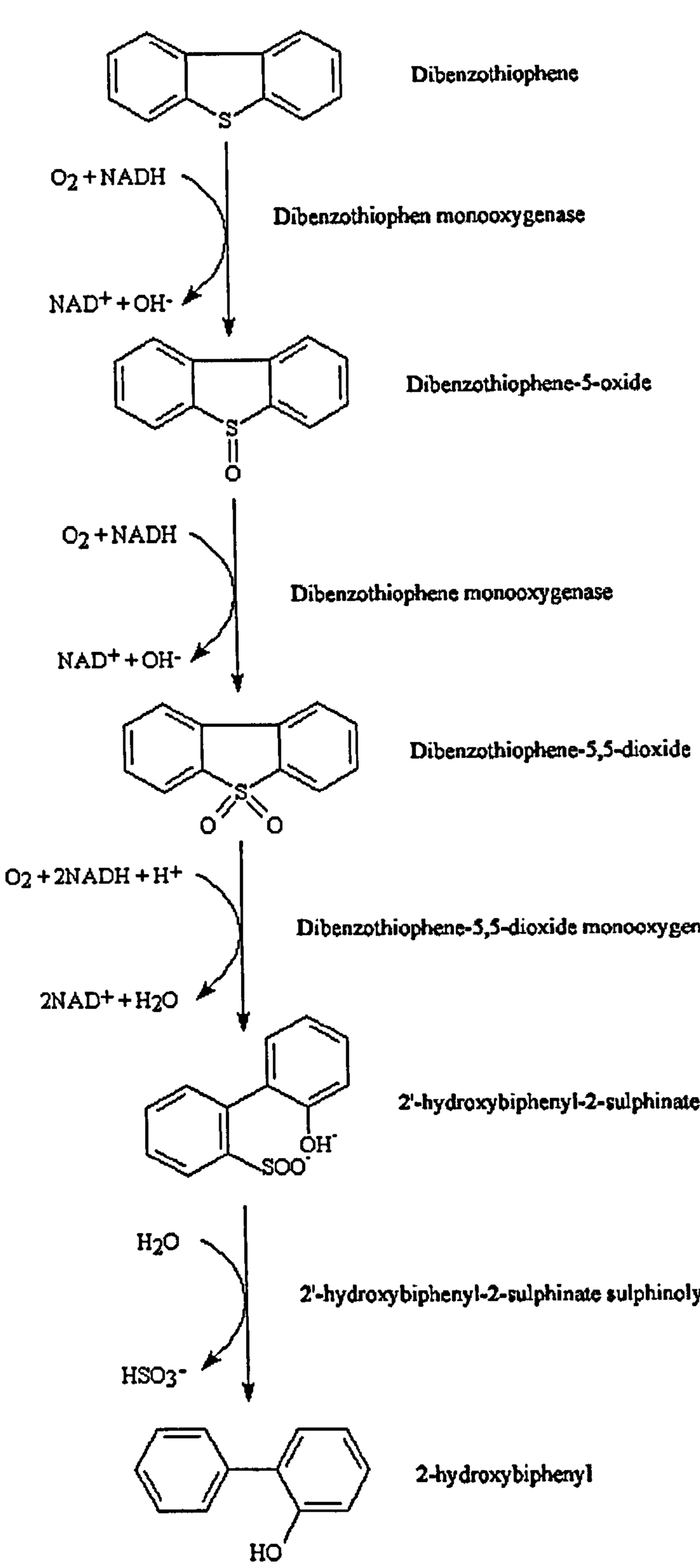


Figure 2.13 The metabolic pathway of DBT degradation by *R. erythropolis* IGTS8 (Gray *et al.*, 1996).

DBT may also be desulphurised by a minor route in which HBPSi is oxidised to 2-hydroxybiphenyl 2'-sulphonate (HBPSo). HBPSo spontaneously cyclises to biphenylene sultone (BPSo) which will be desulphurised to 2,2'-dihydroxybiphenyl (DHBP) and sulphite. It seems that HBPSo is produced through a disproportion reaction characteristic of aromatic sulphinate (Oldfield *et al.*, 1998). The pathway is not quantitatively significant in strain IGTS8, less than 1 mol% of DBT carbon is recovered as DHBP. The existence of this pathway in IGTS8 is reminiscent of *Pseudomonas* strain CB1, as the strain CB1 was reported to desulphurise DBT to DHBP (Isbister & Kobylinski, 1985). The DBT degradation pathway by the strain CB1 was not characterised in detail and it appears that the DBT-desulphurisation phenotype has been lost from all isolates of this organism, including the ATCC deposit (Oldfield *et al.*, 1998).

There have been some reports on biodesulphurisation of alkylated dibenzothiophenes. For instance, *Arthrobacter* species isolated by Lee *et al.* (1995) were demonstrated to desulphurise the sterically hindered compound 4,6-diethyldibenzothiophene, yielding 2-hydroxy-3,3'-diethylbiphenyl as the sulphur-free product. Similarly, *R. erythropolis* H-2 isolated by Ohshiro *et al.* (1996) was able to remove the sulphur atom from 3,4-benzo DBT, 2,8-dimethy DBT and 4,6-dimethyl DBT. The reaction product from 3,4-benzo DBT was identified as an α-hydroxy-β-phenylnaphthalene whereas the reaction products from structurally symmetrical 2,8 and 4,6-dimethyl DBTs were identified as the corresponding monohydroxy dimethyl biphenyls. In addition, *Mycobacterium* sp. strain G3 was reported to degrade 4,6-dimethyl DBT (Nekodzuka *et al.*, 1997).

To date, a mechanism to selectively remove sulphur from unsubstituted thiophene to that found in the 4S pathway for dibenzothiophenes has not been published. Kurita *et al.* (1971) reported anaerobic degradation of thiophene to hydrogen sulphide by a bacterial culture isolated from oil-contaminated sludge. Moriya & Horikoshi (1993) demonstrated a small amount of aerobic thiophene degradation by the *Bacillus* species isolated from a deep sea sediment. However, neither study provides a degradation pathway of thiophene.

There were some attempts to isolate microorganisms capable of degrading thiophene substituted in the 2-position. *Flavobacterium* sp. (Amphlett & Callely, 1969), *Rhodococcus* sp. (Kanagawa & Kelly, 1987), *Vibrio* YC1. (Evans & Venables, 1990) and yellow gram-negative rod (Cripps, 1973) isolated by enrichment on thiophene-2-carboxylic acid (T2C) were reported to release the sulphur as sulphate but they utilised the rest of the compound as a source of carbon for growths. In addition, there is no successful article yet on bioremoval of sulphur from thiophenes substituted in the 3-position (Shennan, 1996).

To achieve significant sulphur removal from thiophene, mutagenically altered bacterium might be involved. A genetically modified strain of *Pseudomonas alcaligenes* was shown to be capable of oxidising thiophene (Hartdegen *et al.*, 1983). Successive mutations of the facultative anaerobe *E. coli* yielded a strain able to degrade thiophene. However, even with these strains, the sulphur was not completely removed and the reaction was slow (Alam & Clark, 1991).

Similarly, initial attempts on bioremoval of sulphur from benzothiophene (BT) were not successful. *Pseudomonas putida* strain RE204 was reported to oxidise BT, but no subsequent release of the sulphur heteroatom (Eaton & Nitterauer, 1994). The 1-methylnaphthalene-degrading strain of *Pseudomonas* was demonstrated to transform BT and each of the six isomers of methyl BT by oxidation (Kropp *et al.*, 1994). The sulphur heteroatom was oxidised to the corresponding sulphoxide and sulphone when the thiophene ring was substituted (2- and 3-methyl BT) and 2,3-diones when the benzene ring was substituted (at positions 4, 5 or 6). Oxidation of 7-methyl BT yielded the corresponding sulphoxide, sulphone and 2,3-dione. Therefore, this organism only partially metabolised BT, without achieving sulphur removal.

The first reported bacterium able to remove sulphur from BT via the 4S pathway was *Gordonia* sp. strain 213E (Gilbert *et al.*, 1998). BT-desulphurisation mechanism by this strain is relatively unknown. However, the sulphur specific pathway can be outlined as shown in Figure 2.14.

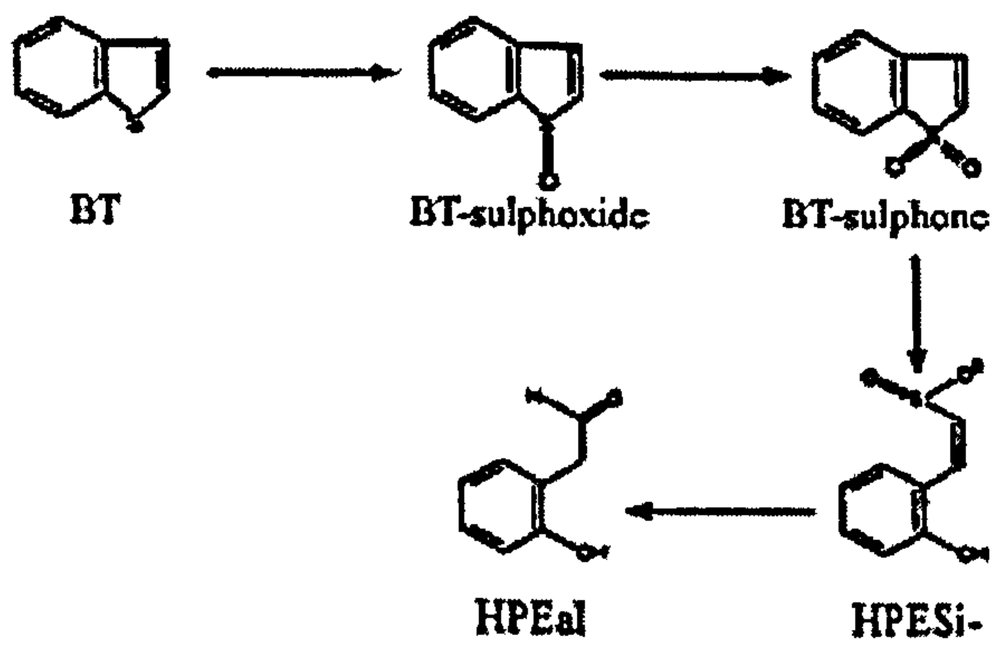


Figure 2.14 Benzothiophene desulphurisation pathway (Gilbert *et al.*, 1998).

Interestingly, even with the obvious chemical similarity of DBT and BT, the *Rhodococcus* species able to desulphurise DBT could not desulphurise BT and the *Gordonia* species able to desulphurise BT could not desulphurise DBT. There are a number of parallels between the BT and DBT desulphurisation pathways (Gilbert *et al.*, 1998).

The first step, BT → BT-sulphoxide → BT-sulphone mirrors the sequence DBT → DBT-sulphoxide → DBT-sulphone. Therefore, strain 213E must produce an S-oxygenase equivalent to *DszC*. The thiophene ring-opening step, BT-sulphone → HPESi[1] likewise mirrors the step DBT-sulphone → HBPSi. In principle, ring opening of the asymmetric BT-sulphone molecule could also proceed by way of cleavage of the alkenyl C-S bond, rather than by way of the aromatic C-S bond. If this were the case, the aryl sulphinate, 2-[z-2'-hydroxyethen] benzene 1-sulphinate, would be the expected product. However, no such compound is present in ethyl acetate extracts of culture media. It means that thiophene ring opening occurs exclusively by cleavage of the aromatic C-S bond of BT-sulphone. Thus the strain 213E must produce an enzyme which is functionally equivalent to *DszA* (Oldfield *et al.*, 1998).

In the final step of this pathway, HPESi is desulphinated to HPEal[2]. The reaction mechanism of DBT- and BT-pathway must differ critically at this step, since the desulphurisation of HPESi cannot be expected to proceed using the hydrolytic

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[1] HPESi = (Z)-2-(2'-hydroxyphenyl)ethan-1-sulphinate

[2] HPEal = 2-(2'-hydroxyphenyl)ethan-1-al

mechanism apparently utilised by *DszB*. It has been proposed that HPESi is desulphinated by attack of the alkenyl C-S carbon by an hydroxylase, resulting in the desulphurisation of HPESi to an enol, either *cis*- or *trans*-2-[2'-hydroxyphenyl]ethan 1-ol depending exactly on the enzymatic mechanism, which will tautomerise to HPEal and sulphite (Oldfield *et al*., 1998). However, the identity of the sulphur-containing product of the reaction has not yet been confirmed.

Recently, a single bacterial strain able to desulphurise alkylated forms of both DBT and BT has been reported (Kobayashi *et al*. 2000). The bacterium was isolated from a soil sample enrichment in DBT. It was classified as *R. erythropolis* strain KA2-5-1. The strain KA2-5-1 is quite similar to the strain IGTS8. The *DszABC* genes in IGTS8 were also found in KA2-5-1. The bacterium grew well in medium containing 3-methyl, 2-ethyl or 2,7-diethyl benzothiophene as the sole sulphur source, suggesting that KA2-5-1 may release sulphur from some benzothiophene derivatives.

However, no significant growth was observed when BT, 2-methyl BT, 5-methyl BT, 7-methyl BT, 7-ethyl BT or 5,7-dimethyl BT was added to the medium as the sole sulphur source. In conformity, the resting cells of KA2-5-1 also did not significantly attack BT and 5-methyl BT. Nevertheless, the monooxygenase *DszC* from KA2-5-1 can convert all these benzothiophenes to corresponding sulphones. These results show that there is the possible involvement of the same enzyme in the bacterial degradation of benzothiophenes and dibenzothiophenes (Kobayashi *et al*., 2000).

Ring cleavage of heterocyclic sulphur was studied in more detail. From calculations by Sabbah (1979), the C-S bond is weaker than the C-C bond as presented in Table 2.7. Furthermore, with aerobic microbial attack on heterocyclic sulphur, the addition of oxygen atoms to the molecule can weaken nearby bonds. For example, the strength of the H-C bond in methane is 438 kJ/mol; the addition of an OH group weakens this bond strength to 410 kJ/mol in methanol. Further oxidation to formaldehyde decreases the H-C bond strength to 364 kJ/mol. In addition, the oxidation of the sulphur atom in dimethyl sulphide (CH_3-SCH_3) to give dimethyl sulphone decreases the C-S bond strength from 308 to 280 kJ/mol. Similarly, oxidation of methyl tolyl sulphide to the corresponding sulphone decreases the C-S bond strength from 257 to 221 kJ/mol. Thereby, the C-S bond in a heterocyclic sulphur is likely the weakest bond in the molecule (Bressler *et al.*, 1998).

Removal of organic sulphur is more difficult than removal of inorganic sulphur. There were several bacterial cultures proclaimed to be useful for removing organic sulphur, however, their abilities were unstable and the reproducibility of results was poor: almost every research group involved reports of problems with stability or reproducibility. Although extensive studies have been done on bioremoval of organic sulphur, most of these were carried out using model compounds which are recognised to behave differently to sulphur in coal. The experiment using specific coal types, which has been carried out in the present study, is undoubtedly a requirement to enable an assessment of this technology.

Table 2.7 Bond strengths in selected compounds (Bressler *et al.*, 1998).

Bond	Bond strength (kJ/mol)	Reference
C-C bonds		
H_3C-CH_3	376	Lide, 1995
H_2C=CH_2	733	Lide, 1995
C-C in benzene	505	Sabbah, 1979
C-S bonds		
C-S in thiophene	341	Sabbah, 1979
C-S in benzothiophene	339	Sabbah, 1979
C-S in dibenzothiophene	338	Sabbah, 1979
HS-CH_3	312	Lide, 1995
H_3C-SCH_3	308	Lide, 1995
H_3C-SO_2CH_3	280	Lide, 1995
H_3C-$SCH_2C_6H_5$	257	Lide, 1995
H_3C-$SO_2CH_2C_6H_5$	221	Lide, 1995
H-C bonds		
H-CH_3	438	Lide, 1995
H-CH_2OH	410	Lide, 1995
H-CHO	364	Lide, 1995

2.5.4 Biodesulphurisation in hydrophobic media

There are relatively few reports in the literature describing biodesulphurisation in non-aqueous media. An unclassified aerobic, gram-positive, soil bacterium, designated as FE-9, was reported to desulphurise DBT in 100% dimethylformamide. This microorganism converted DBT to biphenyl and hydrogen sulphide under hydrogen, and to biphenyl, hydroxybiphenyl and sulphate in air (Finnerty, 1993). Recently, DBT desulphurisation by *Rhodococcus* species in two-phase system (solvent:water) was investigated. The results show that DBT desulphurisation rates were increased in the presence of 40-50% n-tetradecane or kerosine (Ohshiro *et al.*, 1996), 96% hexadecane (Kaufman *et al.*, 1998), or 50% diesel (Pacheco *et al.*, 1999).

DBT desulphurisation in *Rhodococcus* appears to occur intracellularly with DBT uptake from the oil phase possibly occurring after transient adsorption to the cell (Oldfield *et al.*, 1997). The oil phase and cuff layer emulsions were found to contain significant amounts of *Rhodococcus* in 1-10 μm droplets during desulphurisation of DBT in high hexadecane concentration (Kaufman *et al.*, 1998). In addition, Kayser *et al.* (1993) reported that the desulphurisation activity of *R. erythropolis* IGTS8 is associated with the external surface of the cell wall/membrane. Since membranes are hydrophobic environments, the desulphurisation enzymes should function in non-aqueous solvents which in turn would facilitate contact with coal and increase mass transfer during biodesulphurisation (Patel *et al.*, 1997).

Desulphurisation of coal using bacteria or bacterial extracts emulsified in mineral oil, or in mineral oil and solvent mixtures seems to be an alternative approach. Lee & Yen (1990) demonstrated biodesulphurisation of coal using reverse micelle solutions (finely dispersed water in oil emulsions) containing *T. ferrooxidans* cells, or their cell-free enzyme extracts. A reduction in total sulphur as high as 48% could be achieved within 24-hour treatment; cell free enzyme extracts outperformed the whole-cell preparations. With longer times, as much as 70% of the total sulphur was removed. Not all the sulphur reduction is attributed to biological activity because abiotic controls can reduce the total sulphur by as much as 25%.

Finnerty (1993) also support this idea, the advantages of biodesulphurisation in hydrophobic systems over biodesulphurisation in aqueous systems were proposed as follows:

1) The enhanced efficiency of the biocatalyst in the removal of heteroatoms from such substrata;
2) Elimination of water-dependent side reactions which may downgrade the fuel value of the final product;
3) Improved efficiencies in product recovery through the use of low boiling point organic solvents;
4) Process cost benefits, which are derived from organic solvent recycling;
5) Facilitated integration of bioprocessing systems into the existing fossil industry process infrastructure.

However, more research is needed to establish the capabilities, limitations and optimum conditions for coal biodesulphurisation in hydrophobic media.

2.5.5 Desulphurising bacteria

Several microorganisms have been suggested for the coal biodesulphurisation process. Sulphate-reducing bacteria were reported to desulphurise sulphur compounds in coal to hydrogen sulphide. However, no significant reduction in the sulphur content of coal was observed in any work (McFarland, 1999). The mesoacidophilic, chemolithotropic bacteria have been considered to be the most important organisms for coal depyritisation. Three species including *Thiobacillus ferrooxidans*, *T. thiooxidans*, and *Leptospirillum ferrooxidans* are mainly involved (Schippers & Sand, 1999). All species of the genus *Thiobacillus* can derive energy from the oxidation of reduced sulphur compounds. Unlike other members of this genus, *T. ferrooxidans* can also use ferrous iron as an electron donor (Nemati *et al.*, 1998).

In the industrial processes for removal of inorganic sulphur, *L. ferrooxidans* is thought to be more dominant than *T. ferrooxidans*. The major reason is a greater affinity for ferrous iron, and less sensitivity to inhibition by ferric iron on prolonged aeration of *L. ferrooxidans*. In addition, the optimum pH for growth of *T. ferrooxidans* is within the range of 1.8-2.5 whereas *L. ferrooxidans* is more acid resistant since it can grow at a pH of 1.2. With regard to temperature, *T. ferrooxidans* is considered to be more tolerant of low temperature and less tolerant of high temperature than *L. ferrooxidans* (Rawlings *et al.*, 1999). Some strains of *T. ferrooxidans* are able to oxidise pyrite at temperaturs as low as 10°C (Norris, 1990) however, 30-35°C is considered to be optimal. While, *L. ferrooxidans* has an upper limit of around 45°C (Norris *et al.*, 1986), and a lower limit of about 20°C (Sand *et al.*, 1993).

Although mesoacidophilic bacteria are the most successful microorganisms for the removal of inorganic sulphur, they do not work well for the removal of organic sulphur. Thermoacidophilic bacteria, such as *Sulfolobus acidocaldarius*, *Sulfolobus brierleyi* (reclassified as *Acidianus brierleyi*), *Metallosphaera sedula* and *Thiobacillus caldus* were later suggested for the process (Schippers *et al.*, 1999). These bacterial species can grow in temperature ranges of 40-90°C, and a pH range of 1.0-5.8 (Karavaiko & Lobyreva, 1994). The biodepyritisation rates by thermoacidophilic bacteria are higher than the rates by mesoacidophilic bacteria, however, from many results in the literature it is accepted that mesophiles are more suitable for coal depyritisation than thermophiles. Even though *S. acidocaldarius* and *A. brierleyi* were reported to degrade some organic sulphur compounds, their degradation is in a C-C-bond-targeted fashion which is not preferable as sulphur still remains in the compounds and the caloric value of hydrocarbon is reduced (Konishi *et al.*, 1997). Bioremoval of organic sulphur was thought not to work at that time.

Pseudomonas species were of great interest in early success of organic sulphur removal. For instance, the mutagenically altered bacterium CB1 isolated from coal-contaminated soil, using medium containing DBT for the selection, was reported to remove organic sulphur from model compounds and various coal samples without reduction of the fuel value (Arctech, 1988). Unfortunately, this strain is apparently no longer available to the research community due to viability loss (Oldfield *et al.*, 1998). *Pseudomonas putida* was also claimed to remove both organic and inorganic sulphur. However, conflicting results were obtained from the literature (McFarland *et al.*, 1998). It seems that there are no well-characterised strains of *Pseudomonas* species available for further research.

Indeed, the ability to remove both organic and inorganic sulphur has been found in *Rhodococcus* species which are aerobic, mesophilic, chemoorganotropic, and gram-positive bacteria (Warhurst & Fewson, 1994). A number of DBT-desulphurising *Rhodococcus* species have been reported. These include *Rhodococcus erythropolis* IGTS8 formerly called *R. rhodochrous* IGTS8 (Kayser *et al.*, 1993), *R. erythropolis* D-1 (Izumi *et al.*, 1994), *Rhodococcus* sp. SY1 (Omori *et al.*, 1995) first reported as *Corynebacterium* sp. SY1 (Omori *et al.*, 1992), *R. erythropolis* H-2 (Ohshiro *et al.*, 1996), *R. erythropolis* N-36 (Wang & Krawiek, 1996), *Rhodococcus* sp. strain B1, If, Ig and Ih (Denis-Larose *et al.*, 1997) and *Rhodococcus* sp. ECRD-1 (Grossman *et al.*, 1999) which was initially classified as *Arthrobacter* (Lee *et al.*, 1995) and sometimes classified as *R. erythropolis* X310 (Denis-Larose *et al.*, 1997). All these strains are considered very closely related. Among them *R. erythropolis* IGTS8 is the most widely studied.

Other DBT-desulphurising isolates have been classified as *Agrobacterium* strain MC501 (Constanti *et al.*, 1996), *Mycobacterium* strain G3 (Nekodzuka *et al.*, 1997) and *Paenibacillus* strain A11-2 (Konishi *et al.*, 1997). *Agrobacterium* strain MC501 is the first gram-negative bacterium reported to have DBT desulphurisation ability. *Paenibacillus* sp. strain A11-2 is a thermophile with an apparent maximum specific DBT-desulphurisation activity at 50°C. All of these other isolates were reported to desulphurise DBT to HBP as well, but are otherwise poorly characterised.

All the above DBT-desulphurising bacteria seem to have little activity toward thiophenes and benzothiophenes. *Gordonia* sp. strain 213E able to remove sulphur from BT was later isolated from soil sample (Gilbert *et al.*, 1998). The strain is an aerobic, mesophilic, gram-positive chemoorganotroph, and subsequently recognised as the new species, *Gordonia desulfuricans* (Kim *et al.*, 1999). The *Gordonia desulfuricans*, however, do not have activity toward DBT.

From the literature, it can be pointed out that the most successful desulphurising bacteria now a day are *Rhodococcus* species, which are able to selectively remove sulphur from several model compounds. However, the bacteria are not versatile for all sulphur compounds. More active microbial cultures are still desirable. Recently, it has been found that when using *Shewanella putrefaciens* strain NCIMB 8768 in clay biodesulphurisation sulphur odour was reduced (Whittles, personal communication). *Shewanella putrefaciens* species are facultatively anaerobic mesophiles, and have several notably properties, e.g. they are versatile with regard to the use of electron acceptors, including oxygen, nitrate, nitrite, dimethyl sulphoxide, thiosulphate, fumarate, and metal oxide. Some strains of *S. putrefaciens* are iron reducing bacteria, and are able to grow by elemental sulphur reduction (Moser & Nealson, 1996). However, there are no reports on sulphur removal by the strain NCIMB 8768. This stimulated the work to investigate the desulphurisation ability of the strain NCIMB 8768, which may provide greater desulphurisation efficiency than the current desulphurising bacteria.

2.6 AIMS AND OBJECTIVES

Biological process presents many advantages over physical and chemical processes, and it is believed to be a suitable system that could constitute the basis of an economically favourable pre-combustion coal desulphurisation process by many researchers. However, from the literature there are many obstacles associated with the development of coal biodesulphurisation process, e.g. the lack of microorganisms that present the abilities to desulphurise a wide range of sulphur compounds, and the lack of stability of desulphurisation abilities in the microorganisms. It is thereby necessary to find better bacterial cultures with improved stability and efficiency.

Another obstacle to the development of coal biodesulphurisation process is the lack of data on biodesulphurisation of coal, especially data on organic sulphur removal. Most of investigations in the literature were carried out using model compounds, not coal samples. The experiments performed with a range of coal types are certainly required. There is also a need for more convenient and accurate analytical techniques for measuring sulphur in coal as the standard methods used are not very consistent: replicate analyses performed by the same laboratory may differ by 10% and replicate analyses performed by different laboratories by 20% (Kilbane, 1989).

Moreover, investigations of biodesulphurisation have revealed a number of critical variables which affect the reactions and hence any potential biodesulphurisation process. These variables include biological parameters such as nutrient supplies and supplements, control of the physical environment (and such factors as pH, and

temperature), and the particular strain of microorganism used. This thesis is therefore aimed to carry out a further study on biodesulphurisation process.

Previous work at Cranfield Biotechnology Centre reported that when using *Shewanella putrefaciens* (NCIMB 8768) in clay biodesulphurisation, sulphur odour was reduced (Whittles, personal communication). This presented a challenging task to investigate the desulphurisation ability of this strain of *S. putrefaciens* and compare it to the current desulphurising bacteria, *Rhodococcus erythropolis* strain IGTS8 and strain X309. The objectives of the work can be identified as follows:

1. to examine the abilities of the bacteria on utilisation of a wide range of sulphur model compounds (both organic and inorganic forms);
2. to investigate the effect of environmental parameters on the abilities of the bacteria in biodesulphurisation of sulphur model compounds;
3. to perform biodesulphurisation of coal samples with the three specific bacteria and with bacteria inherent in the coal;
4. to study the desulphurisation abilities of isolated bacteria that inherent in the coal; and
5. to improve analytical methods for measuring sulphur in coal.

Finally, the implications of the results obtained from the experiments are discussed and interpreted as a basis for future work. Figure 2.15 shows the structure of the work programme of this study.

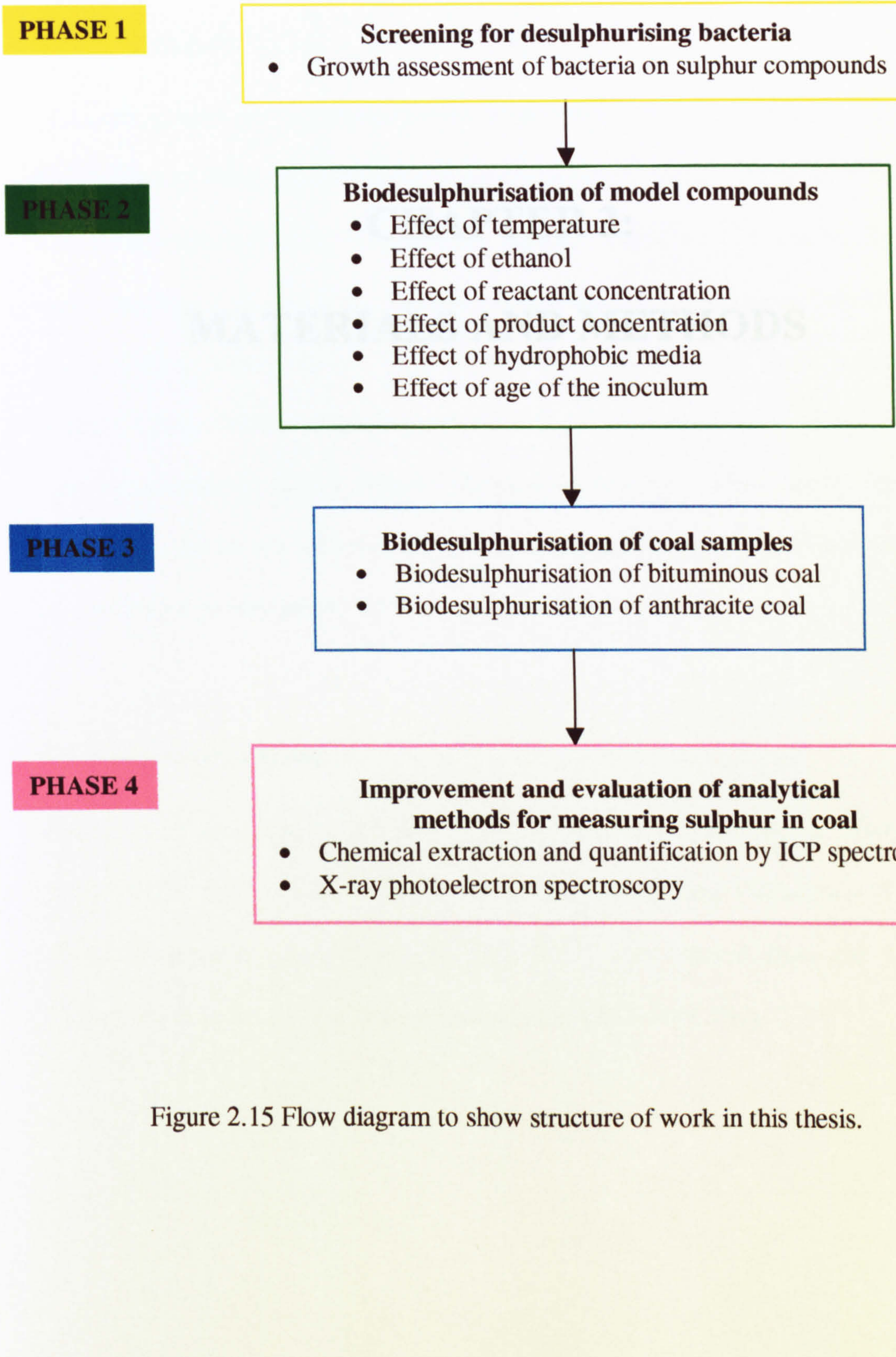


Figure 2.15 Flow diagram to show structure of work in this thesis.

CHAPTER 3:

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals

Dibenzothiophene (99%), dibenzothiophene-sulphone (97%), benzothiophene (sold as thianaphthene; 99%), thiophene-2-carboxylic acid (97%) and diphenyl disulphide were from Aldrich (Dorset, UK). Eschka mixture, Ringer's tablet and nutrient agar were from Merck (Poole, UK). Growth media components were from Lab M (Bury, UK) or Oxoid (Milton Keynes, UK). Tryptic soy broth was from Difco Laboratories (Detroit, USA). Sulphite oxidase [suspension in 3.2 M ammonium sulphate], bovine-heart cytochrome *c*, Gibb's reagent (2,6-dichloroquinone 4-chloroimide), dimethyl sulphoxide, and all other chemicals were from Sigma (Dorset, UK). Water used was purified by ion exchange and reverse osmosis (Elga Ltd, UK).

3.1.2 Microorganisms

Rhodococcus erythropolis IGTS8 (ATCC 53968) and *Rhodococcus erythropolis* X309 (ATCC 55309) were from the American Type Culture Collections (Virginia, USA). *Shewanella putrefaciens* (NCIMB 8768) was ordered from the National Collections of Industrial and Marine Bacteria Ltd (Aberdeen, UK).

3.1.3 Media

Tryptic soy broth purchased from Difco Laboratories (Detroit, USA) contains the components as shown in Table 3.1. Composition of nutrient agar from Merck (Poole, UK) is presented in Table 3.2. Nutrient medium was prepared as shown in Table 3.3 (Whittles, 1998). Basic salt medium was prepared as shown in Table 3.4 (Patel *et al.*, 1997). All media were sterilised by autoclaving at 121°C for 15 minutes.

Table 3.1 Composition of tryptic soy broth.

Component	Amount
Bacto tryptone	17.0 g
Bacto soytone	3.0 g
Bacto dextrose	2.5 g
Sodium chloride	5.0 g
Dipotassium phosphate	2.5 g
Water	1.0 L
pH	7.3 ± 0.2

Table 3.2 Composition of nutrient agar.

Component	Amount
Peptone	5.0 g
Meat extract	3.0 g
Agar-agar	12.0 g
Water	1.0 L
pH	7.0 ± 0.2

Table 3.3 Composition of nutrient medium (Whittles, 1998).

Component	Amount
Lab-Lemco powder	1.0 g
Yeast extract	2.0 g
Peptone	5.0 g
Sodium chloride	5.0 g
Water	1.0 L
pH	7.4 ± 0.2

Table 3.4 Composition of basic salt medium (Patel *et al.*, 1997).

Component	Amount
KH_2PO_4	2.44 g
Na_2HPO_4	5.57 g
NH_4Cl	2.00 g
$MgCl_2.6H_2O$	0.20 g
$CaCl_2.2H_2O$	0.001 g
$FeCl_3.6H_2O$	0.001 g
$MnCl_2.4H_2O$	0.004 g
Glycerol	1.84 g
Water	1.0 L
pH	7.0 ± 0.2

3.2 METHODS

3.2.1 Bacteria revival method

Freeze-dried cultures were rehydrated and revived according to the instructions of The National Collections of Industrial and Marine Bacteria Ltd. (Aberdeen, UK). An opened vial was rehydrated with 0.5 ml of medium (tryptic soy broth for *R. erythropolis* strain IGTS8 and X309, and nutrient medium for *S. putrefaciens*). The contents were mixed without frothing. The suspension was divided into two aliquots and each aliquot was sub-cultured into 5 ml of medium in a Universal bottle. All the Universal bottles were incubated on an orbital shaker (L.H. Engineering Co. Ltd., UK) at 100 rpm and 30°C for 24 hours. The cultures were then transferred to 250-ml flasks containing 100 ml of medium and incubated under the same condition as described above.

3.2.2 Bacteria maintenance and storage methods

The bacteria were maintained by sub-culturing into liquid medium or plating on solid medium (nutrient agar) every week. For long-term storage, 0.85 ml of culture was transferred to 0.15 ml of sterile glycerol (sterilised by autoclave at 121°C for 15 minutes) in a screw cap tube. All tubes were mixed by vortex to ensure that the glycerol was evenly dispersed. The tubes were kept in a labelled airtight gasket and frozen at -80°C. The bacteria can be recovered by scraping the frozen surface of the cultures with a sterile inoculating needle, and then immediately streaking the cells that adhere to the needle onto the surface of a nutrient agar plate or transferring the defrosted cells straightaway to fresh liquid medium.

3.2.3 Bacteria harvest method

The cultures grown until mid-log phase in liquid medium were harvested by centrifugation at 7500 × g for 15 minutes with a Beckman Model J2-21 Centrifuge (Beckman, UK). The supernatant was discarded and the cell pellets were washed twice with Ringer's solution. The Ringer's solution was prepared by dissolving 1 Ringer's tablet (purchased from Merck, containing NaCl, KCl, $CaCl_2$ and N-2-hydroxyethylpiperazine-N'-2-ethansulphonic acid (HEPES)) in 500 ml of water. The cells were resuspended in the same solution to a required concentration and used on the day of harvesting, or kept at –80°C until used.

3.2.4 Bacterial characterisation

3.2.4.1 GROWTH IN COMPLEX MEDIUM

One ml of actively grown cell of each culture in complex medium (tryptic soy broth for *R. erythropolis* strain IGTS8 and X309, and nutrient medium for *S. putrefaciens*) was inoculated into 100 ml of complex medium and incubated at 30°C and 100 rpm. Bacterial growth was measured from optical density at 600 nm (A_{600}) using Camspec M350 Double Beam UV-Visible Spectrophotometer (Camspec, UK).

3.2.4.2 GRAM STAIN

Gram staining allows bacteria to be divided into two major groupings, gram-positive and gram-negative (Jones *et al.*, 1998). A smear of bacterium was prepared on a microscope slide by flaming an inoculating loop and picking up a loop full of culture. The culture was mixed with a drop of water on the slide and then the suspension was dragged along the slide to make a smear using a second slide. The smear was air dried and fixed by passing the slide through a Bunsen flame. The smear was stained with a drop of 0.5% methyl violet for 30 seconds. The smear was rinsed with water. The sample was stained with a drop of 2%KI/1%I (Lugol's iodine) for 1 minute. The slide was rinsed with acetone for a short time until the violet colour no longer ran out. The slide was rinsed with water. The sample was counterstained with 0.1% saffranin for 2-3 minutes. The slide was rinsed briefly with water and allowed to dry. The sample was observed under the microscope (Olympus BH2 Microscope by using UplanFI 100*/1.30 oil lense). Gram-positive bacteria are indicated by blue/violet coloration while gram-negative cells show the pink colour of the counterstain.

3.2.5 Biodesulphurisation of sulphur model compounds

3.2.5.1 ENHANCEMENT OF SHEWANELLA PUTREFACIENS

The original culture of *S. putrefaciens* (NCIMB 8768) did not grow using dimethyl sulphoxide (DMSO) or dibenzothiophene (DBT) as a sole source of sulphur. Therefore, experiments were carried out to enhance bacterial growth on these compounds. Basic salt medium (BSM) which is a sulphur deficient medium (checked by AtomScan 16 Sequential ICP Spectrometer from Thermo Jarrell Ash Corporation, USA) was used. A sulphur source was added aseptically to a 250-ml flask containing 100 ml of BSM at a final concentration of 0.3 mM. DBT is partially soluble in water, so it was dissolved in ethanol to 100 mM concentration before addition. One ml of inoculum (cells grown in nutrient medium) was inoculated into the flask and incubated at 30°C and 100 rpm for 3 days. A further 2 ml of inoculum was added to the same flask after 3 days to get good growth of the culture.

3.2.5.2 DBT DESULPHURISATION

The bacteria grown in BSM with DMSO as a sole source of sulphur were harvested as described in Section 3.2.3 and resuspended in Ringer's solution to A_{600} = 1.0. One ml of inoculum was added to 250-ml flasks containing 100 ml of BSM with 0.3 mM of DBT-ethanol solution and incubated at 30°C (100 rpm). Control flasks without bacterial cells were also performed. Samples (5 ml) were taken daily. They were monitored for bacterial growth at A_{600} and analysed for DBT using high-pressure liquid chromatography (HPLC), and hydroxybiphenyl (HBP) using Gibb's assay. Sulphite in the samples was monitored by spectrophotometric assay.

Effect of different parameters on bacterial growth and DBT desulphurisation was examined as follows:

A. ***Effect of temperature:*** inoculum was added to 250-ml flasks containing 100 ml of BSM with 0.3 mM of DBT-ethanol solution and incubated on a shaker, 100 rpm at three different temperatures (25, 30 and 35°C).

B. ***Effect of Ethanol:*** BSM (100 ml) containing 0.3 mM of DBT-ethanol solution or 0.0055 g of DBT powder was used as growth medium.

C. ***Effect of DBT concentration:*** BSM (100 ml) containing a wide range concentration of DBT-ethanol solution (0.1, 0.2, 0.3, 0.4, 0.5, 10, 15 or 20 mM) was used as growth medium.

D. ***Effect of HBP concentration:*** BSM (100 ml) containing:

0.3 mM of DBT-ethanol solution;

0.3 mM of DBT-ethanol solution + 0.10 mM of HBP; or

0.3 mM of DBT-ethanol solution + 0.20 mM of HBP; or

0.3 mM of DBT-ethanol solution + 0.30 mM of HBP

was applied as growth medium.

E. ***Effect of sulphate:*** BSM (100 ml) containing:

0.3 mM of DBT-ethanol solution;

0.3 mM of sodium sulphate; or

0.3 mM of DBT-ethanol solution + 0.05, 0.10, 0.15 or 0.30 mM of sodium sulphate

was used as growth medium.

3.2.5.3 DBT DESULPHURISATION IN TWO-PHASE SYSTEM

Biodesulphurisation assays were carried out in water/solvent emulsions according to improve biodesulphurisation activity in hydrophobic media as described in the literature review (Chapter 2). Hexadecane was chosen as a representative of solvent based on work by Patel *et al.* (1997). The bacteria grown in BSM with DMSO as the sole source of sulphur were harvested as previously reported, and resuspended in Ringer's solution to A_{600} = 3.0 (inoculum). The assays were carried out in 15-ml sterile centrifuge tubes by adding 1.0 ml of inoculum to 0.325 ml of hexadecane containing 1.0 mg of DBT. Control tubes without bacterial cells were also performed. All tubes were incubated at 30°C (100 rpm). Samples were taken daily for DBT and HBP analyses using HPLC method.

In addition, the effect of age of the inoculum on desulphurisation activity was examined. In the same way, the inoculum kept at –80°C for 2 weeks, or the inoculum kept at –80°C for 4 weeks was used instead of fresh inoculum.

3.2.5.4 DBT-SULPHONE DESULPHURISATION

The bacterial cultures grown in BSM with DMSO were harvested and resuspended in Ringer's solution to A_{600} = 1.0. One ml of inoculum was added to 250-ml flasks containing 100 ml of BSM with 0.3 mM of DBT-sulphone-acetone solution and incubated at 30°C (100 rpm). It was noted that DBT-sulphone is partially dissolved in water or ethanol. Control flasks without bacterial cells were also performed. Samples (5 ml) were taken daily. The bacterial growth was monitored at A_{600}. DBT-sulphone was analysed using HPLC, HBP using Gibb's assay and sulphite using spectrophotometric assay.

3.2.5.5 BT DESULPHURISATION

The bacterial cultures grown in BSM with DMSO were harvested and resuspended in Ringer's solution to A_{600} = 1.0. One ml of inoculum was added to 250-ml flasks containing 100 ml of BSM with 0.3 mM of BT-ethanol solution and incubated at 30°C (100 rpm). Control flasks without bacterial cells were also performed. Samples were taken at day interval. Bacterial growth was monitored at A_{600}. BT was analysed using HPLC. Presence of 2-(2'-hydroxyphenyl)ethan 1-al (HPEal) was indicated using Gibb's assay and mass spectrometry (performed by Kent Mass Spectrometry, Kent, UK).

3.2.5.6 GROWTH ON SULPHUR MODEL COMPOUNDS

The abilities of the bacteria to use a variety of sulphur compounds (DMSO, DBT, DBT-sulphone, BT, diphenyl disulphide (DPDS), thiophene-2-carboxylic acid (T2C), sodium sulphate and elemental sulphur) as the sole sulphur source for their growth were monitored. The bacteria grown in complex media were harvested and resuspended in Ringer's solution to A_{600} = 1.0. One ml of inoculum was added to 250-ml flasks containing 100 ml of BSM with a sulphur source (sulphur compound dissolved in acetone to 100 mM concentration) at a final concentration of 0.3 mM. Control flasks without bacterial cells were also performed. BSM lacking glycerol was used to determine if the bacteria could use these sulphur compounds as the carbon sources. All flasks were incubated at 30°C (100 rpm). Samples were taken at one day intervals, and bacterial growth was observed at A_{600}.

3.2.6 Biodesulphurisation of coal samples

The nature of coal samples used in this study is summarised in Table 3.5. The coal samples were ground in a ball mill and sized to <106 μm by sieve before applied in biodesulphurisation experiments because increased desulphurisation rates have been observed with smaller coal particle sizes (Bayram *et al.*, 1999).

Table 3.5 The nature of coal samples used in biodesulphurisation experiments.

Name	Description	Type	Source
House Cobbles	Lumps Approx. 50-100 mm	Bituminous coal	Thoresby Colliery, Nottinghamshire
Anthracite Grains	Grains Approx 10 mm	Anthracite	Bryn Bach West Wales

3.2.6.1 BIODESULPHURISATION ASSAY

The bacteria grown in their suitable media were harvested and resuspended in Ringer's solution to A_{600} = 1.0. Five ml of inoculum was added to 250-ml flasks containing 100 ml of BSM and 2.0 g of sterile coal (autoclaved at 121°C for 15 minutes). The cultures were incubated at 30°C, 100 rpm. In the same way, uninoculated control flasks were performed. For the desulphurisation by bacteria inherent in the coal, the flasks containing 100 ml of BSM and 2.0 g of non-sterile coal were incubated without adding bacterial culture.

Samples (coal powder mixed thoroughly with liquid media, 15 ml) were taken periodically and transferred to sterile centrifuge tubes. The samples were centrifuged at 10,000 × g for 15 minutes. Immediately following centrifugation, the supernatant was transferred to other sterile centrifuge tubes and kept for further analyses (free bacterial cells, soluble sulphur and soluble iron). The coal sediment was kept for analyses of attached bacterial cells and sulphur forms.

The bacterial cells, both free and attached to coal samples were measured by estimating protein concentration (Kilbane & Jackowski, 1992 and Dastidar *et al.*, 2000). Free protein was monitored straightaway in the supernatant samples described above. For attached protein, the coal samples were dissolved in 15 ml of BSM, 50 µl of 0.1N NaOH was added, and the samples were placed in a boiling water bath for 30 minutes. Upon cooling to room temperature, 25 µl of 1N HCl was added to precipitate soluble material and the samples were centrifuged at 10,000 × g for 15 minutes (Kilbane & Jackowski, 1992). Immediately following centrifugation, the supernatant was transferred to other sterile centrifuge tubes and kept for attached protein analysis.

The tubes containing coal sediment were then put into a freeze-dried system to get rid of the moisture following by extraction processes for sulphur forms analyses.

Soluble sulphur and soluble iron were monitored using inductively coupled plasma (ICP) spectroscopy. Total sulphur, sulphate sulphur and pyritic sulphur in coal samples were extracted by wet chemical techniques followed by quantification with ICP spectroscopy. Organic sulphur was determined by two methods: a standard method and a proposed method. The direct detection of sulphur was performed by X-ray photoelectron spectroscopy (XPS) analysis. The surface of the coal samples before and after treatment with biodesulphurisation processes was studied by scanning electron microscopy (SEM) with energy-dispersive X-ray (EDX) analysis.

3.2.6.2 FREEZE-DRIED METHOD

The freeze-drier system used was from Edward, West Sussex, UK. The refrigeration unit was turned on and waited until the temperature was below -45°C. The samples to be freeze-dried were placed on the platform above the condenser and covered with the perspex dome. The pressure valve was then closed. The vacuum pump was turned on. A vacuum should gradually register on the gauge. If the stage 1 pump is being used the vacuum should be about 9 on the mBar gauge. The stage 2 pump should reach between 7 and 4 on the mBar guage. It might take an hour to get to the maximum vacuum. At the end of freeze-drying, the vacuum pump was turned off. The pressure valve was slowly opened and then the refrigeration unit was turned off. Finally, the condenser was defrost and dried.

3.2.6.3 ISOLATION OF BACTERIA INHERENT IN THE COAL

The bacteria inherent in the coal were isolated after bacterial growth in the enrichment solution was observed. Aliquots of the enrichment solution were plated on nutrient agar and growth was followed by the formation of colonies. Each colony was then transferred to 250-ml flasks containing 100 ml of nutrient broth. Further investigation on the desulphurisation abilities were carried out by transferring 2 ml of the culture to other 250-ml flasks containing 100 ml of BSM and 0.3 mM of sulphur model compound. The bacterial growth was determined at A_{600}.

3.2.7 Analytical methods

3.2.7.1 GIBB'S ASSAY

Aromatic hydroxyl compounds produced as a consequence of biodesulphurisation of DBT, DBT-sulphone or BT were monitored using Gibb's assay. The principle of this assay is that Gibb's reagent (2,6-dichloroquinone-4-chloroimide) can react with aromatic hydroxyl groups to form a blue-coloured complex that can be monitored spectrophotometrically at 610 nm. (Kayser *et al.*, 1993).

Samples (1.5 ml) taken daily from experiments described in Sections 3.2.5.2, 3.2.5.4 and 3.2.5.5 were transferred to 15-ml centrifuge tubes and centrifuged (7700 × g for 15 minutes) to remove the bacterial cells. Supernatant (1.0 ml) from each tube was transferred to a disposable spectrophotometer cuvette and stored at 4°C until analysed. To analyse, 10 µl of 10 mM Gibb's reagent in acetone was added to each cuvette. A blank solution (BSM plus 10 µl Gibb's reagent in acetone) was also prepared. The assay was incubated overnight at 30 °C, for full colour development,

and the A_{610} was measured. A_{610} was converted to HBP (a product from DBT or DBT-sulphone desulphurisation) concentration using a standard curve prepared with authentic HBP in the range of 0-0.4 mM (Figure 3.1).

It is noted that 2-(2'-hydroxyphenyl)ethan 1-al (HPEal) which is a product from BT desulphurisation is not commercially available and it was not possible to construct a standard curve. However, development of blue colour produced by the reaction between Gibb's reagent and aromatic hydroxyl group can be used as an indication of the present of HPEal.

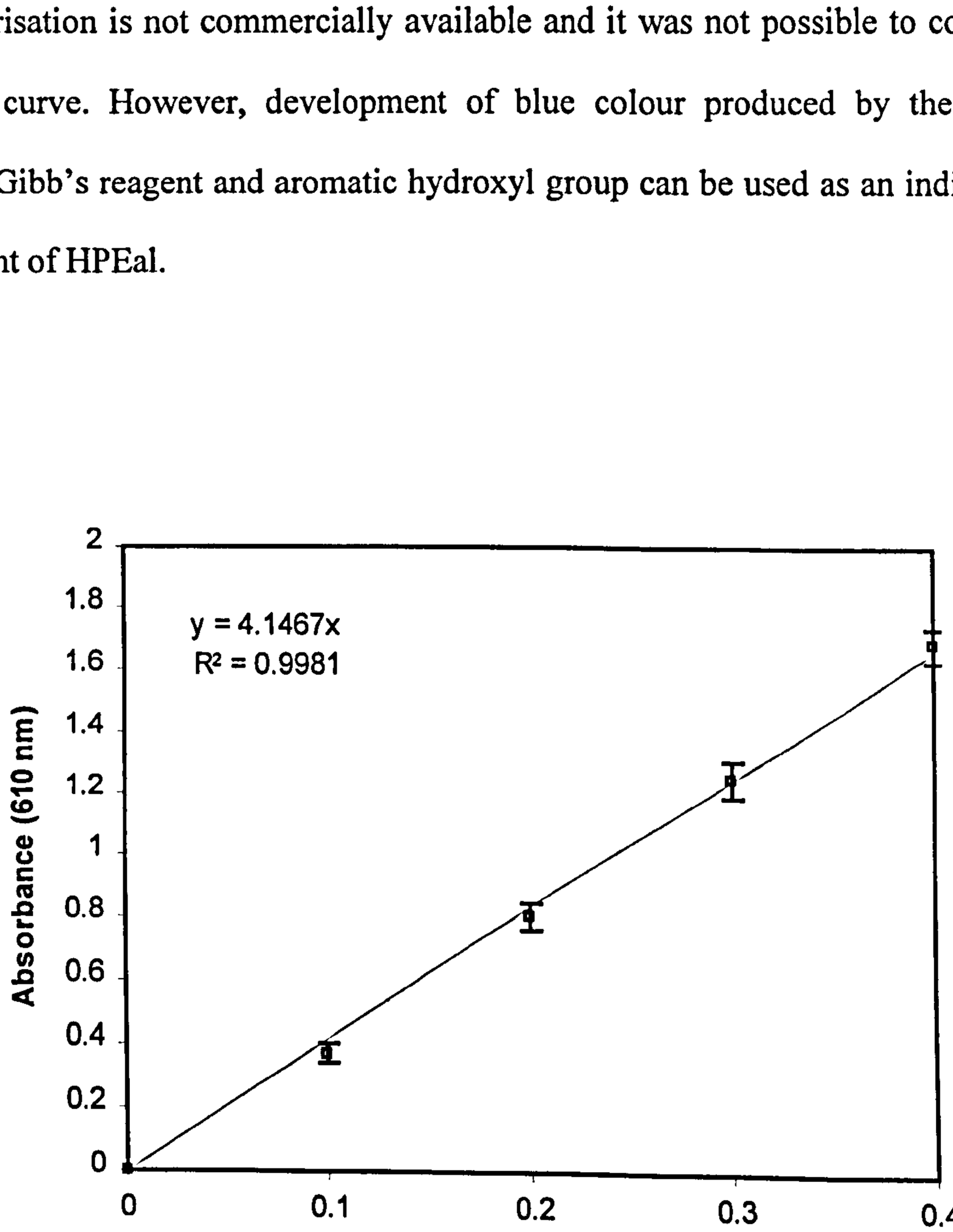


Figure 3.1 Standard curve of HBP obtained by Gibb's assay. Each point is a mean of 3 replicates, and error bar is a standard deviation.

3.2.7.2 DEVELOPMENT OF A HIGH-PRESSURE LIQUID CHROMATOGRAPHY METHOD

High-pressure liquid chromatography (HPLC) was developed to analyse the concentrations of DBT, DBT-sulphone, HBP and BT. Samples from experiments described in Sections 3.2.5.2-3.2.5.5 (1.0 ml) were transferred to 15-ml centrifuge tubes containing 1.0 ml of acetonitrile. All tubes were mixed thoroughly by vortex. The bacterial cells were removed by centrifugation at 7700 × g for 15 minutes. The supernatant from each tube was then filtered through a 0.2 μm inorganic membrane filter (Whatman International Ltd.) and kept in a vial (Fisher, UK) stored at 4°C until analysed.

The analysis was performed with a Gilson HPLC Model 715 from Anachem (Luton, UK) equipped with a Nova Pak phenyl column (3.9×150 mm) with a guard column from Waters (Milford, USA). The detector used was a 117 UV detector fixed at 233 nm wavelength. The mobile phase, a mixture of HPLC grade of water and acetonitrile was sonicated for 10 minutes, and de-gassed with helium, before use. In the beginning isocratic elution with 70% acetonitrile and 30% water at 1.5 ml/min based on work by Patel *et al.* (1997) was used, but with this elution a chromatogram peak of each compound was not distinguished. The mobile phase was then changed to 60% acetonitrile and 40% water, a distinguished peak of each compound was observed. However, this elution presented the problems of column contamination: changes in retention time and increase of system backpressure after only few uses. Following several changes of ratio of acetonitrile:water and flow rate, a suitable gradient of mobile phase was achieved as shown in Table 3.6.

Table 3.6 Mobile phase gradient for HPLC analysis.

Time (min)	Flow rate (ml/min)	% Water
0.00	1.30	57.0
1.00	1.30	60.0
2.00	1.30	60.0
5.00	1.30	55.0
7.50	1.30	0.0
8.50	1.60	0.0
12.00	1.60	0.0
12.50	1.30	57.0
15.00	1.30	57.0

Total running time of each sample was 15 minutes, from 7.50 to 12.00 minute was a cleaning procedure (flushing with pure acetonitrile). The contamination problems were relieved with this procedure and the retention time of each compound can be summarised as shown in Table 3.7.

Table 3.7 Retention time of chemical compounds in HPLC analysis.

Compound	T_R (min)
DBT-sulphone	3.50
HBP	4.60
BT	5.90
DBT	7.90

The concentration of DBT, DBT-sulphone, HBP or BT in each sample was calculated by HPLC software using calibration curves as shown in Appendix A.

3.2.7.3 SOLID-PHASE EXTRACTION PROCEDURE

Solid-phase extraction (SPE) was applied as a method for separation of the desulphurisation metabolites of BT before analysis with mass spectrometry. The compounds to be analysed have polar functional groups such as S=O or OH. Therefore, a silica cartridge was selected to trap the compounds quantitatively (Onaka *et al.*, 1997). Figure 3.2 represents the scheme of SPE isolation. Firstly, the SPE column was conditioned by washing with 3.0 ml of *n*-hexane. One ml of sample (from Section 3.2.5.5) was pipetted onto the column. After the sample was absorbed onto the column cartridge, it was washed with 3.0 ml of *n*-hexane again. The eluent, which had a 7:3 composition of ethyl acetate (EtOAc) and iso-propanol (IPA) was added to elute the absorbed substances. Finally, the solution was collected in a vial and sent to Kent Mass Spectrometry (Kent, UK) for mass spectrometry analysis.

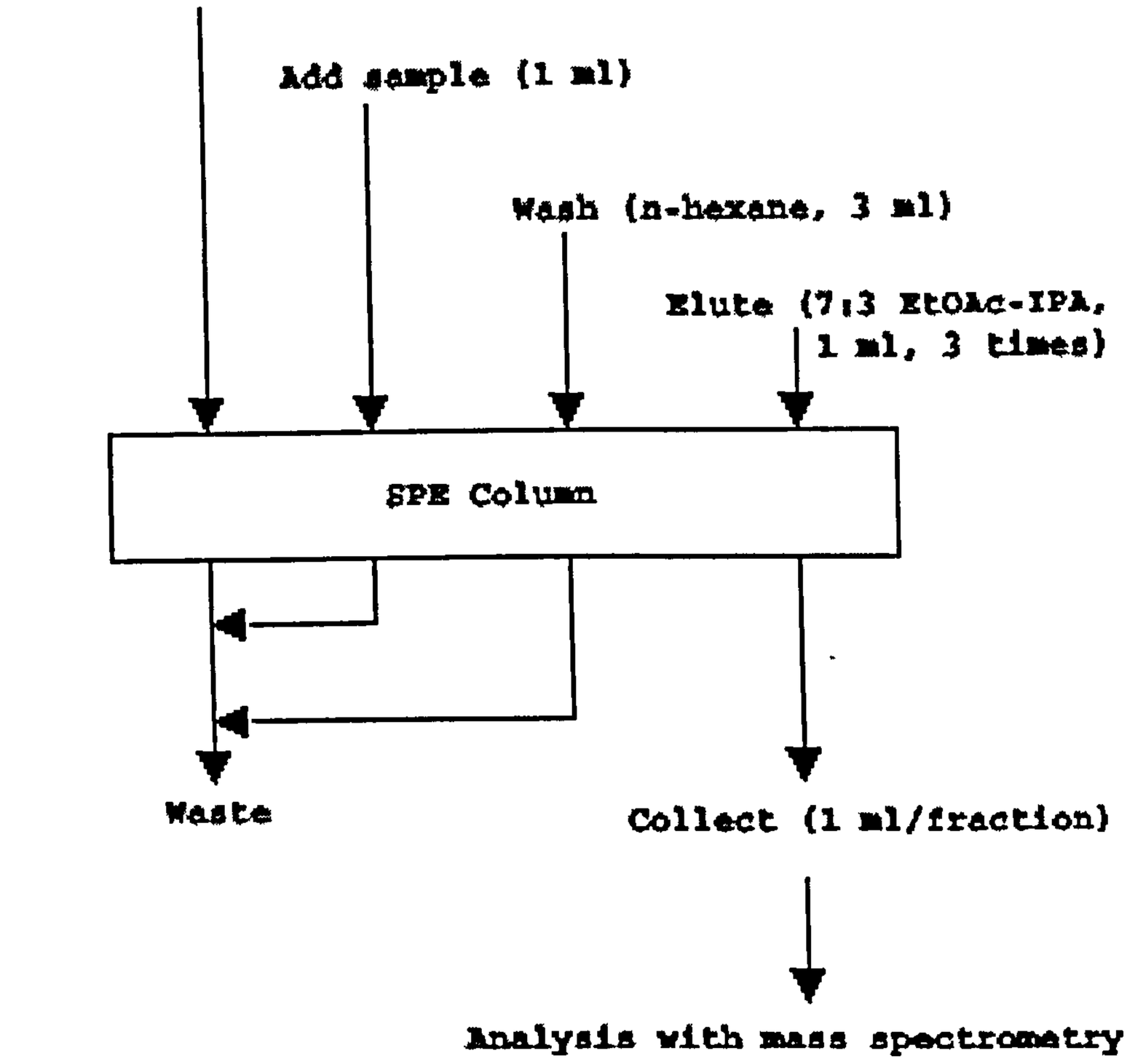


Figure 3.2 The solid phase extraction scheme (Onaka *et al.*, 1997). This procedure was applied for separation of the desulphurisation metabolites of BT before analysis with mass spectrometry.

3.2.7.4 SPECTROPHOTOMETRIC ASSAY FOR SULPHITE

Sulphite was estimated using a modification of the sulphite-oxidase-linked assay (oxidation of sulphite to sulphate, linked to the reduction of cytochrome *c*) which is described by Beutler (1987). Firstly, 1.5 ml of each sample (from Sections 3.2.5.2 and 3.2.5.4) was added to a 15-ml centrifuge tube and centrifuged (7700 × g, 15 min) to remove the bacterial cells. Then 1.0 ml of the supernatant was transferred to a disposable spectrophotometer cuvette and stored at 4°C until analysed. To analyse, cytochrome *c* (20 µl of 5 mM solution in 50 mM of N-[2-Hydrocyethyl]piperazine-N'-[3-propanesulphonic acid] (HEPPS), pH 8) was added to each cuvette (Oldfield *et al.*, 1997). The assay was incubated at room temperature for 5 minutes and the A_{550} was read. Two microlitres of the Sigma sulphite oxidase suspension was then added to each cuvette and the set was incubated at room temperature for further 5 minutes. The A_{550} was read again and the ΔA_{550} was converted to sulphite concentration using a standard curve prepared with sodium sulphite as shown in Figure 3.3.

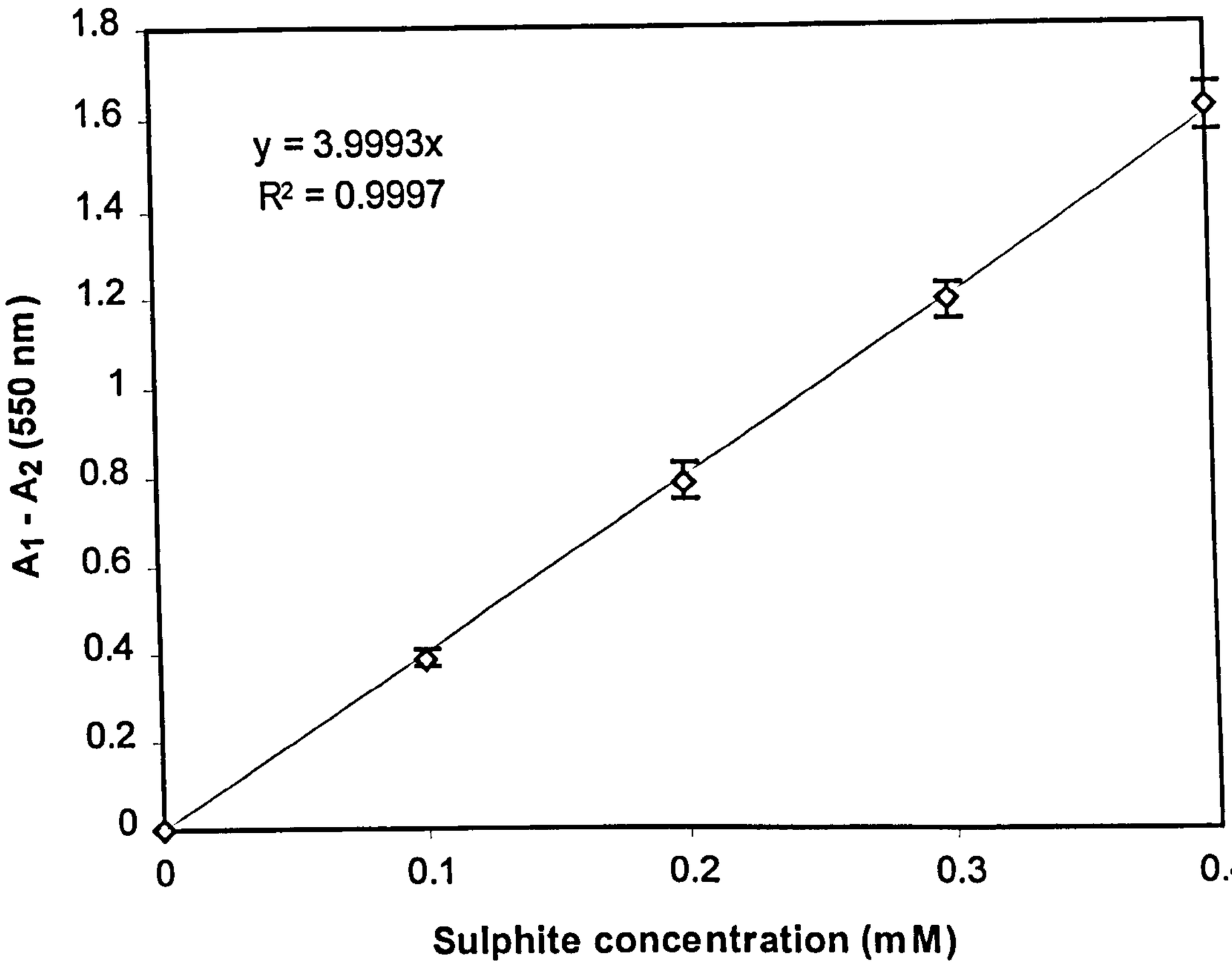


Figure 3.3 Standard curve of sulphite obtained by spectrophotometric assay. Each point is a mean of 3 replicates, and error bar is a standard deviation.

3.2.7.5 PROTEIN ASSAY

The protein concentration was estimated using a Micro BCA Protein Assay Reagent Kit from Pierce, USA. The kit contained:

1. Micro BCA Reagent A (MA), 240 ml; containing sodium carbonate, sodium bicarbonate and sodium tartrate in 0.2 N NaOH;
2. Micro BCA Reagent B (MB), 240 ml; containing bicinchoninic acid(4.0%) in water;
3. Micro BCA Reagent C (MC), 12 ml; containing 4.0% cupric sulphate, pentahydrate in water; and
4. Albumin Standard, 10 × 1 ml ampules; containing bovine serum albumin (BSA) at a concentration of 2.0 mg/ml in a solution of 0.9% saline and 0.05% sodium azide.

The Micro BCA Protein Assay method is highly sensitive for the quantitative colorimetric determination of total proteins in dilute aqueous solutions. This unique reagent system utilises bicinchoninic acid (BCA) as the detection reagent for Cu^{+1}, which is formed when $Cu^{+2}$is reduced by protein in an alkaline environment (Smith *et al.*, 1985). The purple coloured reaction product is formed by the chelation of two molecules of BCA with one cuprous ion (Cu^{+1}). This water-soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentrations.

Firstly, the BCA working reagent (WR) was prepared by mixing 25 parts of MA and 24 parts of MB with 1 part of MC. Then 0.1 ml of each sample (from Section 3.2.6.1) was pipetted into a disposable spectrophotometer cuvette and diluted to 1 ml by BSM. One ml of WR was added and mixed well. All cuvettes were incubated at 60°C for 60

minutes. After cooling down to room temperature A_{562} was read. A_{562} was converted to protein concentration using a standard curve (Figure 3.4). Standard curve was prepared using BSA as a standard protein.

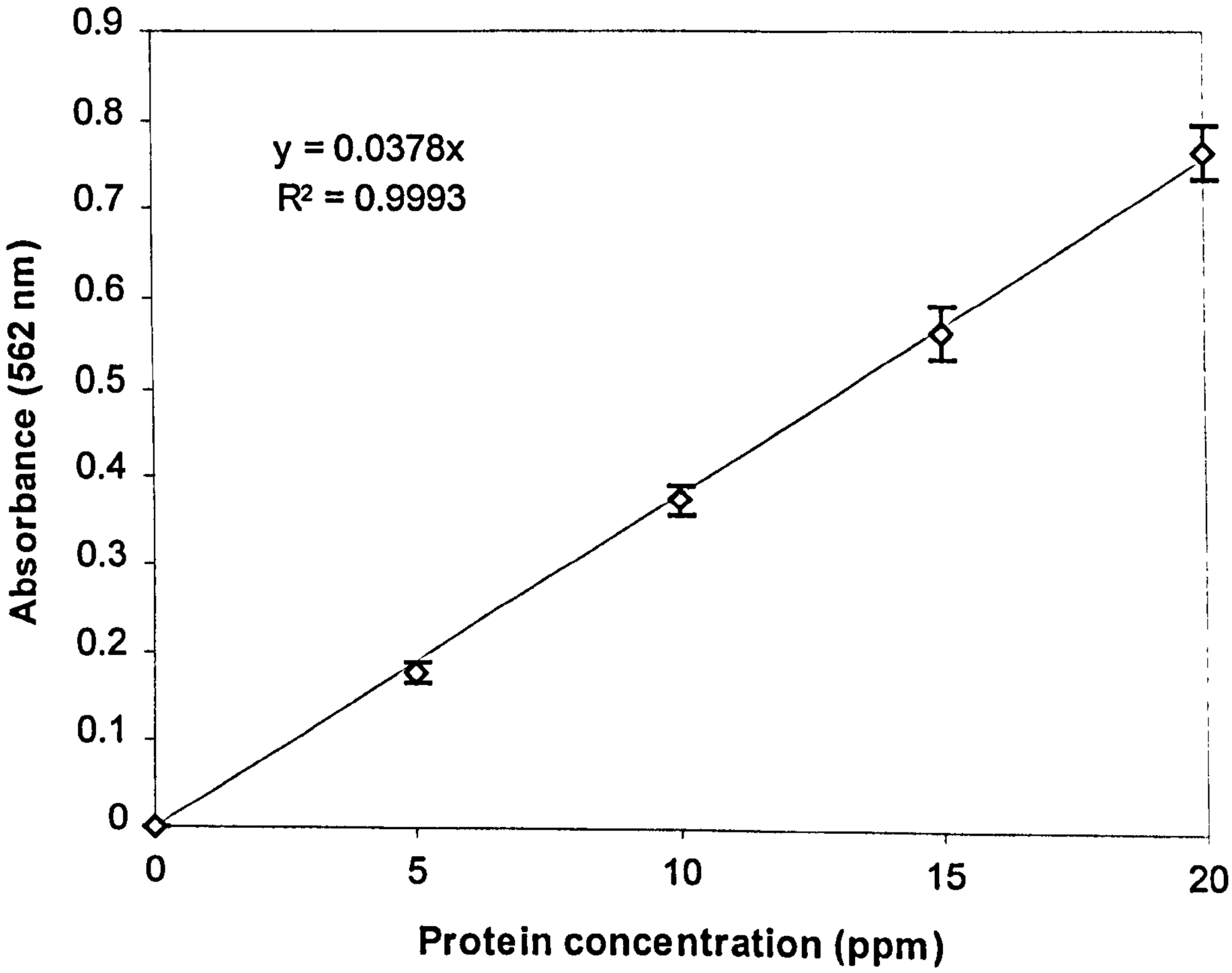


Figure 3.4 Standard curve of bovine serum albumin obtained using the micro BCA protein assay. Each point is a mean of 3 replicates, and error bar is a standard deviation.

3.2.7.6 COAL EXTRACTION METHODS

Coal was extracted for sulphur determination following the procedure adapted from Eames & Cosstick (1992), Laban & Atkin (2000) and Davidson (1994).

Total sulphur

An accurately weighed 0.1 g portion of a <106 μm coal was transferred into a porcelain crucible, charged with approximately 0.1 g of Eschka mixture and mixed well. A further portion of Eschka mixture was added to bring the total mass added to 0.8 ± 0.02 g. The crucible was placed in a muffle furnace, and the temperature raised to 800°C over about 1 h and then kept at 800°C for 1 h. After the crucible had been cooled, the contents were transferred quantitatively into a 100-ml beaker containing approximately 30 ml of water and dispersed with a stirring rod. 4 ml of concentrated hydrochloric acid (HCl) was added and the solution was boiled gently to dissolve all the sulphate. The cool solution was transferred into a 100-ml calibrated flask and diluted to the mark with water. A blank was also prepared by carrying through the same procedure, but without the addition of coal. Control standard was prepared by using Na_2SO_4 instead of coal. The sulphur concentration in blank, control standard and samples were analysed by ICP spectroscopy.

Sulphate sulphur

A <106 μm coal was weighed to 0.1 g and transferred into a 100-ml beaker. After the addition of approximately 0.2 ml of methanol to wet the sample, 10 ml of 5 M HCl was added. The beaker was covered with a watch-glass, and the contents were boiled gently for 15 min. The product was cooled before being filtered on a Durapore membrane filter (0.22 μm GV from Millipore), and the residue was washed with 5 M

HCl. The filtrate was collected in a vial and adjusted to 30 ml with water. The sulphur concentration was then measured by ICP spectroscopy.

Pyritic sulphur

The residue from the sulphate sulphur determination was transferred to a centrifuge tube. The nitric acid used in this stage may form an aggressive acidic mixture with any traces of HCl remaining on the residue from sulphate sulphur analysis. This can result in the partial attack of organic phases, rather than the intended dissolution of pyritic minerals alone. To ensure the dilution of any remaining HCl, 45 ml of distilled water was added to the residue which was then centrifuged for 5 minutes at 5000 × g. The clear solution was discarded. The residue was then transferred into a 100-ml beaker and 10 ml of 2 M nitric acid (HNO_3) was added. The beaker was covered with a watch-glass and the contents were gently boiled for 30 min. The digested sample was cooled before being filtered on a membrane filter and washed with 2 M HNO_3. The filtrate was collected in a vial and adjusted to 30 ml with water. The resultant solution was analysed for sulphur by ICP spectroscopy.

Organic sulphur

Organic sulphur was determined by two methods; the standard method and a proposed new method. In the standard method, organic sulphur was calculated by subtracting the sum of sulphate and pyritic sulphur from the total sulphur. In the proposed method, the residue from the pyritic sulphur determination was transferred into a porcelain crucible, charged with approximately 0.1 g of Eschka mixture and a procedure for total sulphur determination was carried out as described before. The total sulphur was taken as organic sulphur.

3.2.7.7 INDUCTIVELY COUPLED PLASMA SPECTROSCOPY ANALYSIS

The sample solutions from each stage of coal extraction were analysed for sulphur and iron by inductively coupled plasma (ICP) spectroscopy. The measurements were performed on AtomScan 16 Sequential ICP Spectrometer (Thermo Jarrell Ash Corporation, USA) with Thermospec software version 6.20 in School of Water Sciences, School of Industrial and Manufacturing Science, Cranfield University. Instrumental parameters are listed in Table 3.8.

The spectrometer was operated under vacuum with an argon-flushed viewing tube between the plasma and the polychromator. The spectral 180.731 nm was selected for detection of sulphur and the spectral 259.940 nm was selected for detection of iron. No significant matrix interference from sodium and magnesium at the levels present in sample extracts was observed.

Table 3.8 Inductively coupled plasma spectroscopy operating parameters.

Gas Flow Rates	
Torch gas flow	High flow
Auxiliary gas flow	1.0 L/min
Peristaltic Pump Parameters	
Flush pump rate	200 rpm
Relaxation time	10 sec
Pump tubing type	Tygon-Orange
Plasma Parameters	
Approximate RF power	1150 W
Analysis pump rate	100 rpm
Nebulizer pressure	30 psi
Flush time	45 s
Number of repeat	3

3.2.7.8 X-RAY PHOTOELECTRON SPECTROSCOPY ANALYSIS

X-ray photoelectron spectroscopy (XPS) analysis was carried out as a non-invasive method of sulphur detection in coal samples. The coal powder was pressed by an IR Disc Press (School of Mechanical Engineering, Cranfield University) by applying 10 tonnes of weight. The disc size is 1 cm diameter. The XPS analysis was then performed on a Vacuum Generators (VG) Scientific Esca lab 200D system (Advanced Materials Department, School of Industrial and Manufacturing Science, Cranfield University). The XPS spectra were obtained using non-monochromatic Mg Kα radiation. An energy correction was made for sample charging, based on the C(1s) peak position observed at 288 ± 1 eV.

3.2.7.9 SCANNING ELECTRON MICROSCOPY ANALYSIS

The surface of the coal samples before and after treatment with the biodesulphurisation process was studied by scanning electron microscopy (SEM) analysis. The coal sample was mounted on an aluminium stub by double side conductive tape and coated with silver (Ag). The SEM analysis was conducted using Microscope Stereoscan 250 MK3 interfaced with Link System 860 Series (Cambridge, UK) in Advanced Materials Department, School of Industrial and Manufacturing Science, Cranfield University. An electron acceleration voltage of 20 keV was selected. Characteristic X-rays were collected for 100s from each sampling point of the coal particle.

3.2.7.10 REPLICATION AND STATISTICAL ANALYSIS OF DATA

All experiments were replicated at least three times unless otherwise stated. Error bar on graph is a standard deviation. In addition, coefficient of variation (CV) was calculated to check the precision of data.

$$CV = (\text{standard deviation}/ \text{mean}) \times 100\%$$

Analysis of variance (ANOVA) was carried out using Microsoft Excel tool package. Effect of interaction between the ANOVA test was set at 5% significant level. Least significant difference (LSD) was carried out on data following the ANOVA test to identify significant differences between individual treatments.

$$LSD = \text{square root (Within Group Mean Square} \times 2 / \text{replicate per treatment)} \times t$$

Where $t = t$ distribution value at x degrees of freedom (given in Appendix C)

and $x =$ degrees of freedom within treatments.

The LSD value was then applied to all means to establish whether a significant difference existed. In addition, standard deviation was calculated.

CHAPTER 4:

RÈSULTS

4.1 BACTERIAL CHARACTERISTICS

Three bacterial strains investigated in this study are; *Rhodococcus erythropolis* IGTS8, *R. erythropolis* X309 and *Shewanella putrefaciens* strain NCIMB 8768. *R. erythropolis* IGTS8 was formerly classified as *R. rhodochrous* IGTS8. It was patented by the Institute of Gas Technology and licensed for commercial development to Energy Biosystems Corporation (McFarland, 1999). *R. erythropolis* X309 is one of two bacterial strains isolated by Lee *et al.* (1995) which was initially classified as an *Arthrobacter* species. Strain X309 is the mucoid strain while another strain is nonmucoid and reclassified as *R. erythropolis* strain ECRD-1 or strain X310. The strain ECRD-1 was chosen for study by Lee *et al.* (1995). There has been only little previous knowledge of strain X309. *S. putrefaciens* strain NCIMB 8768 (formerly called *Pseudomonas rubescens*) was isolated by Pivnick (1955). The strain NCIMB 8768 has not yet been proved to possess the capacity for desulphurisation. Some characteristics of these three organisms are summarised in Table 4.1. In addition, the relative growth of all strains in the complex medium is shown in Appendix B.

Table 4.1 Characteristics of the three bacteria.

Description	S. putrefaciens[3]	*R.* IGTS8[4]	*R.* X309[5]
Isolation source	Oil emulsion	Soil	Soil
Gram stain[6]	-	+	+
Motility	+	-	-
Catalase	+	+	+
Oxidase	+	-	-

[3] Information from Pivnick (1955); Lysenko (1961).

[4] Information from Kilbane (1990); Finnerty (1993).

[5] Information from Lee *et al.* (1995).

[6] Gram stain was also performed in this study (Section 3.2.4.2).

4.2 BIODESULPHURISATION OF MODEL COMPOUNDS

The abilities of *R. erythropolis* IGTS8, *R. erythropolis* X309 and *S. putrefaciens* in the desulphurisation of a wide range of sulphur model compounds (both inorganic and organic sulphur) are reported in this Section.

4.2.1 DBT desulphurisation

With a supply of dibenzothiophene (DBT) as a sole source of sulphur in BSM, *R. erythropolis* IGTS8 grew with a mean doubling time of 13 hours as shown in Figure 4.1. DBT concentration in the medium declined from 0.3 to 0.03 mM. Production of hydroxybiphenyl (HBP) increased dramatically in the early exponential growth phase, and reached a concentration of 0.25 mM in the stationary growth phase (144 hours). Sulphite release was observed, but in a small concentration of 0.01 mM. There was a transient appearance of DBT-sulphone in the medium. There was no DBT degradation in the control flasks (incubated without bacterial cells).

Figure 4.2 represents DBT desulphurisation by *R. erythropolis* X309. This strain also grew in BSM + DBT with a mean doubling time of 13 hours. DBT concentration in the medium declined from 0.3 to 0.03 mM, and HBP concentraion increased from 0 to 0.24 mM in 144 hours. Sulphite release was observed in a small amount of 0.01 mM. There was a similar transient appearance of DBT-sulphone in the medium, as observed with IGTS8.

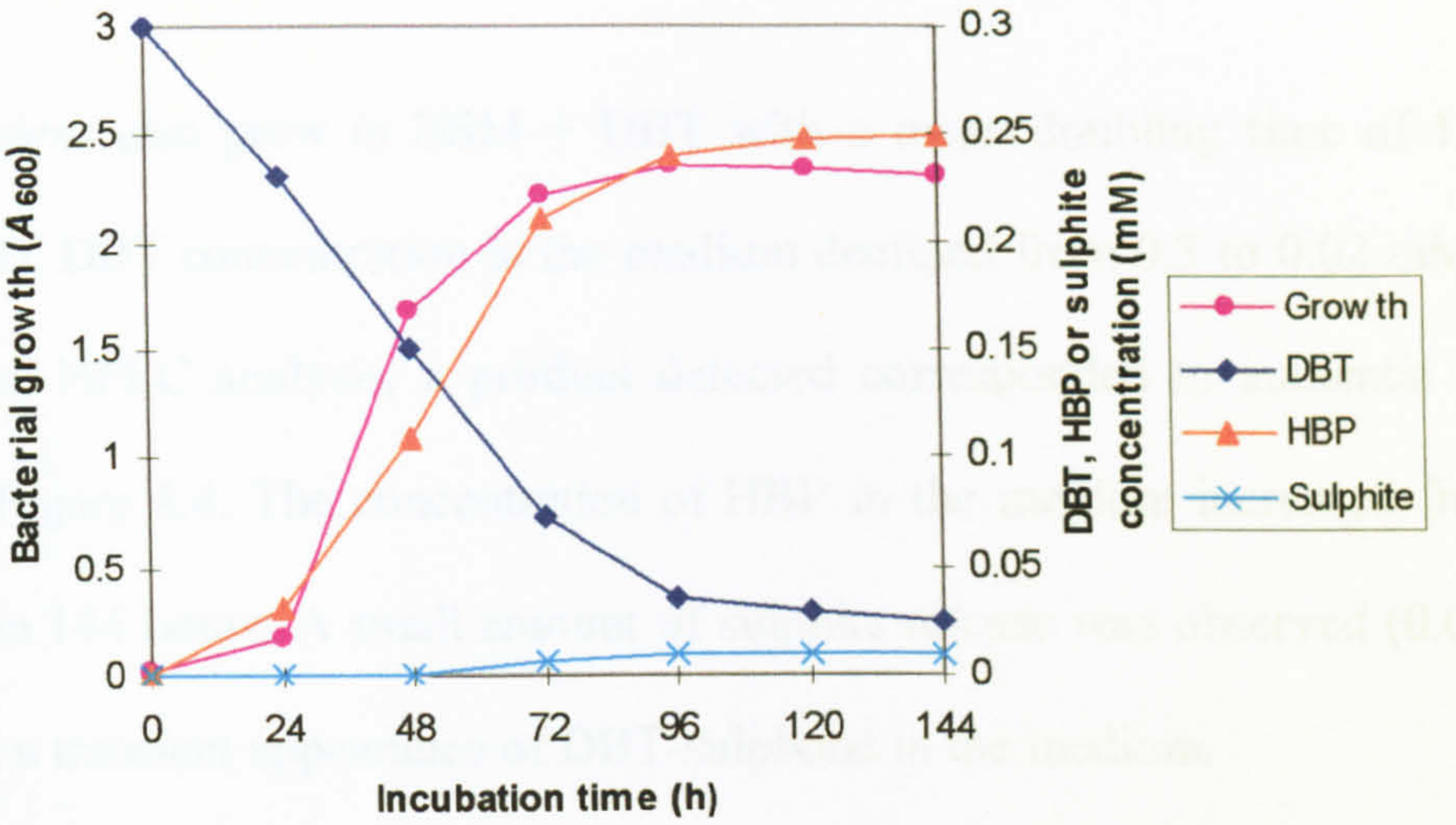


Figure 4.1 DBT desulphurisation by *R. erythropolis* IGTS8. Cell grown in BSM with DMSO as a sole source of sulphur until the mid-log phase, washed twice, and resuspended in Ringer's solution to A_{600} = 1.0. One ml of the cell suspension was inoculated into 100 ml of BSM containing 0.3 mM of DBT-ethanol and incubated at 30°C, 100 rpm. Samples taken daily were monitored for bacterial growth (A_{600}), DBT (HPLC), HBP (Gibb's assay) and sulphite (spectrophotometric assay). Each point is a mean of five replicates, and CV is less than 5%.

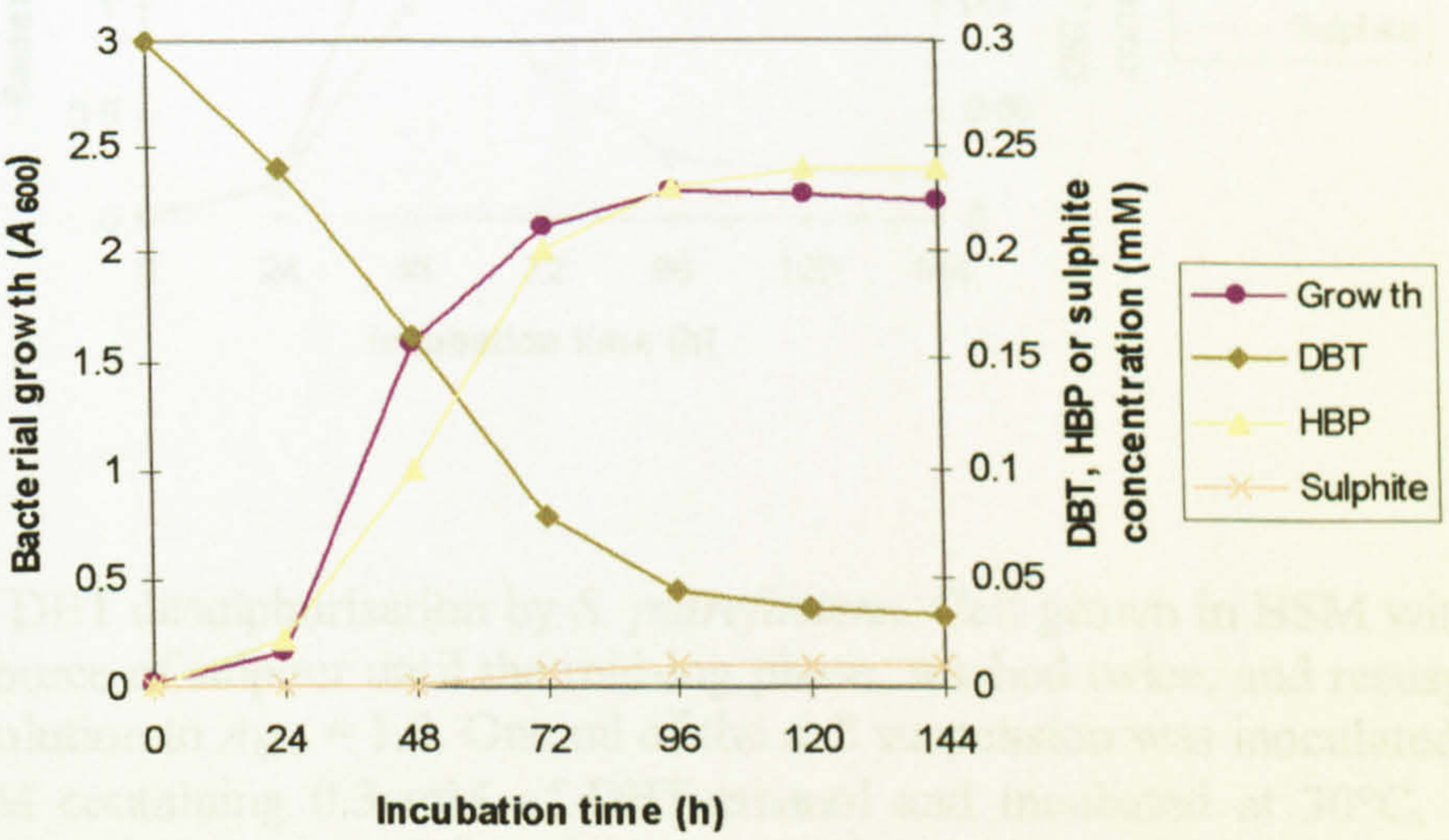


Figure 4.2 DBT desulphurisation by *R. erythropolis* X309. Cell grown in BSM with DMSO as a sole source of sulphur until the mid-log phase, washed twice, and resuspended in Ringer's solution to A_{600} = 1.0. One ml of the cell suspension was inoculated into 100 ml of BSM containing 0.3 mM of DBT-ethanol and incubated at 30°C, 100 rpm. Samples taken daily were monitored for bacterial growth (A_{600}), DBT (HPLC), HBP (Gibb's assay) and sulphite (spectrophotometric assay). Each point is a mean of five replicates, and CV is less than 5%.

S. putrefaciens also grew in BSM + DBT with a mean doubling time of 13 hours (Figure 4.3). DBT concentration in the medium declined from 0.3 to 0.02 mM in 144 hours. With HPLC analysis, a product detected corresponded to authentic HBP as shown in Figure 4.4. The concentration of HBP in the medium increased from 0 to 0.26 mM in 144 hours. A small amount of sulphite release was observed (0.01 mM). There was a transient appearance of DBT-sulphone in the medium.

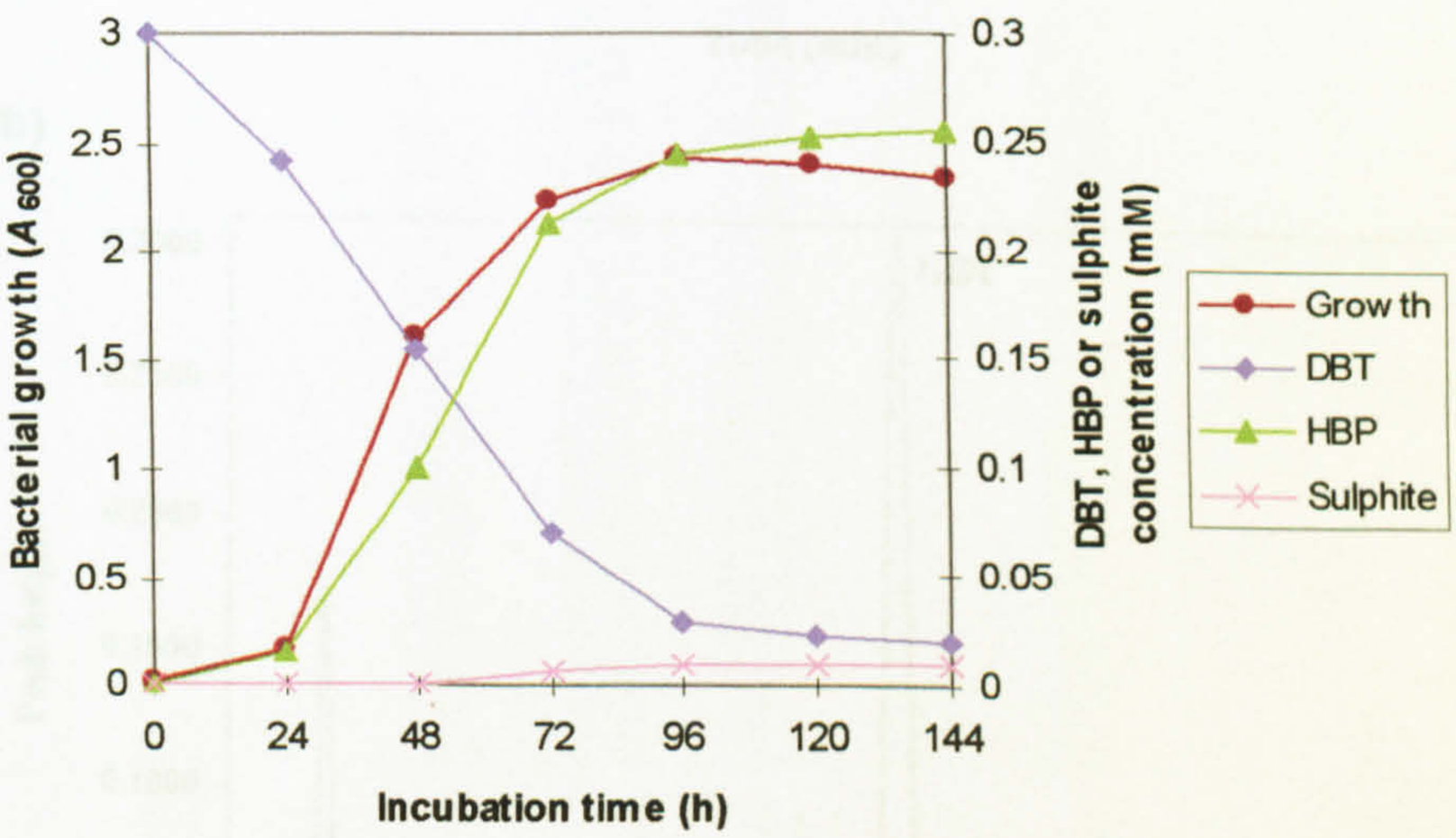


Figure 4.3 DBT desulphurisation by *S. putrefaciens*. Cell grown in BSM with DMSO as a sole source of sulphur until the mid-log phase, washed twice, and resuspended in Ringer's solution to A_{600} = 1.0. One ml of the cell suspension was inoculated into 100 ml of BSM containing 0.3 mM of DBT-ethanol and incubated at 30°C, 100 rpm. Samples taken daily were monitored for bacterial growth (A_{600}), DBT (HPLC), HBP (Gibb's assay) and sulphite (spectrophotometric assay). Each point is a mean of five replicates, and CV is less than 5%.

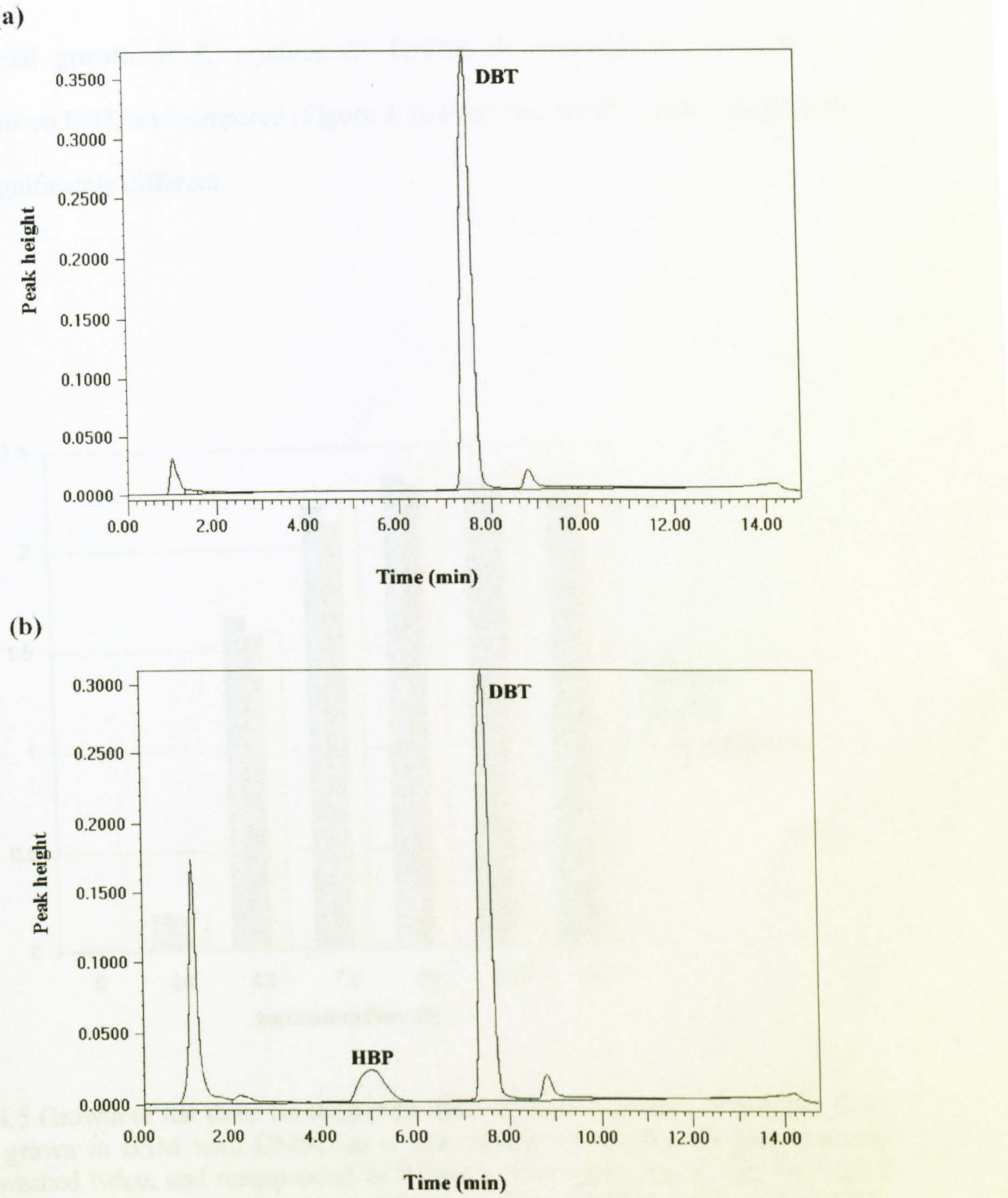


Figure 4.4 HPLC chromatogram of DBT desulphurisation by *S. putrefaciens*: (a) at 0 incubation time; and (b) after 3 days of incubation. Cell grown in BSM with DMSO as a sole source of sulphur until the mid-log phase, washed twice, and resuspended in Ringer's solution to A_{600} = 1.0. One ml of the cell suspension was inoculated into 100 ml of BSM containing 0.3 mM of DBT-ethanol and incubated at 30°C, 100 rpm. Samples taken daily were analysed using HPLC. Peaks at 1.0 and 8.8 min were not related to the analysis.

The bacterial growth of *R. erythropolis* IGTS8, *R. erythropolis* X309 and *S. putrefaciens* on DBT was compared (Figure 4.5). From the ANOVA test, their growth was not significantly different.

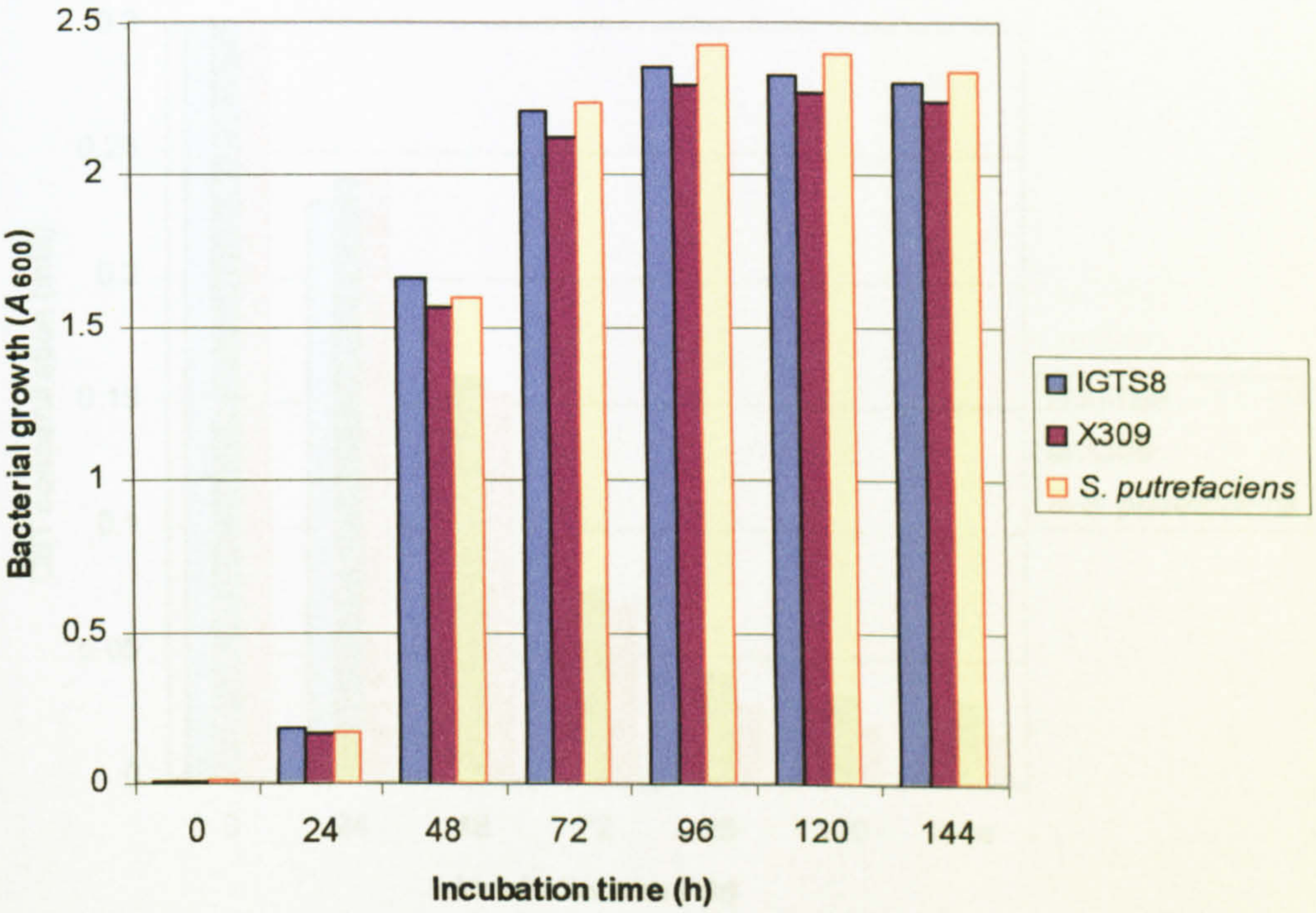


Figure 4.5 Growth of the three bacteria with DBT as the sole source of sulphur. Each culture grown in BSM with DMSO as a sole source of sulphur until the mid-log phase, washed twice, and resuspended in Ringer's solution to A_{600} = 1.0. One ml of the cell suspension was inoculated into 100 ml of BSM containing 0.3 mM of DBT-ethanol and incubated at 30°C, 100 rpm. Samples taken daily were monitored for bacterial growth at A_{600}. Each point is a mean of five replicates, and LSD (P=0.05) = 0.24.

Figure 4.6 compares the degradation of DBT by the three bacteria. From the ANOVA test, DBT degradation by *R. erythropolis* IGTS8, *R. erythropolis* X309 and *S. putrefaciens* was not significantly different.

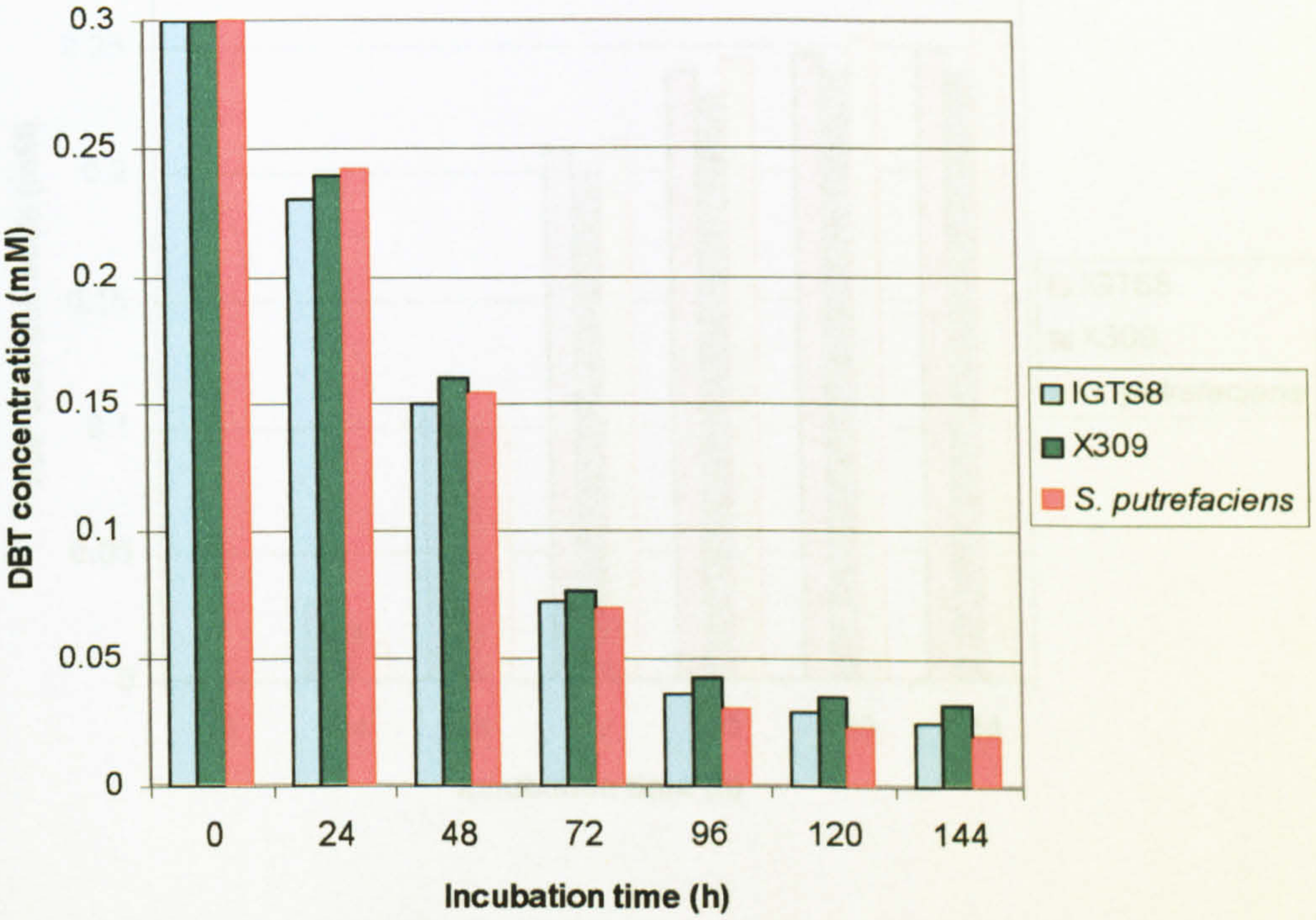


Figure 4.6 Degradation of DBT by the three bacteria. Each culture grown in BSM with DMSO as a sole source of sulphur until the mid-log phase, washed twice, and resuspended in Ringer's solution to A_{600} = 1.0. One ml of the cell suspension was inoculated into 100 ml of BSM containing 0.3 mM of DBT-ethanol and incubated at 30°C, 100 rpm. Samples taken daily were analysed for DBT using HPLC. Each point is a mean of five replicates, and LSD (P=0.05) = 0.01.

Similarly, the amount of HBP produced by the three bacteria was not significantly different from the ANOVA test (Figure 4.7).

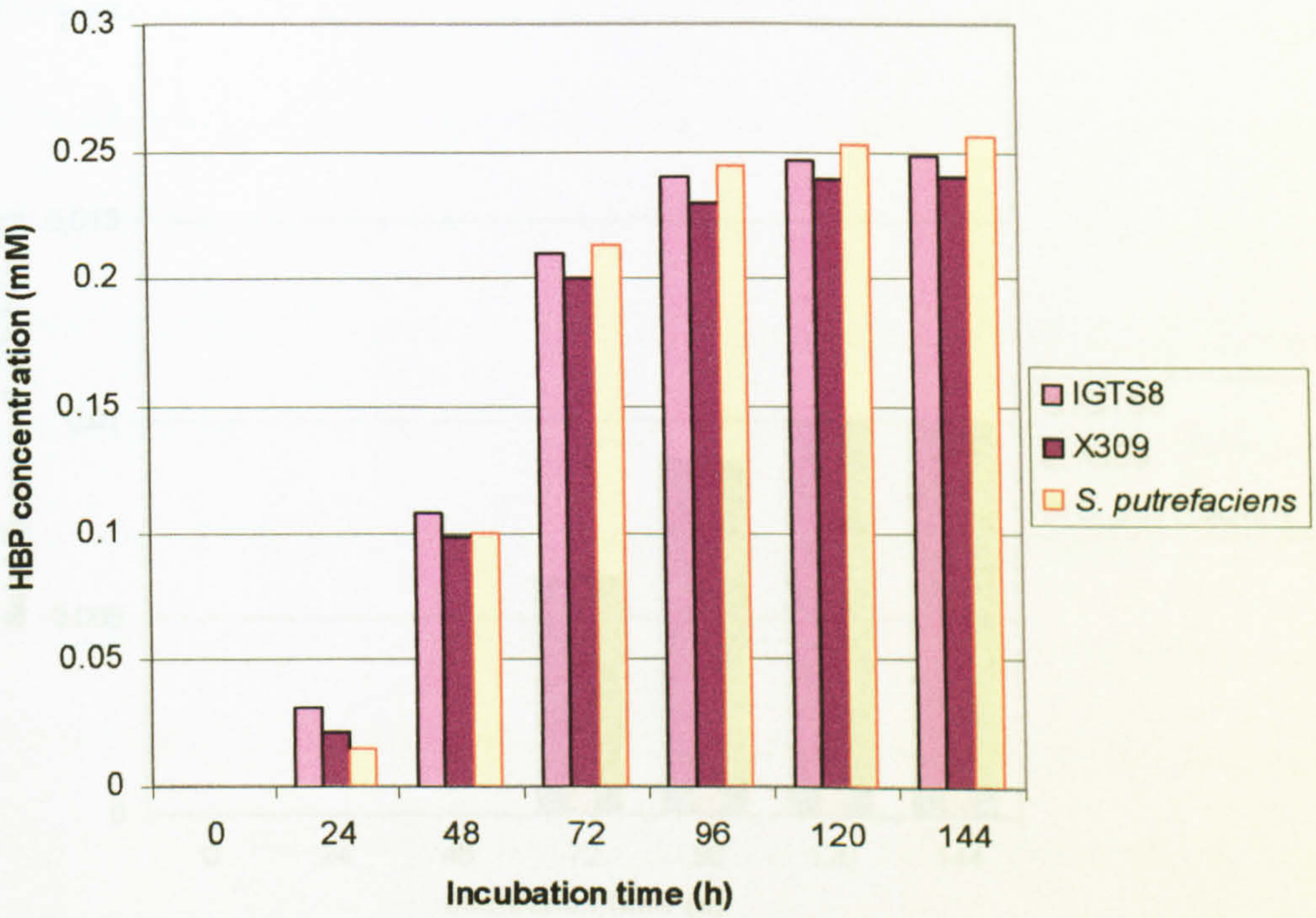


Figure 4.7 Production of HBP from DBT degradation by the three bacteria. Each culture grown in BSM with DMSO as a sole source of sulphur until the mid-log phase, washed twice, and resuspended in Ringer's solution to A_{600} = 1.0. One ml of the cell suspension was inoculated into 100 ml of BSM containing 0.3 mM of DBT-ethanol and incubated at 30°C, 100 rpm. Samples taken daily were analysed for HBP using Gibb's assay. Each point is a mean of five replicates, and LSD (P=0.05) = 0.01.

In addition, it is shown in Figure 4.8 that the amount of sulphite produced by the three bacteria was not different.

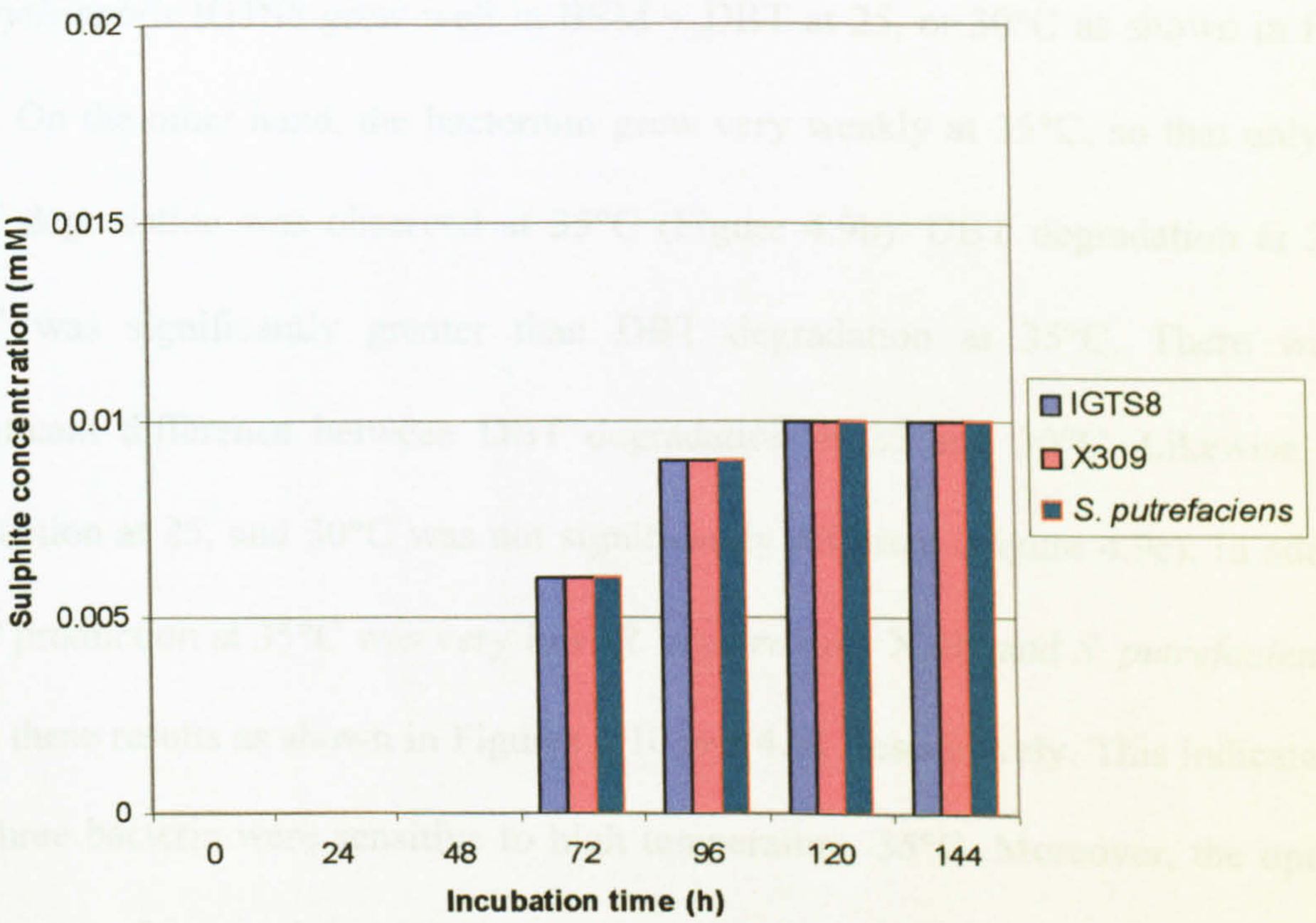
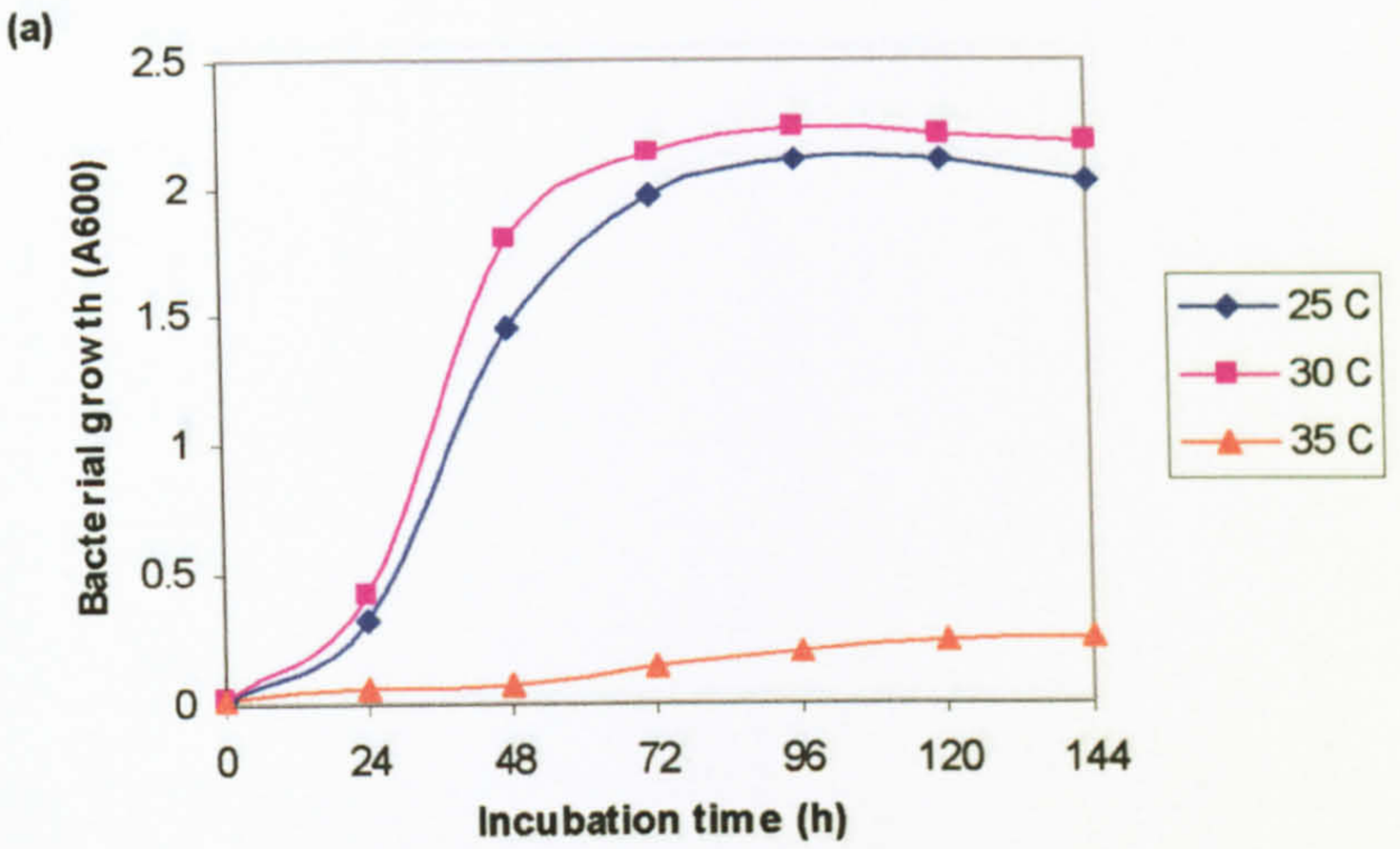


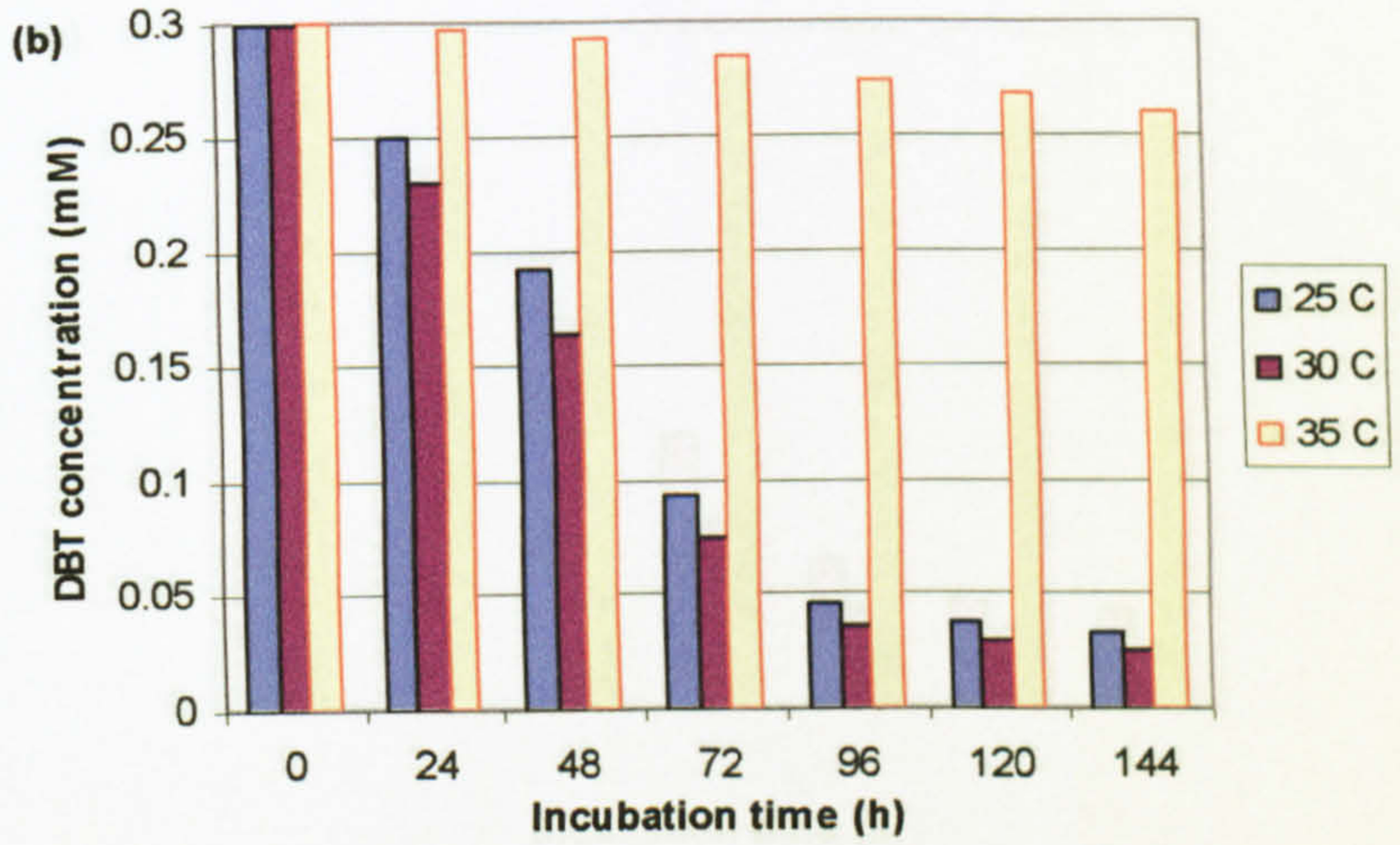
Figure 4.8 Production of sulphite from DBT degradation by the three bacteria. Each culture grown in BSM with DMSO as a sole source of sulphur until the mid-log phase, washed twice, and resuspended in Ringer's solution to A_{600} = 1.0. One ml of the cell suspension was inoculated into 100 ml of BSM containing 0.3 mM of DBT-ethanol and incubated at 30°C, 100 rpm. Samples taken daily were analysed for sulphite using spectrophotometric assay. Each point is a mean of five replicates.

The effect of different parameters on bacterial growth and DBT desulphurisation was examined. The results obtained are presented as follows.

A. Effect of temperature

R. erythropolis IGTS8 grew well in BSM + DBT at 25, or 30°C as shown in Figure 4.9a. On the other hand, the bacterium grew very weakly at 35°C, so that only little DBT degradation was observed at 35°C (Figure 4.9b). DBT degradation at 25, or 30°C was significantly greater than DBT degradation at 35°C. There was no significant difference between DBT degradation at 25 and 30°C. Likewise, HBP production at 25, and 30°C was not significantly different (Figure 4.9c). In addition, HBP production at 35°C was very low. *R. erythropolis* X309 and *S. putrefaciens* also gave these results as shown in Figures 4.10 and 4.11, respectively. This indicated that the three bacteria were sensitive to high temperature, 35°C. Moreover, the optimum temperature for growth and DBT desulphurisation seems to be 30°C.





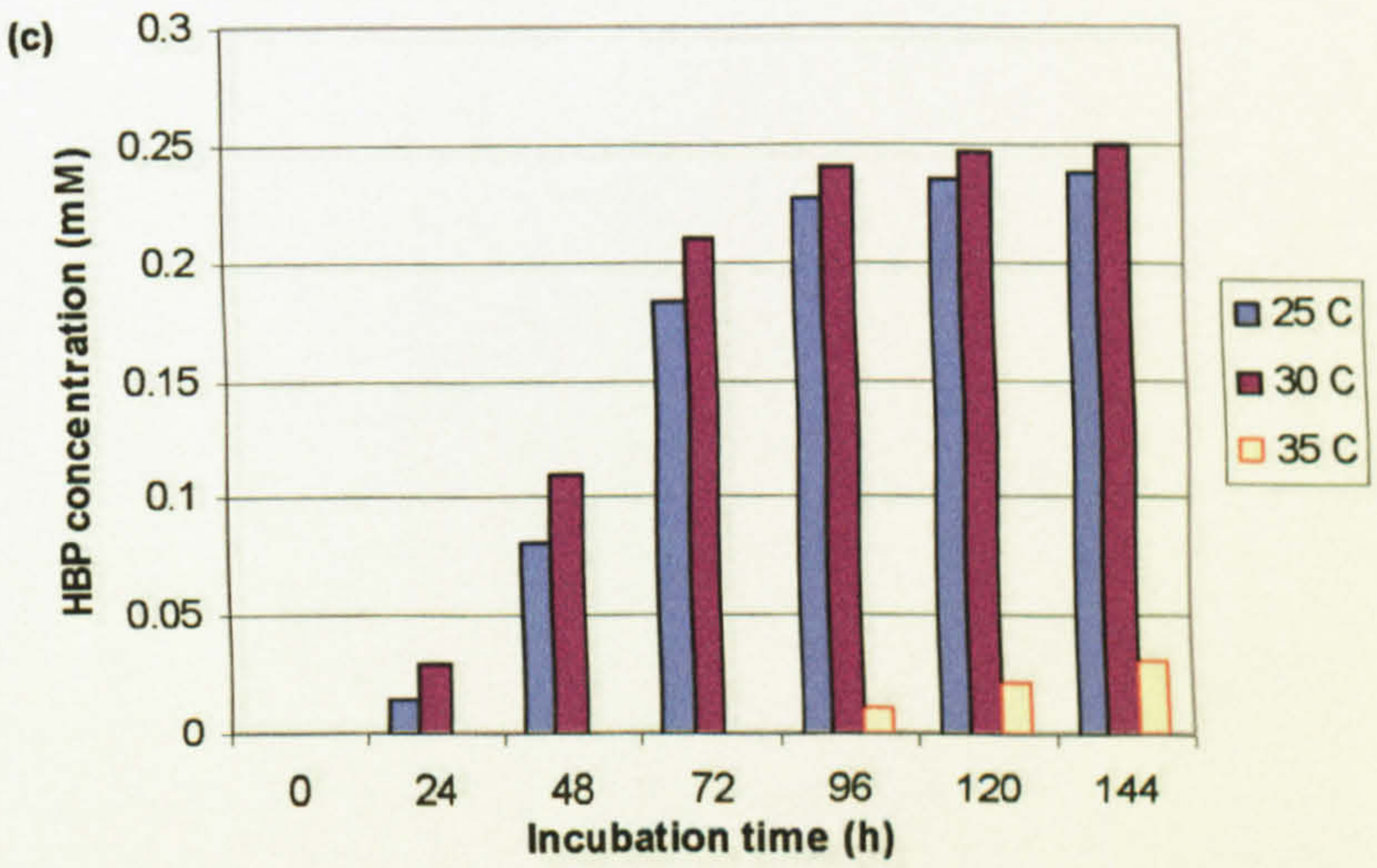


Figure 4.9 Effect of temperature on (a) growth on DBT, (b) DBT degradation, and (c) HBP production by *R. erythropolis* IGTS8. Each point is a mean of three replicates, LSD (P=0.05) of DBT degradation = 0.18, and LSD (P=0.05) of HBP production = 0.17.

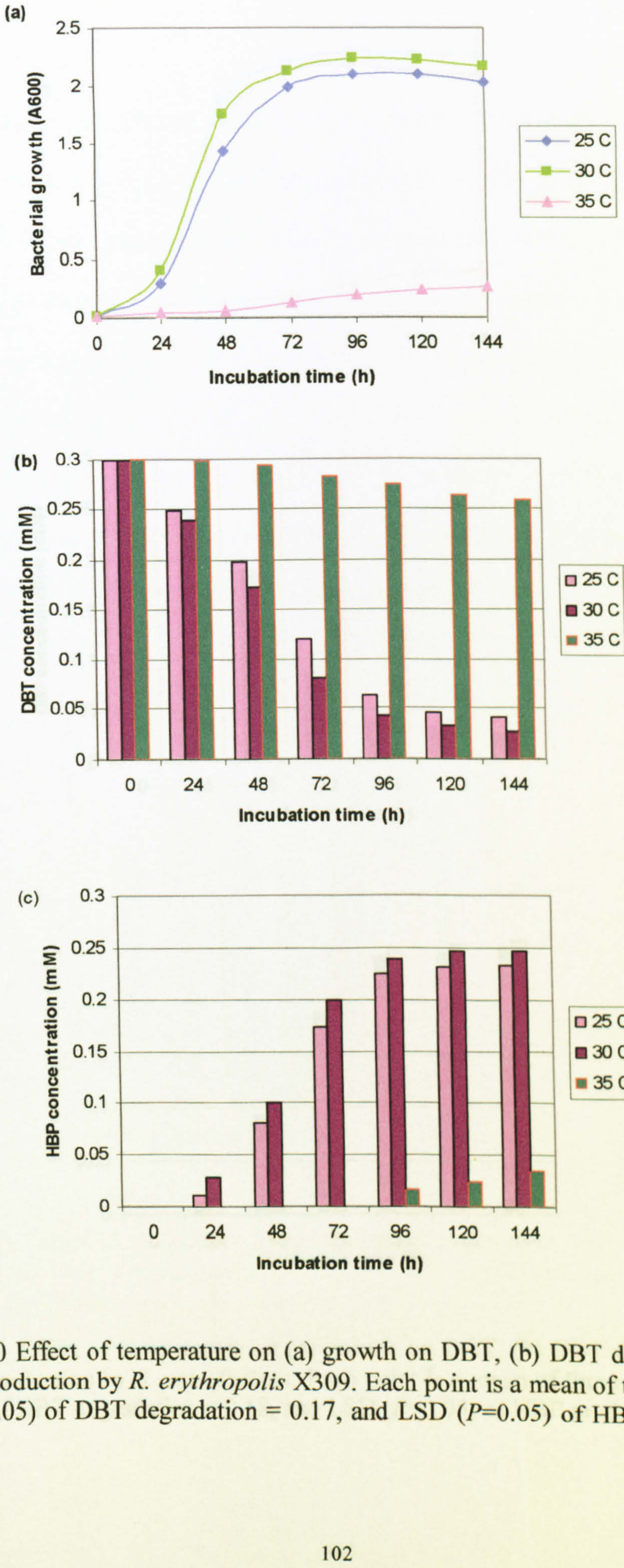


Figure 4.10 Effect of temperature on (a) growth on DBT, (b) DBT degradation, and (c) HBP production by *R. erythropolis* X309. Each point is a mean of three replicates, LSD (P=0.05) of DBT degradation = 0.17, and LSD (P=0.05) of HBP production = 0.16.

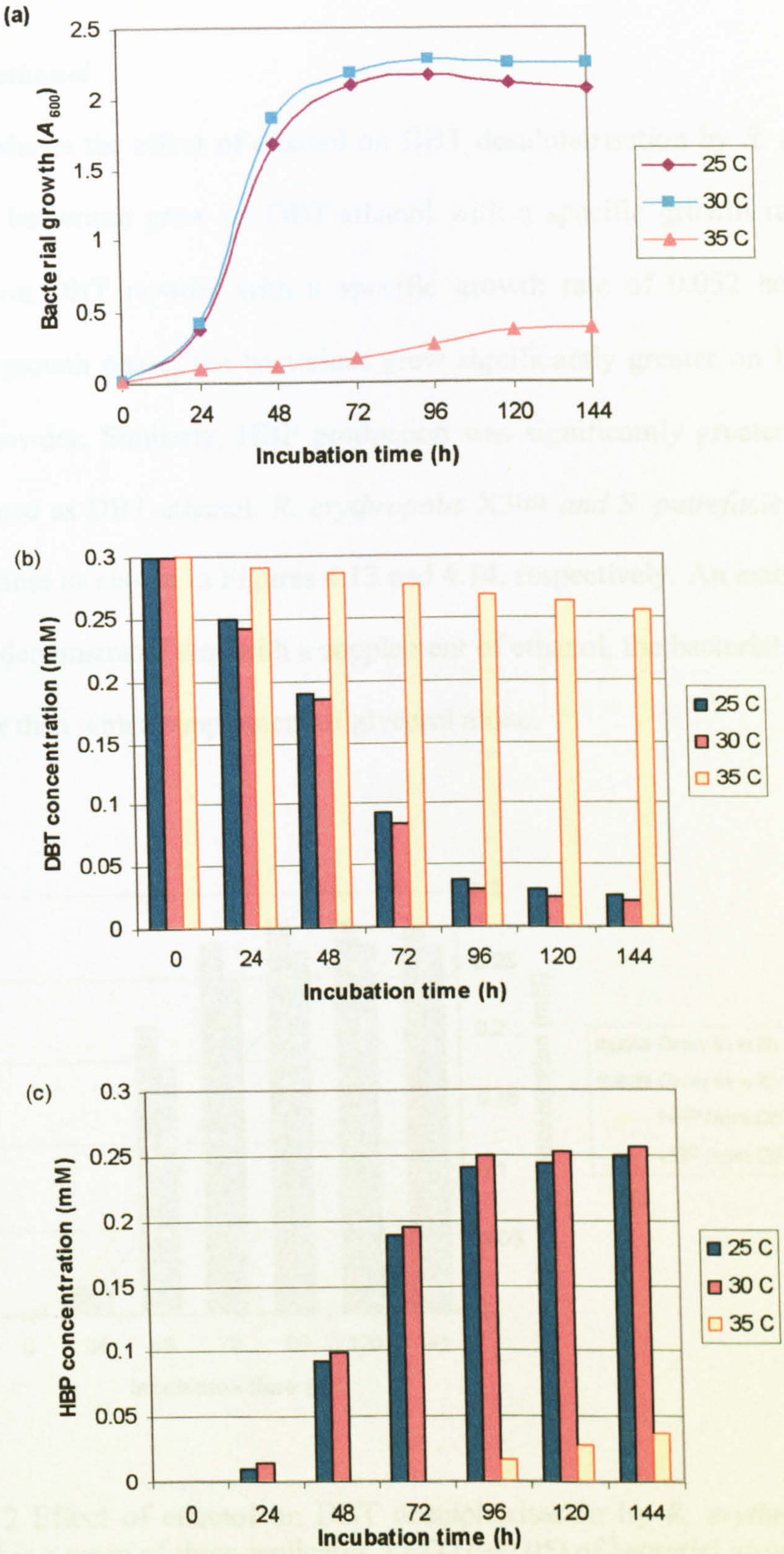


Figure 4.11 Effect of temperature on (a) growth on DBT, (b) DBT degradation, and (c) HBP production by *S. putrefaciens*. Each point is a mean of three replicates, LSD (P=0.05) of DBT degradation = 0.18, and LSD (P=0.05) of HBP production = 0.17.

B. Effect of ethanol

Figure 4.12 shows the effect of ethanol on DBT desulphurisation by *R. erythropolis* IGTS8. The bacterium grew on DBT-ethanol with a specific growth rate of 0.053 $hour^{-1}$, and on DBT powder with a specific growth rate of 0.052 $hour^{-1}$. In the exponential growth phase, the bacterium grew significantly greater on DBT-ethanol than DBT powder. Similarly, HBP production was significantly greater when DBT was introduced as DBT-ethanol. *R. erythropolis* X309 *and S. putrefaciens* also gave similar response as shown in Figures 4.13 and 4.14, respectively. An extension of this experiment demonstrated that with a supplement of ethanol, the bacterial growth rates were greater than with a supplement of glycerol alone.

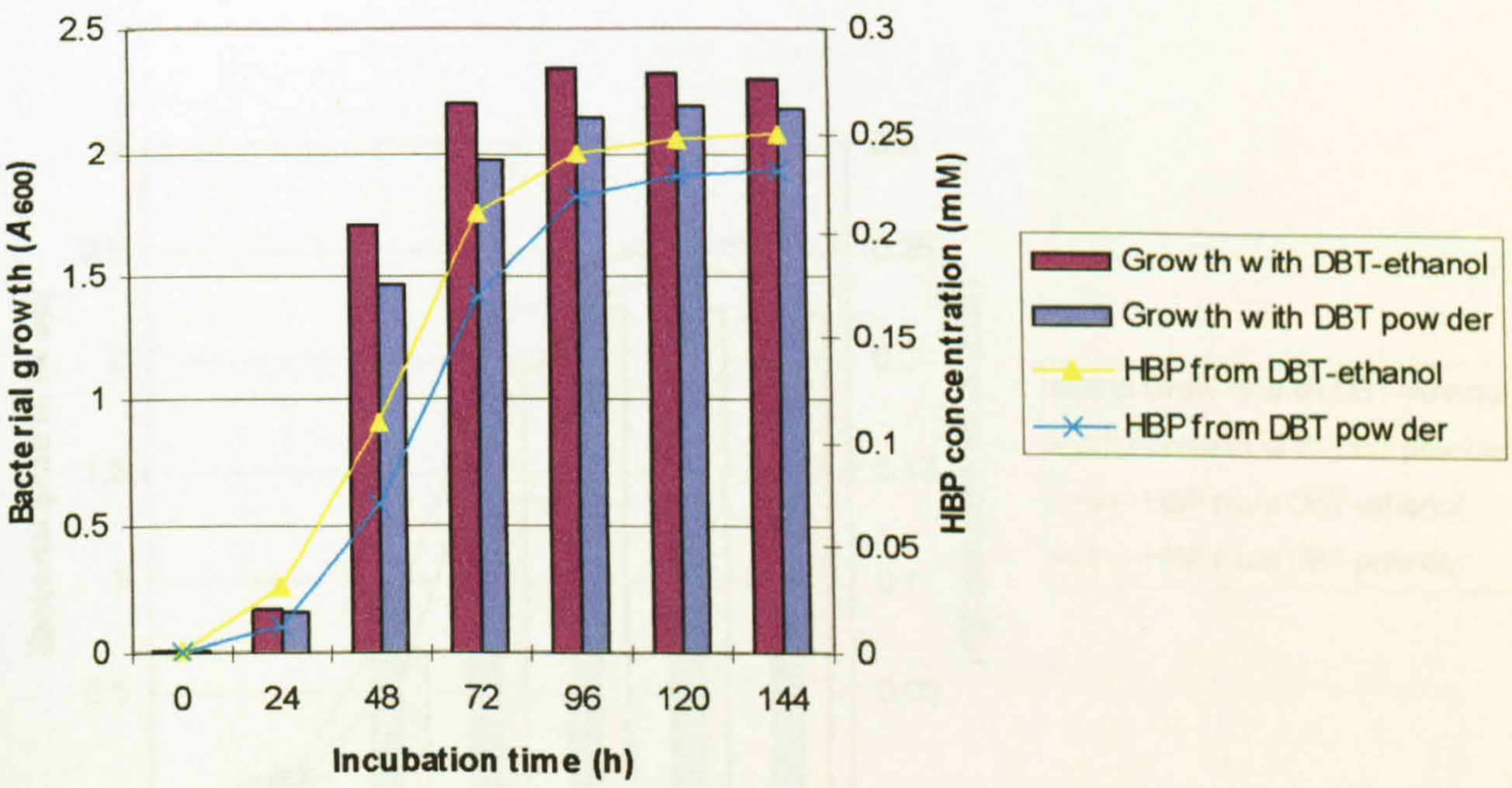


Figure 4.12 Effect of ethanol on DBT desulphurisation by *R. erythropolis* IGTS8. Each point is a mean of three replicates, LSD (P=0.05) of bacterial growth = 0.14, and LSD (P=0.05) of HBP production = 0.02.

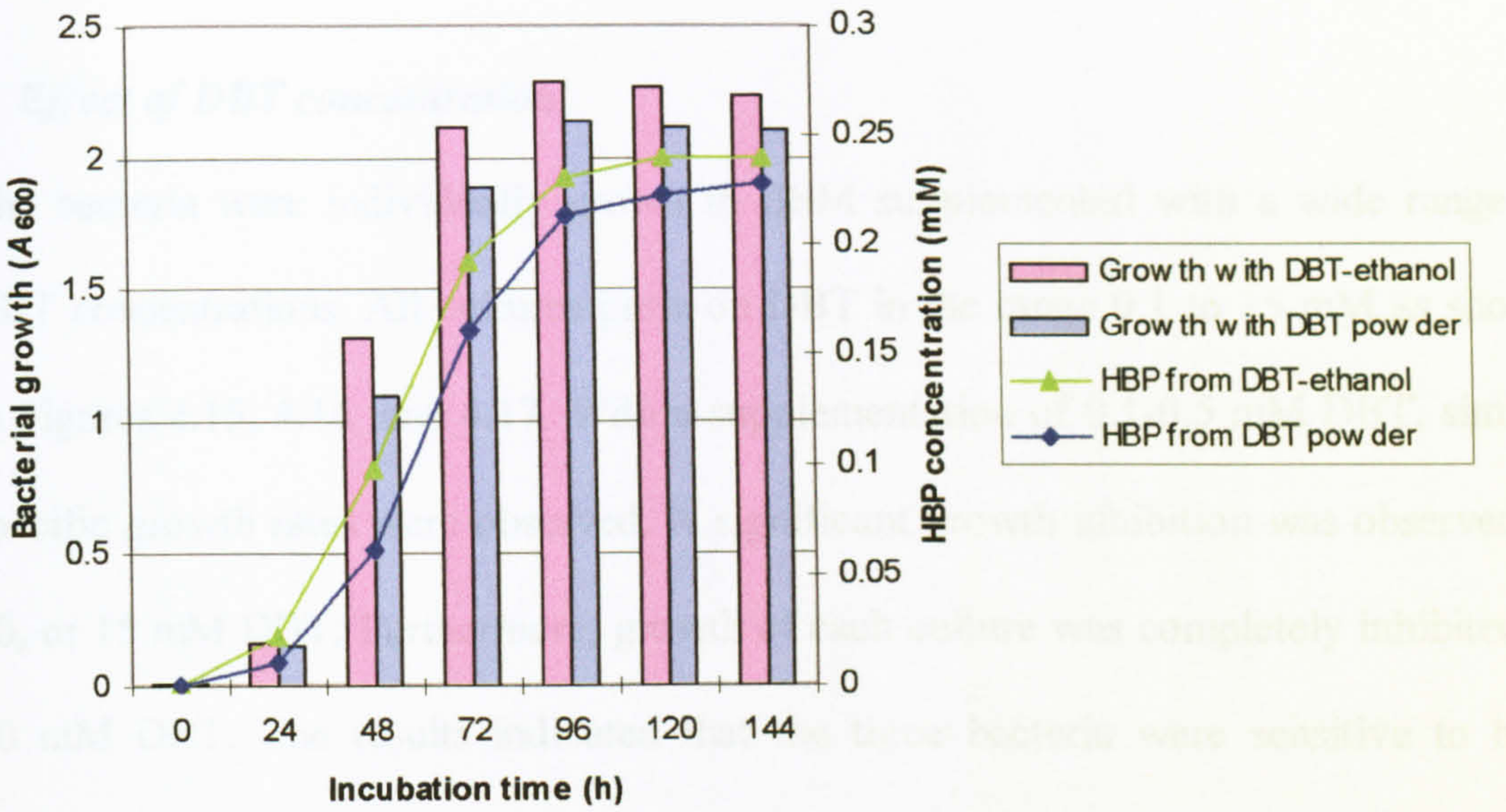


Figure 4.13 Effect of ethanol on DBT desulphurisation by *R. erythropolis* X309. Each point is a mean of three replicates, LSD (P=0.05) of bacterial growth = 0.14, and LSD (P=0.05) of HBP production = 0.02.

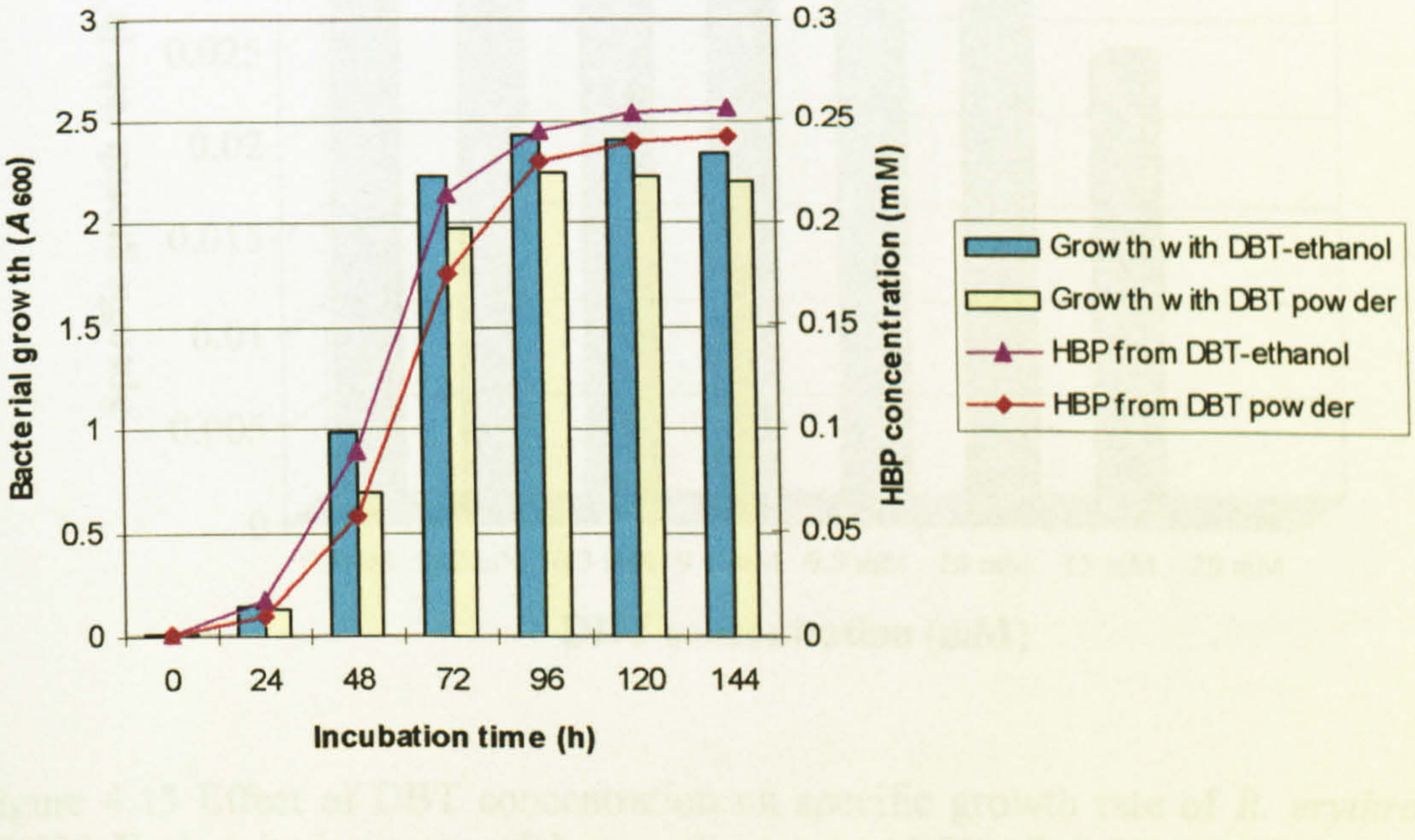


Figure 4.14 Effect of ethanol on DBT desulphurisation by *S. putrefaciens*. Each point is a mean of three replicates, LSD (P=0.05) of bacterial growth = 0.14, and LSD (P=0.05) of HBP production = 0.02.

C. Effect of DBT concentration

The bacteria were individually grown in BSM supplemented with a wide range of DBT concentrations. All cultures grew on DBT in the range 0.1 to 15 mM as shown in Figures 4.15, 4.16, and 4.17. With a supplementation of 0.1-0.5 mM DBT, similar specific growth rates were observed. A significant growth inhibition was observed at 10, or 15 mM DBT. Furthermore, growth of each culture was completely inhibited at 20 mM DBT. The results indicated that the three bacteria were sensitive to high concentrations of DBT.

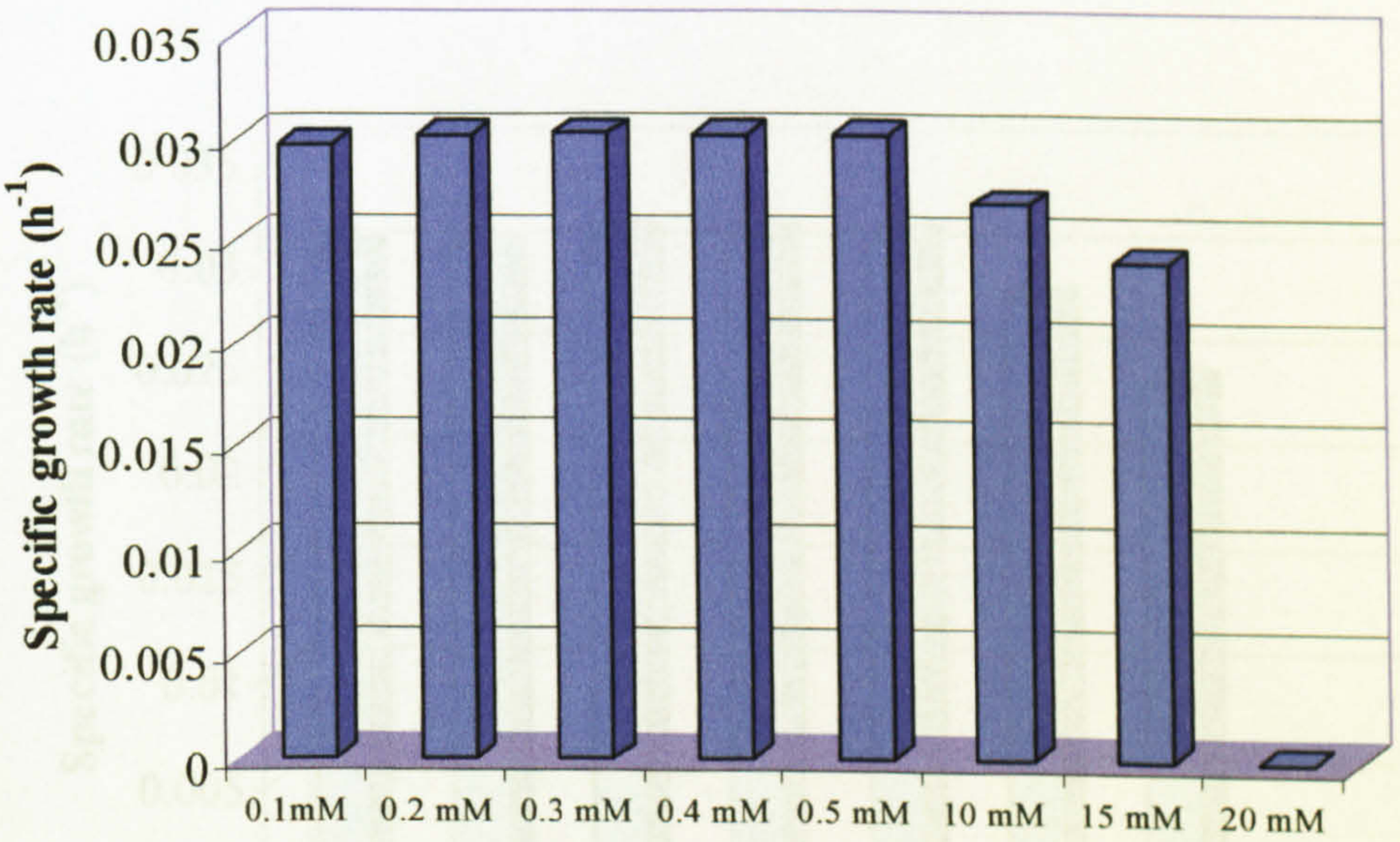


Figure 4.15 Effect of DBT concentration on specific growth rate of *R. erythropolis* IGTS8. Each point is a mean of three replicates, and LSD (P=0.05) = 0.003.

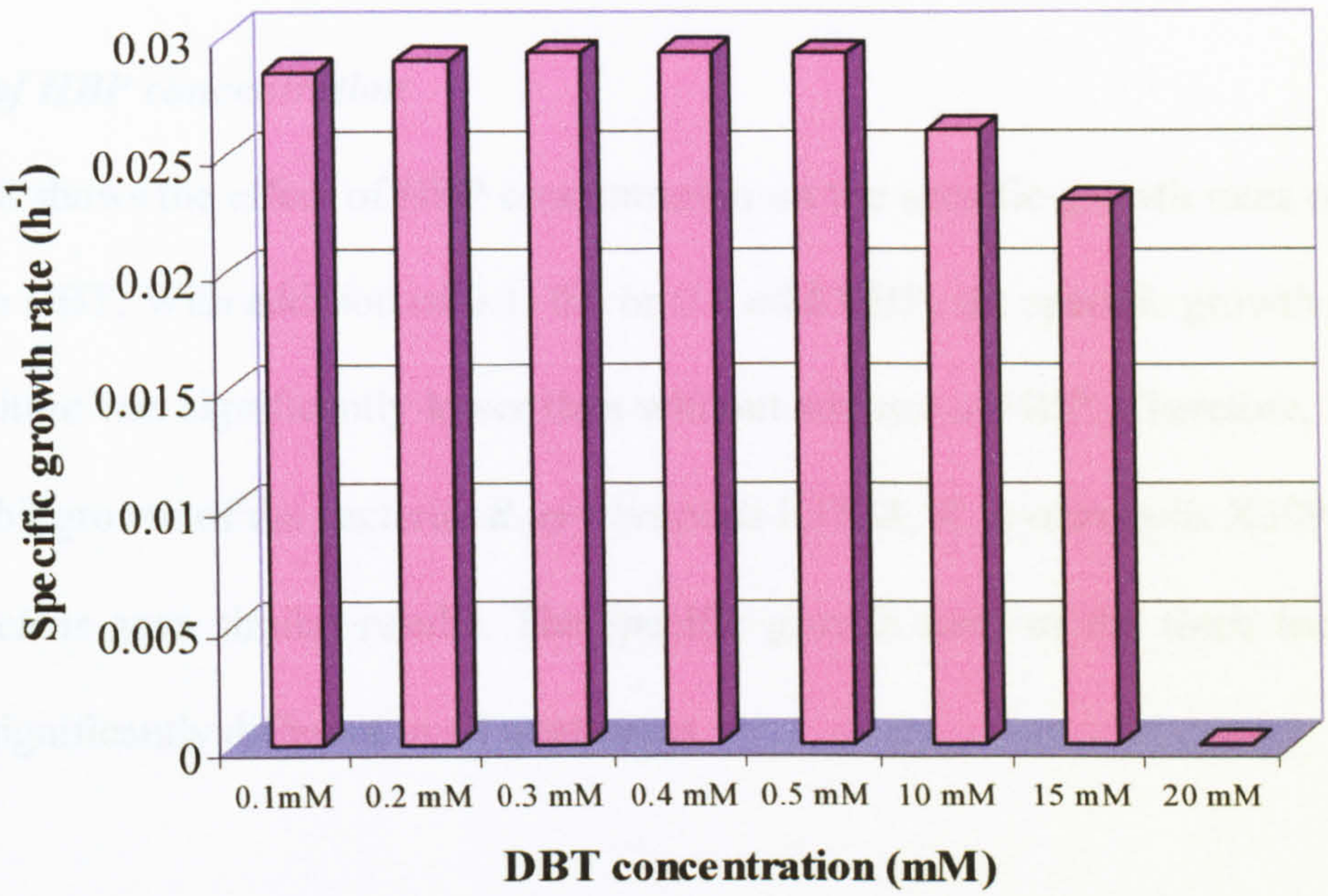


Figure 4.16 Effect of DBT concentration on specific growth rate of *R. erythropolis* X309. Each point is a mean of three replicates, and LSD (P=0.05) = 0.003.

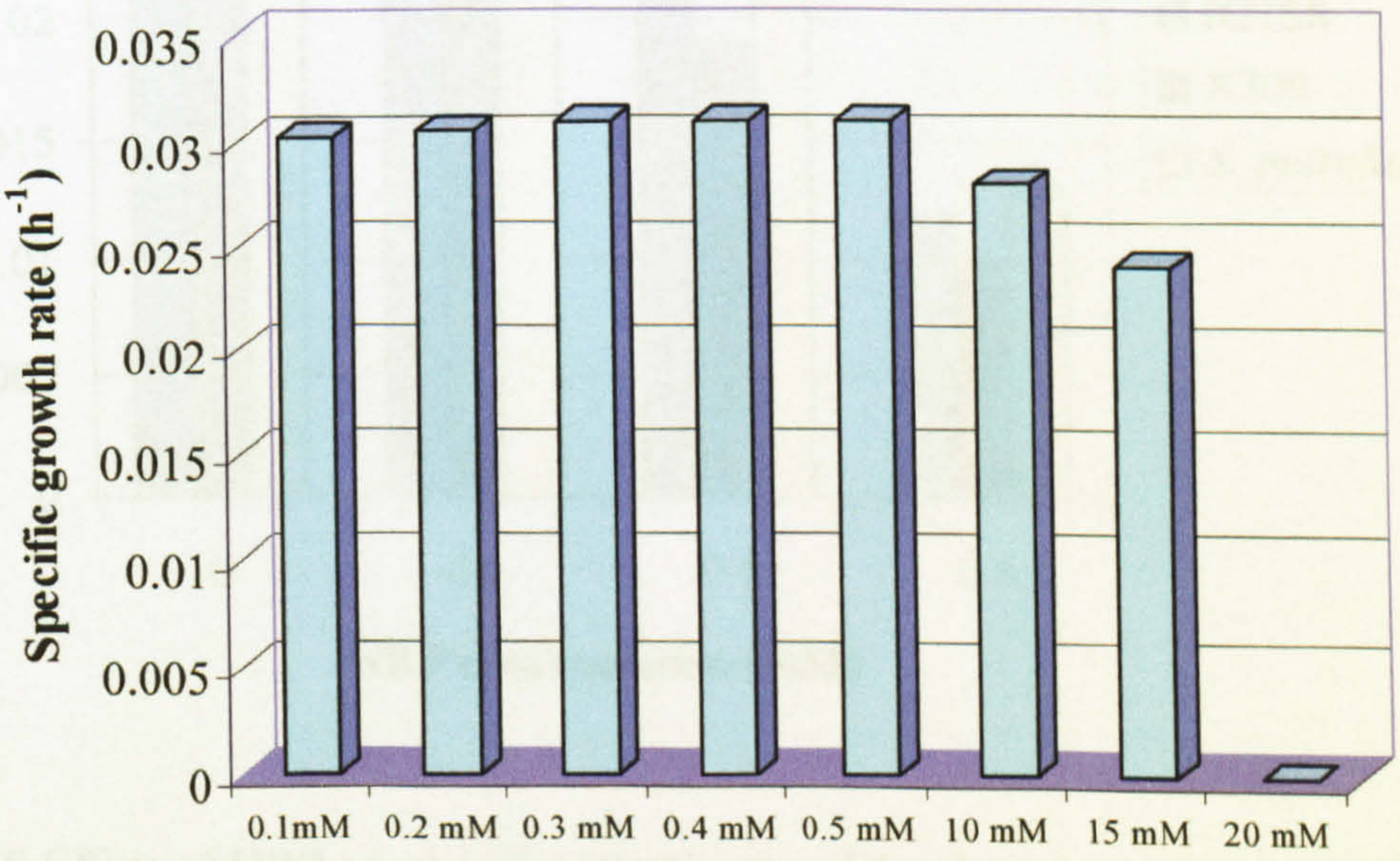


Figure 4.17 Effect of DBT concentration on specific growth of *S. putrefaciens*. Each point is a mean of three replicates, and LSD (P=0.05) = 0.003.

D. Effect of HBP concentration

Figure 4.18 shows the effect of HBP concentration on the specific growth rates of the bacteria on DBT. With addition of 0.1, 0.2 or 0.3 mM HBP, the specific growth rates of each culture was significantly lower than without additon of HBP. Therefore, HBP could inhibit growth of the bacteria. *R. erythropolis* IGTS8, *R. erythropolis* X309, and *S. putrefaciens* gave similar results. The specific growth rates of the three bacteria were not significantly different in all treatments.

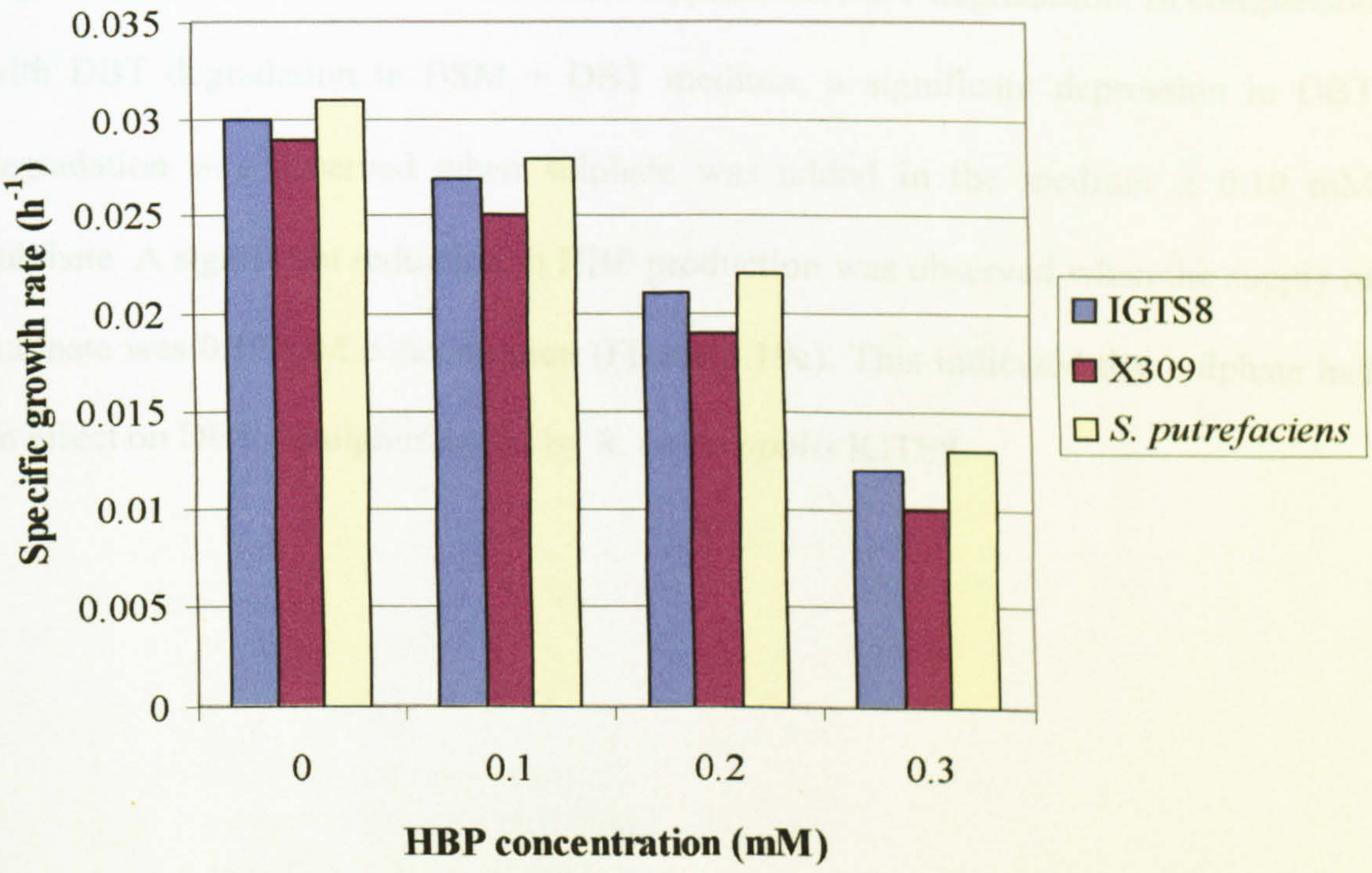


Figure 4.18 Effect of HBP on specific growth rate of the three bacteria on DBT. Cell grown in BSM with DMSO as a sole source of sulphur until the mid-log phase, washed twice, and resuspended in Ringer's solution to A_{600} = 1.0. One ml of the cell suspension was inoculated into 100 ml of BSM containing 0.3 mM of DBT-ethanol with or without HBP and incubated at 100 rpm, 30°C. Samples taken daily were monitored for bacterial growth at A_{600}. Each point is a mean of three replicates, LSD (P=0.05) of strain type = 0.012, and LSD (P=0.05) of HBP concentration = 0.003.

E. Effect of sulphate

With a supply of sodium sulphate in BSM, *R. erythropolis* IGTS8 grew well with a specific growth rate of 0.45 $hour^{-1}$. In BSM + DBT only, a significantly lower specific growth rate (μ = 0.30 $hour^{-1}$) was observed as shown in Figure 4.19a. Moreover, the specific growth rate increased with addition of sodium sulphate in BSM + DBT. From the ANOVA test, the specific growth rate in BSM + DBT was significantly lower than the specific growth rates in BSM + DBT + sulphate ($\geq$ 0.15 mM sulphate).

Figure 4.19b shows the effect of sodium sulphate on DBT degradation. In comparison with DBT degradation in BSM + DBT medium, a significant depression in DBT degradation was observed when sulphate was added in the medium $\geq$ 0.10 mM sulphate. A significant reduction in HBP production was observed when the supply of sulphate was 0.10 mM concentration (Figure 4.19c). This indicated that sulphate had an effect on DBT desulphurisation by *R. erythropolis* IGTS8.

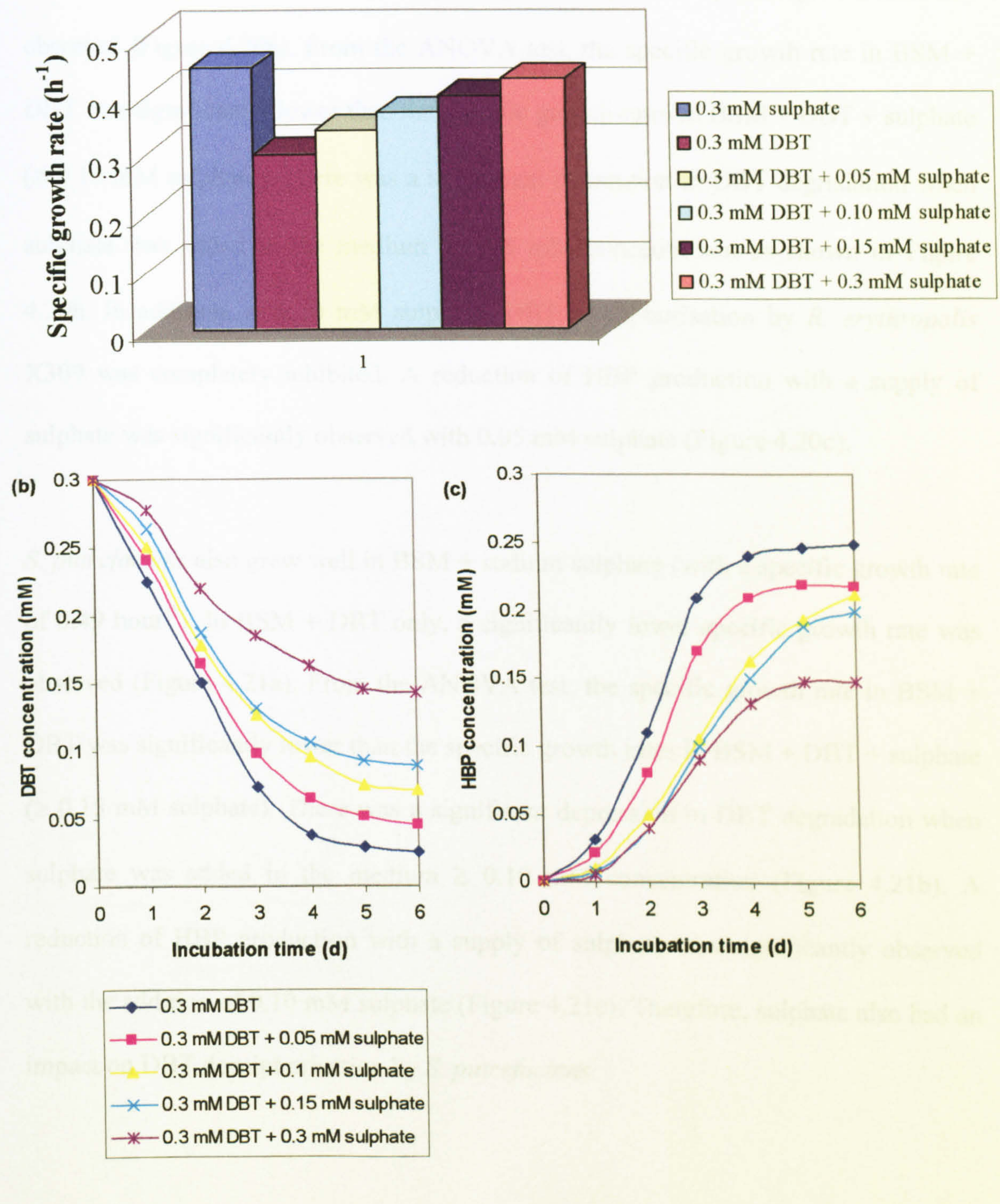
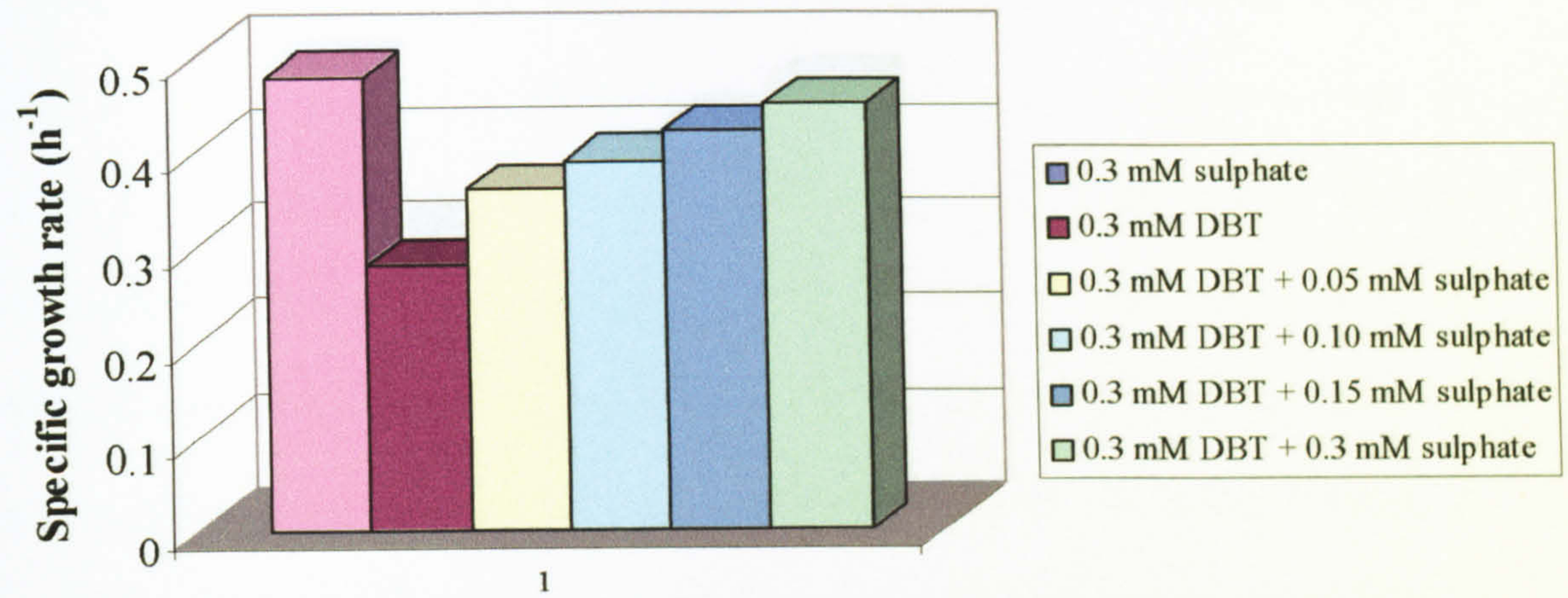


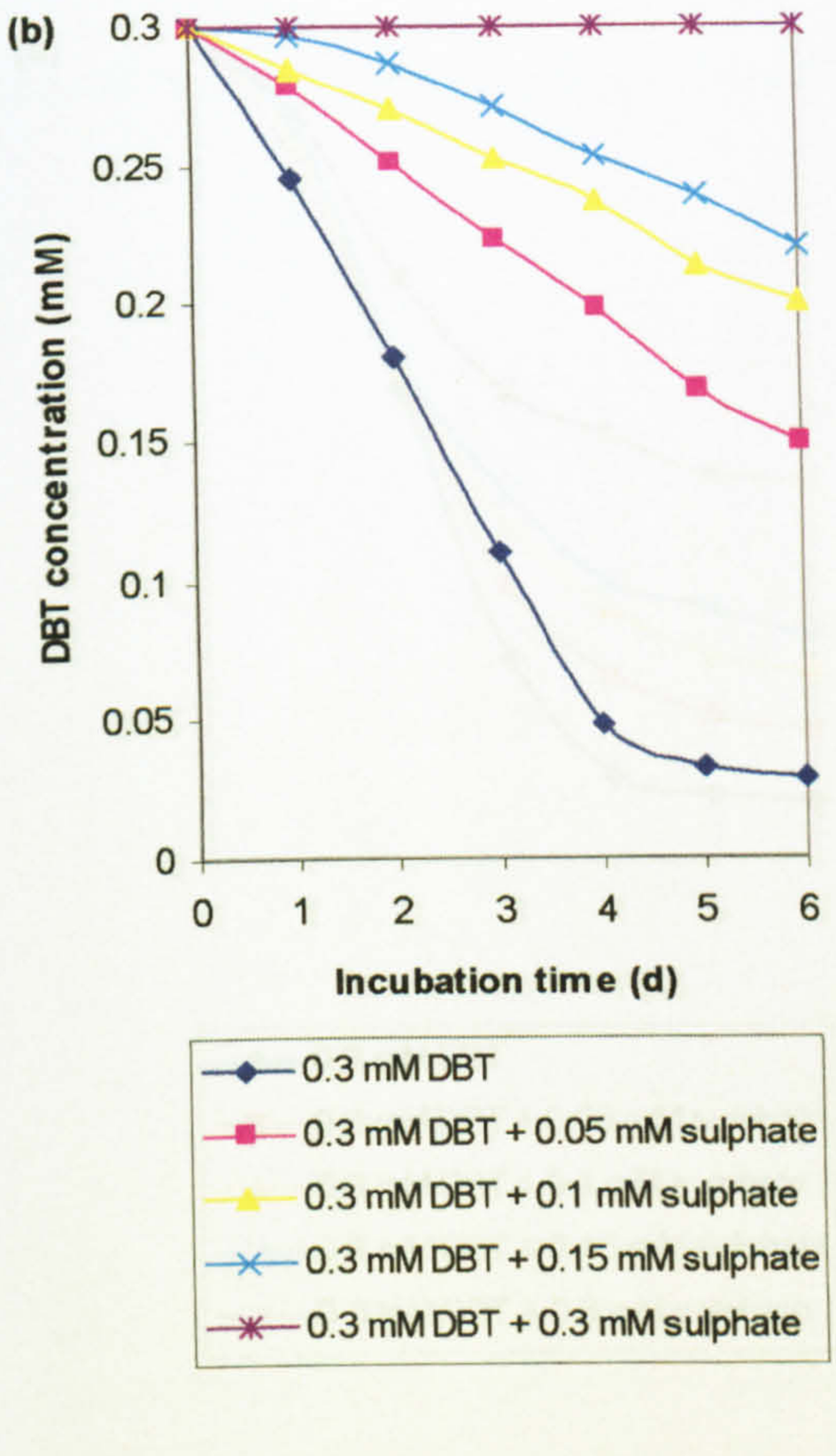
Figure 4.19 Effect of sulphate on (a) specific growth rate on DBT, (b) DBT degradation and (c) HBP production by *R. erythropolis* IGTS8. Each point is a mean of three replicates, LSD (P=0.05) of specific growth rate = 0.07, LSD (P=0.05) of DBT degradation = 0.047, and LSD (P=0.05) of HBP production = 0.043.

R. erythropolis X309 grew with a specific growth rate of 0.48 $hour^{-1}$ in BSM + sodium sulphate. In BSM + DBT only, a significantly lower specific growth rate was observed (Figure 4.20a). From the ANOVA test, the specific growth rate in BSM + DBT was significantly lower than the specific growth rates in BSM + DBT + sulphate (≥ 0.10 mM sulphate). There was a significant depression in DBT degradation when sulphate was added in the medium ≥ 0.05 mM concentration as shown in Figure 4.20b. In addition, at 0.30 mM sulphate, DBT desulphurisation by *R. erythropolis* X309 was completely inhibited. A reduction of HBP production with a supply of sulphate was significantly observed with 0.05 mM sulphate (Figure 4.20c).

S. putrefaciens also grew well in BSM + sodium sulphate (with a specific growth rate of 0.49 $hour^{-1}$). In BSM + DBT only, a significantly lower specific growth rate was observed (Figure 4.21a). From the ANOVA test, the specific growth rate in BSM + DBT was significantly lower than the specific growth rates in BSM + DBT + sulphate (≥ 0.15 mM sulphate). There was a significant depression in DBT degradation when sulphate was added in the medium ≥ 0.10 mM concentration (Figure 4.21b). A reduction of HBP production with a supply of sulphate was significantly observed with the addition of 0.10 mM sulphate (Figure 4.21c). Therefore, sulphate also had an impact on DBT desulphurisation by *S. putrefaciens*.

(b)





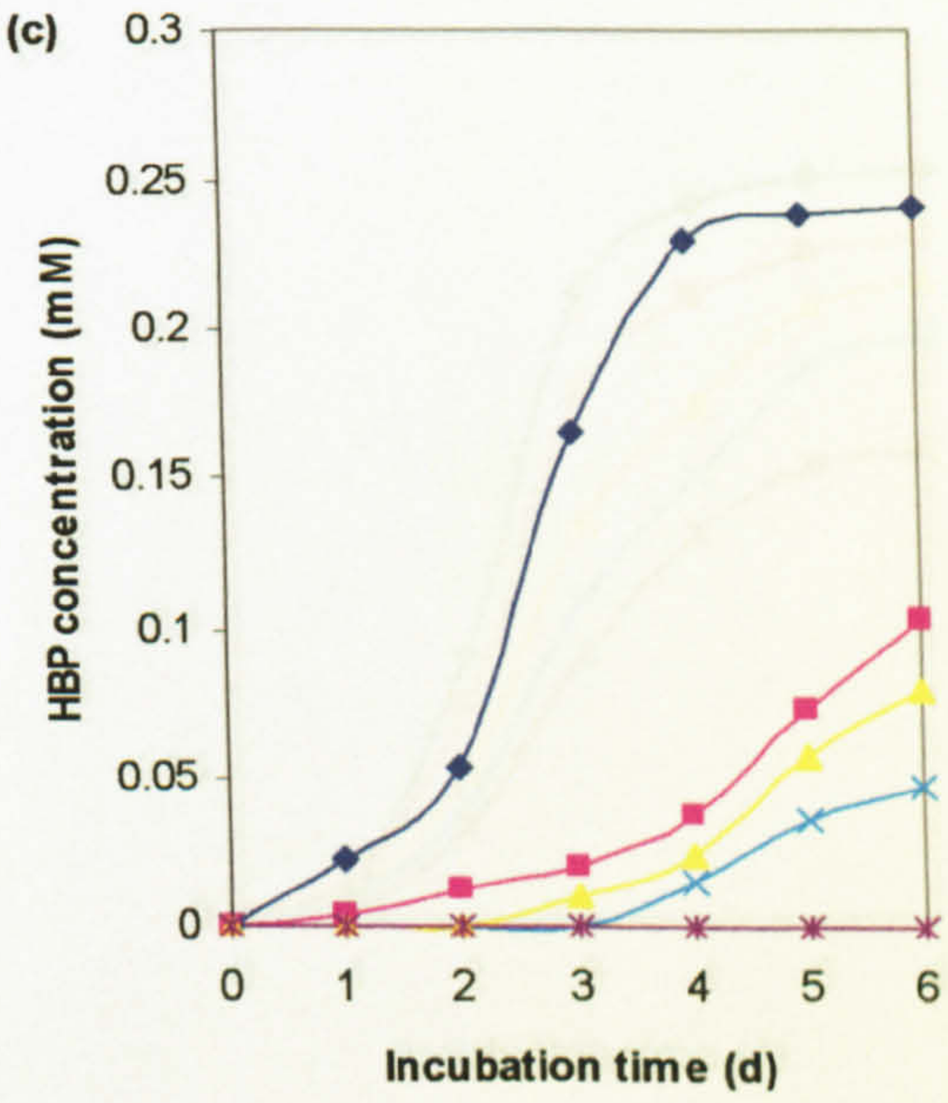


Figure 4.20 Effect of sulphate on (a) specific growth rate on DBT, (b) DBT degradation, and (c) HBP production by *R. erythropolis* X309. Each point is a mean of three replicates, LSD (P=0.05) of specific growth rate = 0.09, LSD (P=0.05) of DBT degradation = 0.095, and LSD (P=0.05) of HBP production = 0.087.

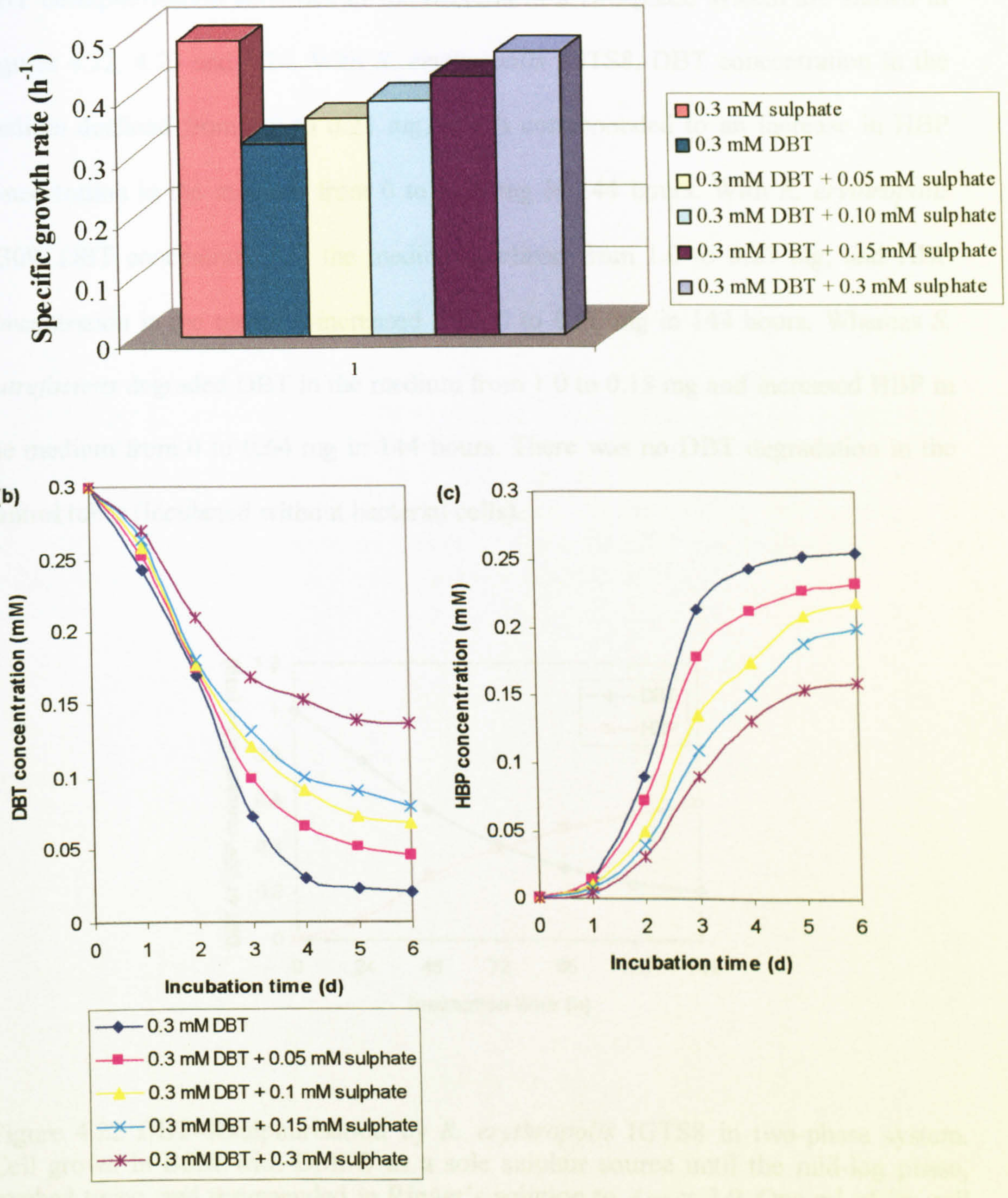


Figure 4.21 Effect of sulphate on (a) specific growth rate on DBT, (b) DBT degradation, and (c) HBP production by *S. putrefaciens*. Each point is a mean of three replicates, LSD (P=0.05) of specific growth rate = 0.08, LSD (P=0.05) of DBT degradation = 0.042, and LSD (P=0.05) of HBP production = 0.042.

4.2.2 DBT desulphurisation in two-phase system

DBT desulphurisation activities of the bacteria in a two-phase system are shown in Figures 4.22, 4.23 and 4.24. With *R. erythropolis* IGTS8, DBT concentration in the medium declined from 1.0 to 0.21 mg, which corresponded to an increase in HBP concentration in the medium from 0 to 0.59 mg in 144 hours. With *R. erythropolis* X309, DBT concentration in the medium declined from 1.0 to 0.23 mg, and HBP concentration in the medium increased from 0 to 0.56 mg in 144 hours. Whereas *S. putrefaciens* degraded DBT in the medium from 1.0 to 0.18 mg and increased HBP in the medium from 0 to 0.64 mg in 144 hours. There was no DBT degradation in the control tubes (incubated without bacterial cells).

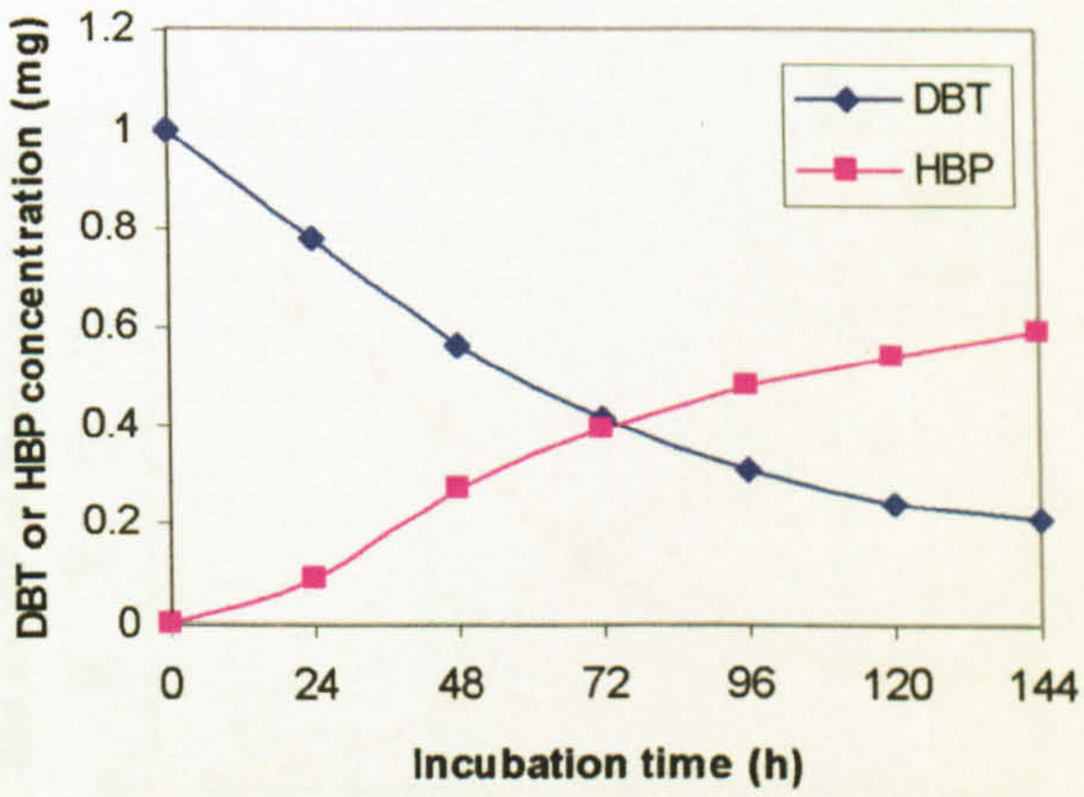


Figure 4.22 DBT-desulphurisation by *R. erythropolis* IGTS8 in two-phase system. Cell grown in BSM with DMSO as a sole sulphur source until the mid-log phase, washed twice, and resuspended in Ringer's solution to A_{600} = 3.0. One ml of the cell suspension was added to 0.325 ml of hexadecane + 1 mg of DBT and incubated at 30°C, 100 rpm. Samples taken daily were analysed for DBT and HBP using HPLC. Each point is a mean of five replicates, and CV is less than 5%.

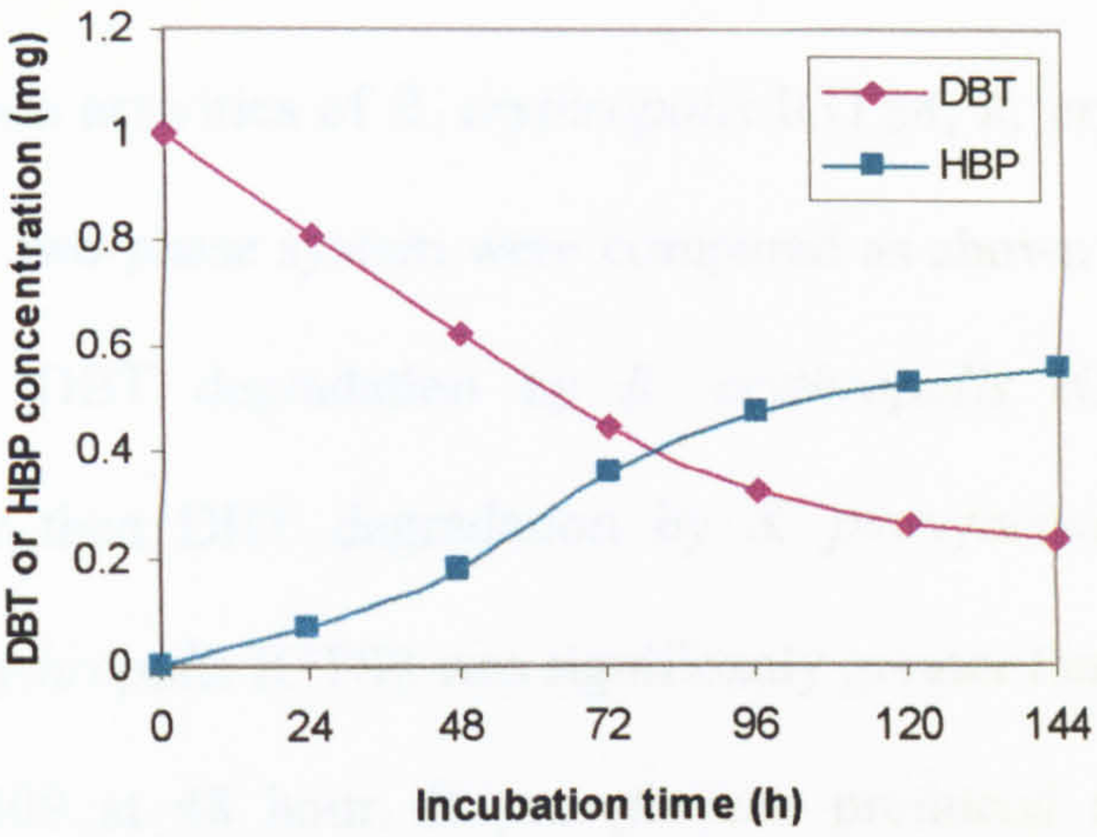


Figure 4.23 DBT-desulphurisation by *R. erythropolis* X309 in two-phase system. Cell grown in BSM with DMSO as a sole sulphur source until the mid-log phase, washed twice, and resuspended in Ringer's solution to A_{600} = 3.0. One ml of the cell suspension was added to 0.325 ml of hexadecane + 1 mg of DBT and incubated at 30°C, 100 rpm. Samples taken daily were analysed for DBT and HBP using HPLC. Each point is a mean of five replicates, and CV is less than 5%.

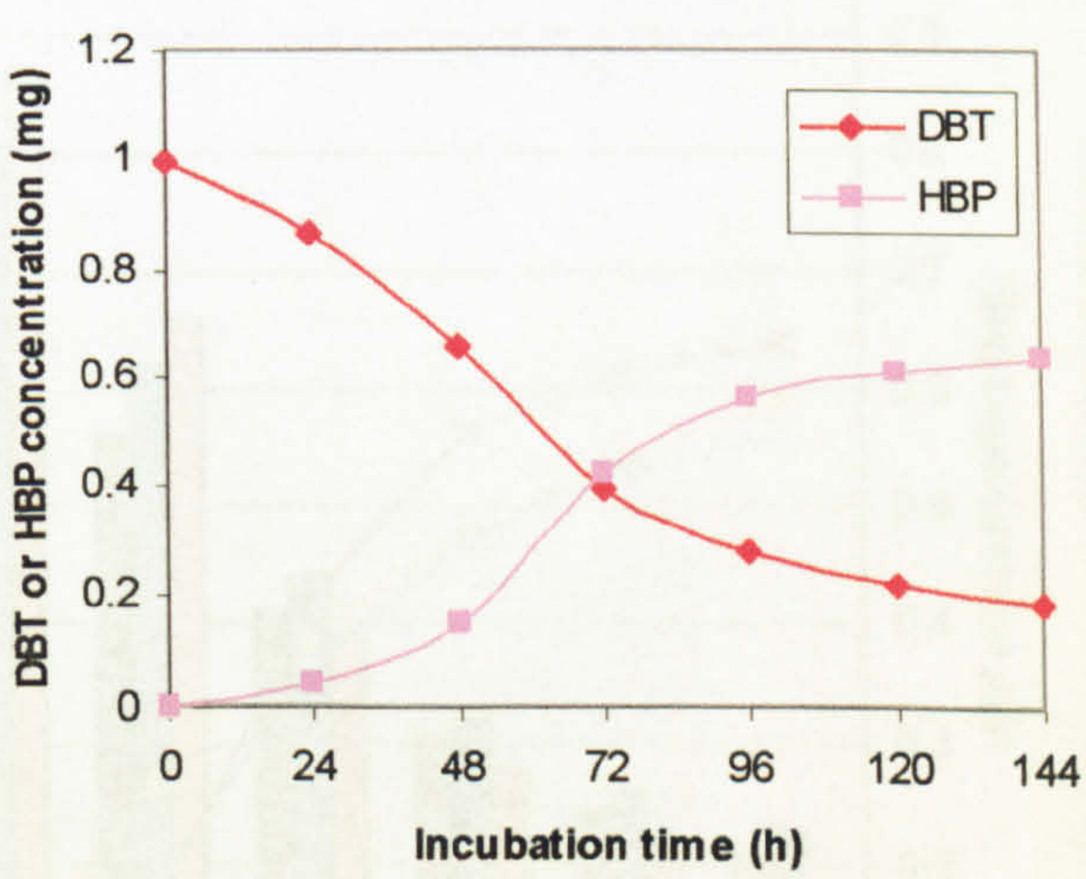


Figure 4.24 DBT-desulphurisation by *S. putrefaciens* in two-phase system. Cell grown in BSM with DMSO as a sole sulphur source until the mid-log phase, washed twice, and resuspended in Ringer's solution to A_{600} = 3.0. One ml of the cell suspension was added to 0.325 ml of hexadecane + 1 mg of DBT and incubated at 30°C, 100 rpm. Samples taken daily were analysed for DBT and HBP using HPLC. Each point is a mean of five replicates, and CV is less than 5%.

DBT desulphurisation activities of *R. erythropolis* IGTS8, *R. erythropolis* X309, and *S. putrefaciens* in a two-phase system were compared as shown in Figure 4.25. From the ANOVA test, DBT degradation by *R. erythropolis* IGTS8 or X309 was significanly greater than DBT degradation by *S. putrefaciens* at 24 hour. HBP production by *R. erythropolis* IGTS8 was significanly greater than HBP production by *R. erythropolis* X309 at 48 hour. *S. putrefaciens* produced a significantly lower amount of HBP than *R. erythropolis* IGTS8 at 48 hour, but a significantly higher amount of HBP at 96 hour. In addition, HBP production by *R. erythropolis* X309 was significanly lower than HBP production by *S. putrefaciens* from 96-144 hours.

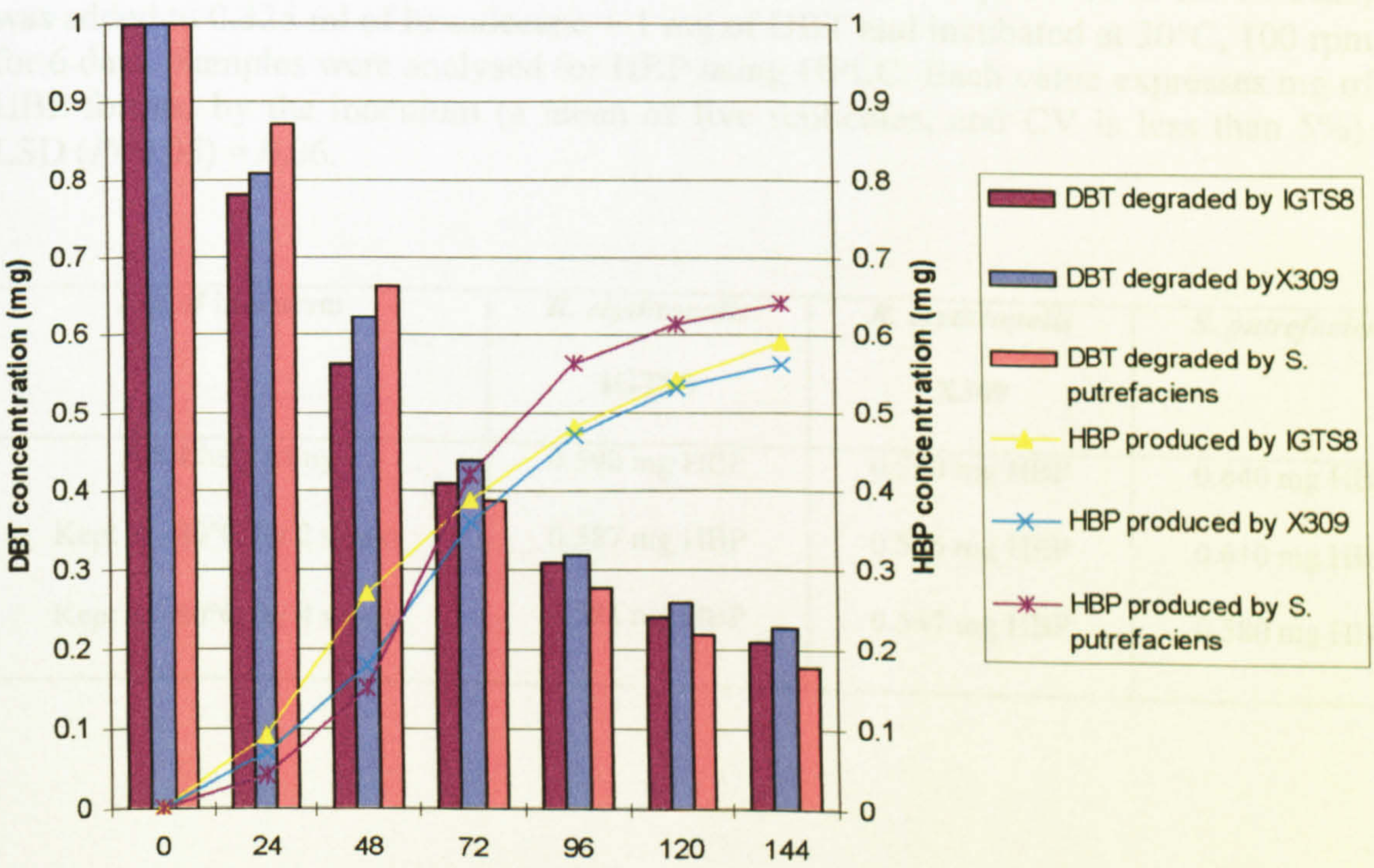


Figure 4.25 DBT-desulphurisation activities of the bacteria in two-phase system. Each point is a mean of five replicates, LSD (P=0.05) of DBT degradation = 0.054, and LSD (P=0.05) of HBP production = 0.071.

Results from investigation of the effect of age of the inoculum on DBT desulphurisation activity of each culture are shown in Table 4.2. Desulphurisation activity is expressed as mg of HBP formed by the inoculum incubated with 1 mg of DBT for 6 days, as determined by HPLC. From the ANOVA test, the desulphurisation activity of *S. putrefaciens* was significantly lost when it was kept at –80°C for 4 weeks. There was no significant loss in activity of both strains *of R. erythropolis* at the time investigated.

Table 4.2 Effect of age of inoculum on desulphurisation activity. One ml of the cell suspension (after harvesting, kept at –80°C for 2 weeks, or kept at –80°C for 4 weeks) was added to 0.325 ml of hexadecane + 1 mg of DBT and incubated at 30°C, 100 rpm for 6 days. Samples were analysed for HBP using HPLC. Each value expresses mg of HBP formed by the inoculum (a mean of five replicates, and CV is less than 5%). LSD (P=0.05) = 0.06.

Age of inoculum	***R. erythropolis*** **IGTS8**	***R. erythropolis*** **X309**	***S. putrefaciens***
After harvesting	0.590 mg HBP	0.560 mg HBP	0.640 mg HBP
Kept at –80°C for 2 weeks	0.587 mg HBP	0.555 mg HBP	0.610 mg HBP
Kept at –80°C for 4 weeks	0.581 mg HBP	0.547 mg HBP	0.580 mg HBP

A comparision of DBT desulphurisation rates in a two-phase system and DBT desulphurisation rates in an aqueous system was made. DBT desulphurisation rates produced by each culture are expressed as μM of DBT depletion hour^{-1} by 1 unit of inoculum (A_{600}), and the results are shown in Table 4.3. This shows that the DBT desulphurisation rates in a two-phase system were significantly greater than the desulphurisation rates in an aqueous system for all bacterial treatments.

Table 4.3 DBT desulphurisation rates in aqueous system and two-phase system. For desulphurisation in aqueous system, 1 ml of the cell suspension (A_{600} = 1.0) was inoculated into 100 ml of BSM containing 0.3 mM of DBT-ethanol and incubated at 30°C, 100 rpm for 6 days. For desulphurisation in two-phase system, 1 ml of the cell suspension (A_{600} = 3.0) was added to 0.325 ml of hexadecane + 1 mg of DBT and incubated at 30°C, 100 rpm for 6 days. Each value is a mean of five replicates, and CV is less than 5%.

System	*R. erythropolis* IGTS8 (μM/h*1unit inoculum)	*R. erythropolis* X309 (μM/h*1unit inoculum)	*S. putrefaciens* (μM/h*1unit inoculum)
Aqueous	1.88	1.88	1.94
Two-phase	7.49	7.30	7.77

4.2.3 DBT-sulphone desulphurisation

To examine whether the DBT-sulphone was an intermediate in the DBT desulphurisation pathway, DBT-sulphone was supplied as a sole source of sulphur in BSM. *R. erythropolis* IGTS8 grew well reaching the stationary phase in 96 hours before the growth decelerated (Figure 4.26). The concentration of DBT-sulphone in the medium declined from 0.3 to 0.02 mM, which corresponded to an increase in HBP concentration in the medium from 0 to 0.25 mM in 144 hours. Sulphite release was observed in a small amount of 0.01 mM.

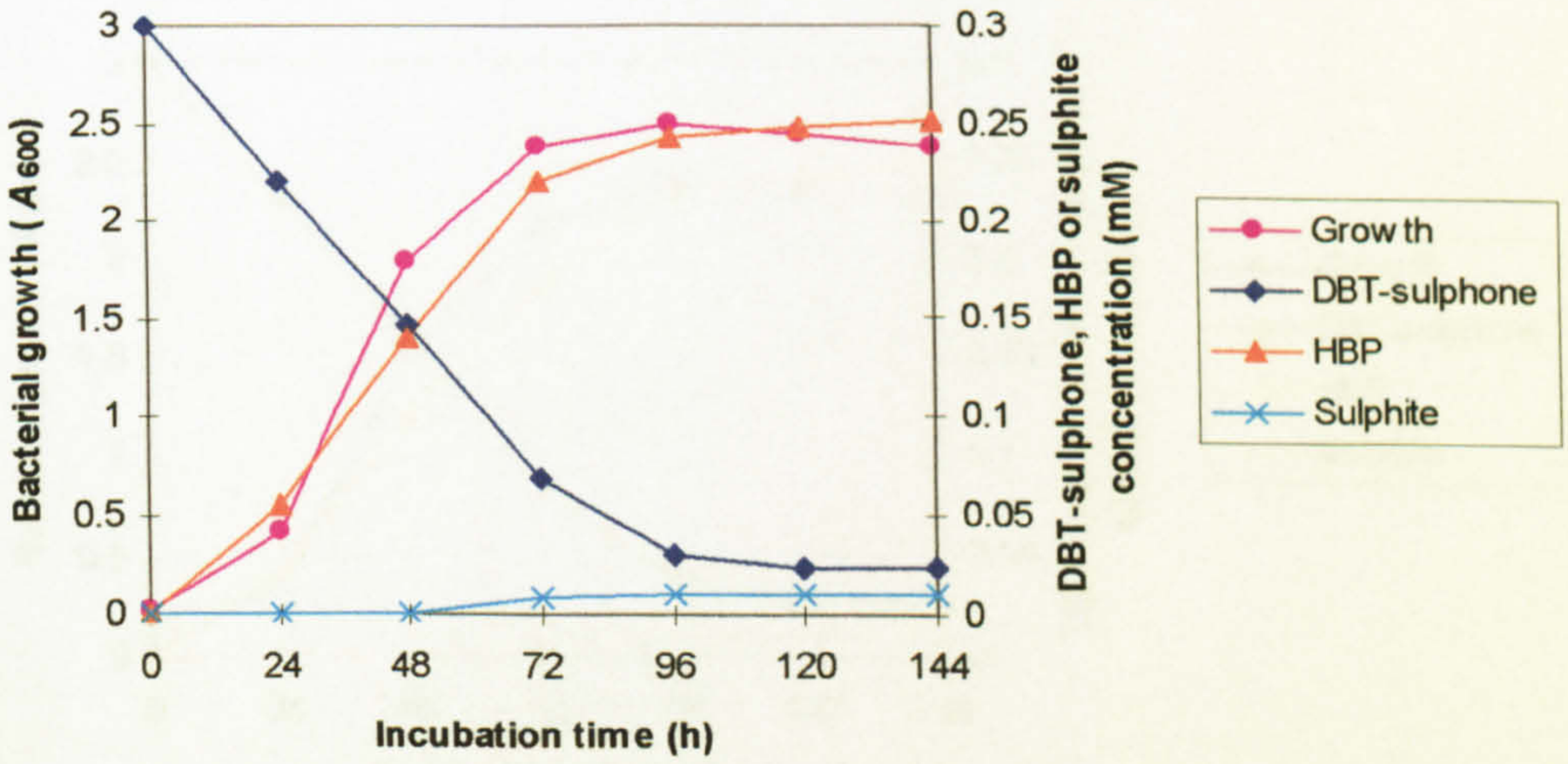


Figure 4.26 DBT-sulphone desulphurisation by *R. erythropolis* IGTS8. Cell grown in BSM with DMSO as a sole source of sulphur until the mid-log phase, washed twice, and resuspended in Ringer's solution to A_{600} = 1.0. One ml of the cell suspension was added to 100 ml BSM + 0.3 mM DBT-sulphone-acetone and incubated at 100 rpm, 30°C. Samples taken daily were analysed for bacterial growth (A_{600}), DBT-sulphone (HPLC), HBP (Gibb's assay), and sulphite (spectrophotometric assay). Each point is a mean of five replicates, and CV is less than 5%.

Similarly, *R. erythropolis* X309 grew well with DBT-sulphone as a sole source of sulphur. Figure 4.27 represents DBT-sulphone desulphurisation by this bacterial strain. The bacterial growth reached the stationary phase in 96 hours before the growth reduced. DBT-sulphone concentration in the medium declined from 0.3 to 0.03 mM, which corresponded to an increase of HBP concentration in the medium from 0 to 0.25 mM in 144 hours. Only a small amount of sulphite was released. There was no DBT-sulphone degradation in the control flasks (incubated without bacterial cells).

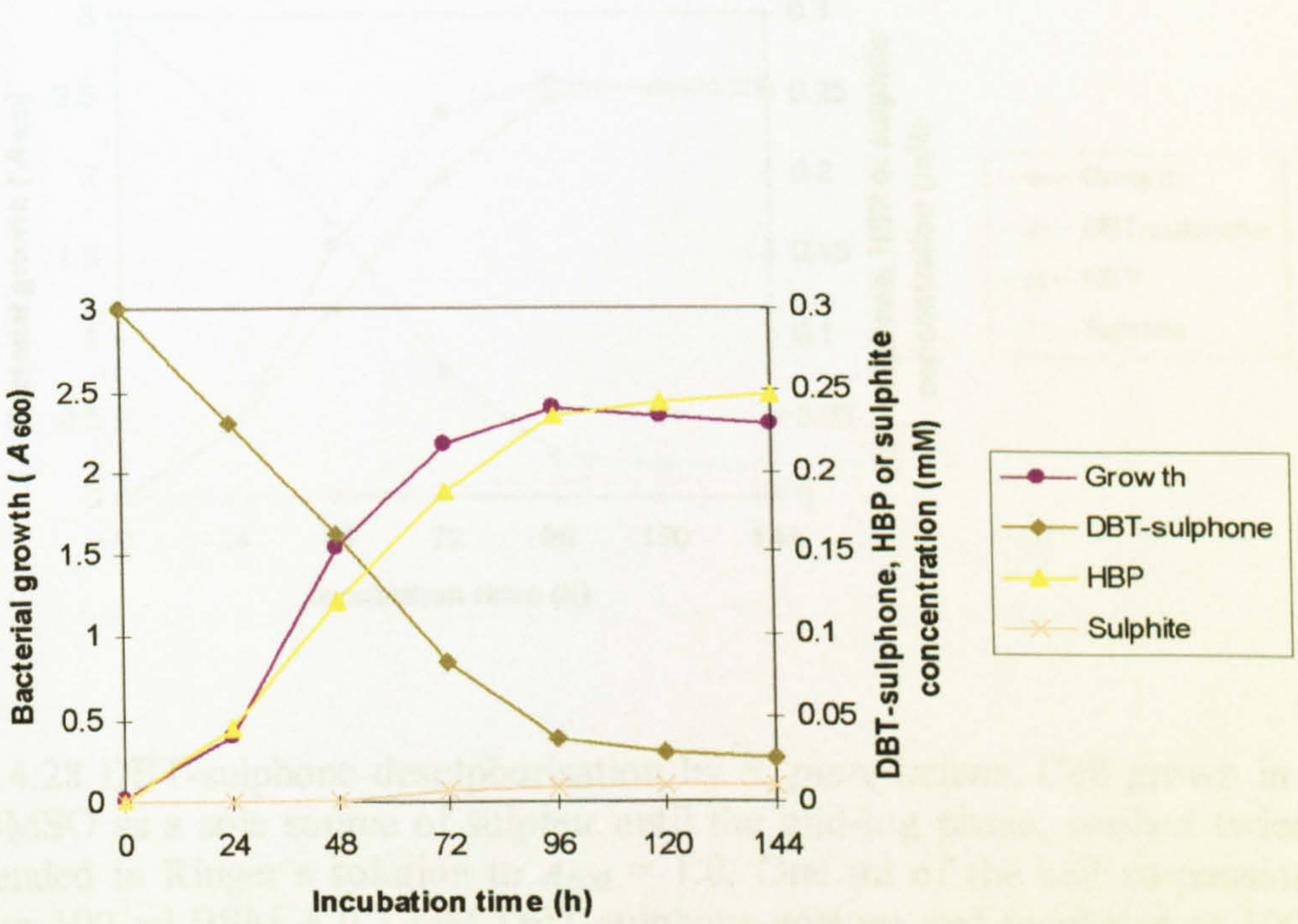


Figure 4.27 DBT-sulphone desulphurisation by *R. erythropolis* X309. Cell grown in BSM with DMSO as a sole source of sulphur until the mid-log phase, washed twice, and resuspended in Ringer's solution to A_{600} = 1.0. One ml of the cell suspension was added to 100 ml BSM + 0.3 mM DBT-sulphone-acetone and incubated at 100 rpm, 30°C. Samples taken daily were analysed for bacterial growth (A_{600}), DBT-sulphone (HPLC), HBP (Gibb's assay), and sulphite (spectrophotometric assay). Each point is a mean of five replicates, and CV is less than 5%.

S. putrefaciens also grew well with DBT-sulphone as a sole source of sulphur. The bacterial growth reached the stationary phase in 96 hours before deceleration as shown in Figure 4.28. DBT-sulphone concentration in the medium declined from 0.3 to 0.02 mM in 144 hours. With HPLC analysis, a product detected corresponded to HBP. The concentration of HBP in the medium increased from 0 to 0.26 mM in 144 hours. Only a small amount of sulphite was released.

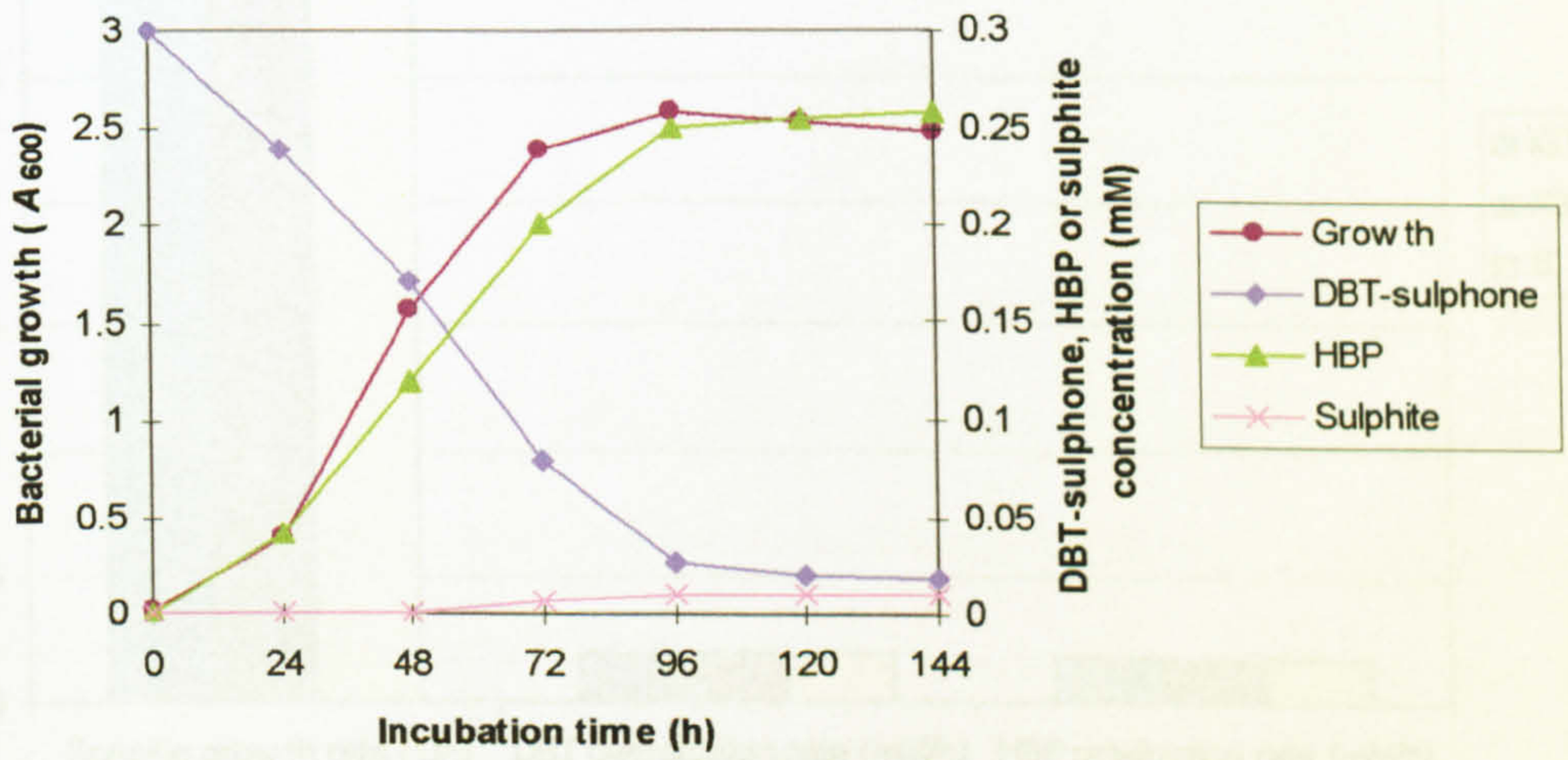


Figure 4.28 DBT-sulphone desulphurisation by *S. putrefaciens*. Cell grown in BSM with DMSO as a sole source of sulphur until the mid-log phase, washed twice, and resuspended in Ringer's solution to A_{600} = 1.0. One ml of the cell suspension was added to 100 ml BSM + 0.3 mM DBT-sulphone-acetone and incubated at 100 rpm, 30°C. Samples taken daily were analysed for bacterial growth (A_{600}), DBT-sulphone (HPLC), HBP (Gibb's assay), and sulphite (spectrophotometric assay). Each point is a mean of five replicates, and CV is less than 5%.

DBT-sulphone desulphurisation activities of the three bacteria were similar as shown in Figure 4.29. From the ANOVA tests, their specific growth rates, DBT degradation rates, or HBP production rates were not significantly different.

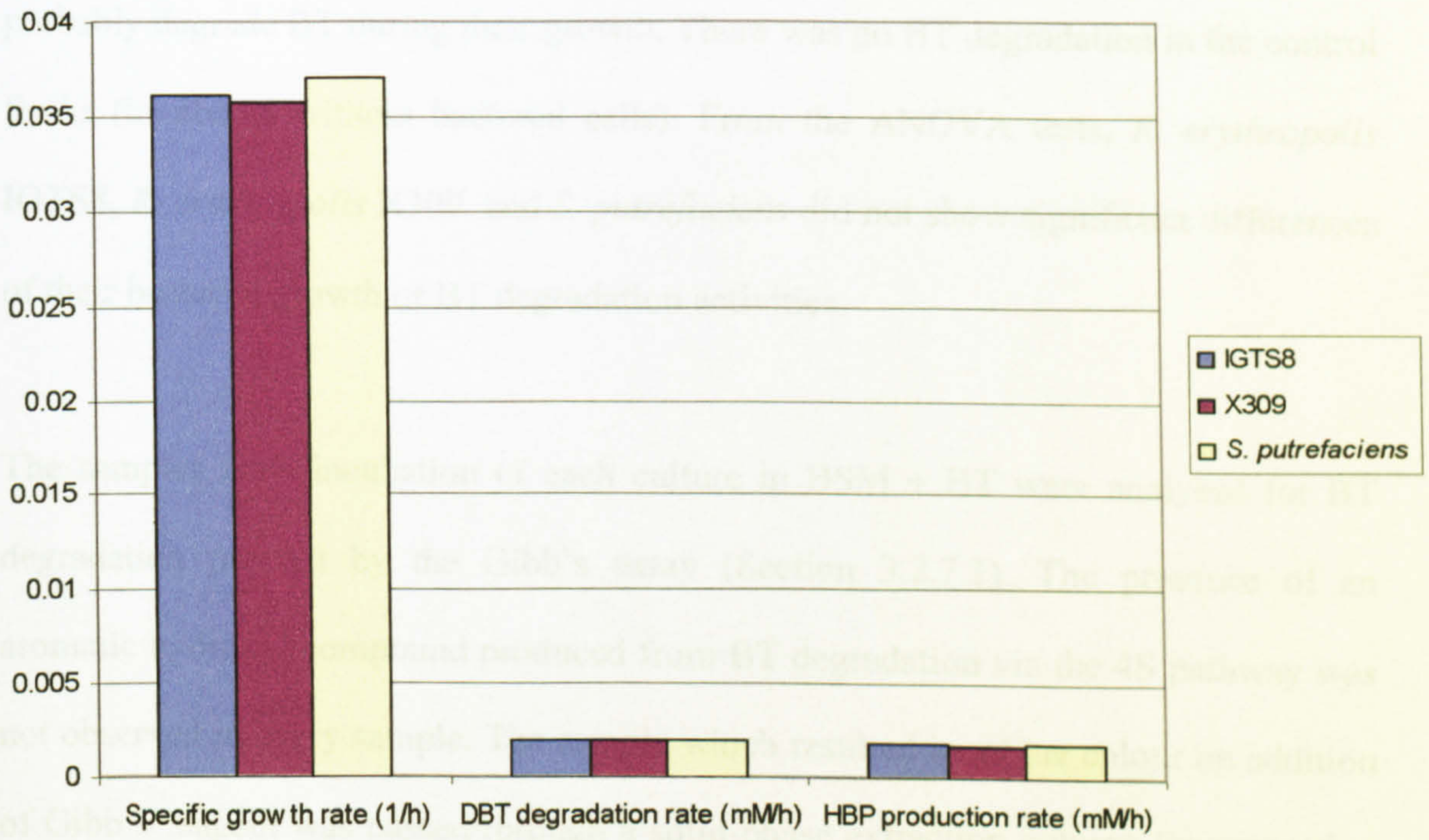
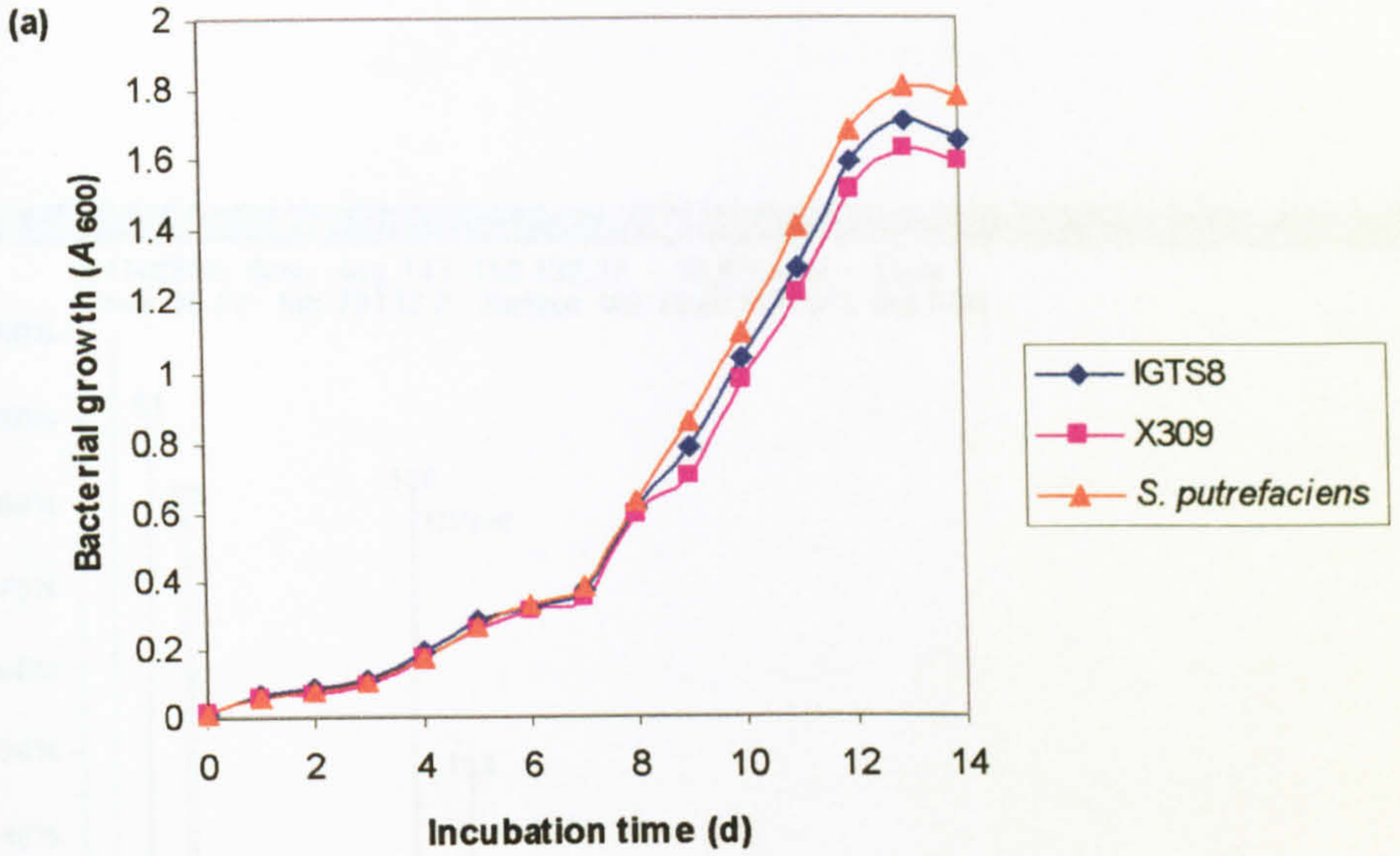


Figure 4.29 DBT-sulphone desulphurisation activities of the bacteria. Each point is a mean of five replicates, LSD (P=0.05) = 0.0008.

4.2.4 BT desulphurisation

Each culture of the three bacteria investigated grew weakly with a supplement of BT as a sole source of sulphur in BSM. A significant growth was observed after 4 days of incubation (Figure 4.30). From HPLC analyses, BT degradation was observed. Therefore, *R. erythropolis* IGTS8, *R. erythropolis* X309, and *S. putrefaciens* could probably degrade BT during their growth. There was no BT degradation in the control flasks (incubated without bacterial cells). From the ANOVA tests, *R. erythropolis* IGTS8, *R. erythropolis* X309, and *S. putrefaciens* did not show significant differences of their bacterial growth or BT degradation activities.

The samples from incubation of each culture in BSM + BT were analysed for BT degradation product by the Gibb's assay (Section 3.2.7.1). The presence of an aromatic hydroxyl compound produced from BT degradation via the 4S pathway was not observed in every sample. The sample which resulted in a blue colour on addition of Gibb's reagent was passed through a solid-phase extraction column for separation of the desulphurisation metabolites and then sent to Kent Mass Spectrometry (Kent, UK) for mass spectrometry analysis. A peak of 2-(2'-hydroxyphenyl)ethan 1-al (HPEal) which is a product of BT desulphurisation via the 4S pathway was observed by each culture. Figure 4.31 presents mass spectrometry analysis of the sample incubated with *S. putrefaciens*. This indicated that *R. erythropolis* IGTS8, *R. erythropolis* X309 and *S. putrefaciens* could possibly desulphurise BT via the 4S pathway, but with little efficiency.



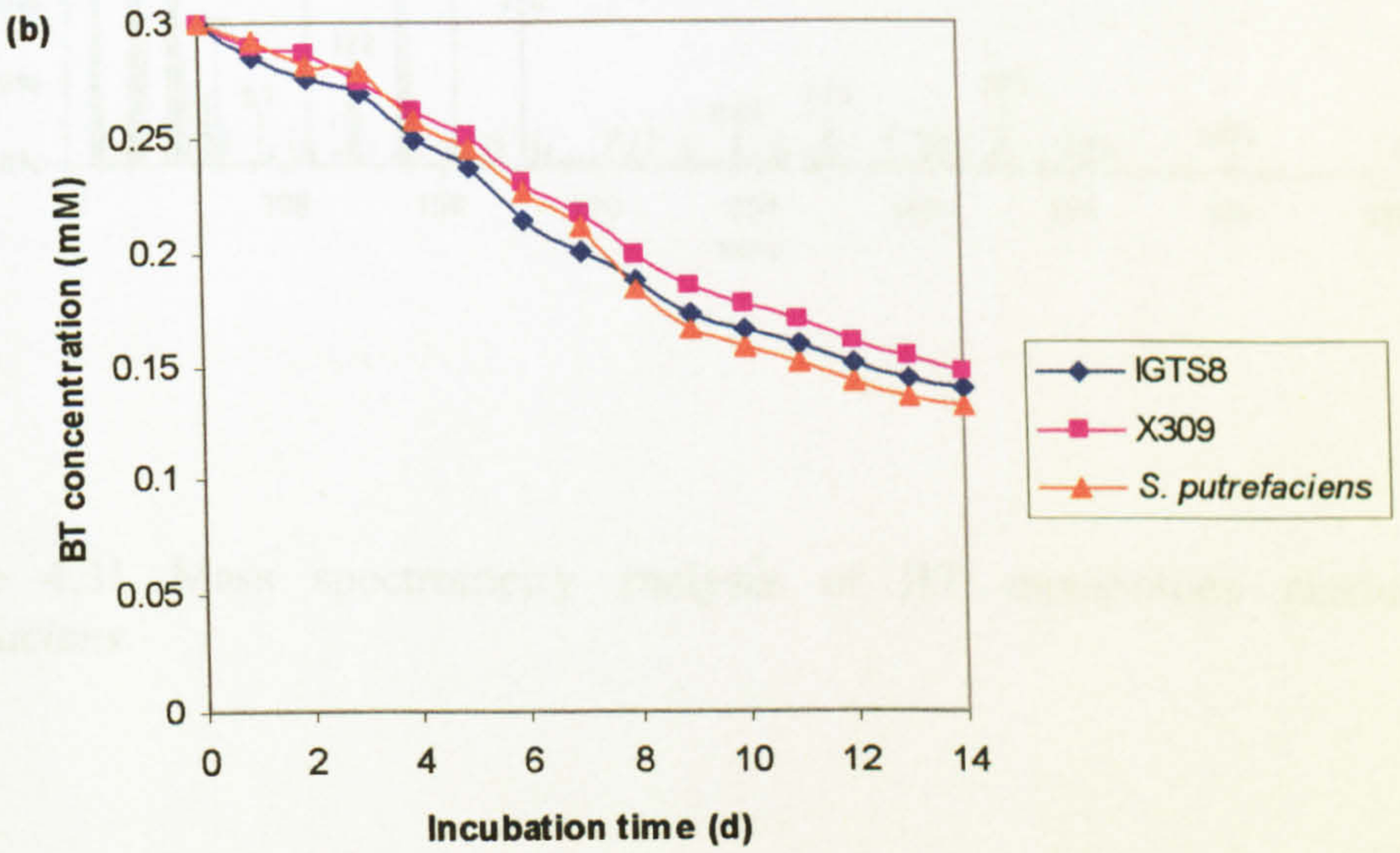


Figure 4.30 Desulphurisation of BT by the three bacteria (a) bacterial growth on BT and (b) degradation of BT. Cell grown in BSM with DMSO as a sole source of sulphur until the mid-log phase, washed twice, and resuspended in Ringer's solution to A_{600} = 1.0. One ml of the cell suspension was added to 100 ml BSM + 0.3 mM BT-ethanol and incubated at 100 rpm, 30°C. Samples taken daily were monitored for bacterial growth at A_{600}, and analysed for BT using HPLC. Each point is a mean of three replicates, and CV is less than 10%. LSD (P=0.05) of bacterial growth = 0.096, and LSD (P=0.05) of BT degradation = 0.013.

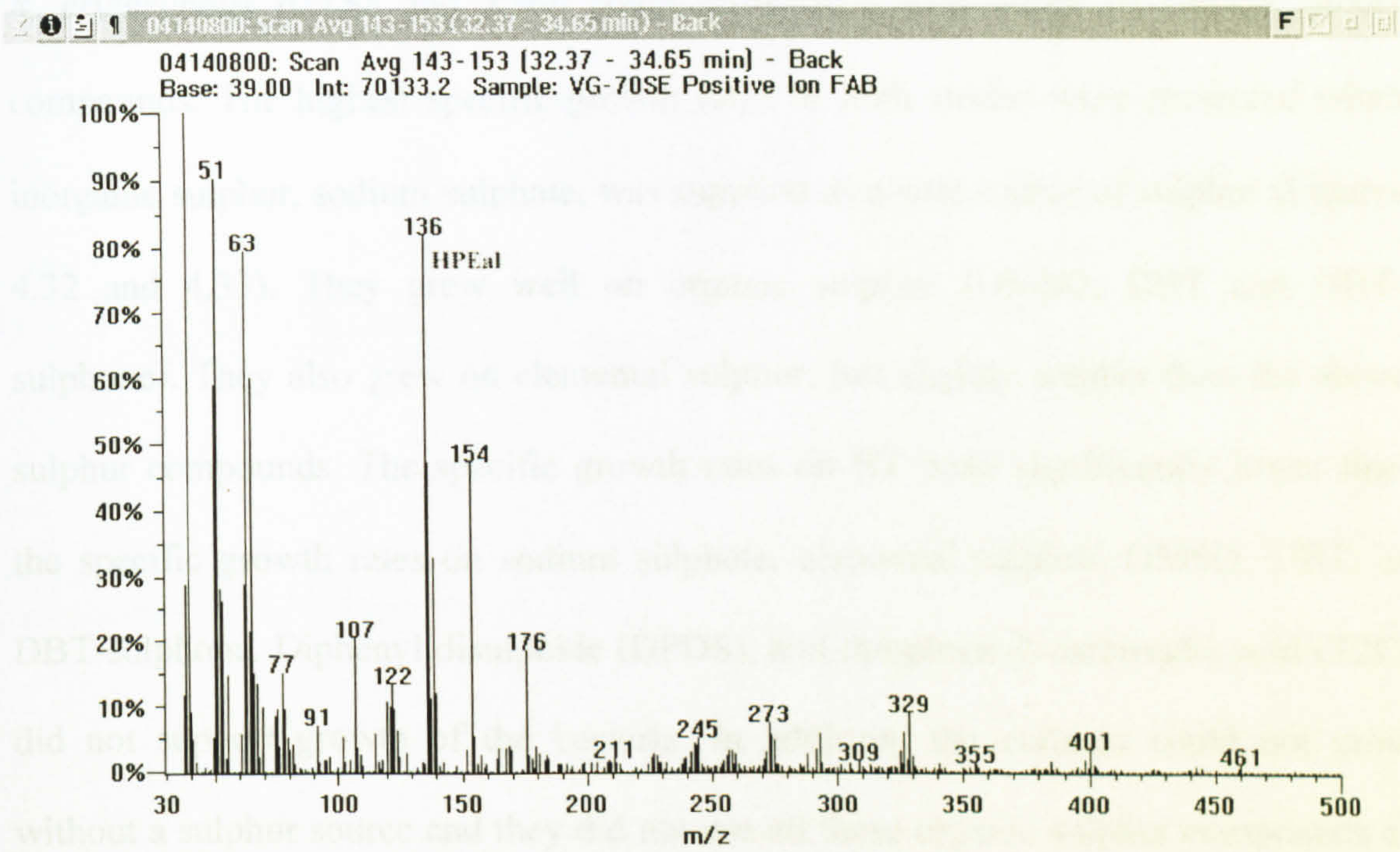


Figure 4.31 Mass spectrometry analysis of BT metabolites produced by *S. putrefaciens*.

4.2.5 Growths on sulphur model compounds

R. erythropolis IGTS8 and X309 could grow on a wide range of sulphur model compounds. The highest specific growth rates of both strains were presented when inorganic sulphur, sodium sulphate, was supplied as a sole source of sulphur (Figures 4.32 and 4.33). They grew well on organic sulphur (DMSO, DBT and DBT-sulphone). They also grew on elemental sulphur, but slightly weaker than the above sulphur compounds. The specific growth rates on BT were significantly lower than the specific growth rates on sodium sulphate, elemental sulphur, DMSO, DBT, or DBT-sulphone. Diphenyl disulphide (DPDS), and thiophene-2-carboxylic acid (T2C) did not support growth of the bacteria. In addition, the cultures could not grow without a sulphur source and they did not use all these organic sulphur compounds as the carbon source for growth.

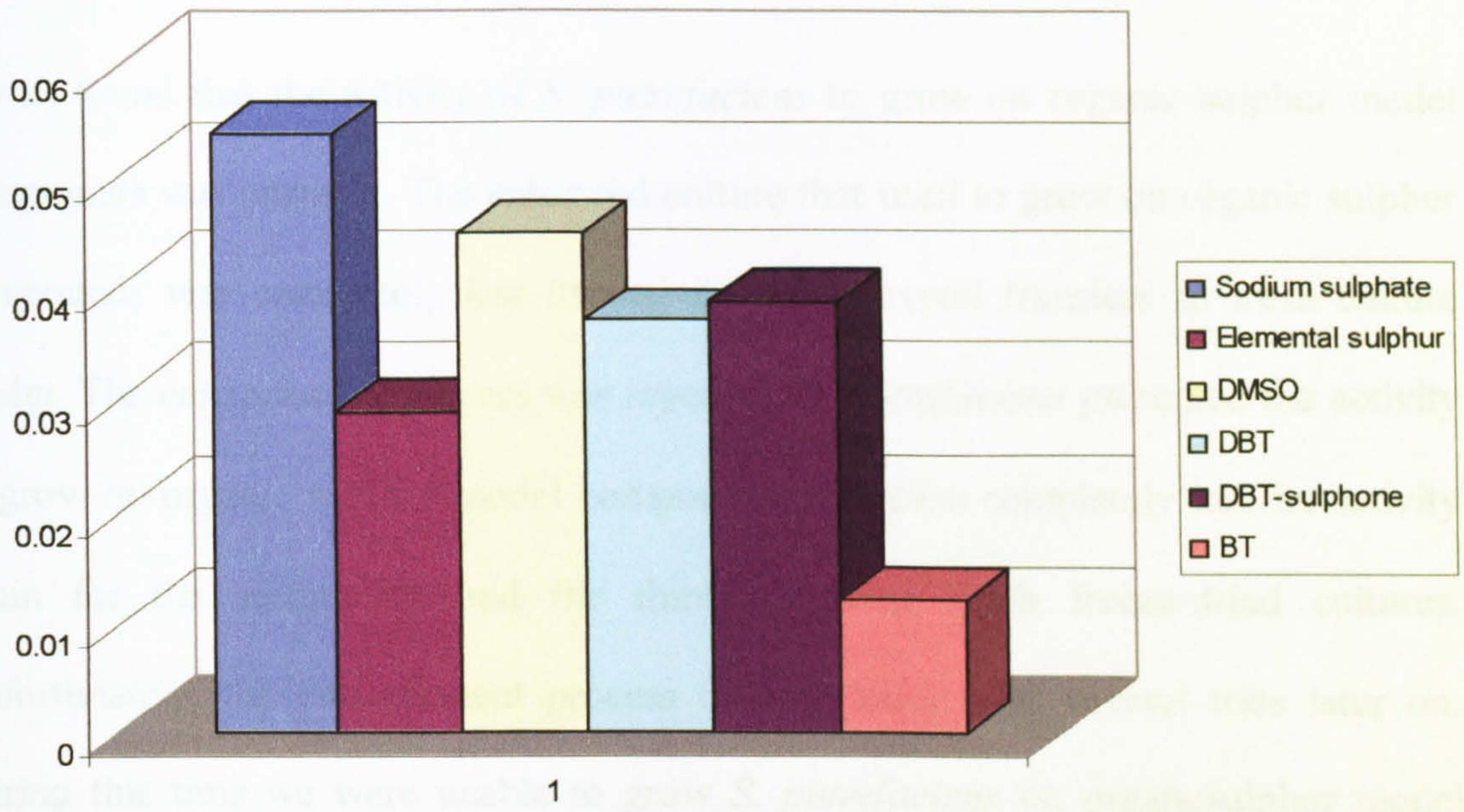


Figure 4.32 Specific growth rates of *R. erythropolis* IGTS8 on sulphur model compounds. LSD (P=0.05) = 0.02.

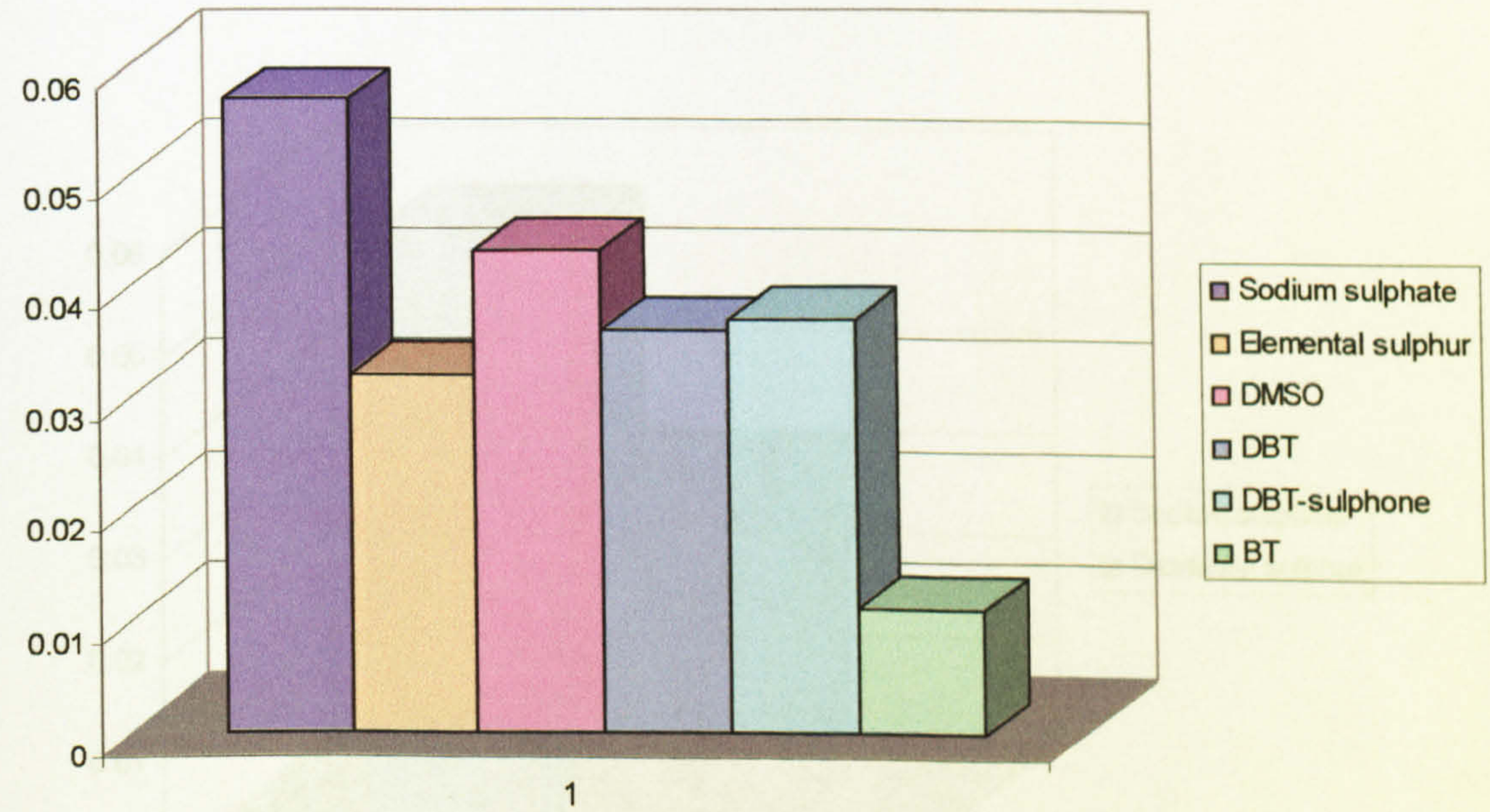


Figure 4.33 Specific growth rates of *R. erythropolis* X309 on sulphur model compounds. LSD (P=0.05) = 0.02.

It was found that the activity of *S. putrefaciens* to grow on organic sulphur model compounds was unstable. The enhanced culture that used to grow on organic sulphur compounds was completely lost its activity after several transfers to fresh culture media. The enhancement process was repeated. *S. putrefaciens* presented the activity to grow on organic sulphur model compounds, and then completely lost its activity again for the second try and the third try using fresh freeze-dried cultures. Unfortunately, the enhancement process did not work after several tries later on. During this time we were unable to grow *S. putrefaciens* on organosulphur model compounds. However, *S. putrefaciens* still presented growth on inorganic sulphur (sodium sulphate), and elemental sulphur. From the ANOVA test, the specific growth rate on sodium sulphate was significantly greater than the specific growth rate on elemental sulphur as shown in Figure 4.34.

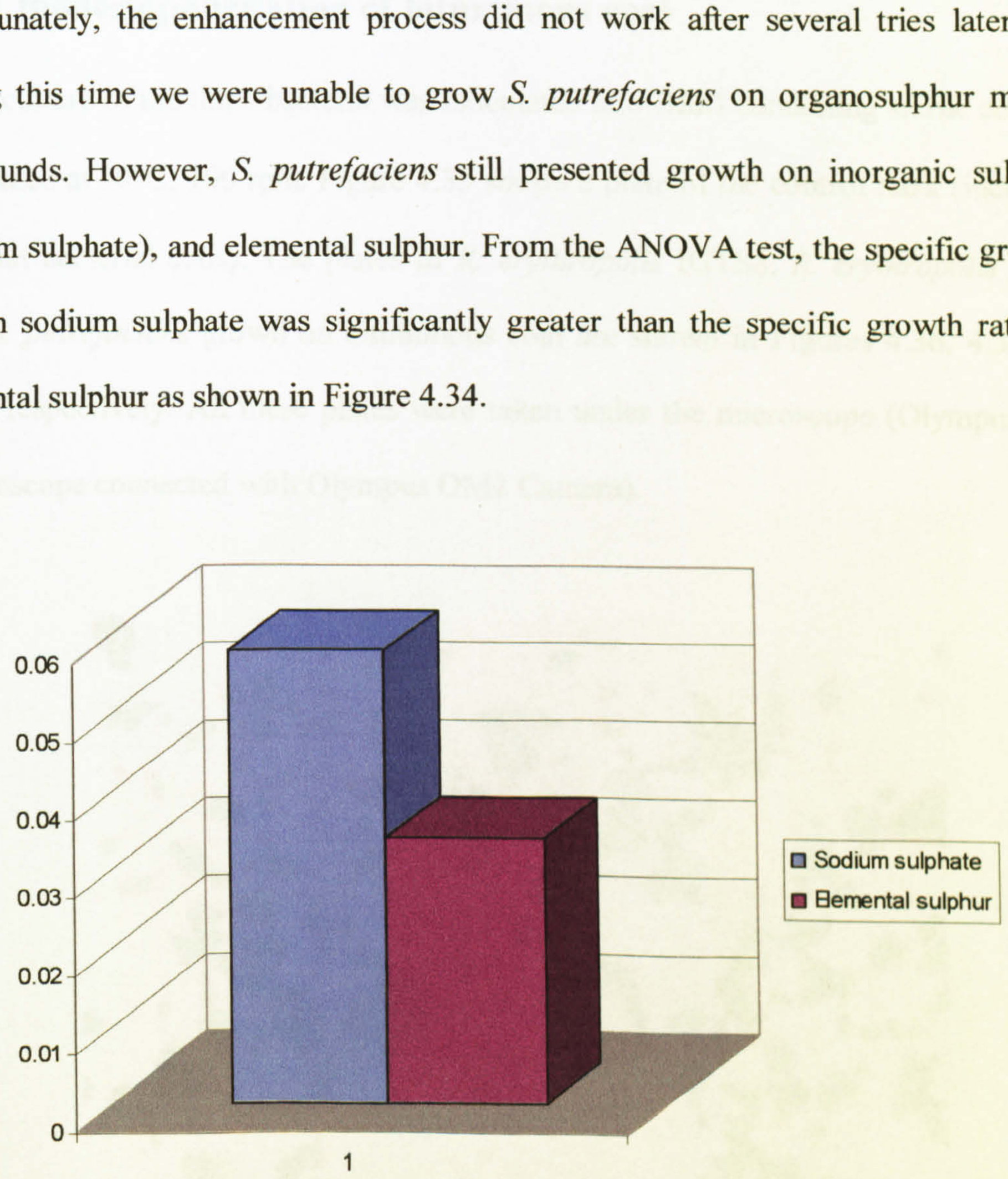


Figure 4.34 Specific growth rates of *S. putrefaciens* on sulphur model compounds. LSD (P=0.05) = 0.02.

4.3 BIODESULPHURISATION OF COAL SAMPLES

In this Section, the results from biodesulphurisation of two types of coal (bituminous and anthracite) by the three bacterial strains and bacteria inherent in the coal are reported. In addition, the desulphurisation activities of the bacteria isolated from coal samples are presented.

4.3.1 Biodesulphurisation of bituminous coal

Each culture of the three bacteria was inoculated into BSM containing sterile coal and incubated at 30°C, 100 rpm. Figure 4.35 shows a plate of the control flask (incubated without bacterial cells). The plates of *R. erythropolis* IGTS8, *R. erythropolis* X309, and *S. putrefaciens* grown on bituminous coal are shown in Figures 4.36, 4.37, and 4.38, respectively. All these plates were taken under the microscope (Olympus BH2 Microscope connected with Olympus OM2 Camera).

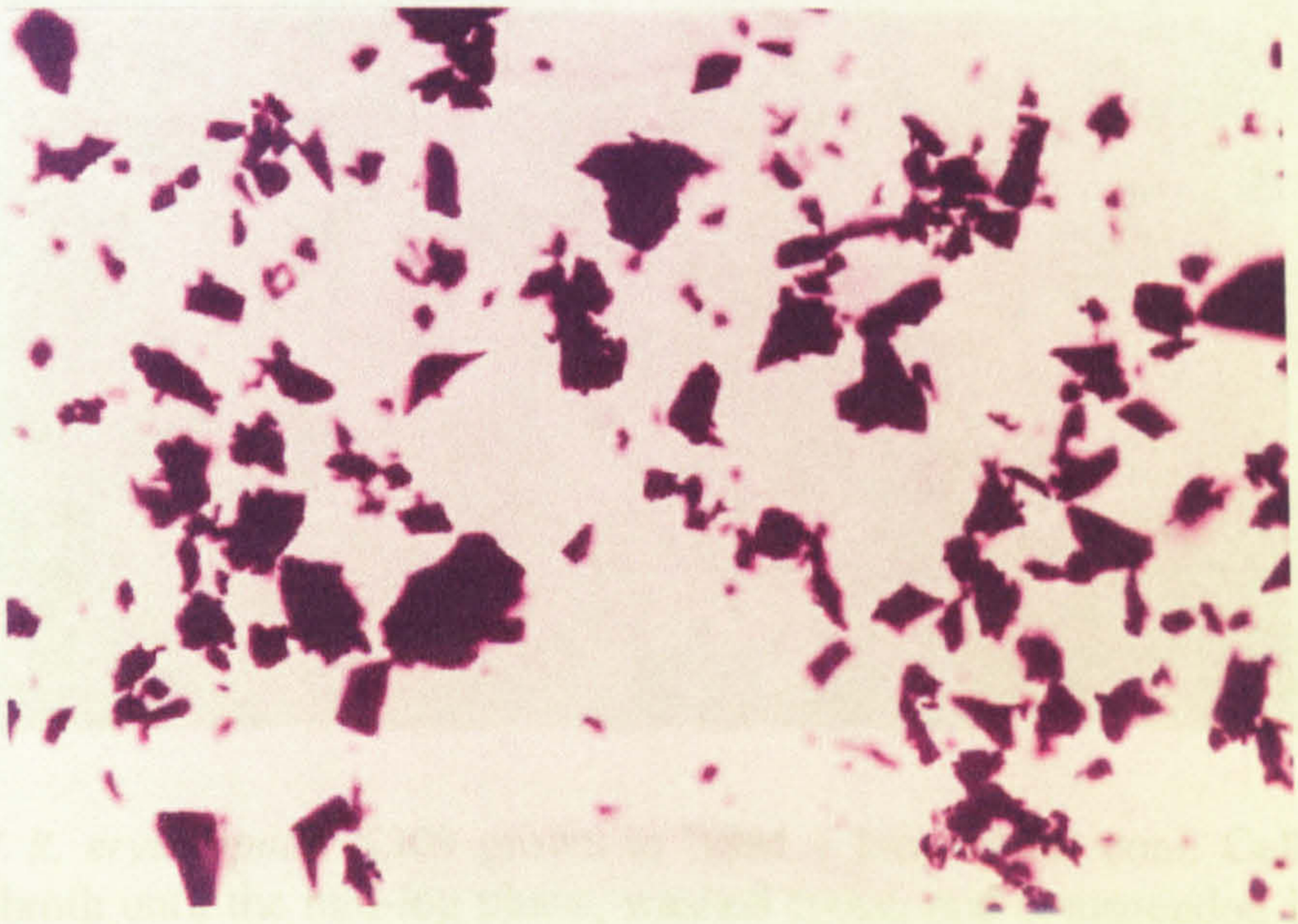


Figure 4.35 The sterile bituminous coal in basic salt medium (control flask). Two g of sterile bituminous coal was inoculated into 100 ml of BSM without inoculum and incubated at 30°C, 100 rpm for 5 days.

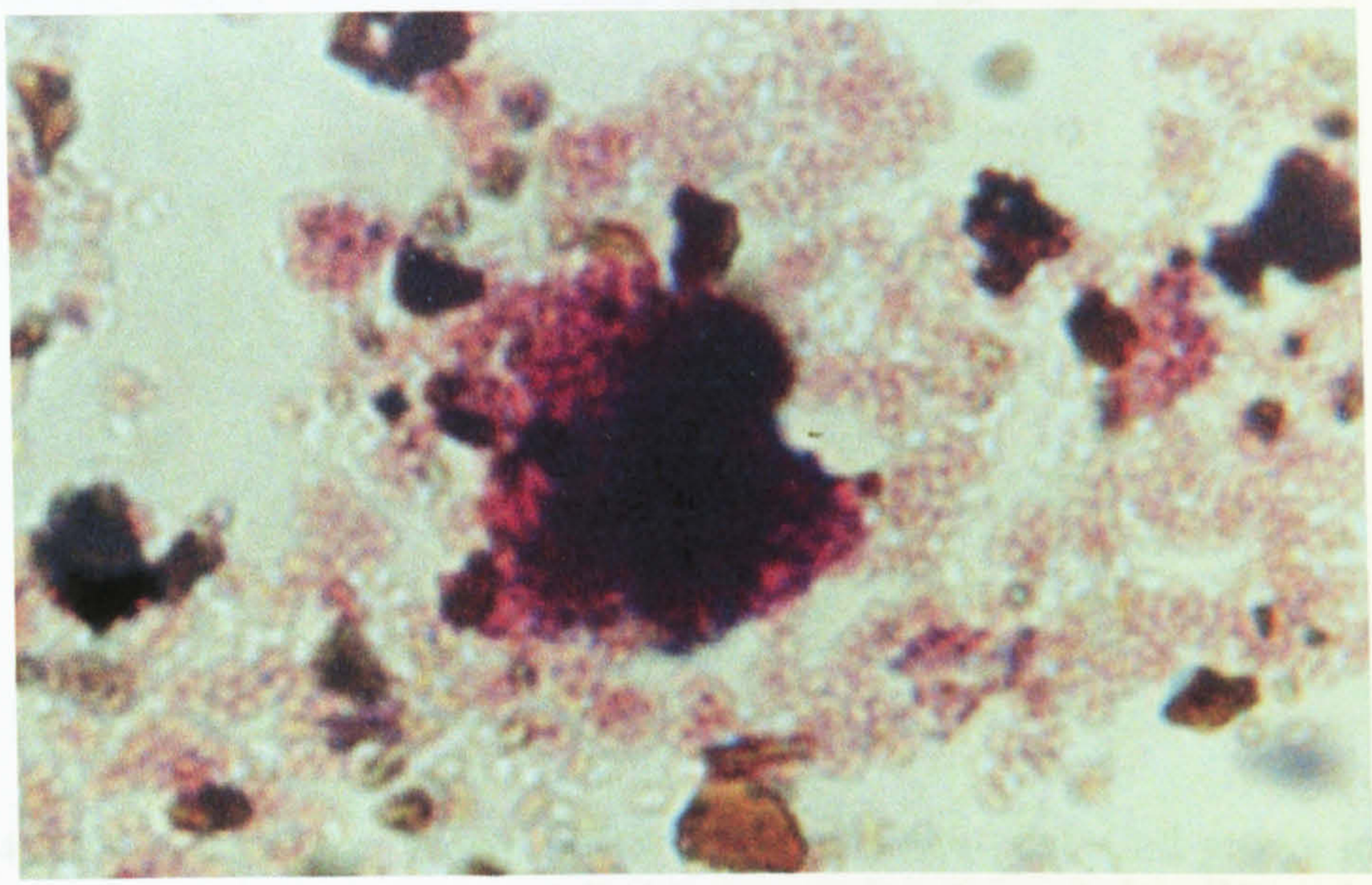


Figure 4.36 *R. erythropolis* IGTS8 grown in BSM + bituminous coal. Cell grown in tryptic soy broth until the mid-log phase, washed twice, and resuspended in Ringer's solution to A_{600} = 1.0. Five ml of the cell suspension was inoculated into 100 ml of BSM + 2 g of sterile coal and incubated at 30°C, 100 rpm for 5 days.

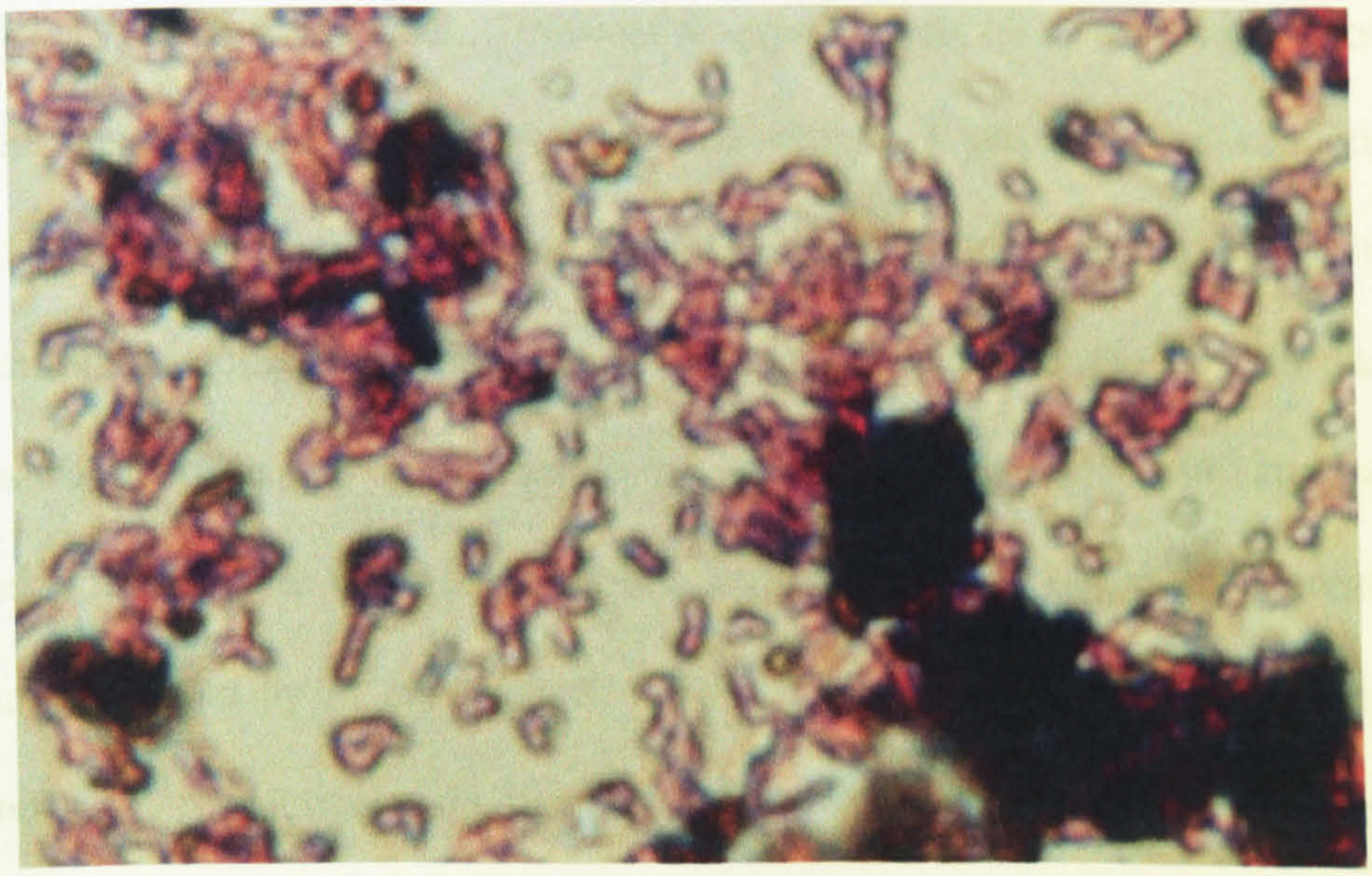


Figure 4.37 *R. erythropolis* X309 grown in BSM + bituminous coal. Cell grown in tryptic soy broth until the mid-log phase, washed twice, and resuspended in Ringer's solution to A_{600} = 1.0. Five ml of the cell suspension was inoculated into 100 ml of BSM + 2 g of sterile coal and incubated at 30°C, 100 rpm for 5 days.

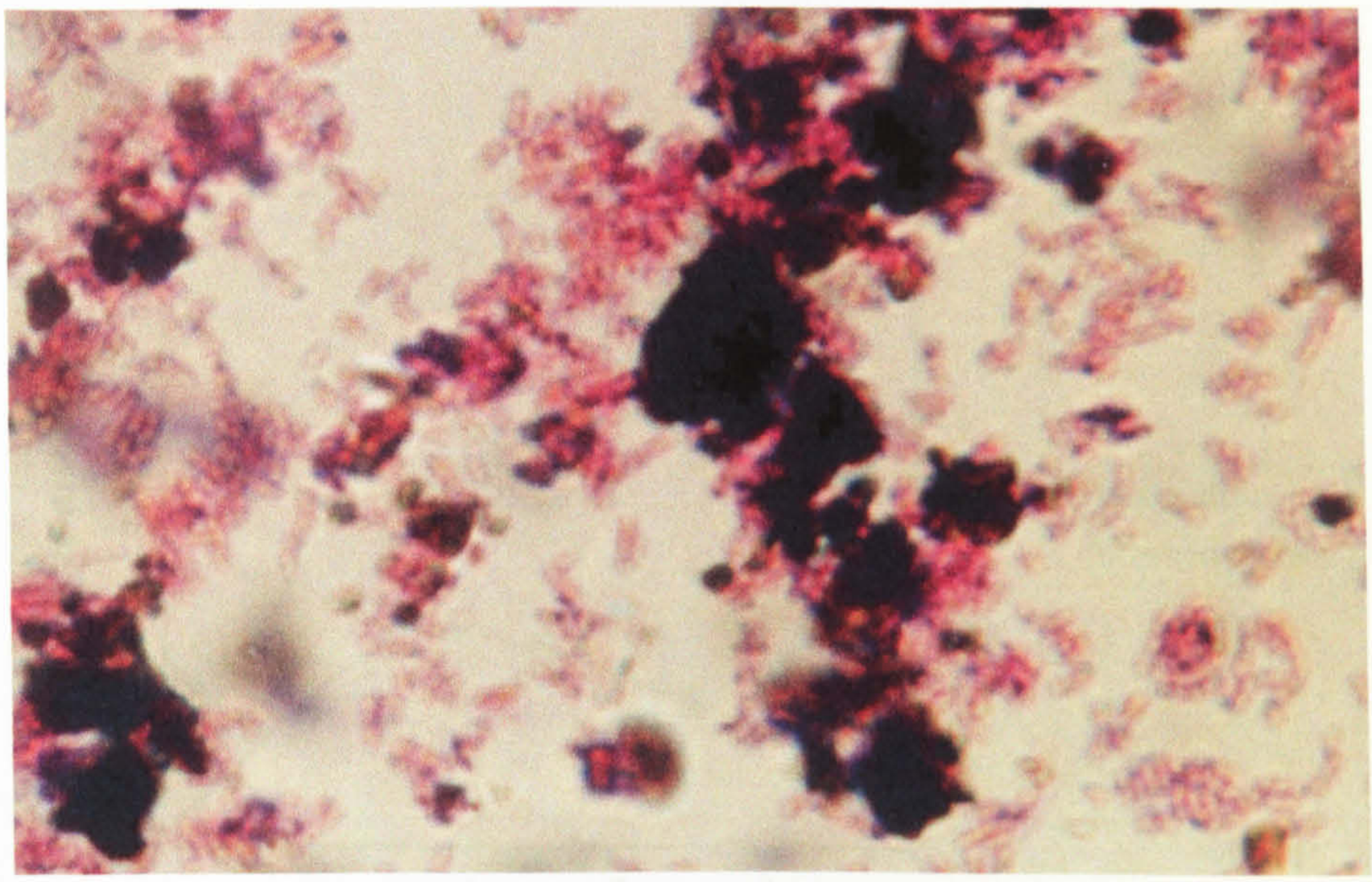


Figure 4.38 *S. putrefaciens* grown in BSM + bituminous coal. Cell grown in nutrient medium until the mid-log phase, washed twice, and resuspended in Ringer's solution to A_{600} = 1.0. Five ml of the cell suspension was inoculated into 100 ml of BSM + 2 g of sterile coal and incubated at 30°C, 100 rpm for 5 days.

Figure 4.39 shows bacterial growth (determined as protein concentration, Kilbane & Jackowski, 1992; Dastidar *et al.*, 2000) of *R. erythropolis* IGTS8 on bituminous coal. It can be seen that the free cells increased within 3 days before reduction was observed. This result indicated that the bacterium could probably grow by using soluble sulphur leached from the coal sample to BSM in its early growth phase, and then absorb itself to the coal sample for more sulphur since the attached cells increased from day 0 to day 10. This phenomenon was also observed in treatment of the bituminous coal by *R. erythropolis* X309 and *S. putrefaciens* as seen in Figures 4.40, and 4.41, respectively. In the treatment with natural bacteria inherent in the bituminous coal, the free cells increased from day 0 to day 10, and the attached cells reduced from day 0 to day 10 (Figure 4.42). This may be because the bacteria were detached from the coal to utilise more bioavailable sulphur in the medium.

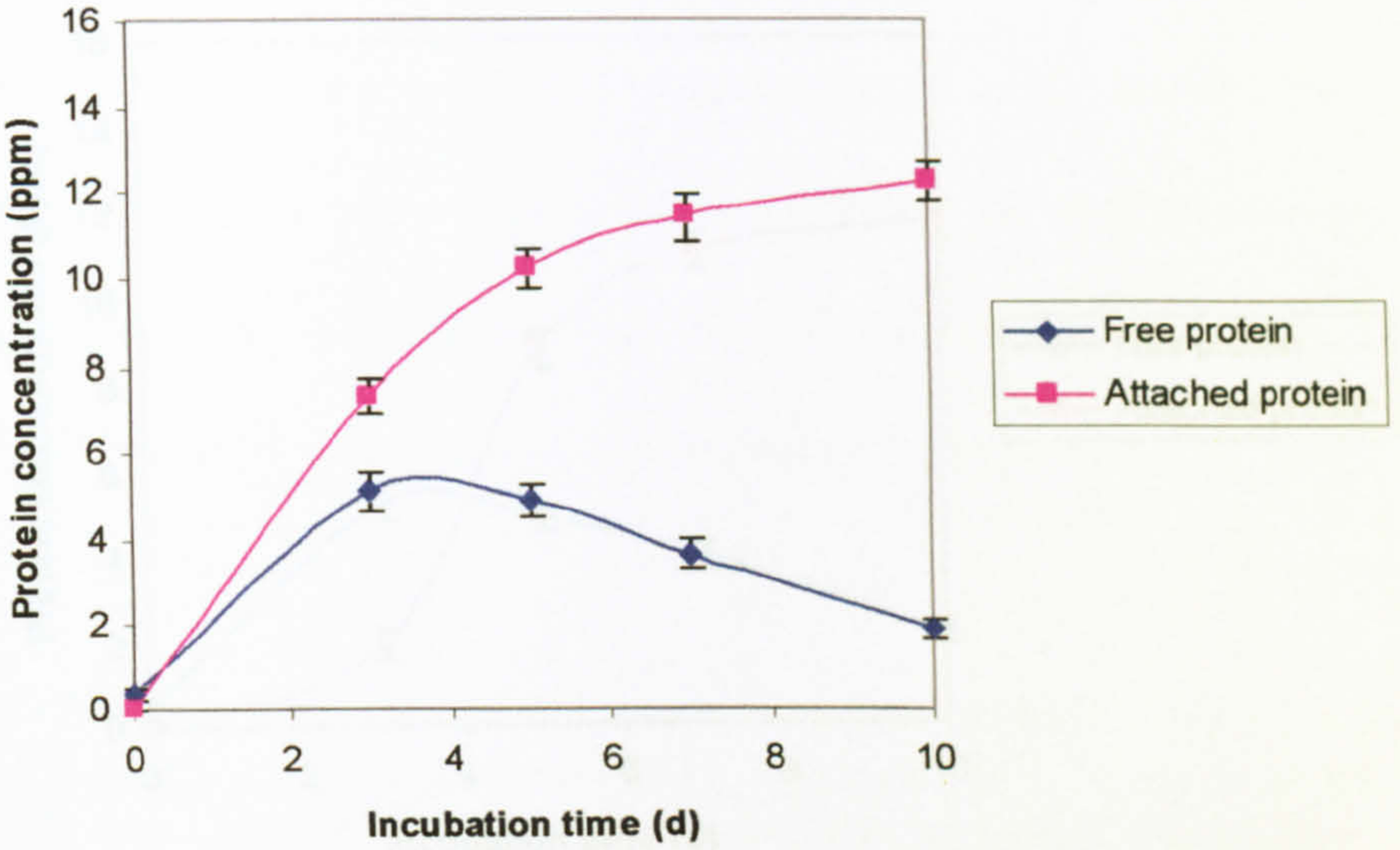


Figure 4.39 Growth of *R. erythropolis* IGTS8 on bituminous coal. Free protein and attached protein were analysed using protein assay. Each point is a mean of three replicates, error bar is a standard deviation, and LSD (P=0.05) = 10.04.

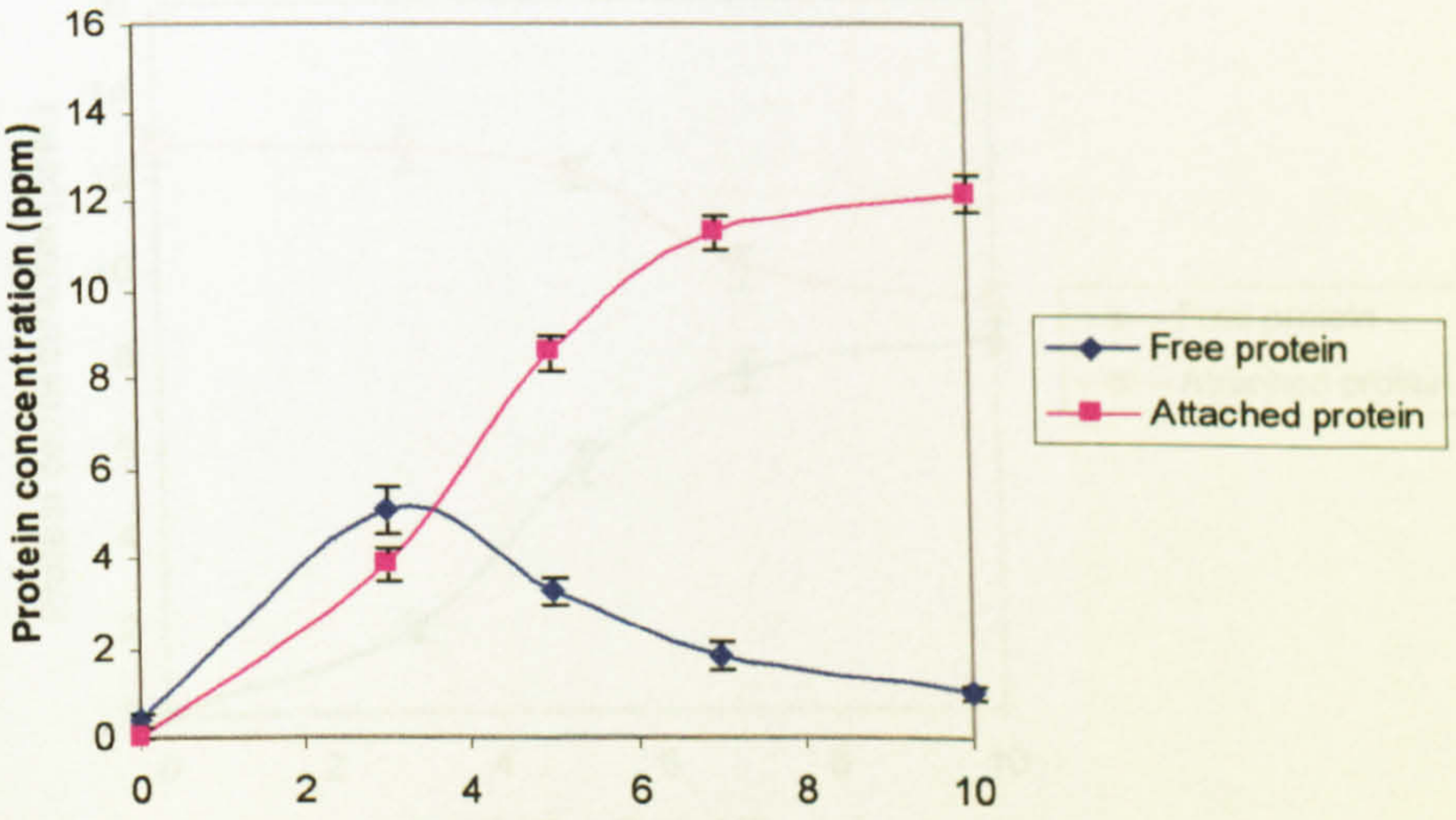


Figure 4.40 Growth of *R. erythropolis* X309 on bituminous coal. Free protein and attached protein were analysed using protein assay. Each point is a mean of three replicates, error bar is a standard deviation, and LSD (P=0.05) = 11.04.

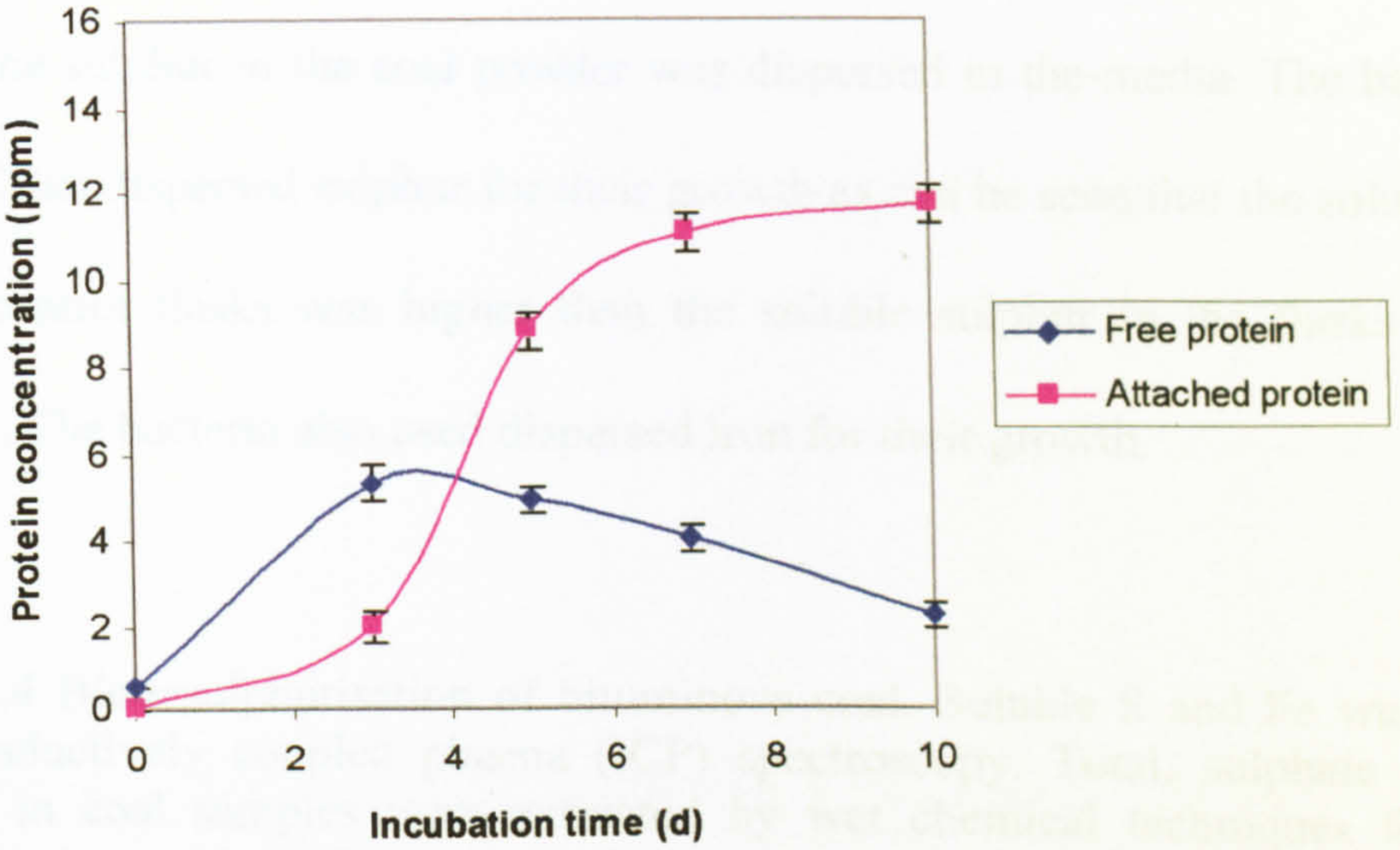


Figure 4.41 Growth of *S. putrefaciens* on bituminous coal. Free protein and attached protein were analysed using protein assay. Each point is a mean of three replicates, error bar is a standard deviation, and LSD (P=0.05) = 9.13.

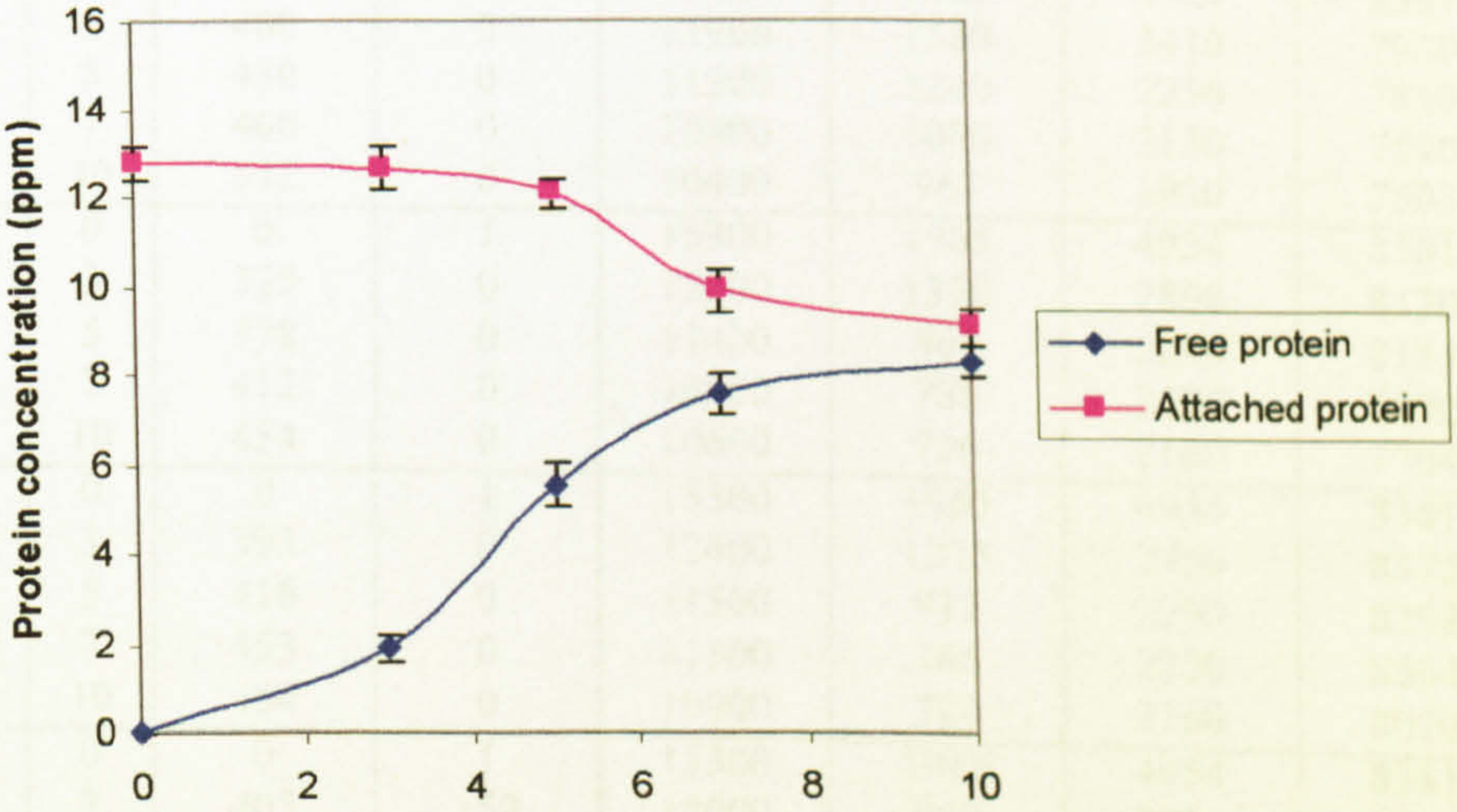


Figure 4.42 Growths of the bacteria inherent in bituminous coal. Free protein and attached protein were analysed using protein assay. Each point is a mean of three replicates, error bar is a standard deviation, and LSD (P=0.05) = 12.78.

The assumption regarding growth was confirmed with the results from ICP spectroscopy analyses of soluble sulphur in the culture media as presented in Table 4.4. Some sulphur in the coal powder was dispersed in the media. The bacteria used some of that dispersed sulphur for their growth as can be seen that the soluble sulphur in the control flasks was higher than the soluble sulphur in the flasks containing bacteria. The bacteria also used dispersed iron for their growth.

Table 4.4 Biodesulphurisation of bituminous coal. Soluble S and Fe were analysed using inductively coupled plasma (ICP) spectroscopy. Total, sulphate and pyritic sulphur in coal samples were extracted by wet chemical techniques followed by quantification with ICP spectroscopy. Organic sulphur was determined by two methods; the standard method[7], and a proposed method[8]. Each value is a mean of six replicates.

Inoculum	Day	Soluble S (ppm)	Soluble Fe (ppm)	Total S (ppm)	Sulphate S (ppm)	Pyritic S (ppm)	Organic S[7] (ppm)	Organic S[8] (ppm)
		CV=1%	CV=1%	CV=5%	CV=7%	CV=9%	CV=19%	CV=10%
IGTS8	0	0	1	15300	1965	4954	8381	8150
	3	400	0	11900	1580	2410	7910	8100
	5	430	0	11300	1240	2250	7810	7730
	7	460	0	10800	1080	2130	7590	7500
	10	532	0	10400	967	1930	7503	7400
X309	0	0	1	15300	1965	4954	8381	8150
	3	329	0	12300	1370	2800	8130	8130
	5	378	0	11400	965	2280	8155	8000
	7	412	0	10900	788	2220	7892	7700
	10	454	0	10600	736	2160	7704	7500
NCIMB 8768	0	0	1	15300	1965	4954	8381	8150
	3	393	0	12400	1275	2750	8375	8140
	5	416	0	11500	912	2290	8298	8050
	7	453	0	11300	766	2230	8304	7900
	10	484	0	10900	720	2160	8020	7800
Bacteria inherent in the coal	0	0	1	15300	1965	4954	8381	8150
	3	405	159	12000	997	2720	8283	8140
	5	458	0	11500	819	2340	8341	8090
	7	533	0	11200	778	2250	8172	8020
	10	589	0	11000	760	2170	8070	7980
Control (without bacteria)	0	0	1	15300	1965	4954	8381	8150
	3	1561	464	12500	892	3270	8338	8140
	5	1770	475	11700	780	2580	8340	8130
	7	1800	484	11400	743	2310	8347	8120
	10	2010	484	11300	700	2280	8320	8130

In relation to determination methods of sulphur forms in coal, the precision of data was expressed as a coefficient of variation (CV). As seen in Table 4.4, the CV of total sulphur, sulphate sulphur, and pyritic sulphur was less than 10%, implying that the analytical methods for these forms of sulphur are reliable. Regarding determination of organic sulphur, our proposed method (CV=10%) was more reliable than the standard method (CV=19%). Therefore, interpretation of data using the proposed method was reliable.

Reduction of sulphur against incubation time is presented in Table 4.5. It can be seen that longer incubation times resulted in greater reduction of each form of sulphur in all treatments. Comparison of reduction of sulphur by each treatment after 10 days is shown in Figure 4.43. *R. erythropolis* IGTS8 actively resulted in significantly greater desulphurisation of total sulphur, pyritic sulphur, or organic sulphur when compared to the control. *R. erythropolis* X309 actively gave a significantly greater desulphurisation of organic sulphur over the control. *S. putrefaciens* or bacteria inherent in the coal did not show significantly greater desulphurisation efficiency than the control.

Reduction of sulphate sulphur by *R. erythropolis* IGTS8 was significantly lower than reduction of sulphate sulphur by other treatments. There were no significant differences between reduction of total sulphur, pyritic sulphur, or organic sulphur by the three bacteria. Regarding reduction of organic sulphur, both strains of *R. erythropolis* gave significantly greater desulphurisation over bacteria inherent in the coal.

Table 4.5 Reduction of sulphur in bituminous coal after biodesulphurisation. Sulphur reduction was calculated from data in Table 4.4. Organic sulphur was determined by the proposed method.

Inoculum	Day	Total S Reduction (%)	Sulphate S Reduction (%)	Pyritic S Reduction (%)	Organic S Reduction (%)
IGTS8	3	22.2	19.6	51.4	0.6
	5	26.1	36.9	54.6	5.2
	7	29.4	45.0	57.0	8.0
	10	32.0	50.8	61.0	9.2
X309	3	19.6	30.3	43.5	0.3
	5	25.5	50.9	54.0	1.8
	7	28.8	59.9	55.2	5.5
	10	30.7	62.5	56.4	8.0
NCIMB 8768	3	19.0	35.1	44.5	0.1
	5	24.8	53.6	53.8	1.2
	7	26.1	61.0	55.0	3.1
	10	28.8	63.4	56.4	4.3
Bacteria inherent in the coal	3	21.6	49.3	45.1	0.1
	5	24.8	58.3	52.8	0.7
	7	26.8	60.4	54.6	1.6
	10	28.1	61.3	56.2	2.1
Control (without bacteria)	3	18.3	54.6	34.0	0.1
	5	23.5	60.3	47.9	0.3
	7	25.5	62.2	53.4	0.4
	10	26.1	64.4	54.0	0.3

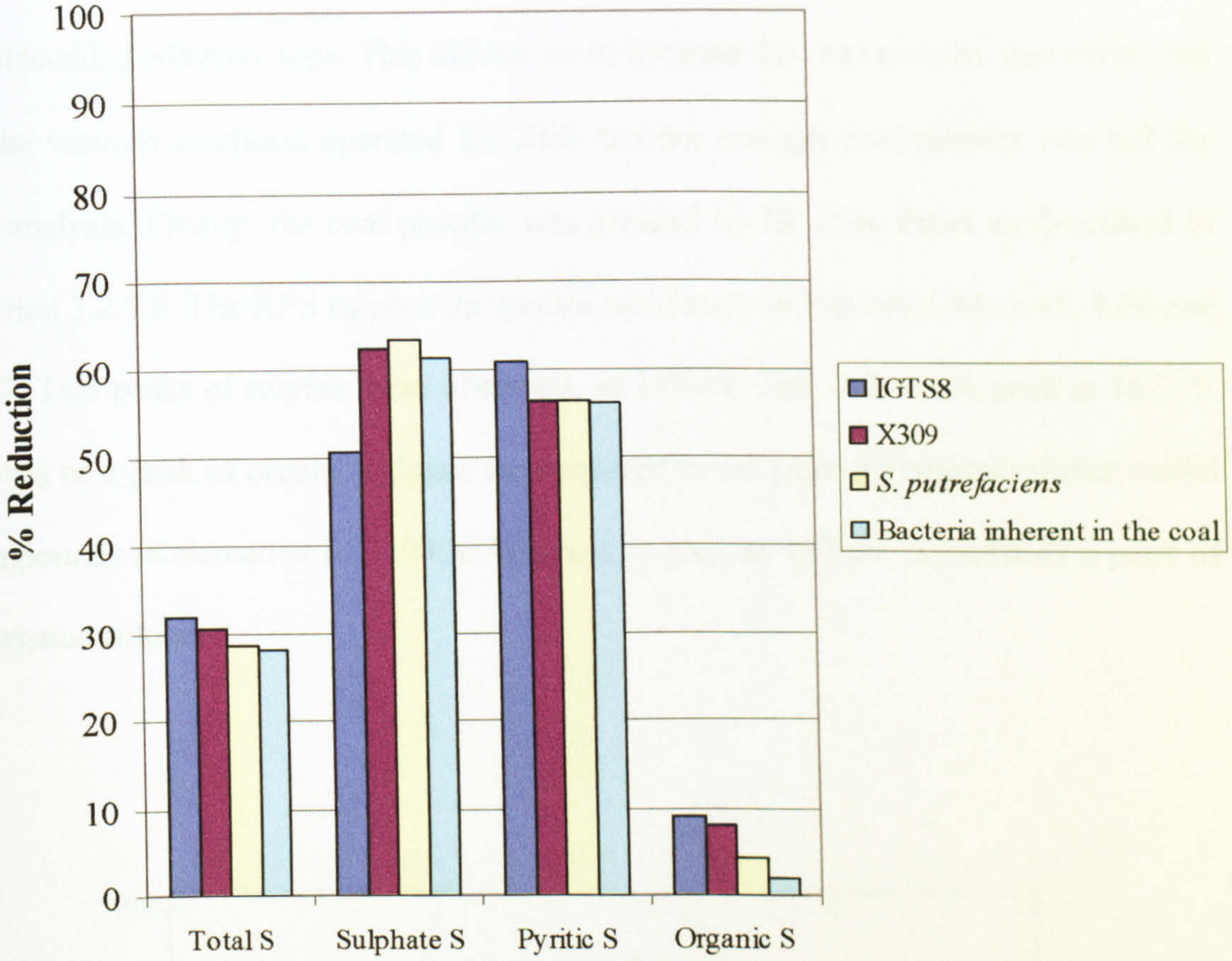


Figure 4.43 Reduction of sulphur in bituminous coal at 10 day incubation. LSD (P=0.05) = 5.0.

Determination of sulphur was also attempted with a non-destructive method, X-ray photoelectron spectroscopy (XPS) analysis. The problem with the XPS analysis was the preparation of coal samples. At the beginning the coal powder was mounted on double-sided adhesive tape. This did not work because the coal powder was blown out in the vacuum condition operated for XPS and not enough coal powder was left for the analysis. Finally, the coal powder was pressed by IR Disc Press as described in Section 3.2.7.8. The XPS sulphur 2p spectra are shown in Figures 4.44, 4.45, 4.46 and 4.47. Two peaks of sulphur were observed; at 157 eV, and 167 eV. A peak at 167 eV should be a peak of organic sulphur as compared to the peak for organosulphur model compounds (Keleman *et al.*, 1990). Whereas, a peak at 157 eV is probably a peak of inorganic sulphur.

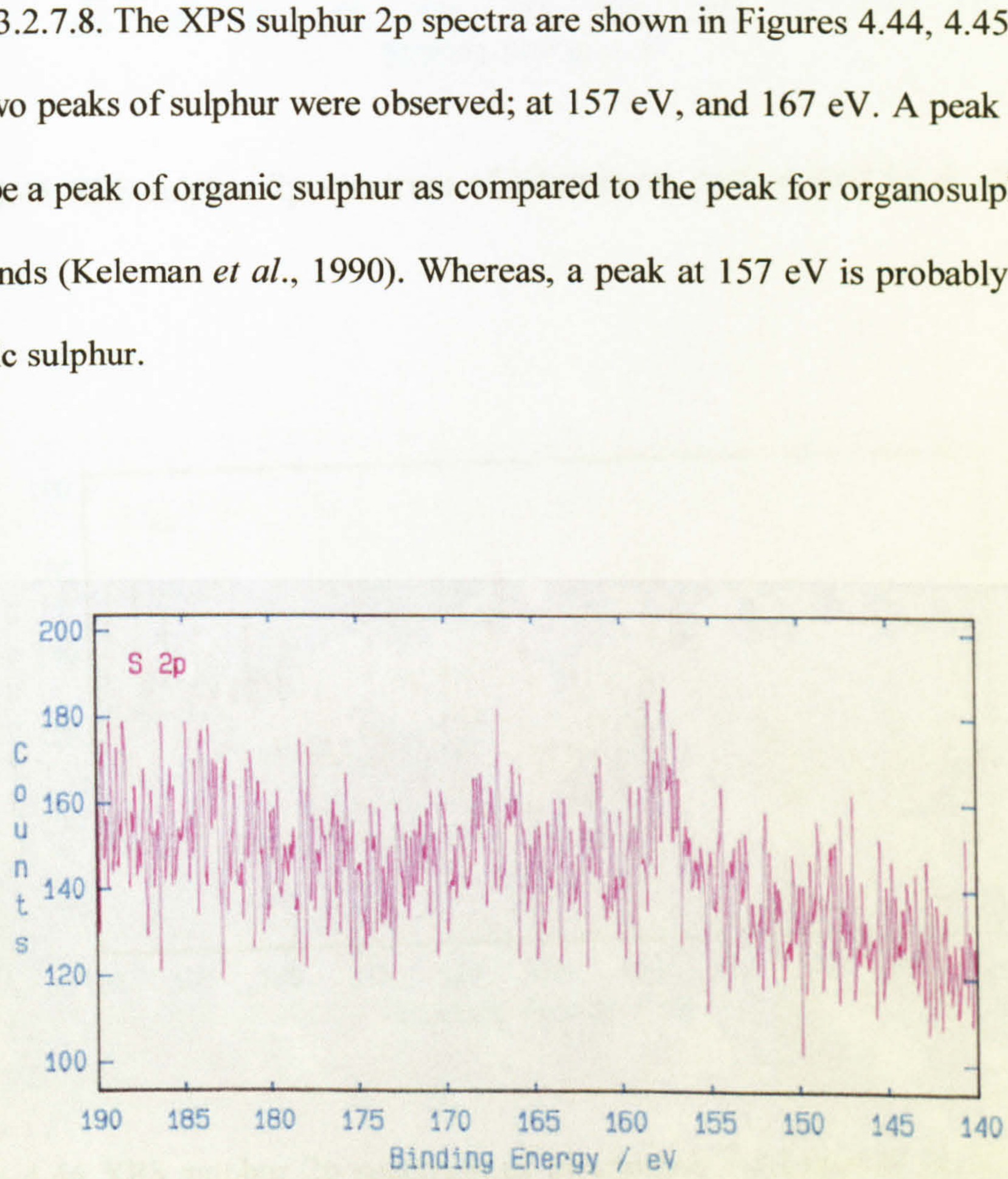


Figure 4.44 XPS sulphur 2p spectrum of untreated bituminous coal.

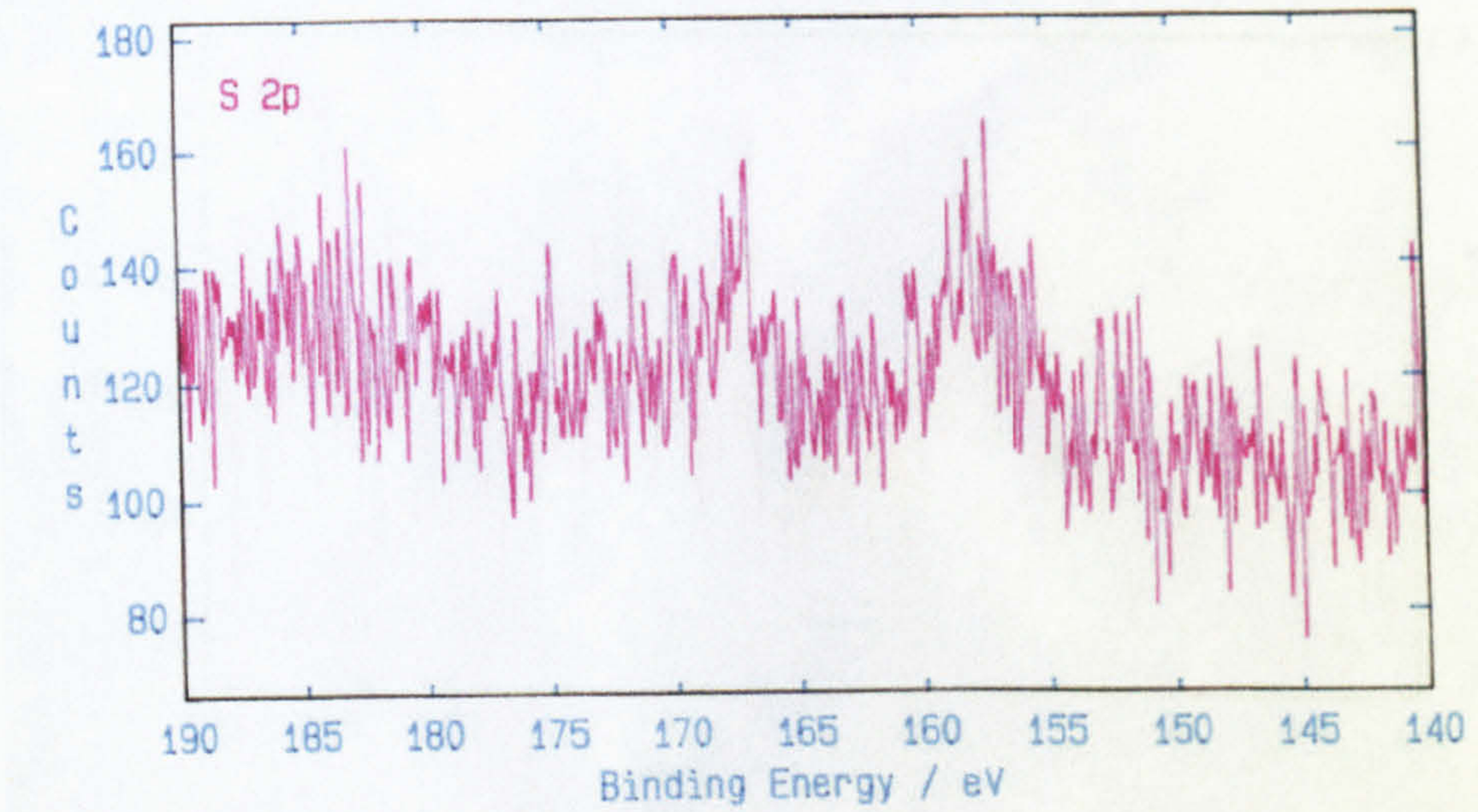


Figure 4.45 XPS sulphur 2p spectrum of bituminous coal treated by *R. erythropolis* IGTS8.

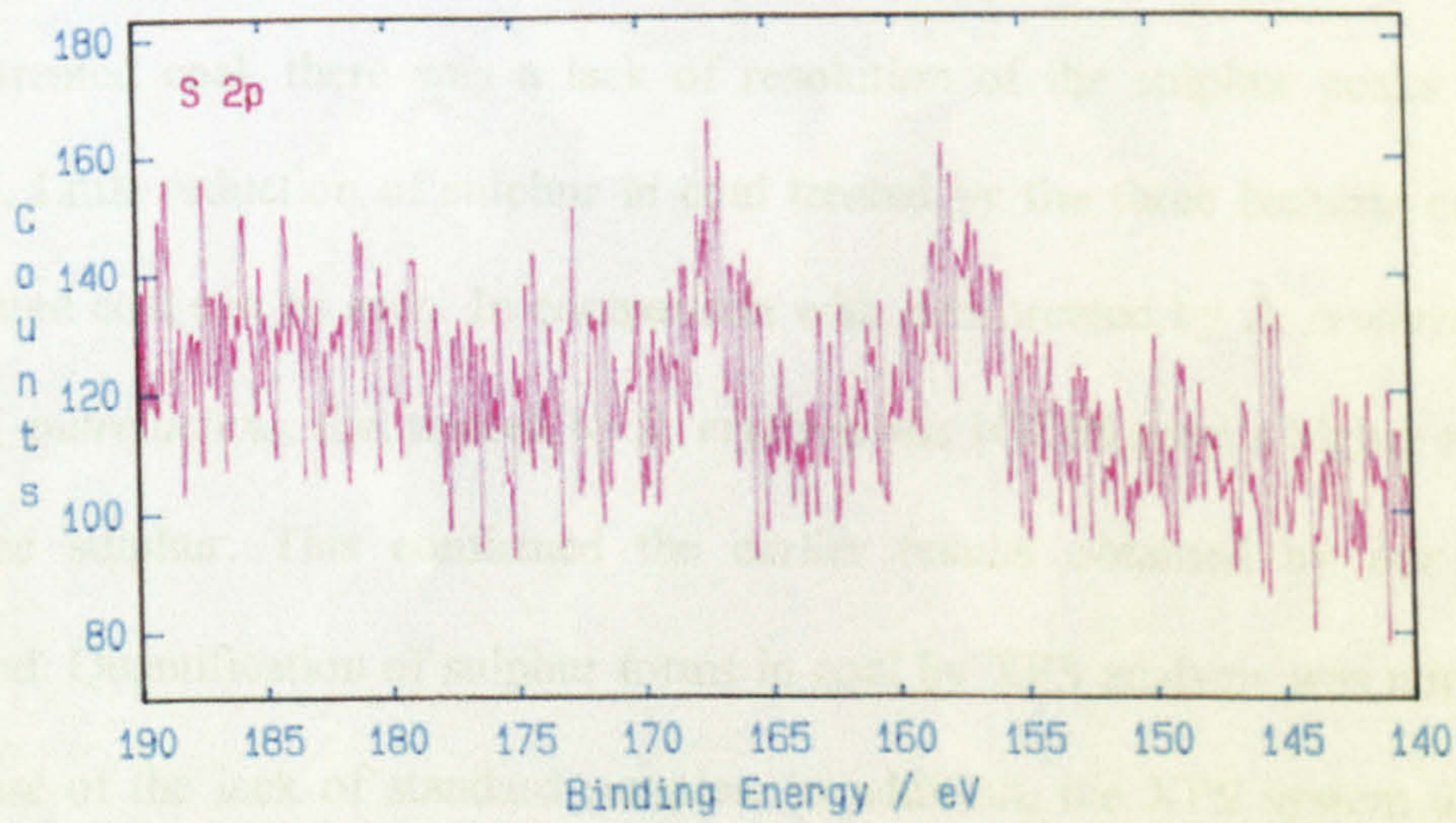


Figure 4.46 XPS sulphur 2p spectrum of bituminous coal treated by *R. erythropolis* X309.

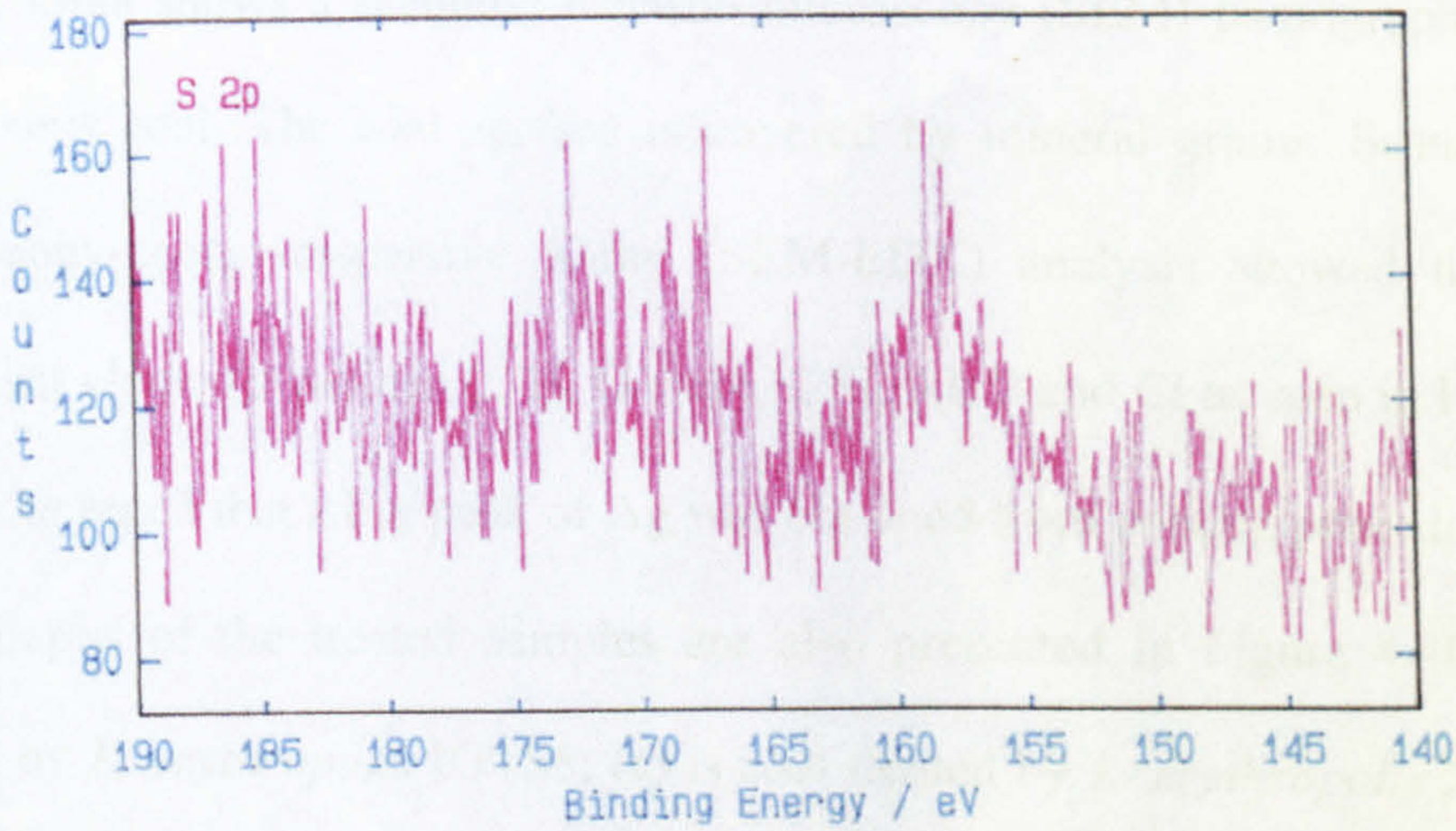


Figure 4.47 XPS sulphur 2p spectrum of bituminous coal treated by *S. putrefaciens*.

In untreated coal, there was a lack of resolution of the sulphur peaks (Davidson, 1994). Little reduction of sulphur in coal treated by the three bacteria compared to untreated coal can be seen. In comparison with coal treated by *R. erythropolis* X309 and *S. putrefaciens*, that treated by *R. erythropolis* IGTS8 gave a higher reduction of organic sulphur. This confirmed the earlier results obtained by our destructive method. Quantification of sulphur forms in coal by XPS analysis was not carried out because of the lack of standard samples. In addition, the XPS system used did not give a high resolution of the sulphur peak. The quantification of each sulphur form in coal carried out in this work was therefore relied on wet chemical extraction followed by ICP spectroscopy analyses.

Figure 4.48a shows a scanning electron microscopy (SEM) photograph of untreated bituminous coal. The coal surface is covered by mineral grains. Scanning electron microscopy-energy-dispersive X-ray (SEM-EDX) analysis showed that the most important elements besides C, H, O were S, Si, Al, P and Cl as seen in Figure 4.49. It should be noted that a big peak of Ag was obtained from coating procedure. The SEM photographs of the treated samples are also presented in Figure 4.48: (b) is coal treated by *R. erythropolis* IGTS8; (c) is coal treated by *R. erythropolis* X309; and (d) is coal treated by *S. putrefaciens*. In comparison with untreated coal, treatment with the bacteria resulted in little physical changes. The most noticeable alteration seems to be that the coal surface was covered by smaller and less mineral grains. These are probably due to the dissolution of minerals and the removal of sulphur as indicated by ICP spectroscopy analyses.

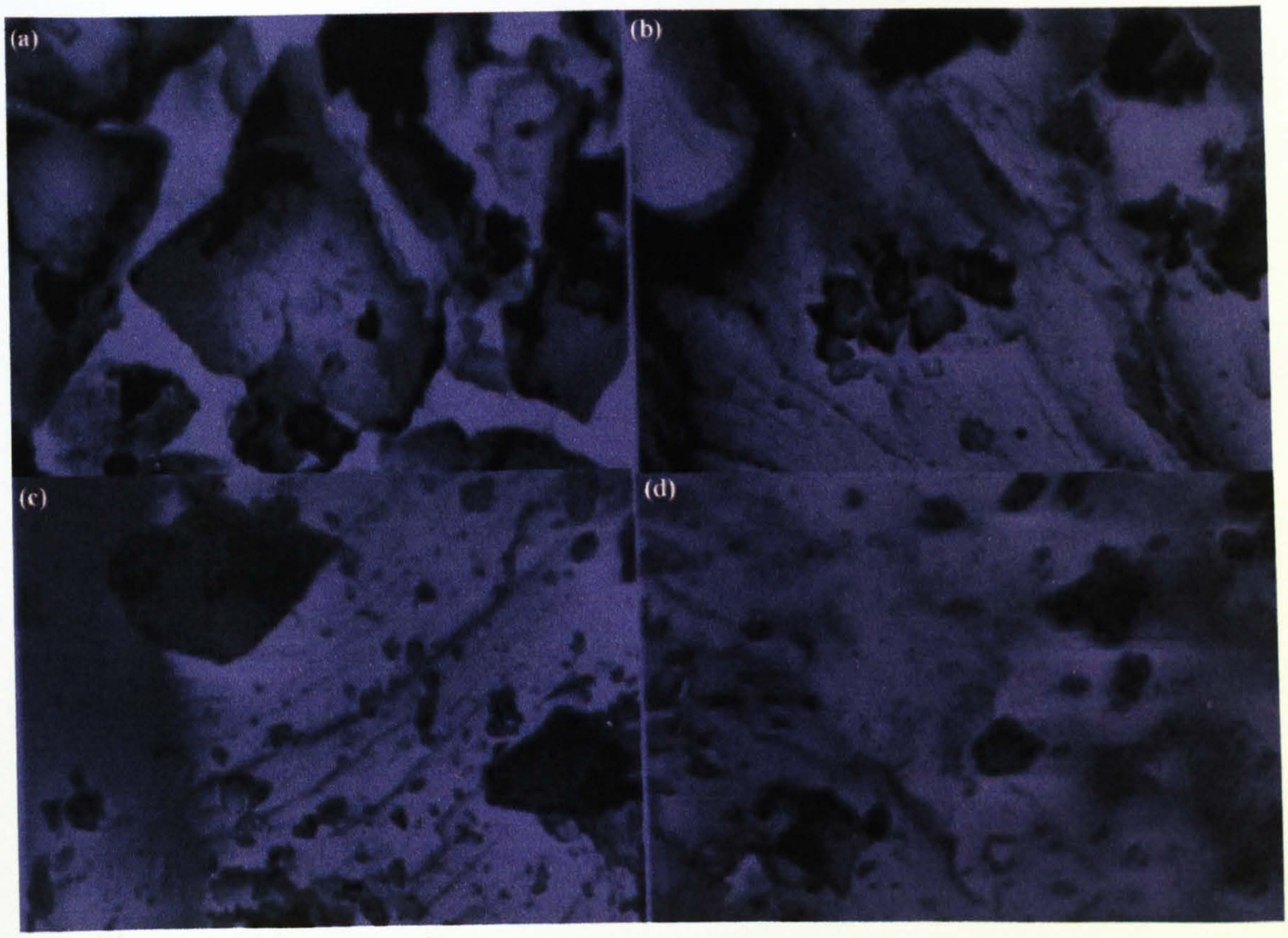


Figure 4.48 Scanning electron microscopy photographs: (a) untreated coal; (b) coal treated by *R. erythropolis* IGTS8; (c) coal treated by *R. erythropolis* X309; and (d) coal treated by *S. putrefaciens*.

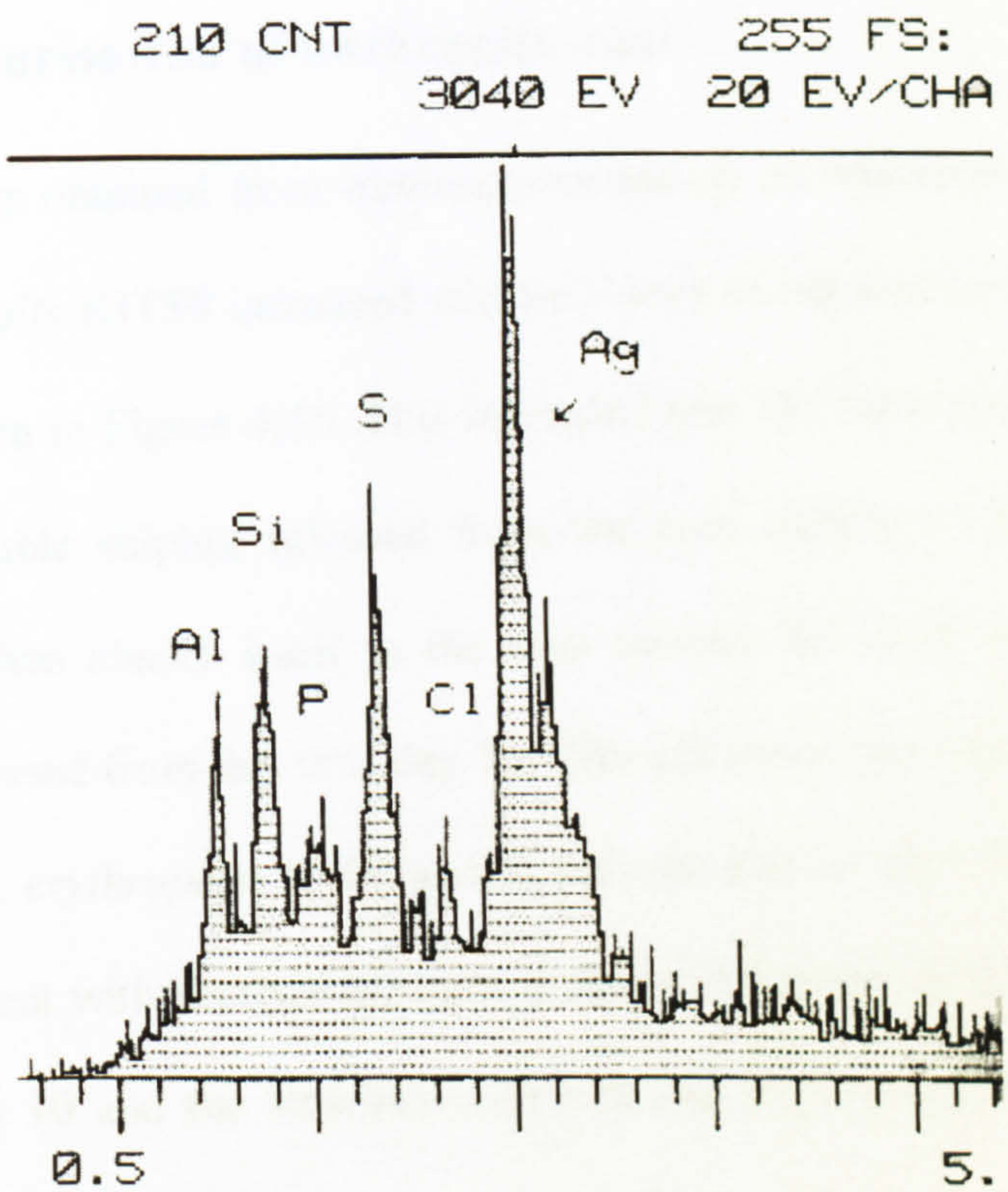


Figure 4.49 Scanning electron microscopy-energy dispersive X-ray analysis.

The bacteria inherent in the bituminous coal were isolated. Two colonies were presented on nutrient agar. One colony was round, regular, smooth, shiny and cream. Another colony was round, regular, smooth, shiny and pale yellow. The two isolates were gram-negative, aerobic, and able to grow at pH 7 and 30°C. With further investigation on the desulphurisation activity of each bacterium, both cream and pale yellow bacteria could grow with elemental sulphur or inorganic sulphur (sodium sulphate) as the sole source of sulphur. But they could not grow with the organic sulphur compounds as the sole source of sulphur.

4.3.2 Biodesulphurisation of anthracite coal

Similar to the results obtained from biodesulphurisation of bituminous coal, the free cells of *R. erythropolis* IGTS8 increased within 3 days of incubation before reduction was observed as seen in Figure 4.50. This indicated that the bacterium could possibly grow by using soluble sulphur released from the coal sample to BSM in its early growth state and then absorb itself to the coal sample for more sulphur since the attached cells increased from day 0 to day 10. This phenomenon was also observed in the treatment by *R. erythropolis* X309 and *S. putrefaciens* as seen in Figure 4.51 and 4.52. In the treatment with bacteria inherent in anthracite coal, the free cells increased from day 0 to day 10 and the attached cells reduced from day 0 to day 10 (Figure 4.53).

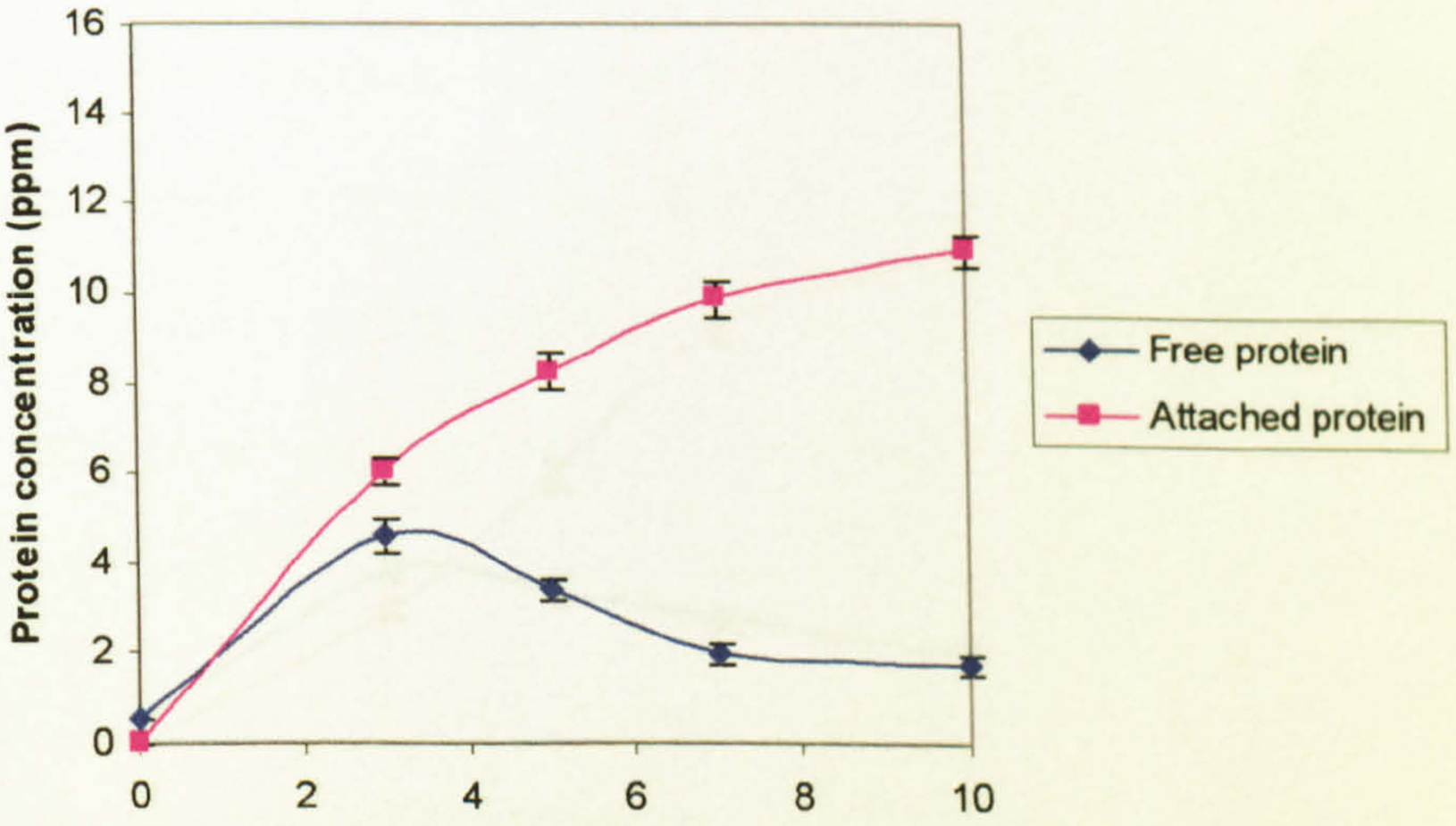


Figure 4.50 Growth of *R. erythropolis* IGTS8 on anthracite coal. Free protein and attached protein were analysed using protein assay. Each point is a mean of three replicates, error bar is a standard deviation, and LSD (P=0.05) = 9.24.

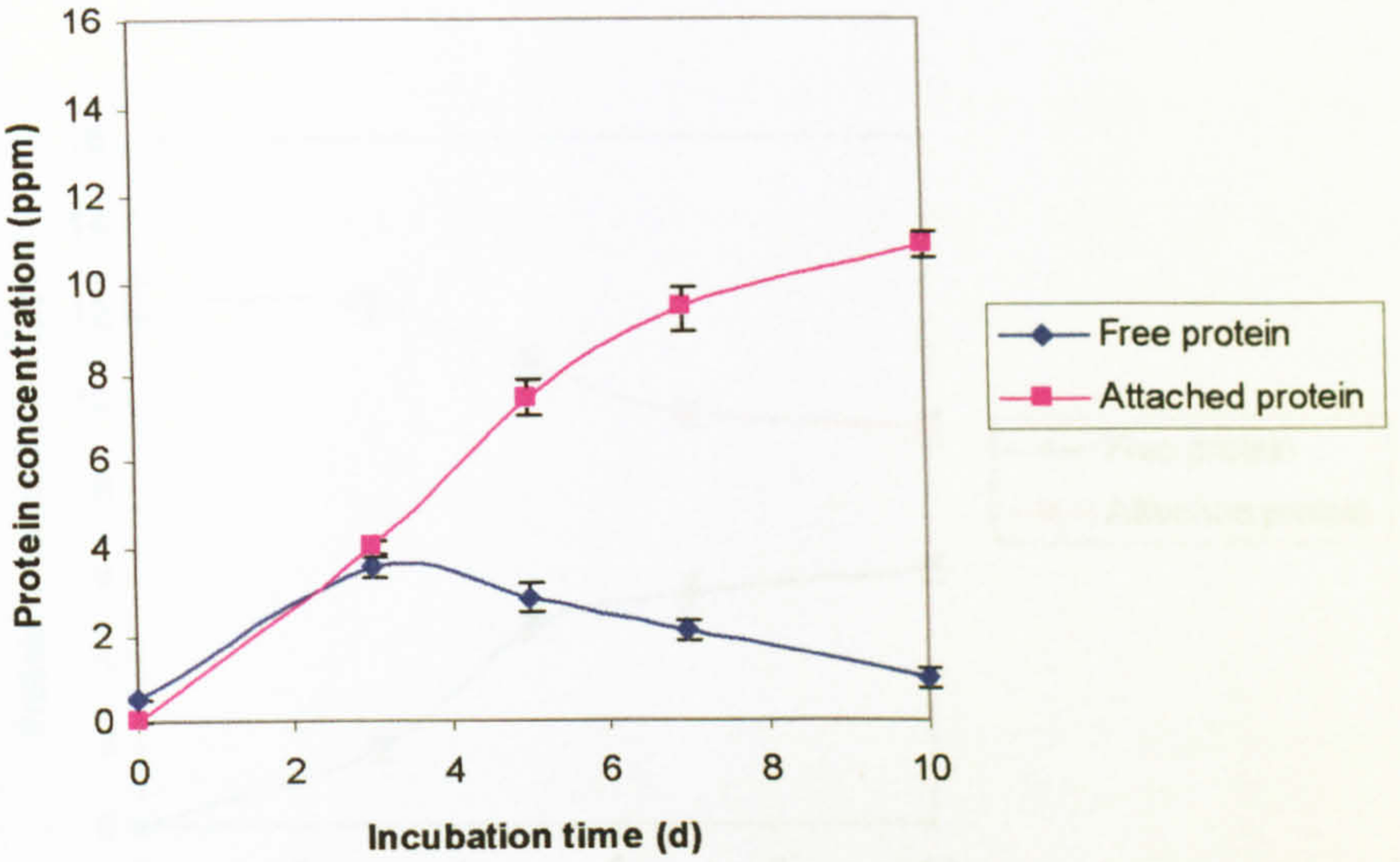


Figure 4.51 Growth of *R. erythropolis* X309 on anthracite coal. Free protein and attached protein were analysed using protein assay. Each point is a mean of three replicates, error bar is a standard deviation, and LSD (P=0.05) = 7.18.

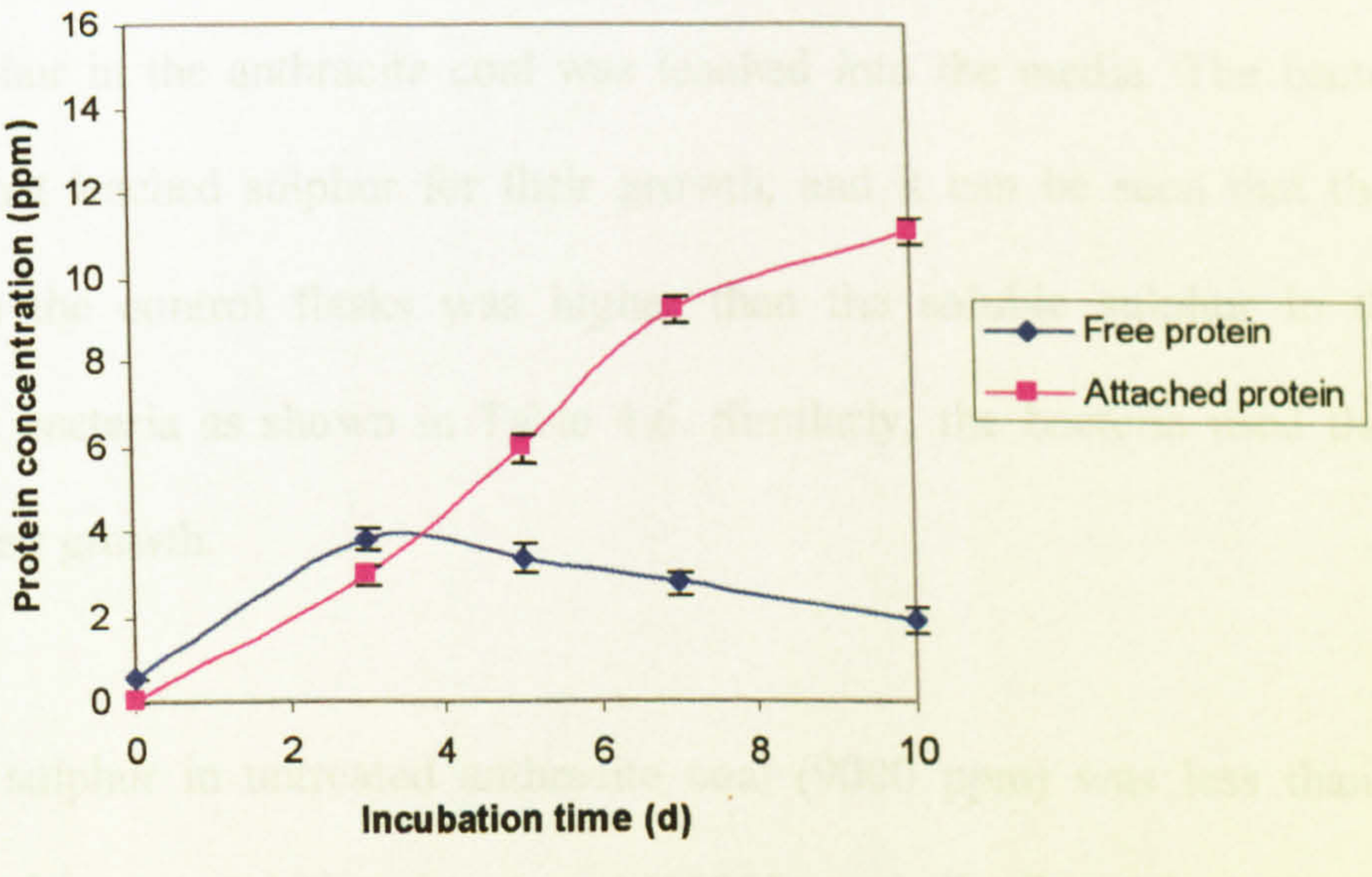


Figure 4.52 Growth of *S. putrefaciens* on anthracite coal. Free protein and attached protein were analysed using protein assay. Each point is a mean of three replicates, error bar is a standard deviation, and LSD (P=0.05) = 7.41.

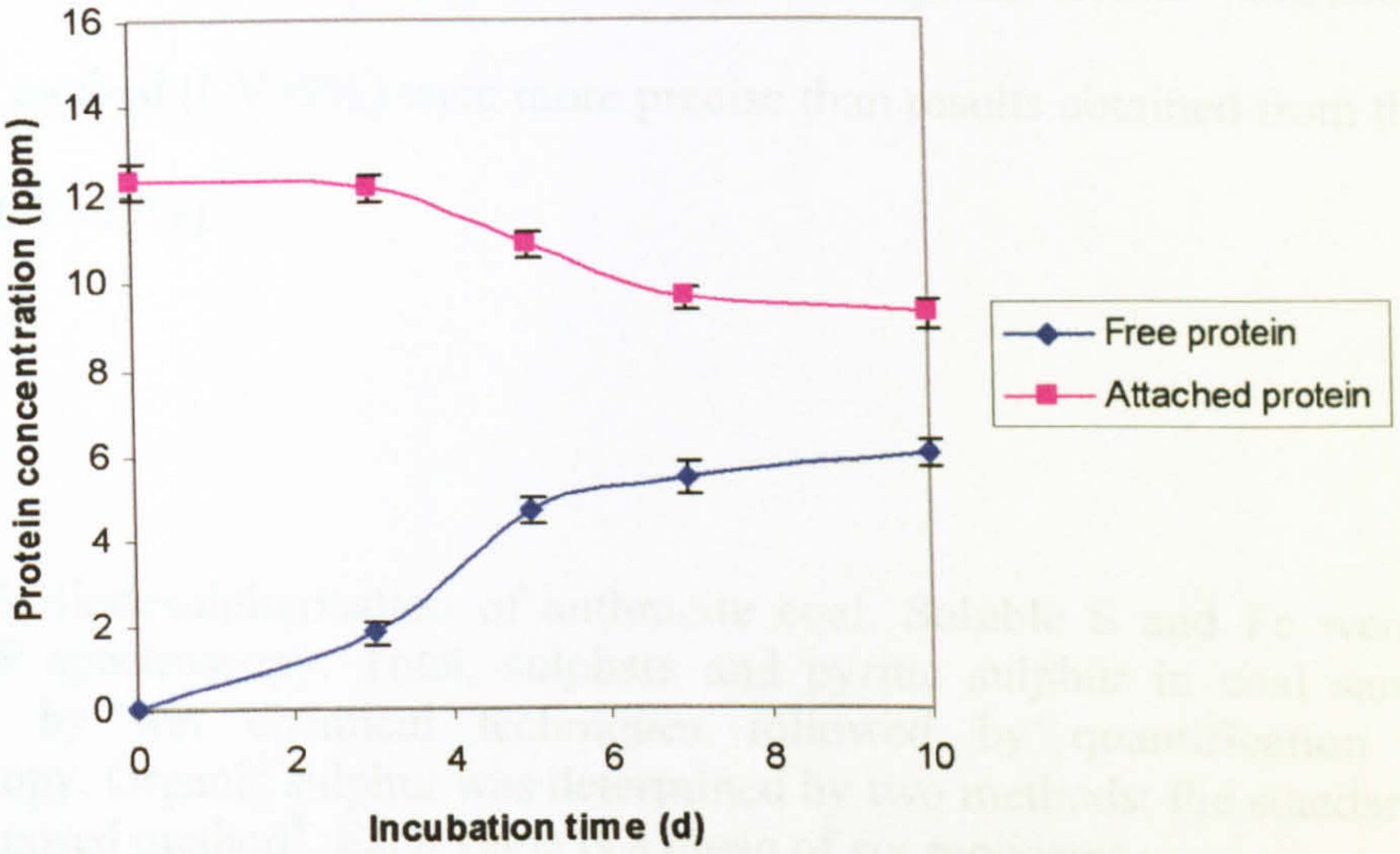


Figure 4.53 Growths of the bacteria inherent in anthracite coal. Free protein and attached protein were analysed using protein assay. Each point is a mean of three replicates, error bar is a standard deviation, and LSD (P=0.05) = 4.65.

Some sulphur in the anthracite coal was leached into the media. The bacteria used some of that leached sulphur for their growth, and it can be seen that the soluble sulphur in the control flasks was higher than the soluble sulphur in the flasks containing bacteria as shown in Table 4.6. Similarly, the bacteria used the leached iron for their growth.

The total sulphur in untreated anthracite coal (9000 ppm) was less than the total sulphur in the untreated bituminous coal (15300 ppm). By *R. erythropolis* IGTS8, the total sulphur was reduced to 7100 ppm in 10 days which was a 21.1% reduction (Table 4.7). For *R. erythropolis* X309, the total sulphur was reduced to 7130 ppm in 10 days (20.8% reduction). *S. putrefaciens* decreased the total sulphur to 7140 ppm in

10 days (20.7% reduction). With bacteria inherent in the coal, the total sulphur reduction was 20.1%, and without bacteria the total sulphur reduction was 18.6% in 10 days. Regarding determination of organic sulphur, results obtained from the proposed method (CV=9%) were more precise than results obtained from the standard method (CV=17%).

Table 4.6 Biodesulphurisation of anthracite coal. Soluble S and Fe were analysed using ICP spectroscopy. Total, sulphate and pyritic sulphur in coal samples were extracted by wet chemical techniques followed by quantification with ICP spectroscopy. Organic sulphur was determined by two methods; the standard method[7], and a proposed method[8]. Each value is a mean of six replicates.

Inoculum	Day	Soluble S (ppm) CV=1%	Soluble Fe (ppm) CV=1%	Total S (ppm) CV=4%	Sulphate S (ppm) CV=7%	Pyritic S (ppm) CV=7%	Organic S[7] (ppm) CV=17%	Organic S[8] (ppm) CV=9%
IGTS8	0	0	1	9000	1500	1700	5800	5700
	3	120	0	8300	1220	1430	5650	5670
	5	157	0	7390	950	1020	5420	5600
	7	172	0	7200	880	940	5380	5450
	10	190	0	7100	850	910	5340	5420
X309	0	0	1	9000	1500	1700	5800	5700
	3	120	0	8350	1210	1490	5650	5680
	5	152	0	7400	934	1076	5390	5630
	7	164	0	7240	868	990	5382	5550
	10	180	0	7130	832	935	5363	5490
NCIMB 8768	0	0	1	9000	1500	1700	5800	5700
	3	113	0	8320	1190	1480	5650	5680
	5	161	0	7340	900	1090	5350	5640
	7	198	0	7190	842	994	5354	5590
	10	212	0	7140	814	941	5385	5530
Bacteria inherent in the coal	0	0	1	9000	1500	1700	5800	5700
	3	125	0	8300	1120	1440	5740	5690
	5	176	0	7380	888	1032	5460	5660
	7	210	0	7230	852	999	5379	5620
	10	240	0	7190	824	955	5411	5590
Control (without bacteria)	0	0	1	9000	1500	1700	5800	5700
	3	310	90	8370	1110	1630	5630	5693
	5	795	94	7530	855	1167	5508	5695
	7	852	100	7350	838	1095	5417	5685
	10	880	106	7330	805	1042	5483	5680

Table 4.7 Reduction of sulphur in anthracite coal after biodesulphurisation. Sulphur reduction was calculated from data in Table 4.6. Organic sulphur was determined by the proposed method.

Inoculum	Day	Total S Reduction (%)	Sulphate S Reduction (%)	Pyritic S Reduction (%)	Organic S Reduction (%)
IGTS8	3	7.8	18.7	15.9	0.6
	5	17.9	36.7	40.0	1.9
	7	20.0	41.3	44.7	4.8
	10	21.1	43.3	46.5	5.4
X309	3	7.2	19.3	12.4	0.4
	5	17.2	37.7	36.7	1.4
	7	19.6	42.1	41.8	2.9
	10	20.8	44.5	45.0	4.0
NCIMB 8768	3	7.6	20.7	12.9	0.4
	5	18.4	40.0	35.9	1.1
	7	20.1	43.9	41.5	1.9
	10	20.7	45.7	44.7	3.0
Bacteria inherent in the coal	3	7.8	25.3	15.3	0.2
	5	18.0	40.8	39.3	0.7
	7	19.7	43.2	41.2	1.4
	10	20.1	45.1	43.8	1.9
Control (without bacteria)	3	7.0	26.0	4.1	0.1
	5	16.3	43.0	31.4	0.1
	7	18.3	44.1	35.6	0.3
	10	18.6	46.3	38.7	0.4

As seen in Table 4.7, biodesulphurisation of anthracite coal incresed with the incubation time, comparison of reduction of sulphur by each treatment was then carried out after a 10 day of incubation (Figure 4.54). There was no significant difference between reduction of total sulphur by the bacteria and control. *R. erythropolis* IGTS8, *R. erythropolis*, or *S. putrefaciens* gave significantly greater desulphurisation of pyritic sulphur, and organic sulphur over the control. Bacteria inherent in the coal gave a significantly greater desulphurisation of pyritic sulphur over the control.

There were no significant differences between reduction of total sulphur, sulphate sulphur, pyritic sulphur, or organic sulphur by the three bacteria. In comparison with treatment by bacteria inherent in the coal, only *R. erythropolis* IGTS8 gave significantly greater reduction of pyritic sulphur and organic sulphur.

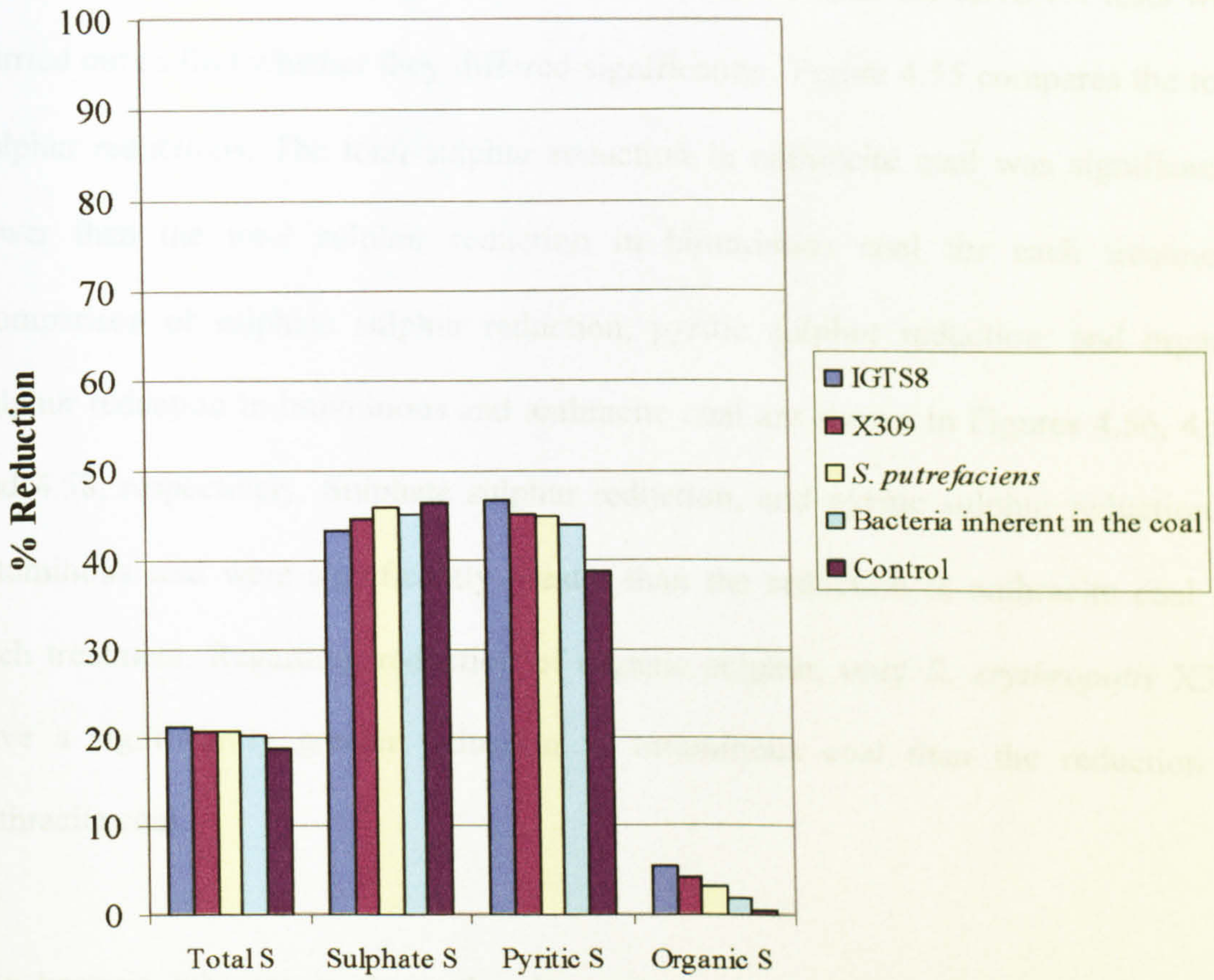


Figure 4.54 Reduction of sulphur in anthracite coal at 10 day incubation. LSD (P=0.05) = 2.6.

To compare the results obtained from biodesulphurisation of anthracite coal with those obtained from biodesulphurisation of bituminous coal, the ANOVA tests were carried out to find whether they differed significantly. Figure 4.55 compares the total sulphur reductions. The total sulphur reduction in anthracite coal was significantly lower than the total sulphur reduction in bituminous coal for each treatment. Comparison of sulphate sulphur reduction, pyritic sulphur reduction, and organic sulphur reduction in bituminous and anthracite coal are shown in Figures 4.56, 4.57, and 4.58, respectively. Sulphate sulphur reduction, and pyritic sulphur reduction in bituminous coal were significantly greater than the reduction in anthracite coal for each treatment. Regarding reduction of organic sulphur, only *R. erythropolis* X309 gave a significantly greater reduction in bituminous coal than the reduction in anthracite coal.

The bacteria inherent in the anthracite coal were isolated. Only one colony was presented on nutrient agar. It was round, regular, smooth, shiny and cream. It was gram-negative, aerobic, and able to grow at pH 7 and 30°C. With further investigation on the desulphurisation activity of this bacterium, it could grow with elemental sulphur or sodium sulphate as the sole source of sulphur. However, it could not grow with the organic sulphur compounds as the sole source of sulphur.

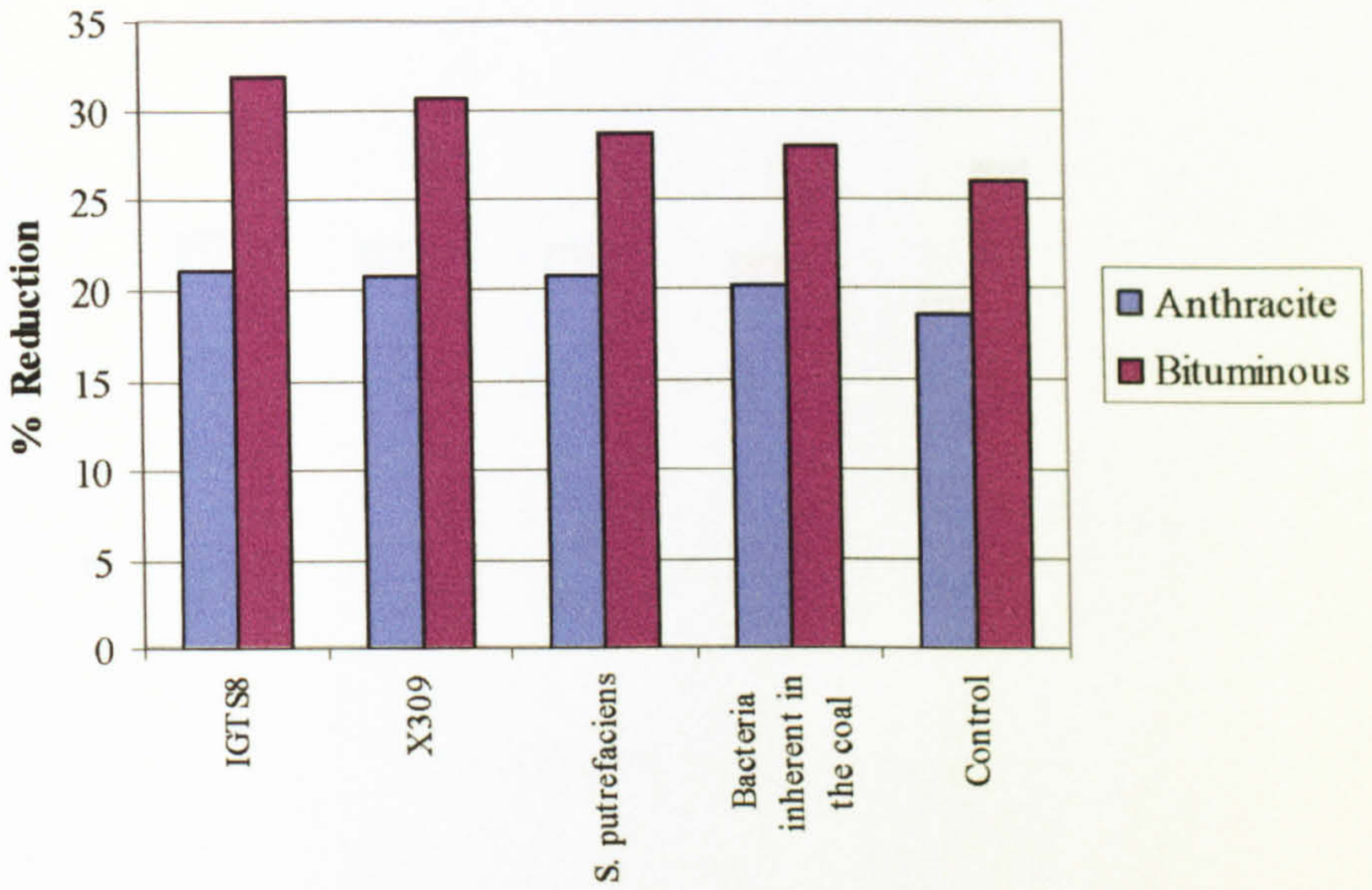


Figure 4.55 Reduction of total sulphur in anthracite and bituminous coal. LSD (P=0.05) = 2.6.

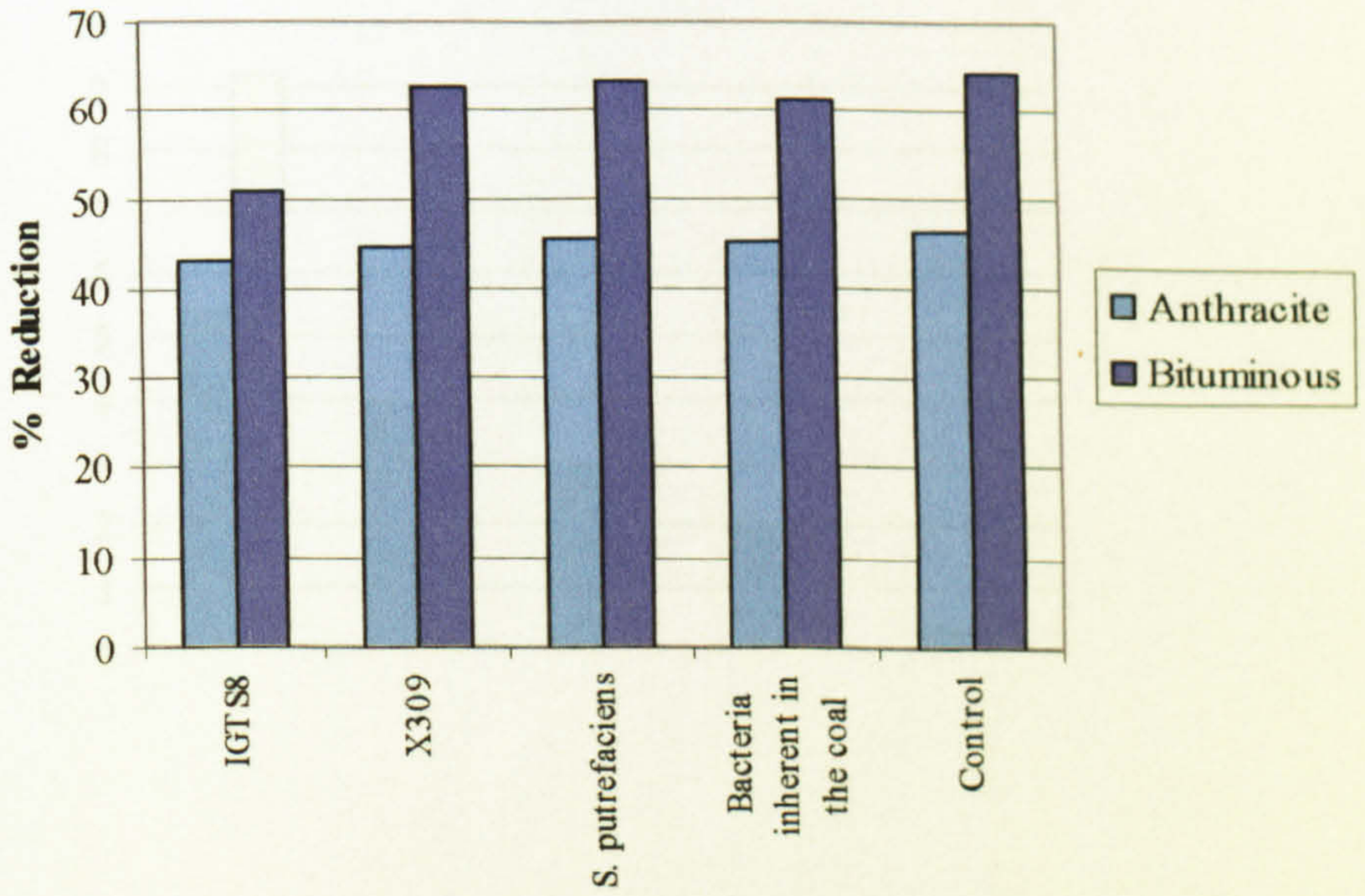


Figure 4.56 Reduction of sulphate sulphur in anthracite and bituminous coal. LSD (P=0.05) = 5.8.

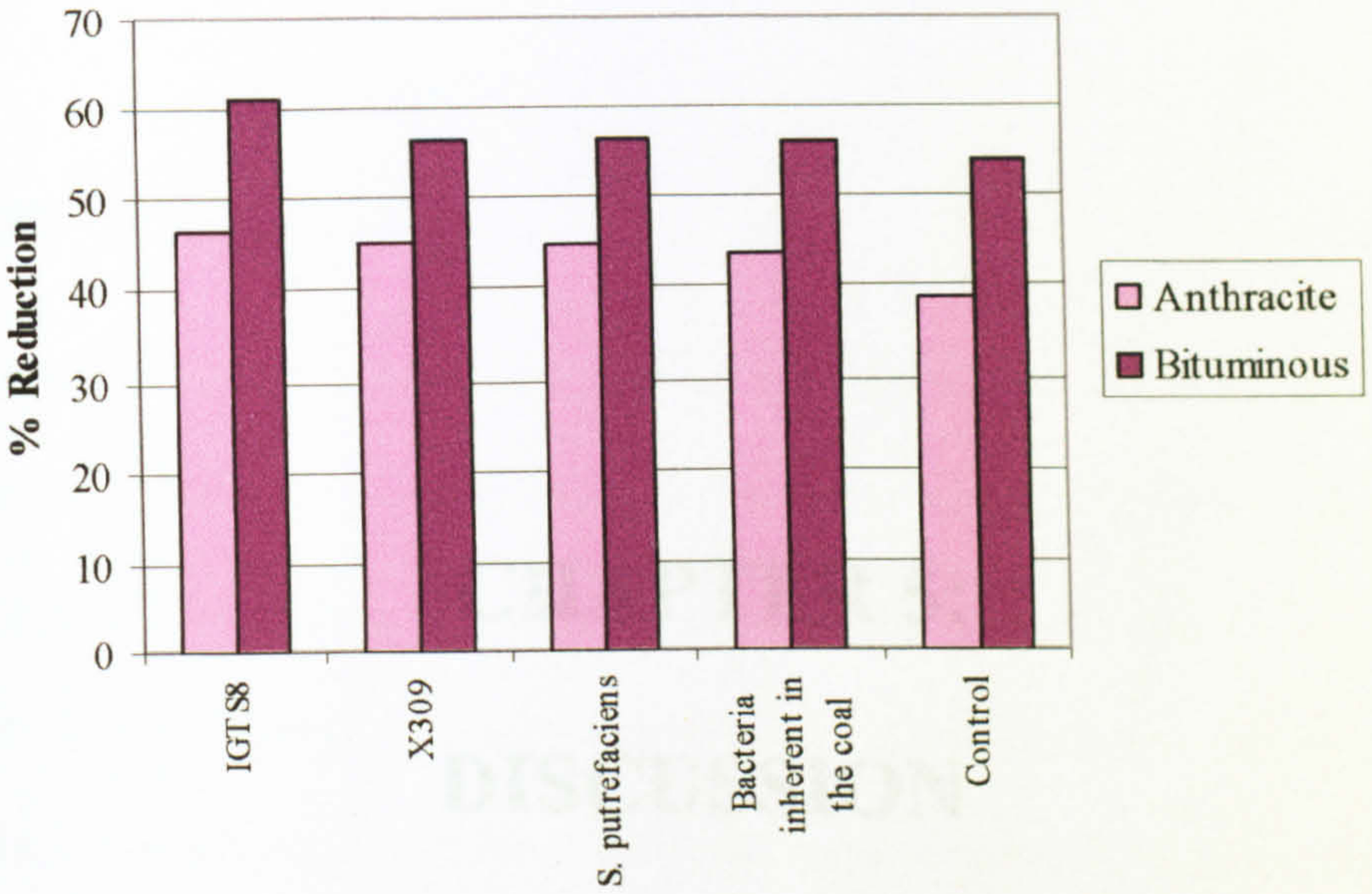


Figure 4.57 Reduction of pyritic sulphur in anthracite and bituminous coal. LSD (P=0.05) = 4.1.

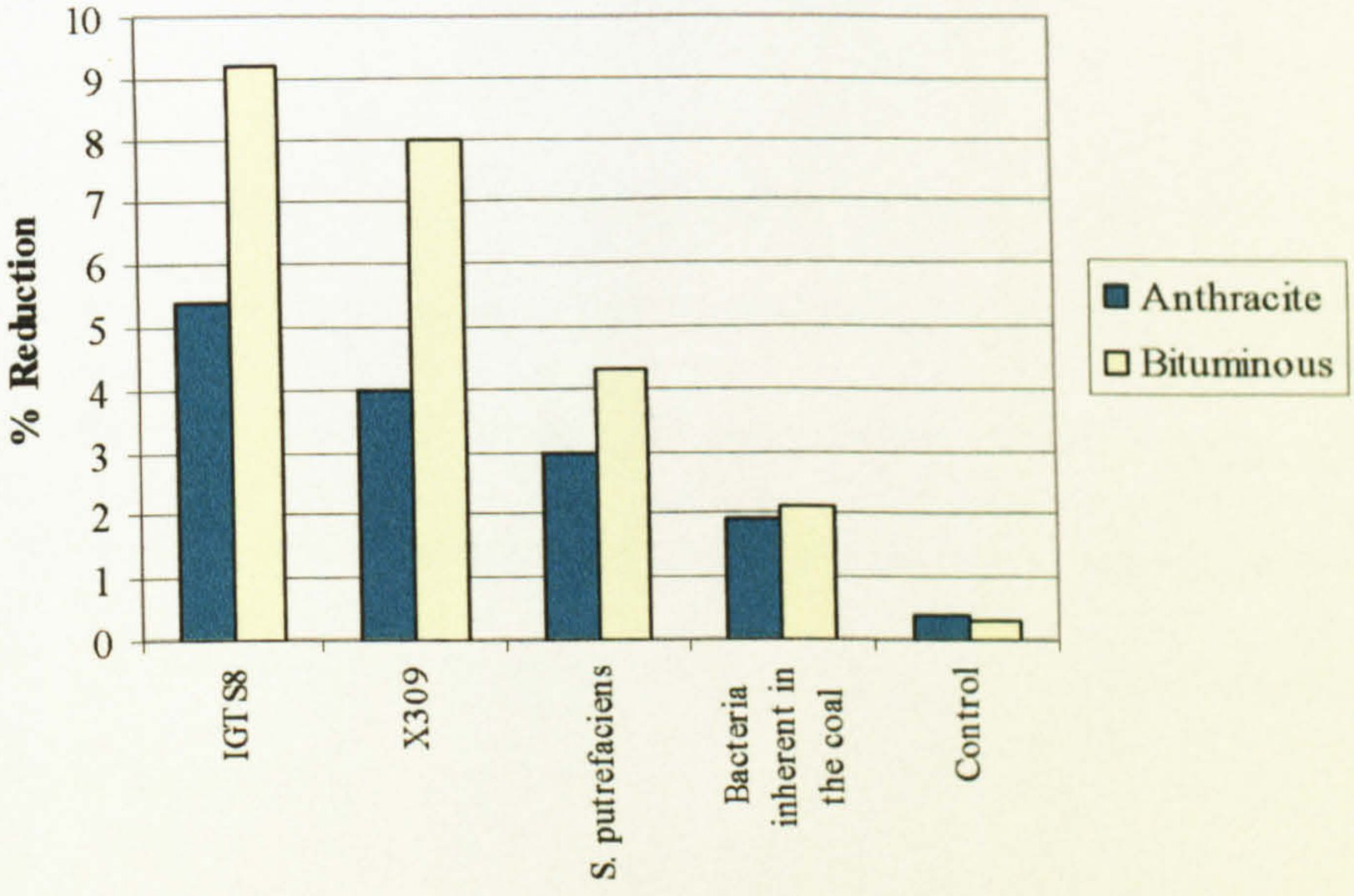


Figure 4.58 Reduction of organic sulphur in anthracite and bituminous coal. LSD (P=0.05) = 4.4.

CHAPTER 5:

DISCUSSION

5.1 BIODESULPHURISATION OF MODEL COMPOUNDS

5.1.1 DBT desulphurisation

In the present study, *R. erythropolis* strains IGTS8 and X309 could grow by using DBT as a sole source of sulphur. Products detected were 2-hydroxybiphenyl (HBP), and sulphite. DBT-sulphone was transiently observed in the medium. Degradation of DBT related to the growth of bacteria. HBP production increased dramatically in the early exponential growth phase before the rate was reduced in the stationary growth phase. Nevertheless, HBP accumualtion did not decrease during the stationary growth phase, implying that HBP was a dead end product of DBT degradation. According to this, the DBT degradation pathway was:

DBT → DBT-sulphone → HBP + sulphite.

This pathway is similar to the 4S pathway proposed by Bressler *et al.* (1998). The difference is that two metabolites (DBT-sulphoxide, and 2-hydroxybiphenyl 2'-sulphinate) in the 4S pathway are missing in our pathway. It has to be noted that these two metabolites were hardly detected by experiments previously (Lee *et al.*, 1995; Maxwell & Yu, 2000). This is not surprising because the two metabolites are intermediates in the pathway.

In the present study, HBP appearance in the medium was almost equimolar to DBT degradation. The concentration of sulphite detected was low and not stoichiometric to the amount of DBT converted to HBP. This may be because the bacteria used some sulphite in the medium for their growth. Kayser *et al.* (1993) also demonstrated that DBT was converted to HBP in near-stoichiometric amount by *R. erythropolis* IGTS8. In addition, they reported that sulphur liberated from DBT was chiefly found associated with, or incorporated into the bacterial cells in experiments involving growing cells. This phenomenon was also observed in DBT desulphurisation by *R. erythropolis* D-1 (Izumi *et al.*, 1994).

Regarding comparison of DBT desulphurisation efficiency of the three bacterial strains, *R. erythropolis* strains IGTS8 and X309 gave similar DBT desulphurisation efficiency. In addition, these two strains of *R. erythropolis* were considered to have better desulphurisation efficiency than *S. putrefaciens* (NCIMB 8768), which lost its desulphurisation ability during the investigation. Further discussion of the loss of the ability of *S. putrefaciens* will be given later.

The effect of some parameters on DBT desulphurisation was studied and the results obtained can be discussed as follows:

A. Effect of temperature

At three different temperatures investigated: 25, 30, and 35°C, the optimum temperature for growth and DBT desulphurisation activity of *R. erythropolis* IGTS8, *R. erythropolis* X309, or *S. putrefaciens* was found to be 30°C. However, the bacteria did not show significant differences of bacterial growth or DBT desulphurisation activities between 25 and 30 °C. In contrast, the bacteria showed lower bacterial growth or DBT desulphurisation activities at 35°C. The reason why DBT desulphurisation activities of these bacteria were inhibited at 35°C is probably because the three bacteria are mesophilic, and not tolerant to higher temperatures. Desulphurisation abilities of some strains of other mesophilic bacteria, *Thiobacillus ferrooxidans*, were also reported to be limited at 35°C (Norris, 1990). The experiments elsewhere on *R. erythropolis* IGTS8 were generally performed at 30°C (Patel *et al.*, 1997; Kayser *et al.*, 1993; Gilbert *et al.*, 1998). *R. erythropolis* X309 was isolated at 25°C (Lee *et al.*, 1995), and *S. putrefaciens* was isolated at 27°C (Pivnick, 1955). Most of these previous studies did not provide further information on the effect of temperature.

B. Effect of ethanol

DBT as a sole source of sulphur supported more rapid growth of the bacteria when it was introduced as a solution (by dissolving in ethanol before addition) rather than as a powder into the medium. The amount of HBP produced from DBT degradation was also higher when DBT was introduced as a solution. This is probably because water solubility of DBT is low, 5.7 μM (Marzona *et al.*, 1997). Its bioavailability in aqueous medium was increased when it was dissolved in ethanol before addition. Furthermore,

ethanol was an effective carbon source for the three bacteria. With a supplementation of ethanol, the bacterial growth rates were greater than with a supplementation of glycerol alone.

Wang & Krawiec (1996) working with *R. erythropolis* N1-36 also reported that DBT or DBT-sulphone as a sole source of sulphur supported more rapid growth of N1-36 if the DBT or DBT-sulphone was introduced in ethanol rather than as a powder or enmeshed in nylon filters. The cultures containing 0.1 or 1% ethanol exhibited a shorter lag time and more rapid exponential growth than cultures grown with glucose alone. Nevertheless, the presence of ethanol at concentrations higher than 1% produced progressively decreased exponential growth rates and slightly reduced overall growth. Therefore, it can be concluded that ethanol can promote bacterial growth and desulphurisation activities at low concentrations and can inhibit bacterial growth and desulphurisation activities at high concentrations.

C. Effect of DBT concentration

At low concentrations of reactant (<10 mM of DBT), growth of each culture was not related to DBT concentration. However, the bacterial growth was inhibited at higher concentrations of DBT. This was probably because the bacteria could not tolerate high concentrations of DBT, 10 mM seems to be a toxic level.

Kayser *et al.* (1993) also reported that the desulphurisation activity of *R. erythropolis* IGTS8 was inhibited at 20 mM of DBT. Moreover, they cited that the minimum sulphur requirement for growth of *R. erythropolis* IGTS8 was 0.1 mM. According to work by Wang & Krawiec (1996), neither DBT nor DBT-sulphone inhibited cell

growth of *R. erythropolis* N1-36 at concentrations up to 0.2 mM. No experiment on the effect of higher concentrations of DBT or DBT-sulphone (>0.2 mM) was reported by Wang & Krawiec (1996). There are no earlier reports on the maximum concentration of DBT that *R. erythropolis* X309 and *S. putrefaciens* can tolerate. At this time, we can only say that the three bacteria are sensitive to high concentration of the reactant, DBT.

D. Effect of HBP concentration

In the present study, bacterial growth and DBT desulphurisation activity of each strain investigated were inhibited with additon of external HBP at 0.1 mM or higher concentrations. The greater addition of HBP was the greater inhibition. This might be attributed to an inhibition effect of HBP on desulphurisation enzymes of the bacteria.

Kayser *et al.* (1993) also proposed that HBP could possibly inhibit the cell growth and DBT desulphurisation activity of *R. erythropolis* IGTS8. Similarly, Lee *et al.* (1995) reported that HBP was toxic to *R. erythropolis* ECRD-1 at a concentration of 50 mg/l. Recently, Maxwell & Yu (2000) demonstrated that the bacterial strain UST-3 showed poor growth when 0.4 mM of HBP was added into the medium, and no DBT desulphurisation was observed. Therefore, this confirms that HBP, which is a final product in the mechanism of DBT desulphurisation, can depress growth and the DBT desulphurisation activity of the bacteria.

E. Effect of sulphate

With a supplement of inorganic sulphur, sodium sulphate, the bacteria grew better than with a supplement of organic sulphur, DBT. This is not surprising since sodium sulphate is a more bioavailable sulphur source than DBT. Nevertheless, a supplement of sulphate in the medium containing DBT led to a depression of DBT desulphurisation activity of each bacterium investigated. The DBT desulphurisation activity of *R. erythropolis* IGTS8 was inhibited at 0.10 mM, or higher concentrations of sulphate. The DBT desulphurisation activity of *R. erythropolis* X309 was decreased at 0.05, 0.10, or 0.15 mM sulphate, and completely inhibited at 0.30 mM sulphate.

Depression of the DBT desulphurisation activity of *R. erythropolis* IGTS8 in the presence of sulphate was reported before by Kayser *et al.* (1993). They cited that the strain IGTS8 did not express the DBT desulphurisation trait in the presence of 20 mM sulphate. Lee *et al.* (1995) reported that *R. erythropolis* ECRD-1 was inhibited by 1 mM sulphate. Maxwell & Yu (2000) found that the DBT desulphurisation activity of the bacterial strain UST-3 was inhibited in the presence of sulphate, with 0.1 mM being the critical level.

This fact clearly indicates that sulphate can inhibit the DBT desulphurisation activity of bacteria. The inhibition is attributed to poor induction of desulphurisation enzymes in the bacteria because sulphate is a better sulphur source for most bacteria. In addition, from this investigation *R. erythropolis* strain IGTS8 was less sensitive to inhibit by sulphate than strain X309.

5.1.2 DBT desulphurisation in two-phase system

In the present study, DBT desulphurisation rates in the two-phase system (75% water: 25% hexadecane) were significantly greater than DBT desulphurisation rates in the aqueous system. This supports the earlier observation by some researchers. For instance, DBT desulphurisation rates were increased in the presence of 40-50% n-tetradecane or kerosine (Ohshiro *et al.*, 1996), 96% hexadecane (Kaufman *et al.*, 1998), or 50% diesel (Pacheco *et al.*, 1999). Therefore, it looks promising to carry out the biodesulphurisation process in the presence of a hydrophobic solvent. Further investigation on parameters of the process is thus worthwhile.

Regarding the effect of inoculum age on DBT desulphurisation activity of each culture investigated, it was found that the desulphurisation activity of *S. putrefaciens* was significantly lost when it was kept at –80°C for 4 weeks. There was no significant activity loss of both strains *of R. erythropolis* during this study.

The effect of storage conditions on DBT desulphurisation activity of *R. erythropolis* IGTS8 was studied earlier by Patel *et al.* (1997). In their work the activity was compared between wet bacteria after harvesting and freeze-dried bacteria. Following freeze-drying the strain lost 21% of its desulphurisation activity after harvesting. From our results, the strain IGTS8 lost only 1.5% of its activity observed after 4 weeks of storage at –80°C (kept as wet bacteria). This indicates that the strain lost more activity when prepared as freeze-dried bacteria. The use of freeze-dried bacteria is needed sometimes since they can be produced at one location for use at another location. Nevertheless, to obtain maximum DBT desulphurisation the bacteria should be prepared on site, and kept as wet bacteria rather than freeze-dried bacteria.

5.1.3 DBT-sulphone desulphurisation

Similar to the results obtained from DBT desulphurisation experiment, *R. erythropolis* strains IGTS8 and X309 could grow by using DBT-sulphone as a sole source of sulphur. Products detected were HBP, and sulphite. HBP production increased dramatically in the early exponential growth phase before the rate was reduced in the stationary growth phase. However, HBP accumualtion did not decrease during the stationary growth phase, implying that HBP was a dead end product of DBT degradation. From these results, DBT-sulphone degradation pathway was:

DBT-sulphone → HBP + sulphite.

Regarding this pathway and the DBT desulphurisation pathway (DBT → DBT-sulphone → HBP + sulphite), DBT-sulphone is certainly an intermediate in the DBT desulphurisation pathway. The mechanism of DBT desulphurisation can be simply explained. The sulphur atom in DBT has two electron pairs, and is easily oxidised. DBT was first oxidised with two oxygen atoms to DBT-sulphone as an intermediate that was further converted to HBP, and sulphite by enzymic breakage of two C-S bonds of DBT-sulphone. HBP was a final product of DBT desulphurisation.

5.1.4 BT desulphurisation

Some studies have suggested that *R. erythropolis* IGTS8 could not desulphurise benzothiophene (BT). For instance, Kayser *et al.* (1993) reported that strain IGTS8 did not grow when using prolonged incubation with BT as a sole sulphur source. Gilbert *et al.* (1998) confirmed this earlier observation by incubating DBT-desulphurisation-competent cells of strain IGTS8 in 50 mM HEPPS buffer containing BT overnight. Ethyl acetate extraction yielded none of the expected BT metabolites. Lee *et al.* (1995) reported that *R. erythropolis* ECRD-1 could grow weakly with BT but there was no report on the degradation of BT and production of BT metabolites. Recently, there was a report on BT desulphurisation by *R. erythropolis* strain KA2-5-1 which is quite similar to strain IGTS8 (Kobayashi *et al.*, 2000). This stimulated the present study to investigate biodesulphurisation of BT.

In the present study, *R. erythropolis* IGTS8 and X309 could grow weakly with a supplement of BT as a sole source of sulphur. BT degradation was determined by HPLC analyses. In addition, an aromatic hydroxyl compound produced from BT degradation via the 4S pathway was observed in some samples. These all show the possibility of BT desulphurisation by the two strains of *R. erythropolis*. However, it has to be accepted that BT desulphurisation activities of both IGTS8 and X309 were low and not stable. Therefore, it is a challenging task to investigate the mechanism of BT desulphurisation.

5.1.5 Growth on sulphur model compounds

R. erythropolis strains IGTS8 and X309 could grow on a wide range of sulphur compounds. The greatest growth occurred with inorganic sulphur, sodium sulphate. The bacteria utilised organic sulphur compounds (DMSO, DBT, DBT-sulphone, and BT) as the sole source of sulphur, but not as the carbon source. This implies that these two bacterial strains can selectively remove sulphur from organic sulphur compounds without reduction of value of the compounds. In addition, elemental sulphur supported growth of the bacteria, but less efficient than the above sulphur compounds. The strains did not present desulphurisation activity on some organic sulphur compounds such as diphenyl disulphide (DPDS), and thiophene-2-carboxylic acid (T2C).

Kayser *et al.* (1993) also reported growth of *R. erythropolis* IGTS8 on sulphate, DBT and DBT-sulphone. However, they claimed that the strain IGTS8 did not grow on BT, whereas it did exhibit weak growth on BT from our observation. From work by Lee *et al.* (1995), sulphate, DMSO, DBT and DBT-sulphone supported good growth of *R. erythropolis* ECRD-1, which was isolated from the same source as *R. erythropolis* X309. BT also supported growth of the strain ECRD-1, but less efficient than the above sulphur compounds. DPDS and T2C did not support growth. Therefore, the present results are in agreement with these of Lee *et al.* (1995).

S. putrefaciens strain NCIMB 8768 could grow on sodium sulphate and elemental sulphur. Nevertheless, activity of the strain to grow on organic sulphur model compounds was unstable. The enhanced culture that used to grow on organic sulphur

compounds was completely lost its activity after several transfers to fresh culture media, and we were unable to enhance its activity during this investigation.

Loss of bacterial activity seems to be a big problem in biodesulphurisation processes. There were many reports of problems with stability or reproducibility. Kilbane (1989) cited in his review that the abilities of many bacterial cultures proclaimed useful for removing organic sulphur were unstable and the reproducibility of results was poor. For example, *Pseudomonas* strain coal bug one (CB1) which was reported to selectively remove sulphur from DBT and coal samples lost its activity during a period of transfer to ARCTECH, Inc., a company formed to pursue the commercial development of this strain (McFarland *et al.*, 1998).

5.2 BIODESULPHURISATION OF COAL SAMPLES

5.2.1 Analytical methods

Only few studies have reported bacterial growth on coal samples. Bozdemir *et al.* (1996) monitored the bacterial growth on coal samples by absorbance measurement at 550 nm. However, no information on how they separated the bacterial cells from the coal samples was given. Therefore, it is doubtful if the growth data presented was reliable. In the present study, bacterial growth on coal samples was estimated as protein concentration. The method was developed and shown to be capable of reproducibly measuring the bacterial growth on coal samples. Previously, Kilbane *et al.* (1992) demonstrated a modified protein assay for measuring bacterial growth on coal samples. Recently, Dastidar *et al.* (2000) also measured free and attached bacterial cells on coal samples by estimating the protein concentration. The method

used in work by Dastidar *et al*. (2000) was a Folin-Lowry method, which requires a longer time to prepare than our method.

In relation to analytical methods for sulphur form determination in coal samples, the proposed methods (Section 3.2.7.6) offered the rapid alternative to the standard methods (ASTM, 1993 and 1994), which took approximately three days to process 12 samples for the forms of sulphur (Laban & Atkin, 2000). Furthermore, the principal failing of the standard methods in determination of pyritic sulphur and organic sulphur was overcome in this study.

In the standard method, pyritic sulphur is calculated by assuming pyrite stoichiometry. The assumption that all of the iron sulphides extracted by nitric acid (HNO_3) have the same stoichiometry as pyrite causes inaccuracies in the calculation of pyritic sulphur. In our method, the pyritic sulphur was determined from direct analysis of sulphur in the HNO_3 extract solution

Organic sulphur determination using the standard method is carried out by subtracting the sum of sulphate and pyritic sulphur from the total sulphur in the sample. This difference method is subject to many errors that may have accumulated in the analyses of sulphate, pyritic and total sulphur. The proposed method for determination of organic sulphur in this work was carried out by analysing total sulphur (as organic sulphur) in the same coal residue following sulphate and pyritic sulphur extraction processes. From the results obtained, our proposed method gave more reliable data than the standard method as was shown by the coefficient of variation.

Regarding determination of sulphur by the non-destructive method, X-ray photoelectron spectroscopy (XPS), the method is a highly specialised technique. The XPS system used in this work did not give a good resolution of sulphur peak. Therefore, quantification could not be done. A more specialised system is required. This implies that the technique will hardly become a routine method for determination of sulphur in coal in the long run.

5.2.2 Biodesulphurisation of bituminous and anthracite coal

In comparison with the leaching process (incubated without inoculum), only *R. erythropolis* IGTS8 gave a significantly greater reduction of total sulphur in bituminous coal. There was no significant difference between reduction of total sulphur in anthracite coal by the bacteria and the leaching process. The results indicate that type of coal has an effect on desulphurisation efficiency. In addition, *R. erythropolis* IGTS8 looks more interesting for use in the biodesulphurisation process than *R. erythropolis* X309, and *S. putrefaciens*. However, the desulphurisation activity of *R. erythropolis* IGTS8 was too low for an economical biodesulphurisation process since the total sulphur reduction obtained was only 32.0% for bituminous coal, and 21.1% for anthracite coal.

Interestingly, the greatest reduction of sulphate sulphur in both bituminous and anthracite coal was obtained by the leaching process. However, to remove sulphate sulphur is not a big problem because it is soluable in water, so that it is easily leached to the medium. With the presence of bacteria, sulphate sulphur could be produced from biodegradation of other forms of sulphur. That liberated sulphur was possibly

incorporated into the bacterial cells attached to the coal samples. Therefore, when those coal samples were analysed for sulphate sulphur, the amount of sulphate sulphur obtained was higher than that obtained from coal treated by the leaching process.

R. erythropolis strain IGTS8 and strain X309 could remove organic sulphur from both bituminous and anthracite coal. Little reduction of organic sulphur in both bituminous and anthracite coal was observed in the treatments by *S. putrefaciens*, and bacteria inherent in the coal. This shows the possibility that *S. putrefaciens*, and bacteria inherent in the coal could remove organic sulphur from the coal although the bacteria did not show stable activities on removal of organic sulphur from the model compounds. The organic sulphur removed by *S. putrefaciens*, and bacteria inherent in the coal may be in other forms of organosulphur presented in the coal, such as cystein and cystine (the sulphur containing amino acids).

Reduction of sulphur in bituminous coal was significantly greater than the reduction in anthracite coal. This may be because anthracite coal, which is in a higher rank coal, has smaller coal pores than bituminous coal. Therefore, it is more difficult for leaching process or microorganisms to remove sulphur from anthracite coal than bituminous coal. In addition, it was reported that biodesulphurisation depends on the amount of sulphur in the coal, its composition, and its degree of distribution throughout the coal (Rubiera *et al.*, 1999). The larger the amount of sulphur in the parent coal, the higher the mass of sulphur removed. Since bituminous has a larger amount of sulphur, the higher reduction of sulphur was obtained.

Following isolation of bacteria inherent in the coal samples, there were two isolated bacteria from bituminous coal, and one isolated bacterium from anthracite coal. As bacteria inherent in the coal, they could remove some sulphur from coal samples, but less efficient than the pure cultures. With further investigation after isolation, these isolates were gram-negative, aerobic, and able to grow at pH 7 and 30°C. Generally, bacteria isolated from coal are in genus of *Pseudomonas*, *Xanthomonas*, *Moraxella*, and *Chryseomonas* (Gomez *et al.*, 1999). *Pseudomonas* species seem to be the most extensive bacteria found in coal. Our isolates are possibly in any of the above genus.

In the present study, *R. erythropolis* IGTS8 removed 32.0% total sulphur from bituminous coal in 10 days. Kilbane (1989) found that *R. erythropolis* IGTS7 removed 85% total sulphur from coal in 212 days. Bozdemir *et al.* (1996) reported that *R. erythropolis* IGTS8 removed 30.2% total sulphur from Mengen lignite coal in 4 days. Rubiera *et al.* (1999) cited that bacteria inherent in the coal removed up to 57.7% total sulphur from antracite coal in 12 days. It can be pointed out that the current coal biodesulphurisation process generally requires more than 3 days to remove about 30% total sulphur, whereas chemical desulphurisation process requires less than 1 day to remove about 65% total sulphur. Biodesulphurisation process is therefore certainly needed to minimise the time requirement of the process.

CHAPTER 6:

CONCLUSION AND FURTHER WORK

6.1 INTRODUCTION

One of the major sources of the environmental problem known as acid rain is sulphur emission from the combustion of coal. Coal possesses both inorganic and organic forms of sulphur. Inorganic sulphur can be effectively removed from coal before combustion by physical and chemical methods, the removal of organic sulphur is still problematic. Biological method occurred under mild condition offers an attractive alternative to the conventional methods. However, there are many obstacles to the development of the biodesulphurisation processes. These include the lack of microorganisms that present the abilities to desulphurise a wide range of sulphur compounds, the lack of stability of desulphurisation abilities in microbial cultures, the lack of data on coal desulphurisation, and the lack of convenient accurate analytical techniques for measuring sulphur forms in coal. The intention of this work was to minimise these obstacles. In addition, from literature biodesulphurisation revealed a number of critical variables which affect the reactions and hence any potential biodesulphurisation process. Some parameters were then studied in this work.

The experiments have been set to carry out the work. The results achieved have been discussed to the extent to which the project objectives have been met. The way in which this work has furthered the current knowledge of biodesulphurisation processes are highlighted and concluded. Finally, remaining gaps in biodesulphurisation processes are identified and suggestions of wider future research aims are presented.

6.2 ACHIEVEMENT OF PROJECT OBJECTIVES

Five main objectives were proposed for this work. The results obtained can be interpreted and summarised as follows:

Objective 1: To examine the abilities of the bacteria on utilisation of a wide range of sulphur model compounds (both organic and inorganic forms).

- *R. erythropolis* IGTS8 and X309 could grow on elemental sulphur, or inorganic sulphur (sodium sulphate) as a sole source of sulphur.
- *R. erythropolis* IGTS8 and X309 could grow on organic sulphur (dimethyl sulphoxide, dibenzothiophene, dibenzothiophene-sulphone, and benzothiophene) as the sole source of sulphur, but not as the carbon source for growth.
- The mechanism pathway of dibenzothiophene (DBT) desulphurisation was DBT → DBT-sulphone → hydroxybiphenyl + sulphite. Therefore, *R. erythropolis* IGTS8 and X309 could remove sulphur from DBT, which is generally regarded as a model compound representative of the forms of organosulphur found in coal, without reduction of the compound caloric value.
- The abilities of *R. erythropolis* IGTS8 and X309 to remove sulphur from benzothiophene (BT) were observed for the first time. *R. erythropolis* IGTS8 and X309 grew slowly (12 days optimal growth) with BT as the sole source of sulphur. BT degradation was detected by HPLC analyses. The appearance of 2-(2'-hydroxyphenyl)ethan 1-al (HPEal) which is an end product from BT degradation via the 4S pathway was observed, but not consistently. Nevertheless,

this presented the possibility that *R. erythropolis* IGTS8 and X309 could desulphurise BT via the 4S pathway.

- Desulphurisation ability of *Shewanella putrefaciens* strain NCIMB 8768 was observed for the first time. Unfortunately, ability of the strain to remove sulphur from organic sulphur compounds (DMSO, DBT, DBT-sulphone, and BT) was lost during investigation.
- In comparison, *R. erythropolis* IGTS8 and X309 presented better stability of the abilities to desulphurise organic sulphur than a novel desulphurising bacterium, *S. putrefaciens* stain NCIMB 8768.
- Although the ability of *S. putrefaciens* stain NCIMB 8768 to grow on DMSO, DBT, DBT-sulphone, or BT was lost, the strain still presented stable ability to grow on inorganic sulphur (sodium sulphate) and elemental sulphur.
- *R. erythropolis* IGTS8, *R. erythropolis* X309, and *S. putrefaciens* did not present the abilities to desulphurise some forms of organic sulphur compounds, such as diphenyl disulphide (DPDS) and thiophene-2-carboxylic acid (T2C).
- Among the bacteria investigated in this study, *R. erythropolis* IGTS8 presented the greatest desulphurisation efficiency.

Objective 2: To investigate the effect of environmental parameters on the abilities of the bacteria in biodesulphurisation of sulphur model compounds.

- At three different temperatures investigated: 25; 30; and 35°C, the optimum temperature for growth and desulphurisation activity of *R. erythropolis* IGTS8, *R. erythropolis* X309 or *S. putrefaciens* was 30°C. High temperature as 35°C could inhibit growth and desulphurisation activity of the bacteria.

- Ethanol can promote bacterial growth and biodesulphurisation activity at low concentrations and can inhibit bacterial growth and biodesulphurisation activity at high concentrations.
- The bacterial growth of each culture was completely inhibited at 20 mM of DBT, indicating that the abilities of bacteria to utilise sulphur for growth can be inhibited with a supplement of too high concentration of sulphur.
- HBP, which is an end product from DBT desulphurisation via the 4S pathway, could inhibit the DBT desulphurisation.
- Desulphurisation abilities of the bacteria were depressed with the presence of sulphate. *R. erythropolis* X309 was higher affected than *R. erythropolis* IGTS8.
- Biodesulphurisation rate could be enhanced by addition of hydrophobic solvent in the culture aqueous medium.
- The loss of desulphurisation activity of each bacterial strain was observed after harvested and storage at –80°C for periods of time. This suggests that the fresh harvested culture should be applied in the biodesulphurisation processes.

Objective 3: To perform biodesulphurisation of coal samples with the three specific bacteria and with bacteria inherent in the coal.

- *R. erythropolis* IGTS8, *R. erythropolis* X309 and *S. putrefaciens* could grow with bituminous or anthracite coal as the sole source of sulphur. In the early state, some sulphur in the coal powder was leached to the media. Each bacterium used that leached sulphur for its growth and then absorbed itself to the coal powder for more bioavailable sulphur.

- *R. erythropolis* IGTS8 and *R. erythropolis* X309 could remove sulphate sulphur, pyritic sulphur, and organic sulphur from the coal samples.
- *S. putrefaciens* and the bacteria inherent in the coal could remove sulphate sulphur, and pyritic sulphur from the coal samples. They possibly removed some forms of organic sulphur (cystein and cystine) from the coal samples.
- The organic sulphur in both bituminous and anthracite coal sample was not removed by leaching process (incubated without bacteria).
- Among the three specific bacteria and bacteria inherent in the coal, *R. erythropolis* IGTS8 presented the greatest desulphurisation efficiency. This can be seen from biodesulphurisation of both bituminous and anthracite coal.
- Regarding type of coal, reduction of sulphur in anthracite coal was lower than reduction of sulphur in bituminous coal. This indicates that type of coal has an effect on desulphurisation efficiency.

Objective 4: To study the desulphurisation abilities of isolated bacteria that inherent in the coal.

- There were two isolates from bituminous coal. These two isolates could grow with elemental sulphur or sodium sulphate, but not the organic sulphur compounds as the sole source of sulphur.
- There was one isolate from anthracite coal. This bacterium could grow with elemental sulphur or sodium sulphate, but not the organic sulphur compounds as the sole source of sulphur.

Objective 5: To improve analytical methods for measuring sulphur in coal.

- The proposed analytical methods offered the rapid alternative to the standard methods.
- Pyritic sulphur was determined from direct analysis of sulphur in the extract solution. This should be more accurate than analysis iron, and assuming pyrite stoichiometry as carried out in the standard method.
- Determination of organic sulphur in coal was carried out by analysing the same coal residue following sulphate and pyritic sulphur extraction procedures for its total sulphur content. This method presented more reliable results than the standard method.

6.3 CONCLUSION ON BIODESULPHURISATION OF COAL

The removal of sulphur from coal before combustion by biological processes is technically feasible. There have been several different microorganisms suggested in the literature for the processes and these microorganisms behave differently. Regarding removal of inorganic sulphur, *Leptospirillum ferrooxidans*, *Thiobacillus ferrooxidans*, and mixed cultures (enriched from coal relavant) have been proven to be efficient for the process (Juszczak *et al.*, 1995 and Klein, 1998).

Removal of organic sulphur is a more difficult task. The bacteria proclaimed useful for the process presented unstable activities and the reproducibility of results was poor. Up to date, *Rhodococcus* species seem to be the most successful genus to apply in the process. However, many strains reported were not very effective for a broad range of sulphur types. Most of them have only little efficiency on benzothiophenes and inefficiency on thiophenes. Additionally, the desulphurisation activities of the strains were too low for an economical biodesulphurisation process. For instance, *R. erythropolis* IGTS8 removed only 32.0% of total sulphur from bituminous coal, and 21.1% of total sulphur from anthracite coal (data from the present study). More active microbial cultures with improved desulphurisation efficiency toward a wide variety of sulphur compounds are certainly needed for the process development.

Alternatively, biodesulphurisation can be carried out in inexpensive conditions by using the bacteria inherent in the coal itself. The advantages of using the bacteria inherent in the coal over using the pure isolated cultures are the immediate adaptation of the microorganisms to the coal and the reduced period of latency (Moran *et al.*, 1997). The use of bacteria inherent in the coal could be of special interest for application in coal heaps in the open air (Rubiera *et al.*, 1999). In addition, the problems on bacteria storage conditions could be neglected. Although in the present study the bacteria inherent in both bituminous and anthracite coal samples did not present high organosulphur removal efficiency, but other coal samples might have more efficient desulphurising bacteria.

The type of coal has an important effect on desulphurisation efficiency since the sulphur reduction in bituminous coal, which is in a lower rank than anthracite, was higher than the sulphur reduction in anthracite coal. Moreover, biodesulphurisation depends on the concentration of sulphur present in the coal, its composition, and its degree of distribution throughout the coal (Thoms, 1995). It can be seen that the larger the amount of sulphur in the parent coal, the higher the amount of sulphur removed.

Analytical methods for measuring sulphur in coal have been improved in the present study. The methods offer rapid alternative to the standard methods and the results obtained are satisfied. Data on coal biodesulphurisation are more reliable. The lack of microorganisms that present the abilities to desulphurise a wide range of sulphur compounds, the lack of stability of desulphurisation abilities in microbial cultures, and the slow process time are still the big problems in biodesulphurisation process.

Cost-effectiveness of coal biodesulphurisation process depends mainly on the type of bioreactor used (Raman *et al.*, 1995). According to long residence time requirement of the process, large reactors are needed. Keeping operating conditions such as pH-level, temperature, oxygen concentration, mixing and slurry stabilisation under control in reactors of such dimensions is regarded by many experts as impractical (Klein, 1998). To date, significant removal of organic sulphur from coal has not been demonstrated. From this point of view, the economics of coal biodesulphurisation technology are not yet favourable.

6.4 SOLUTIONS TO THE FUTURE PROCESSES

Improvement of biodesulphurisation processes should be placed on activity, selectivity and stability of microorganisms applied in the processes. For commercial applications, mixtures of microorganisms may be needed. Genetic engineering could perhaps fulfil the need for microbial cultures that present more completely and more rapidly sulphur removal activities. Recently, Energy Biosystems Corporation (EBC) has discovered that removal of the *DszB* enzyme of *Rhodococcus erythropolis* IGTS8 was beneficial to biodesulphurisation of dibenzothiophene (Pacheco *et al.*, 1999). The incomplete strains offered several advantages, including:

- more activity, because the final step in DBT desulphurisation related to *DszB* enzyme is the slowest, so eliminating the *DszB* enzyme speed up the process;
- removal of restrictions to the process reactor design exerted by ortho phenyl-phenol, which is toxic to bacteria even at low concentrations; and
- protection against sulphate ions in the reactor, which repress production and regeneration of the *Dsz* enzymes.

As the genes involved in the sulphur removal pathway are identified and cloned, the efficiency of biological process could be enhanced by increasing the number of copies of the genes, increasing the amount of expression from each gene, and altering the gene to produce a more active or efficient product.

Coal biodesulphurisation in the presence of hydrophobic media should provide higher rate of desulphurisation efficiency and the process time could be reduced. In general point of view, a successful pre-combustion coal desulphurisation process is likely to be a hybrid process that integrates chemical, physical and microbial approaches.

6.5 FUTURE WORK

For future work a set of wider objectives are proposed as follows:

- further study on the enzyme mechanisms involved in biodesulphurisation processes;
- apply genetic engineering to generate the improved bacterial strains which present higher desulphurisation activities and higher resistant to ortho phenyl-phenol and sulphate, sulphite ions;
- carry out coal biodesulphurisation experiments with a wide range of biocatalysts: pure culture, mixtures of bacterial cultures, bacteria inherent in the coal, and genetic engineered strains;
- carry out coal biodesulphurisation in the presence of hydrophobic media; and
- investigate the effect of several parameters such as substrate type in the growth medium, substrate concentration, type of reactor, type of coal, coal size, initial pH, growth temperature, shaking rate and aeration rate in order to find out the optimal conditions for biodesulphurisation processes.

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PUBLISHED WORK

- Prayuenyong, P. & Tothill, I.E. (1999). *Biodesulphurisation of dibenzothiophene and dibenzothiophene-sulphone by Shewanella putrefaciens (NCIMB 8768).* Chem Show Conference. Jacob K. Javits Center, New York, New York November 16-18.

- Prayuenyong, P. & Tothill, I.E. (2001). Coal Biodesulfurization. *Fuel* (submitted).

APPENDIX A: HPLC RESULTS

A chromatogram of the mobile phase when the HPLC system was in steady state and ready to operate is represented in Figure A.1. The chromatograms of DBT and its metabolites in sterile medium growth without bacterial inoculum and with bacterial inoculum are shown in Figures A.2 and A.3, respectively. In addition, the chromatogram of BT in sterile medium growth with bacterial inoculum is shown in Figure A.4.

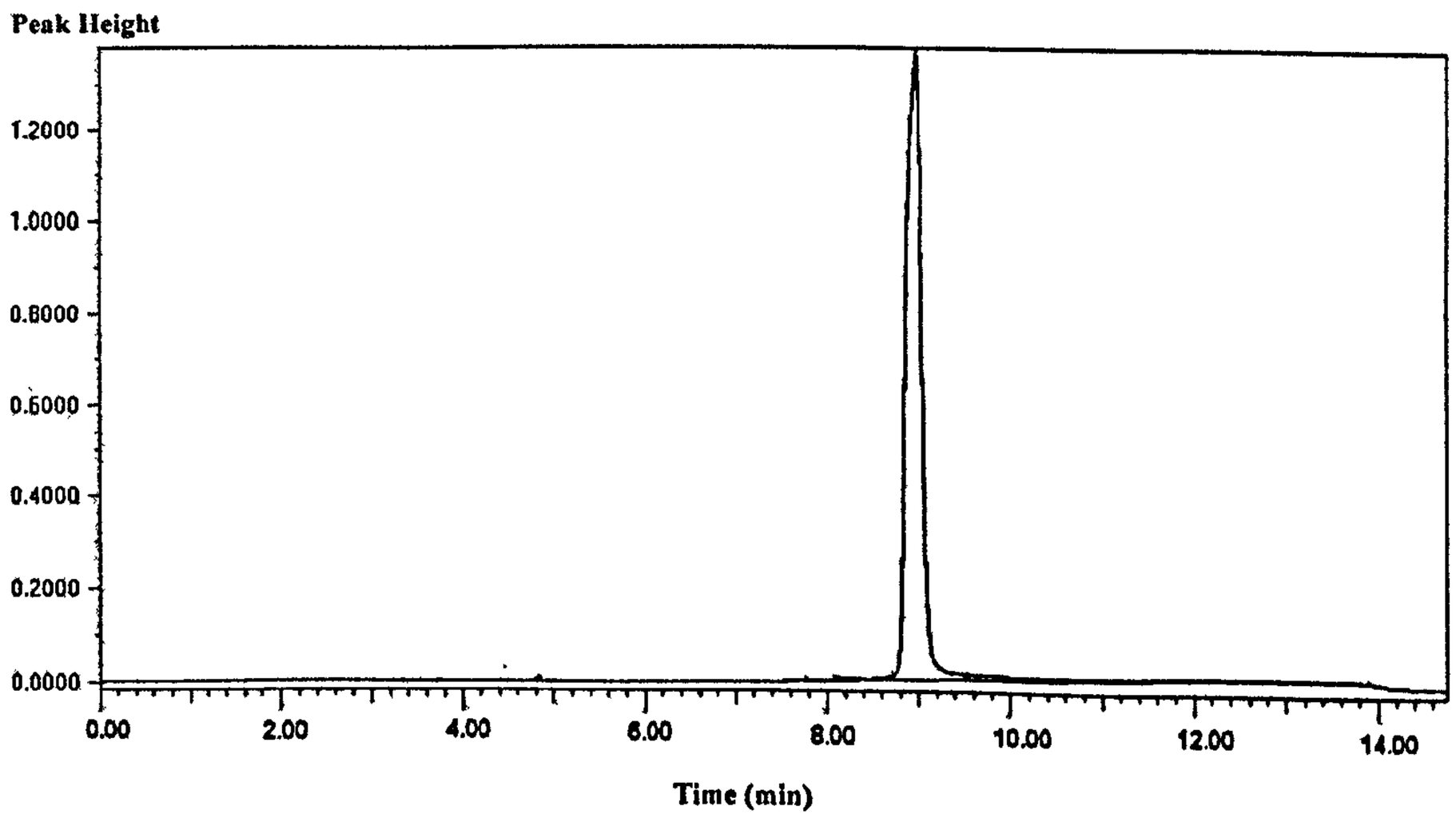

Figure A.1 HPLC chromatogram of mobile phase (water:acetonitrile). The peak was due to change of mobile phase gradient, which is not related to the analysis.

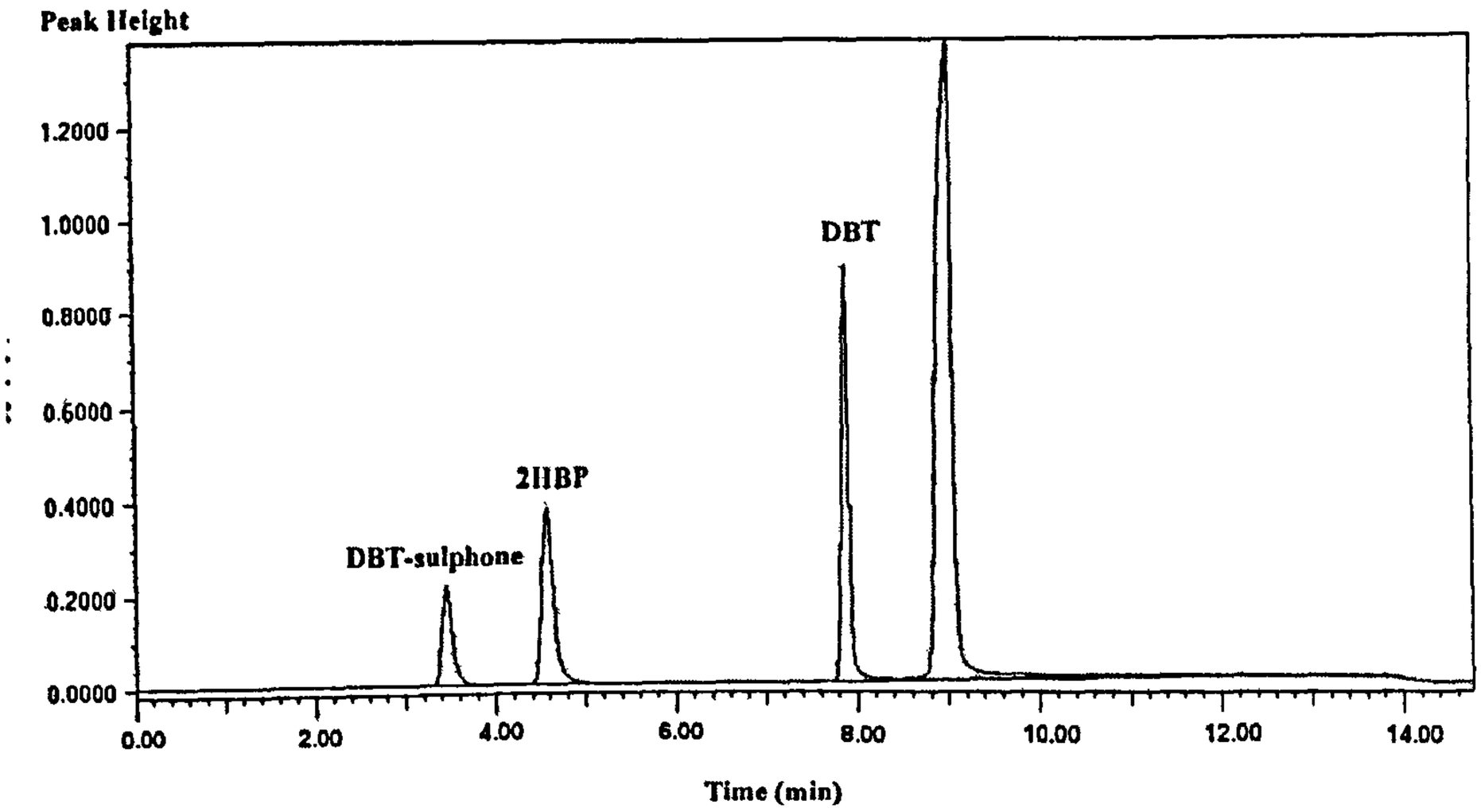

Figure A.2 HPLC chromatogram of DBT and its metabolites in sterile medium growth without bacterial inoculum. Concentration of each compound was 0.1 mM.

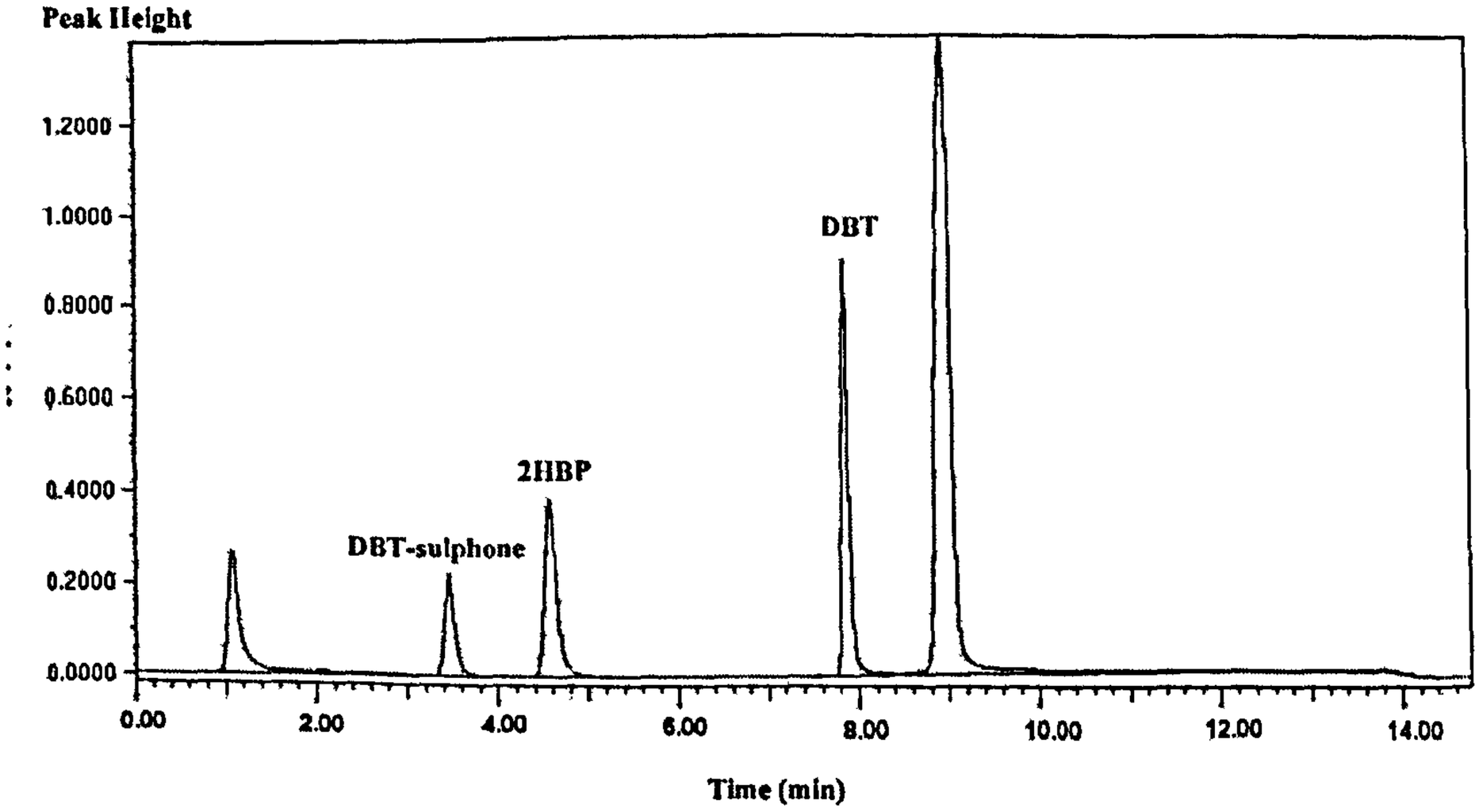

Figure A.3 HPLC chromatogram of DBT and its metabolites in sterile medium growth with bacterial inoculum. Concentration of each compound was 0.1 mM concentration.

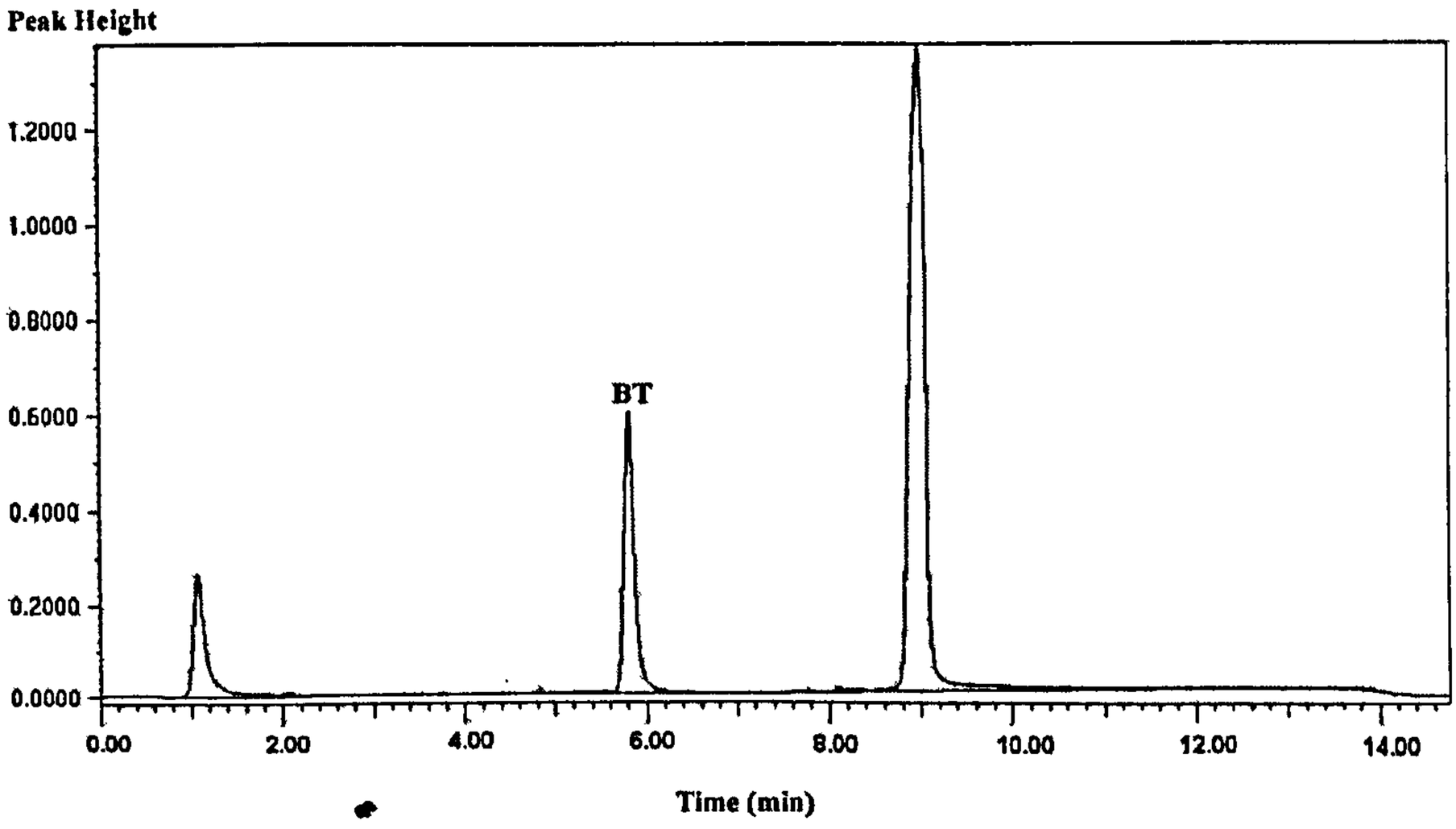

Figure A.4 HPLC chromatogram of BT in sterile medium growth with bacterial inoculum. It presents 0.2 mM concentration of BT.

The concentration of DBT, DBT-sulphone, HBP or BT in each sample was calculated by HPLC software using calibration curves as shown in Figures A.5, A.6, A.7 and A.8.

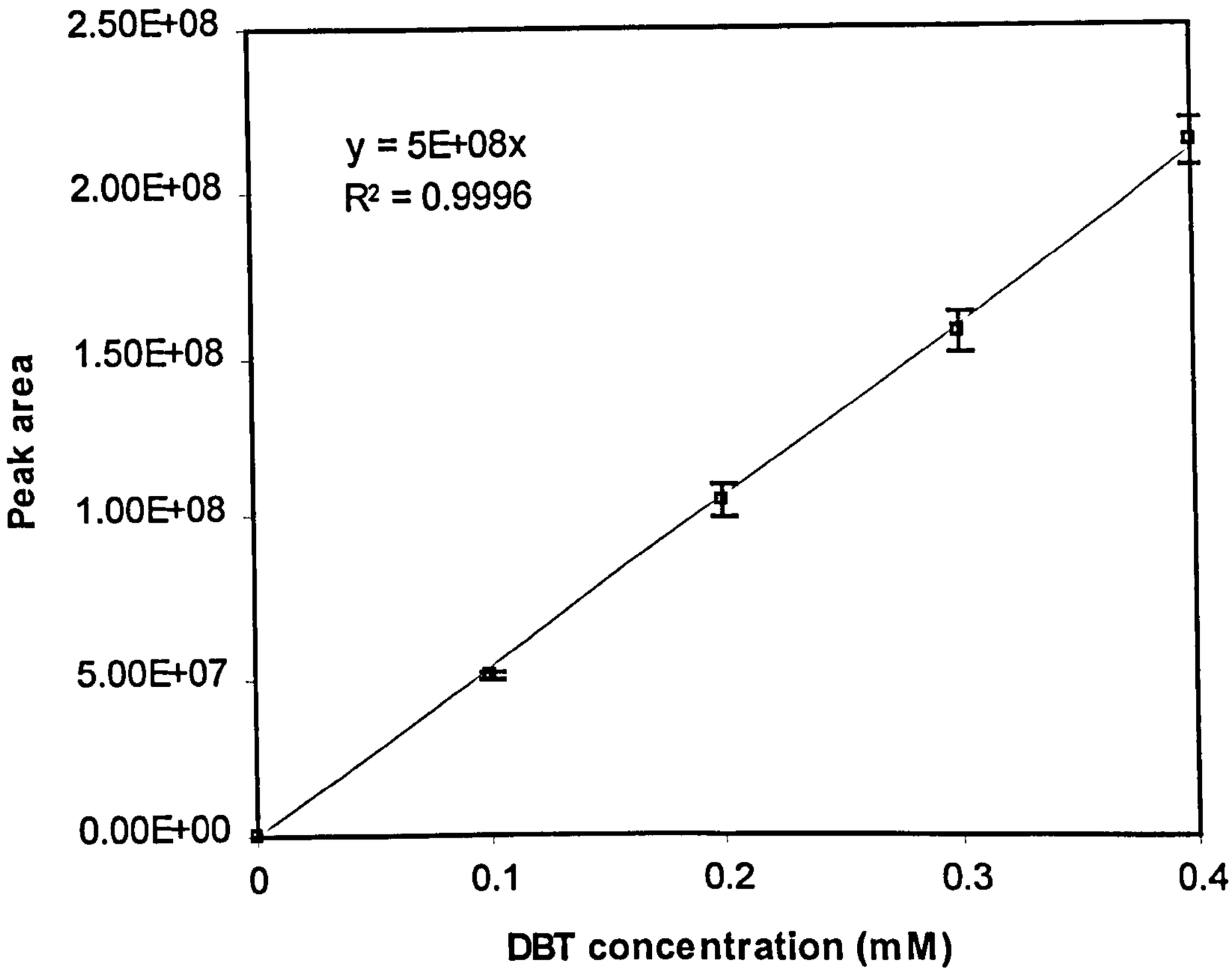

Figure A.5 Calibration curve of DBT by HPLC analysis. Peak area of HPLC chromatogram was converted to DBT concentration. Each point is a mean of 3 replicates and error bar is a standard deviation.

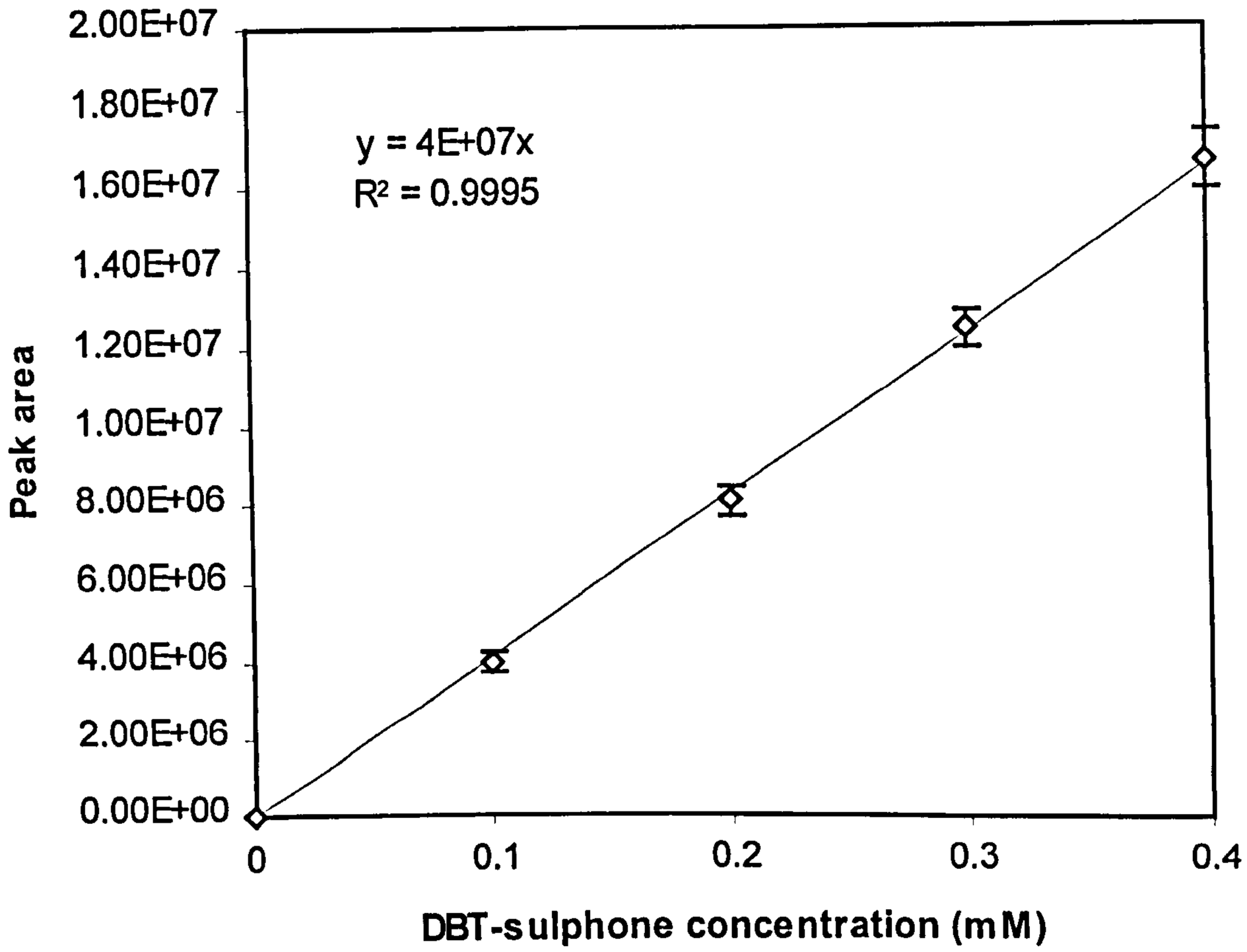

Figure A.6 Calibration curve of DBT-sulphone by HPLC analysis. Peak area of HPLC chromatogram was converted to DBT-sulphone concentration. Each point is a mean of 3 replicates and error bar is a standard deviation.

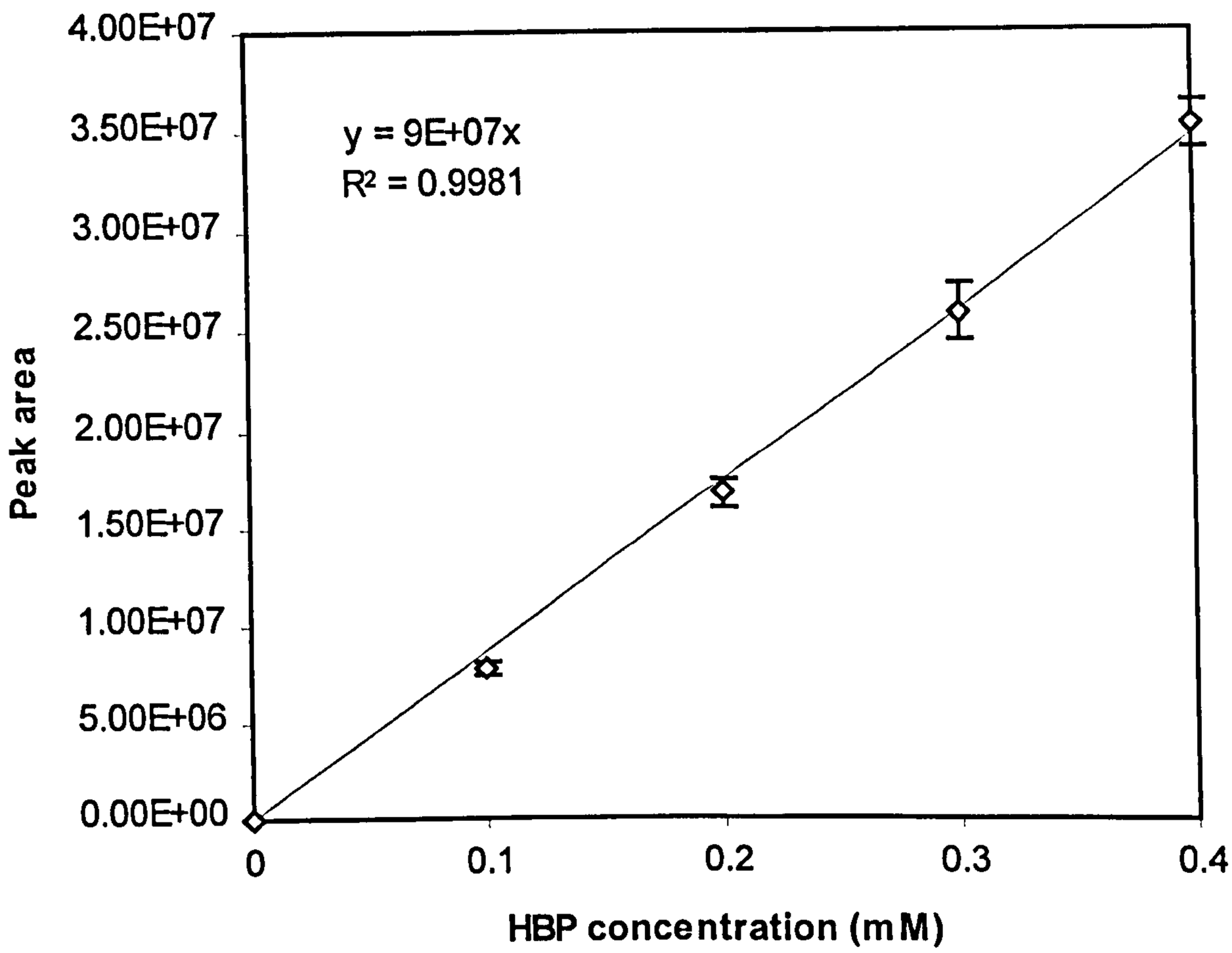

Figure A.7 Calibration curve of HBP by HPLC analysis. Peak area of HPLC chromatogram was converted to HBP concentration. Each point is a mean of 3 replicates and error bar is a standard deviation.

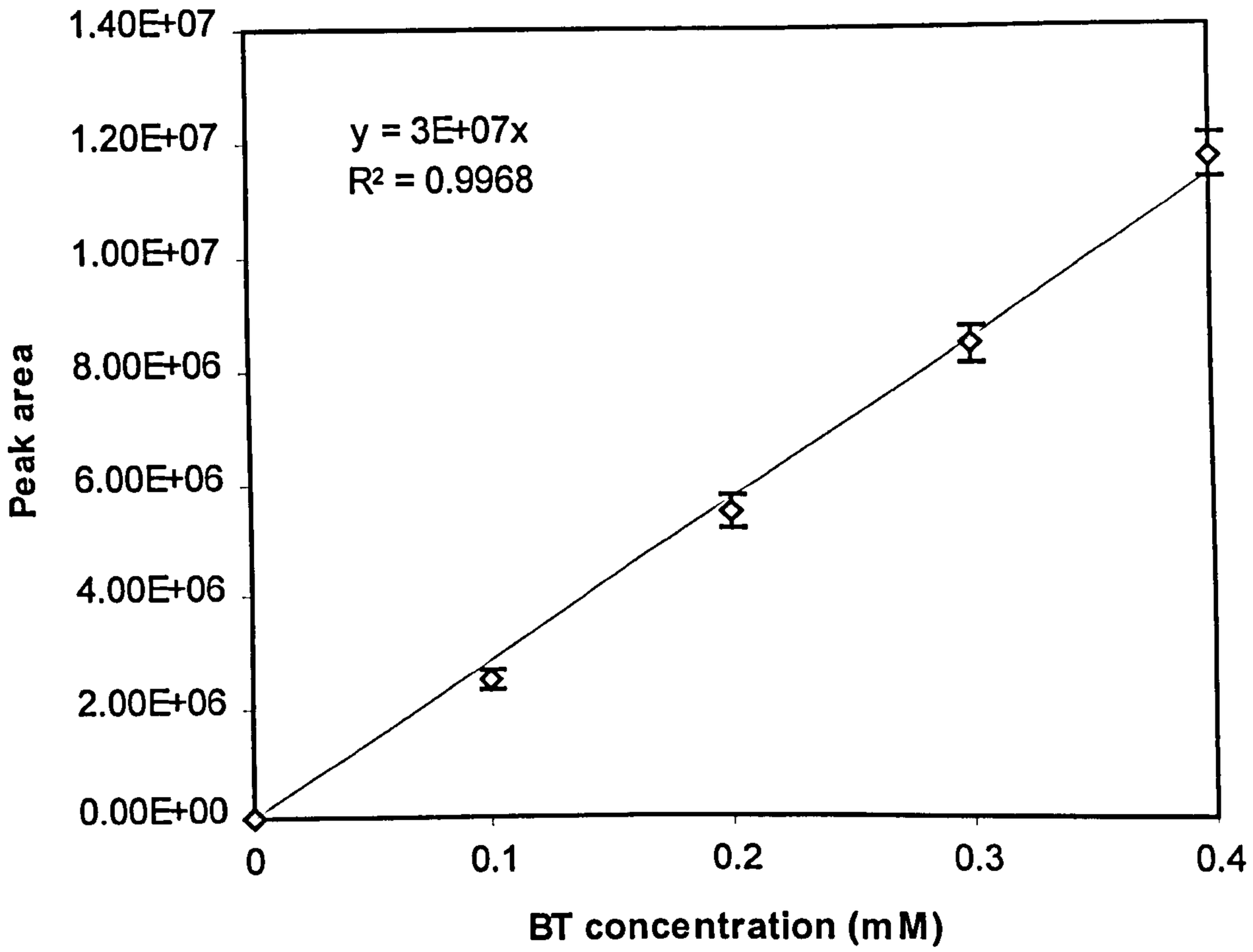

Figure A.8 Calibration curve of BT by HPLC analysis. Peak area of HPLC chromatogram was converted to BT concentration. Each point is a mean of 3 replicates and error bar is a standard deviation.

APPENDIX B: BACTERIAL GROWTH

Bacterial growth in complex medium is shown in Figures B.1, B.2, and B.3. In addition, growth on dimethyl sulphoxide (DMSO) is presented in Figure B.4.

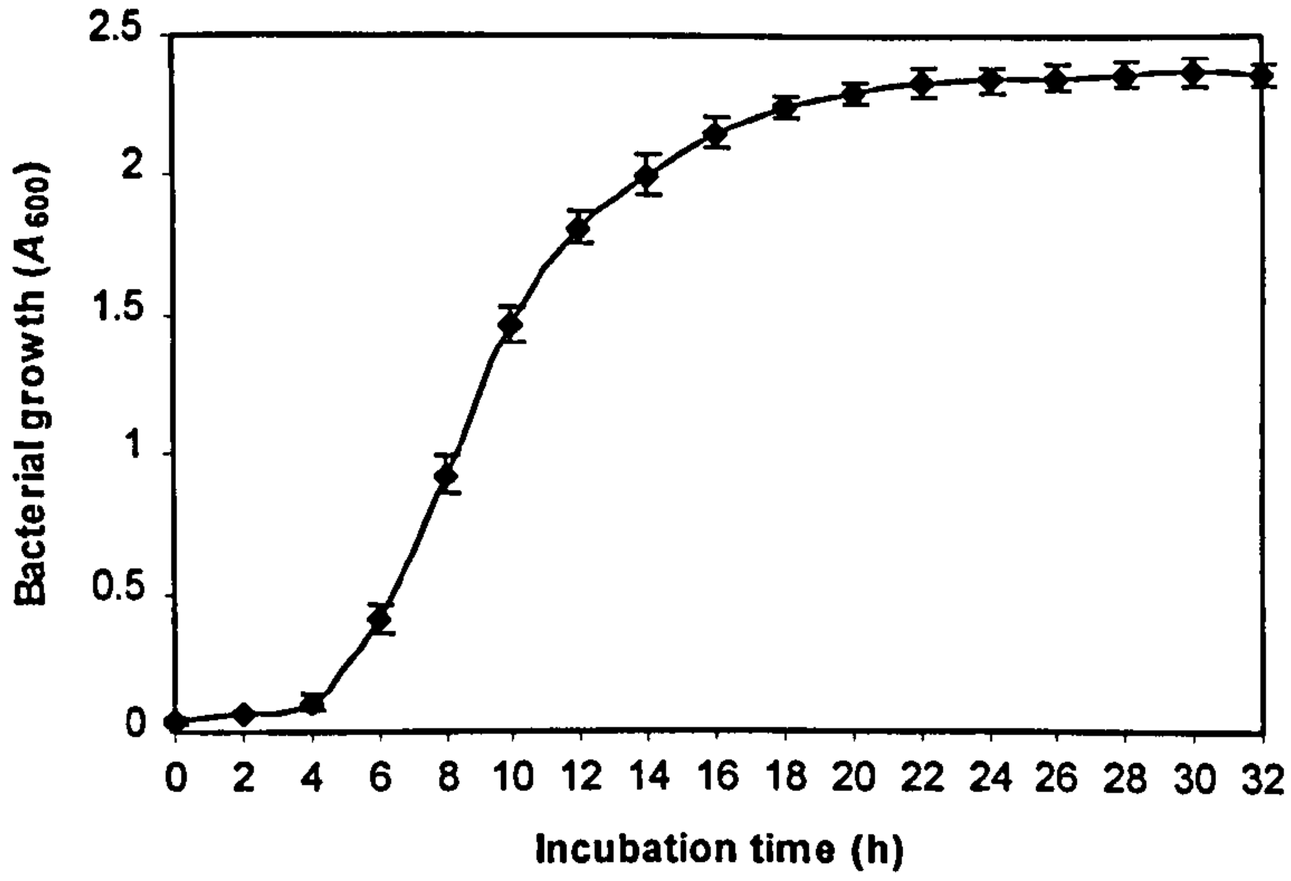

Figure B.1 Growth of *R. erythropolis* IGTS8 in tryptic soy broth. Each point is a mean of three replicates, and error bar is a standard deviation.

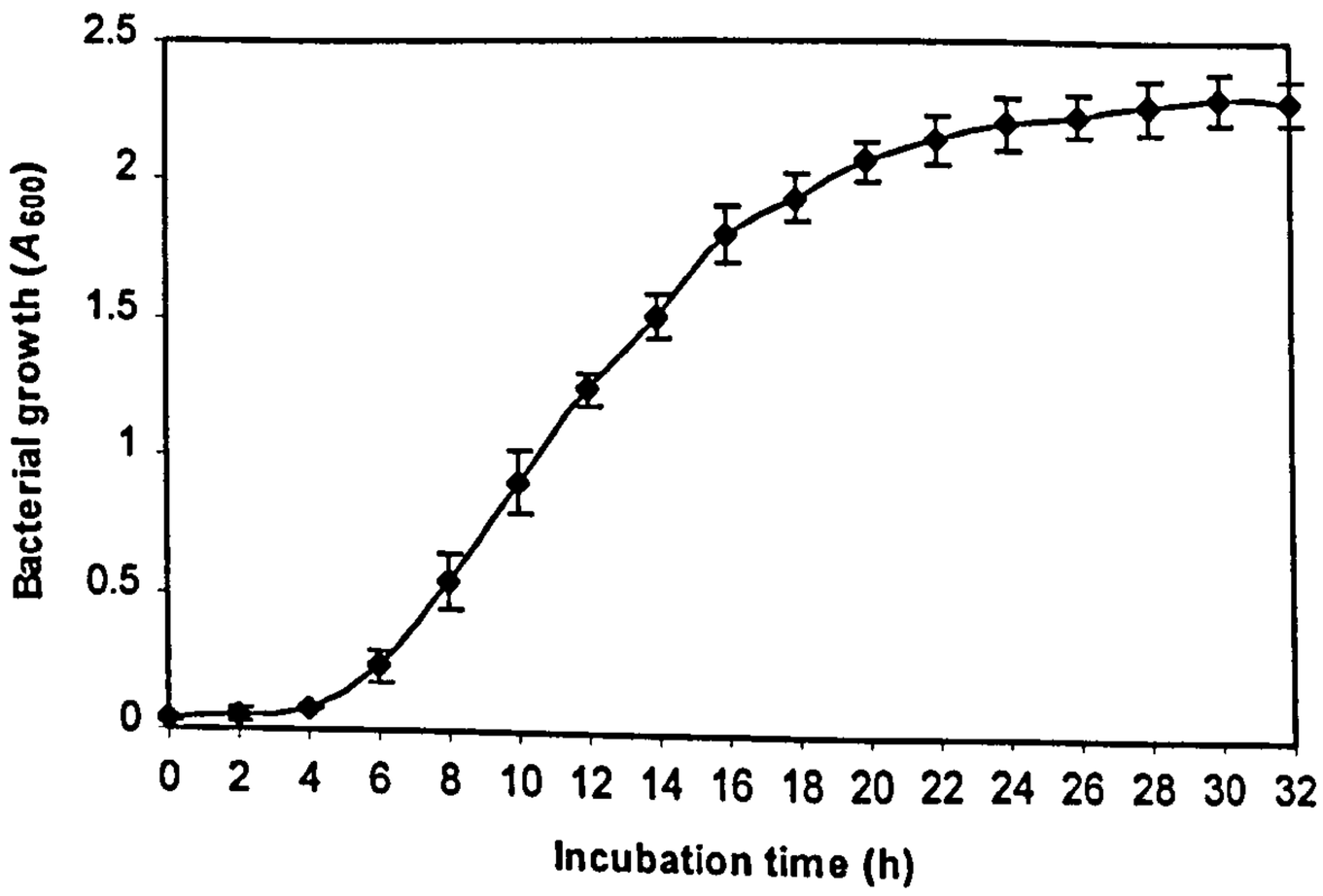

Figure B.2 Growth of *R. erythropolis* X309 in tryptic soy broth. Each point is a mean of three replicates, and error bar is a standard deviation.

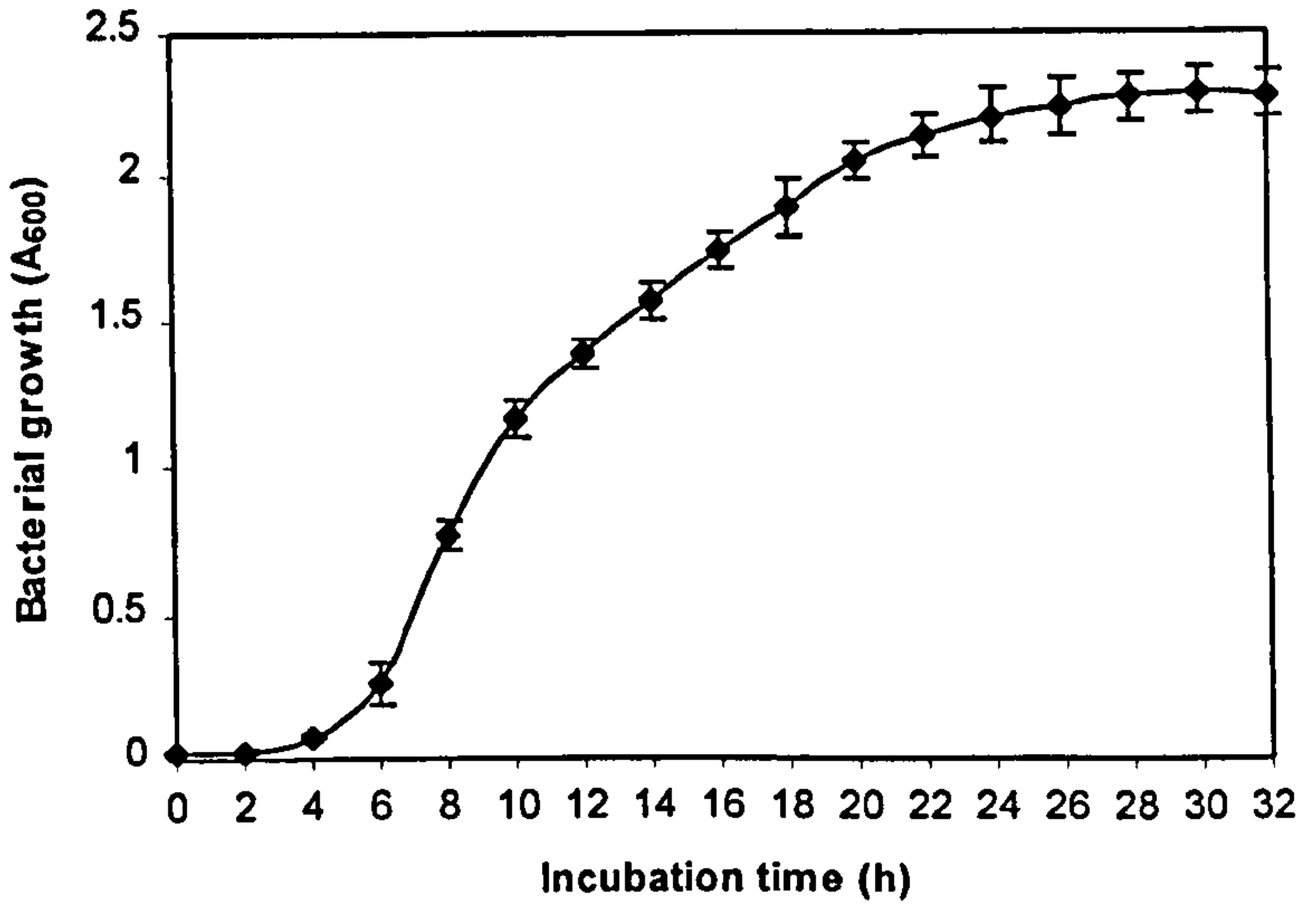

Figure B.3 Growth of S. *putrefaciens* in nutrient medium. Each point is a mean of three replicates, and error bar is a standard deviation.

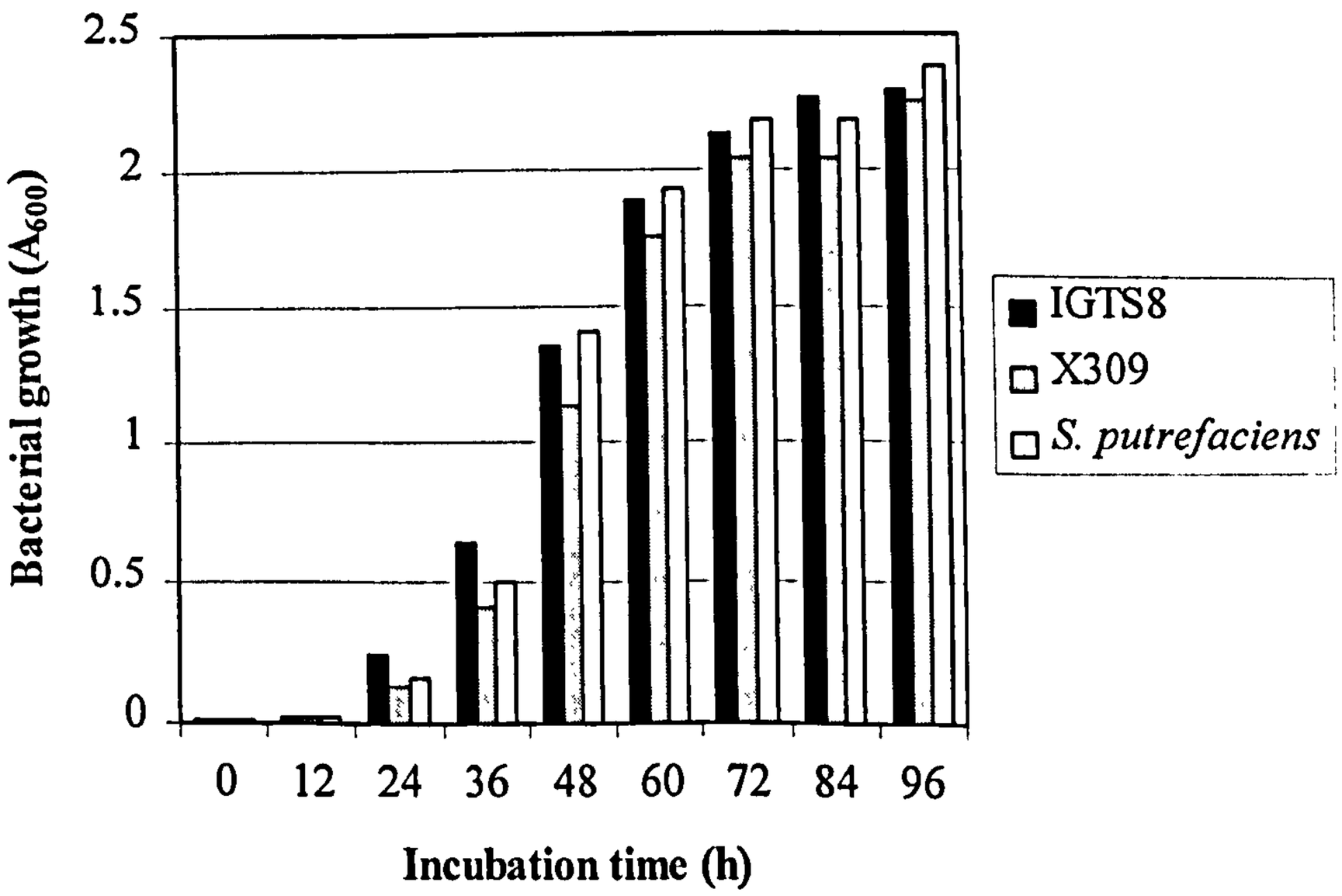

Figure B.4 Growth of the three bacteria in DMSO. Each value is a mean of three replicates.

Each culture grown in complex medium or BSM + DMSO was harvested at mid-exponential growth phase for further experiments.

APPENDIX C: STATISTICAL ANALYSIS

In order to calculate the value of least significant difference (LSD) as explained in Section 3.2.7.10, *t* distribution value at x degrees of freedom is given in Table C.1. Furthermore, example of analysis of variance (ANOVA) is shown in Table C.2.

Table C.1 The *t*-distribution.

Value of t for a confidence interval of Critical value of $\| t \|$ for *P* values of Number of degrees of freedom	**90%** **0.10**	**95%** **0.05**	**98%** **0.02**	**99%** **0.01**
1	6.31	12.71	31.82	63.66
2	2.92	4.30	6.96	9.92
3	2.35	3.18	4.54	5.84
4	2.13	2.78	3.75	4.60
5	2.02	2.57	3.36	4.03
6	1.94	2.45	3.14	3.71
7	1.89	2.36	3.00	3.50
8	1.86	2.31	2.90	3.36
9	1.83	2.26	2.82	3.25
10	1.81	2.23	2.76	3.17
12	1.78	2.18	2.68	3.05
14	1.76	2.14	2.62	2.98
16	1.75	2.12	2.58	2.92
18	1.73	2.10	2.55	2.88
20	1.72	2.09	2.53	2.85
30	1.70	2.04	2.46	2.75
50	1.68	2.01	2.40	2.68
∝	1.64	1.96	2.33	2.58

Table C.2 ANOVA: single factor test.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	3	0.265667	0.088556	0.000206
Column 2	3	2.237333	0.745778	0.034573
Column 3	3	4.441	1.480333	0.08416
Column 4	3	5.690667	1.896889	0.047576
Column 5	3	6.416	2.138667	0.019711
Column 6	3	6.756667	2.252222	0.006411
Column 7	3	6.904667	2.301556	0.002866
Column 8	3	6.922667	2.307556	0.001812

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	14.41146	7	2.058781	83.47146	2.13017E-11	2.657195
Within Groups	0.394632	16	0.024664			
Total	14.8061	23				