BIODEGRADATION OF AZO DYES

by

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CONTENTS

ABSTRACT

The current world production of synthetic organic colorants is estimated to be more than one million tones. Azo dyes are the largest group that exhibit great structural diversity. During manufacturing and using process, a lot of azo dyes have been discharged and deposited in the environment, particularly in rivers that caused serious pollution. This is especially serious in Hong Kong since textile industry is the top industry here. Comparing with other kind of wastewater, an additional concern with the textile industry wastewater is the presence of colour. Since the discharge of coloured water presents an environmental concern due to the loss of aesthetic value of the receiving water, the need is urgent for an economical, effective method to remove the colour exists. Biodegradation is a promising method to counteract the above problem.

A strain ADD 16-2 bas been isolated from Tuen Mun River in the New territories, Hong Kong that has a extraordinary ability to decolour azo dyes. Among 25 kind of dyes tested, 24 azo dyes were decolorized by the strain at different magnitudes. The reaction was more efficient under anaerobic condition. The decolorization reaction resulted in a reduction of the azo linkage. The aromatic amines was accumulated in the medium, which can be toxic and carcinogenous. These products need future treatments.

A strain SAD M-1 was enriched and isolated from the collected samples

mentioned above that can degrade the decolorization intermediate (sulfanific acid) under aerobic condition. A model azo dye Tropaeolin O was completely degraded by two steps. The first is the cleavage the azo bond by strain ADD 16-2 under anaerobic condition and the second step is the mineralization of the intermediates by strain SAD M-1 under aerobic condition. The reaction characteristic of both microbes have been examined and their reaction optima have been identified. The combination of the two strains can substantially de-pollute some azo dyes pollution.

Further improvement of this "de-pollution" potential was studied by immobilizing the cells in gels. The results show that not only the activities can be stabilized but also shifted to an operative condition easier to handle. Finally, a continuous column system was constructed. With this system, more than 80 litres of 100 ppm Tropaeolin O water was treated. The dye was completely degraded. This study provides evidence that the organisms responsible for degradation azo dyes at different conditions, one aerobic and the other anaerobic, can be brought to work together successfully by using immobilization method.

CHAPTER ONE

INTRODUCTION

Light is the source of all colour. Light is comprised of various wavelengths of radiant energy. The human eye, with its marvellous physiology of cones, interprets the wavelengths from 400 to 700 nanometres and transform this into the realization of colour. The absorbance and reflectance of portions of the light wavelengths by an object create in the viewer's mind the distinct and unique colour of that object. Some substance having bright and pure colour are used to improve the appearance of objects. These substances are dyestuffs.

1.1 HISTORY OF DYESTUFFS

The natural world contains a variety of colours. The use of natural colorants can be traced back to prehistoric time. Prehistoric humans used many naturally occurring products to colour their implements, clothing, and dwellings. As far back as 5000 B.C., natural dyes such as organic dyes extracted from plant materials (indigo), from insects cochineal (cochineal) or mollusc (Tyrian Purple) were being used to colour textiles. As early as 3000 B.C. the Egyptians and Chinese were dyeing fabrics, as shown in pictures and other relics. The Indians and Persians were using plant materials to dye silks, brocades and wool before 2000 B.C.. By about 1400 B.C.the Egyptians were producing fine dyed cloth using materials such as Safflower to give a wide

range of colours. From that time on to the mid-nineteenth century, natural dyes and pigments were collected from around the world and applied to various natural fibber substrate and leather in order to obtain colourful dyeing (Boyle, 1973)

In 1856, however, a discovery was made which revolutionized the practice of dyeing and stimulated the whole field of chemical industry. It is the discovery of the first synthetic dye, mauveine, by a young English chemist, William Perkin, using coal tar derivatives. The success of the new coal tar dye attracted other investigators to the field, and the Swiss, French, and Germans quickly joined in the new technology. After that many synthetic dyes were made. In 1858, a diazo compound was isolated from the reaction of nitrous acid and aromatic primary amines by a young German chemist Peter Griess. Meanwhile in 1859 the French chemist Verhuin discovered the first of a brilliant series of triphenylmethane dyestuffs. In 1876 one of the first acid dyes Orange II was produced by coupling diazotized sulphanilic acid and naphthol. These colorants were much cheaper compared to the hitherto used naturally occurring colorants. Although in the first 50 years after the discovery of the first synthetic dye people witnessed the introduction of more new chromogens than in any period since, the true golden age of dye chemistry was arrived only in the 1950s and 60s, During this period, the number of new dye structures introduced onto the market was the highest ever seen. However, from 1970 onwards there has been a steady decline in new introductions because the effects of dyes to the environment have attracted

more and more attention (Houk *et al.*, 1991).

Since the discovery of the first commercially successful synthetic dye to now, synthetic colorants industry has formed one of the most diverse group of fine chemicals manufactured. It has been estimated that at least three million different dyes and pigment structures have been synthesized since the beginnings of the synthetic dye industry.Among them, about 10,000 dyes have been used in commerce.

From historical viewpoint, it can be concluded that at least four distinct developments have made significant impacts on the progression of colour chemistry (Peters, 1991). The initiation was, of course, the discovery of the first synthetic dye, mauveine, by W.H.Perkin in 1856. This historic event ultimately resulted in the commercial development of a vast range of synthetic colorants both for textile and non-textile applications, and which possessed a more favourable cost versus benefit ratio compared to the hitherto used naturally occurring colorants. The second rector was the development over the years of synthetic fibres, an innovation which led to vigorous new research and the addition of the disperse dyes and improved cationic dyes to the extensive volume of synthetic dyestuffs enjoying successful industrial exploitation. The third development can be related to the recognition of the potential adverse effects of certain synthetic dye intermediates on human health.This resulted in the removal of most benzidine and 2-naphthylamine based dyestuffs from the manufacturing processes, and later to guidelines

requiring dyestuff manufacturers to demonstrate safety aspects in all new and certain existing products, and also in the effluent released from manufacturing plants into local waters. The fourth development has been in the application of synthetic colorants in high technology areas involving, for example, lasers, liquid crystalline displays, electro-optical devices and ink-jet printers (Gregory, 1991).

1.2 THE CLASSIFICATION OF DYES

The dyes are not readily classified under conventional chemical headings because of structural variety. Dyes consists of a chromogen and an auxochrome.The chromogen contains the chromophore (colure giver) and is represented by chemical radicals such as azo group, nitroso group, nitro group, ethylene group, carbonyl group etc. The auxochromes normally contain $-NH_2$, -OH, -COOH, or $-SO_3H$. If the dyes are classified by chromophores, ten to twenty classes are obtained. The important chemical groupings are the azo, anthraquinone,indigoid, nitro, nitroso, oxazine,stilbene, thiazole classes etc. An illustration of this is given in Figure 1.1 (Meyer, 1981). From the structural point of view, azo dyes being cost effective due to ease and versatility of synthesis and to relatively high tinctorial strength, constitute the largest group of synthetic colorants.

There are, of course, many varieties in each of these chemical classifications, so that the result is hundreds of individual dyes each with its

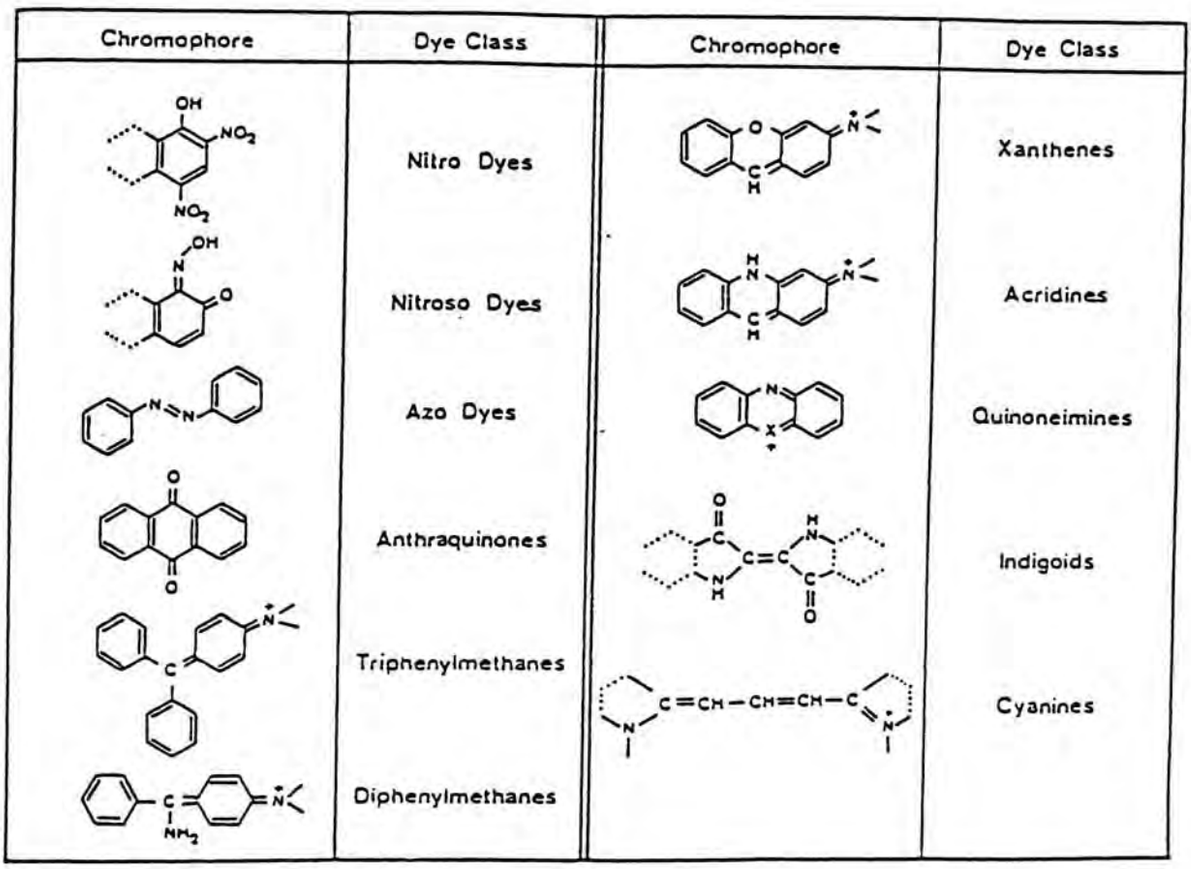


Figure 1-1. Classification of synthetic organic colourants by chromophores (Meyer, 1981)

own specific characteristics for hue and use on substrates. To users it is usually more convenient to consider them according to their method of application and the type of fibre to which they are most suited. Dyers have arranged this very complex and large group of chemical products into ten categories arranged by the method of application to the fibber or substrate. They are: acid dyes, azoic dyes, basic dyes, direct dyes, disperse dyes, reactive dyes, solvent dyes, sulphur dyes, vat dyes, and mordant dyes (Boyle, 1973).

1.3 THE APPLICATION OF DYES

Dyestuffs are widely used for colouring such diverse materials as paper, plastics, leather, wax polish and cosmetics. Some dyes are widely used as colorants in foods such as soft drinks, candy, hot dogs, ice cream, and cereals and in drugs, etc. By far the most important application of dyes, however, is to colour textile fibres.

Although the textile industry is by far the largest user of dyes, in the past few years organic colorants have become increasingly important in the high technology industries (Gregory, 1991). These other areas are diverse but they may be broadly classified into three main types: photographic, electronic/electrical and biological. Colour photography represents a very important commercial outlet for dyes which are used both as photosensitizer and in the image forming process. The use of dyes in Electronic/electrical

includes electrophotography, lasers and liquid crystal. Some biological applications of dyes are used as therapeutic agents (antiseptics and drugs) and, more recently, for protein purification and as structural probes for investigating biochemical processes.

1.4 ECOLOGICAL ASPECTS OF COLOUR CHEMISTRY

Today annual world production of synthetic colorants is estimated to be more than million tones. During manufactural and process operation a lot of dyes have been discharged to the environment that caused serious pollution. For instance, 0.45 million tonnes of dyestuff were produced in 1978, approximately 2% were estimated to be discharged in aqueous effluents during manufacture of the dyestuff and approximately 9% in dye-house operations. Dyes can be found in the effluent of most sewage treatment facilities. It is also known that the dyes are chemically unchanged in treatment plant effluents. The dyes are recalcitrant pollutants. The effect of dyes to environment and biosphere were paid more and more attention by people.

1.4.1. Toxicity to Microorganisms

Many dyes, especially azo dyes, have been found to affect microbial activities and microbial population sizes in aquatic environments.
The study of toxicity to microorganisms was initiated by the ADMI (American Dye Manufacturers Institute, Inc). After that, various studies had been done

to test the effects of dyes to different microorganisms. Azo dyes such as Basic Brown 4, Direst Brown 95, Direct Black 80, etc were inhibitory to microbial oxidation processes in both activated sludge and stream water. Ogawa, *et al* (1981) revealed that the synthesis of DNA was inhibited by *p*-Aminoazobenzene. Fung and Miller (1973) used a rapid screening procedure to test the effect of 42 dyes on growth of 30 bacterial on solid media. The results indicated that many available dyes were inhibitory to specific bacterial groups. The dye Tropaeolin O was found to be toxic to microbial populations in fresh water (Michails *et al.*, 1985).

The activated sludge method is one of the most widely used biological processed in wastewater treatment plants. The behaviour of activated sludge with dyes had also been studied by Idaka *et al* (1985)., Some dyes such as Orange II and Crystal Violet showed an inhibitory effect in the purification function of sewage treatment facility. The toxicity of azo dyes to microorganisms causes a decline in the purification function of the activated sludge process. Inhibitory effects on activity and growth of microorganisms can be found in particular with high dye concentrations; with some cationic species effects are noticeable at low concentration as well (Holme, 1984)

1.4.2 Toxicity to Mammals

Studies from as early as 1895 reported that workers in the dye industry have an incidence of urinary bladder tumours greater than in the general

population. Dyes are some of the first organic compounds to be associated with human cancer. Amaranth has been shown to be carcinogenic in rats (Andrianova 1970). Orange II has been reported to induce bladder tumours in mouse (Bonser 1956). The International Agency for Research on Cancer has published summaries of the literature on azo dyes that are suspected to be human and laboratory animal carcinogens. There are more than 30 kind of dyes in the list.

The correlation of bladder cancer with occupational exposure to azo dyes was studied by Case *et al* (1954). From that time to now, there are hundreds of publications describing the mechanisms of dyes carcinogenicity to man and to laboratory animals. Brown *et al.*(1993) has reviewed the literature that described why some azo dyes may be human carcinogens. Three different mechanisms for azo dye carcinogenicity were identified:

1). Azo dyes that are toxic only after reduction and cleavage of the azo linkage to give aromatic amines.

2). Azo dyes with structures containing free amine groups that can be metabolically oxidized without azo reduction.

3). Azo dyes that may be activated via direct oxidation of the azo linkage to highly reactive electrophilic diazonium salts. The three mechanisms all involve metabolic activation to give reactive electrophilic intermediates that bind DNA covalently.

In addition to their carcinogenic and mutagenic potential, some of the azo

dyes, including Trypan blue and Congo red have embryopathic effects in rodents (Gray *et al.*, 1992). They studied the relationship between gonadal agenesis and fertility in male and female mice exposed *in utero* to the diazo dye Congo Red and found maternal Congo Red treatment inhibited testicular and ovarian function in the offspring.

1.5 COLOUR CONTAMINATION

Apart from the toxicity to living organisms, an additional concern with the wastewater is the presence of colour. High levels of colour are common on textile wastewater. As a general comment, any appreciable quantity of most dyestuff in water is visible. Although it is not possible to give precise concentrations at which dyes would be visible. It has been suggested that 0.1 mg/l may be detectable, and that at 1 mg/l colour will generally be observable. Since the discharge of coloured water presents an environmental concern due to the loss of aesthetic value of the receiving water and the negative effects on aquatic life due to decreased light transmittance, the need for an economical, effective method to remove the colour exists. The discharge of dyestuffs from a specific manufacture site may pose a specific local problem. The remove of colour is therefore a challenge to both the textile industry and the wastewater treatment facilities that must treat these wastewaters.

1.6 TREATMENT OF WASTEWATER CONTAINING DYES

The contamination of the environment with industrial chemicals during the 20th century has overwhelmed the natural cleaning capacities of microorganisms. It caused a serious environment pollution. Scientists are giving effort to find effective methods to solve this problem. Many waste water treatment plants have been built. Measures are taken to satisfy environmental protection requirements include physical, chemical and biological treatments (Bhattachcharya, 1991; Yang, 1990).

Biological method has provided economical and safe solutions for dealing with environmental problems. But in dyestuff industry, dyes have to be more and more resistant to sunlight, washing and microbial action to be successful in the commercial market. It is not surprising that most studies on the biological degradation of dyestuffs yield negative result when dyes are designed to resist this type of treatment. Shaul *et al* (1991) tested 18 azo dyes in activated sludge systems, 11 compounds were found to pass though the activated sludge process substantially untreated, 4 were significantly absorbed onto the waste activated sludge, only three were apparently degraded. Effluents from dyes industrial processes are usually resistant to biological treatment (Paszczynski *et al.*,1991). Costly physical-chemical decontamination processes are often the only treatment alternatives available for such wastewaters.

The conventional methods for treatment of such wastes include neutralization, bleaching and adsorption on activated carbon and cellulose

polymer (Hwang, 1993). Among them coagulation/clarification is an effective process for dye wastewater treatment (Kimberly *et al.*, 1988; Kimberly *et al.*, 1989). Typical coagulants are alum, ferrous sulphate, ferric chloride, cationic, anionic, and non-ionic organic polymers. But the addition of large amounts of chemicals result in the production of significant quantities of waste solids. The ultimate disposal of these wastes may be very expensive. Ozonation is a very effective oxidation process for colour removal. A combination of ozonation with other processes like ultraviolet light was found to be more efficient than individual processes (Beszedits *et al.*, 1980). Other processes especially applicable to colour removal include chlorination, photochemical degradation (Holme, 1984), irradiation, ion exchange, liquid-liquid extraction and etc. These processes may show special advantages for specific, small-volume, dye wastewater. However physical/chemical methods become expensive for large-scale treatment and hence biological methods of destroying these chemical compounds assume considerable interest yet (Kanekar *et al.*, 1991; Holme, 1984).

1.7 STUDIES ON THE FIELD OF BIODEGRADATION OF DYES

1.7.1 Current Knowledge of Biodegradation of Azo Dyes by Bacteria.

In this context the most important microbes by far are the bacteria, capable of utilizing as food---and hence degrading---an infinite variety of organic compounds. This extraordinary adaptability is a result of their

relatively simple organization and structure, allowing revisions of their metabolic capabilities to an extent unapproachable by higher organisms. Given the opportunity to make such revisions, termed *adaptation* or *acclimation*, bacteria can live and propagate indefinitely even upon such improbable foods as gasoline, benzene, phenol, dyes, and other compounds ordinarily considered toxic.

Studies on the metabolism of dyes were begun in the food colorants. As early as 1937, Brohm and Frohwein found decolorization of azo dyes in spoiled dairy products. Amines were found as reaction products so that a reductive cleavage of the azo bridge was assumed. In later work various microorganisms were isolated from a number of sources. Roxon *et al.* (1966) isolated a *Proteus* Sp. from rat gut with azo-reducing ability. Scheline *et al.* (1970) investigated rat faeces and found an *Enterococcus* sp. which could reduce an azo dye. In studies on the anaerobic reduction of the sulfonated water-soluble azo dyes Tartrazine, Amaranth, Sunset Yellow, and New Coccine by suspensions of bacteria isolated from human faeces, it was shown that the bacteria could efficiently reduce all these dyes (Watabe *et al.*, 1980).

However, it was not until recently that colorant-degrading microorganisms were sought on the context of environmental pollution, with more awareness of the pollution problems caused by the dispersal of azo dyes to the environment.

In screening pure cultures for degradation of potential pollutants from dyestuff factories and the textile industry, *B. subtilis* was found to be highly active in reducing p-aminoazobenzone (Toritsu *et al.*, 1977). Conditions were anaerobic and degradation products identified were aniline and *p*-phenylenediamine. Idaka *et al.* (1978) screened for some species by selection from the draining ditches of dyestuff factories. *Aeromonas hydrophila* var 24B was isolated and found to degrade a number of model compounds, mainly derivatives of azobenzene. One isolate from the environmental, *Pseudomonas* S-42 was reported to reduce the azo dyes diamira Brilliant Orange RR, Direct Brown M, and Eriochrome Brown (Lui *et al.*, 1989). They found that oxygen did not inhibit the azo reduction capacity of whole-cell suspensions. However, azo reduction by the purified enzyme was severely inhibited in the presence of oxygen.

As describe above, a wide range of water-soluble azo dyes are reported to be efficiently reduced by bacterial systems under anaerobic condition and the reaction is quite general. Anaerobic transformation by all of these microorganisms begins with reductive fission of the azo linkage, resulting in the formation and accumulation of colourless aromatic amines.
Under anaerobic conditions no further decomposition was observed (Leisinger *et al.*, 1981).

Under aerobic condition, azo dyes has been considered essentially nondegradable by bacteria (Pasti-Grigsby *et al.*, 1992). To achieve complete

degradation, however, scientists studied the factors that might cause the aerobic recalcitrance and searched for ways to circumvent the barriers. Within the last decade, some microorganisms have been found that had the ability of degradating azo dyes under aerobic condition.

After long periods of adaptation in a chemostat under carefully adjusted selective pressure, two *Pseudomonas* strains KF46 and K24 were isolated which utilized carboxy-Orange II as the sole carbon and energy source (Zimmermann *et al.*, 1982). It was found that KF46 had an Orange II-specific azo reductase and K24 had an Orange I-specific azo reductase. The KF46 azo reductase was plasmid-borne and inductase by Orange II carboxy and gratuitously by Orange II. The K24 azo reductase was constitutive. Substrate specificity was tested with a wide range of azo compounds. These strains are highly substrate-specific. Kully *et al.* (1981) observed that a strictly aerobic, soil *Flavobacterivm* species reduced 4,4'-dicarboxyazo-benzene to two molecules of aminobenzoic acid under aerobic condition and used it as sole carbon and energy source. Idaka *et al.* (1987) isolated a strains, *Pseudomonas cepacia* 13NA, from the sludge discharged from azo dye factory. It can completely degradate p-aminoazobenzene. The reductive metabolism of aminoazobenzenes and the oxidative pathway that followed had been studied.

1.7.2 Degradation of Azo Dyes by Fungi and Helminths.

Bacteria play an important role in biodegradation of azo dyes. In addition

to bacteria, fungi, nematodes and cestodes have been reported to reduce azo dyes. In recent years, biodegradation researchers have become increasingly interested in the versatile lignin-degrading white-rot fungus *Phanerochaete chrysosporium.* This fungus is able to mineralize a variety of persistent environmental pollutants, including DDT; lindane; 3,4-dichloroaniline; and dieldrin. A report by Cripps *et al.* (1990) has added an entirely new family of dyes to the long list of organic compounds attacked by *P. chrysosporium.* The dyes include Tropaeoline O, Orange II, and Congo Red. After that, Spadaro *et al.* (1992) utilized radiolabeled substrated to establish that *P. chrysosporium* is capable of mineralizing a wide variety of azo dyes including 4-phenylazophenol, 4-phenylazp-2-methoxyphenol, Disperse Yellow 3, Disperse Orange 3, and Solvent Yellow 14. Twelve days after addition to cultures, these dyes had been mineralized 23.1 to 48.1%. The detailed mechanisms of the biodegradation of azo dyes by fungus is not yet clear.

Besides fungi, nematodes and cestodes have been reported to reduce azo dyes also (Douch and Blair, 1975). Azobenzene, dimethylaminoazobenzene, and 1,2-dimethyl-4-(4-carboxyphenylazo)-5-hydroxybenzene were reduced by the nematode *Ascaris lumbricoides* var. *suum.* Female nematodes were more active in the reduction of these dyes than the males. The younger and smaller nematodes showed a higher azo reduction activity per gram of tissue than mature nematodes (Chung *et al.*, 1993)

1.8 PURPOSE OF STUDY

As described in the introduction, biodegradation of azo dyes can occur in an aerobic and in an anaerobic system. It is important to differentiate clearly between these two conditions. The anaerobic decolorization is relatively easy to achieve by reductive fission of the azo linkage. Under anaerobic conditions no further decomposition was observed. The result are the formation and accumulation of colourless aromatic amines. In most cases, the biodegradation of dyes dose not occur spontaneously in aerobic condition. Lengthy adaptation periods are generally necessary.

It is well known that simple aromatic compounds can be degraded under aerobic conditions via hydroxylation and ring-opening. The breakdown of aromatic compounds is a vital biochemical step in the natural carbon cycle. An aerobic process following the anaerobic process should be able to biodegrade the intermediate(s) formed by an anaerobic degradation of the azo dyes. Such a combination of anaerobic/aerobic processes appears to be a very promising concept.

The present research is based on the concept to isolate bacteria from polluted sites and used these isolates through an anaerobic/aerobic system to degrade azo dyes completely. The following goals are set:

I. Isolate strain from the polluted site that can decolorize azo dyes efficiently under anaerobic condition.
II. Isolate strain from the polluted site that can degrade the aromatic

amines formed in the decolorization step.

III. Optimise the growth and degradation ability of the isolated strains.

IV. Improve the ability of degradation by an immobilization method.

V. Design a bioreactor system that can accommodate a convenient switching in the anaerobic/aerobic reaction process.

CHAPTER 2

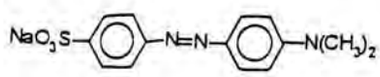
MATERIALS AND METHODS

2.1 MATERIALS

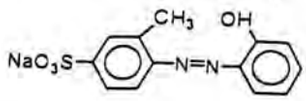
2.1.1 Chemicals

The azo dyes chosen for this investigation were market available and readily soluble in water. They are: Acid Orange 8, Conco Red, Orange I, Orange II, and Tropaeolin O (purchased from Sigma Chemical Co., St.Louis, Mo. USA.); Acid Red 88, Acid Yellow 9, Alizarin Yellow 2G, Chromotrop 2B, Chromotrop 2R, Crocein Orange G, Crocein Scarlet Moo, Direct Black 19, Direct Blue 14, Hydroxynaphthol Blue, Metanil Yellow, Methyl Orange, Nitrazine Yellow, Nitrosulfonazo III, Orange G, Poncean BS, Poncean Xylidine, Reactive Violet 5, Sulfanilic Acid Azochromotrop, Sulfonazo III, and Tropaeolin OO (purchased from Fluka Chemika-Biochemika. Buchs, Switzerland). Figure 2-1 depicts their chemical structures. Both Sulfanific Acid and Sodium alginate (mean molecular weight: 178,000) were purchased from Sigma Chemical Co.. All other chemicals used for the preparation of growth and assay media and buffer solutions were purchased from Sigma Chemical Co., Fluka, or Difco Laboratories. All these chemicals employed were of analytical grade.

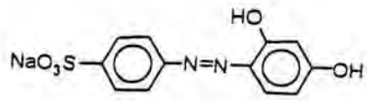
I. Methyl Orange



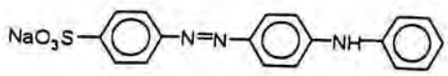
II. Acid Orange 8



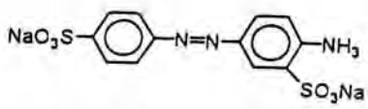
III. Tropaeolin O



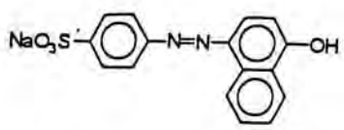
IV. Tropaeolin OO



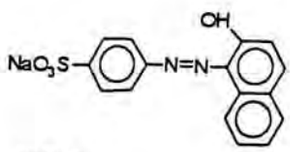
V. Acid Yellow 9



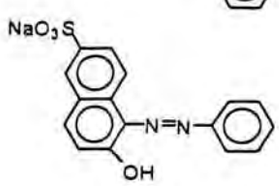
VI. Orange I



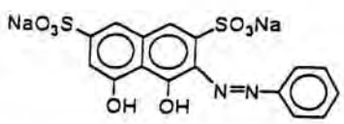
VII. Orange II



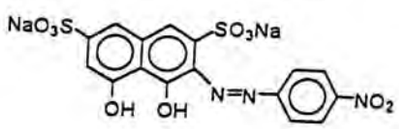
VIII. Crocein Orange G



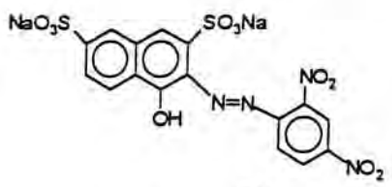
IX. Chromotrop 2R



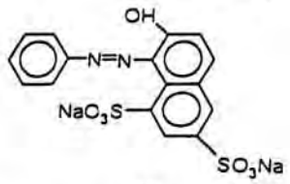
X. Chromotrop 2B



XI. Nitrazine Yellow



XII. Orange G



XIII. Poncean Xylidine

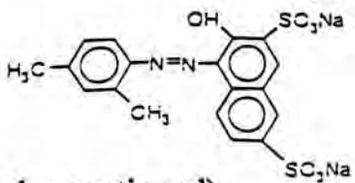
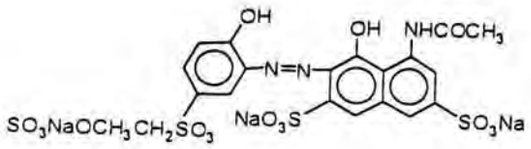
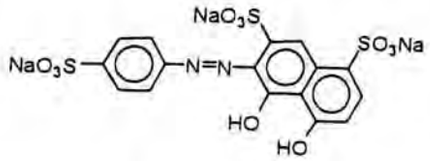


Figure 2-1. Chemical structure of azo dyes (to be continued)

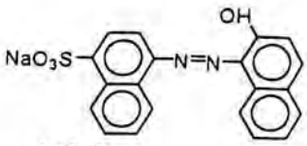
XIV. Reactive Violet 5



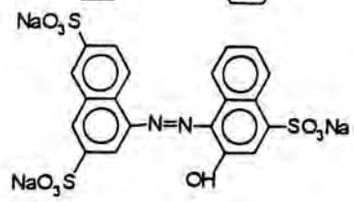
XV. Sulfanilic Acid Azochromotrop



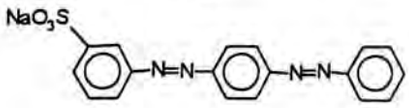
XVI. Acid Red 88



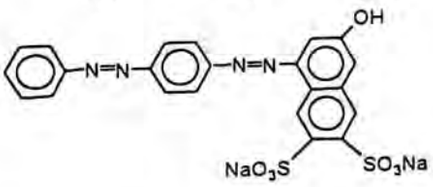
XVII. Hydroxynaphthol Blue



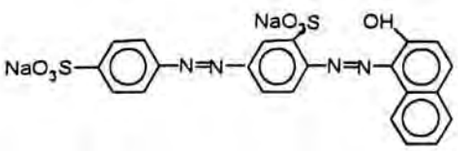
XVIII. Metanil Yellow



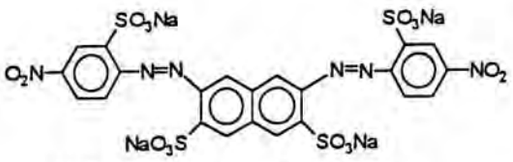
XIX. Crocein Scarlet Moo



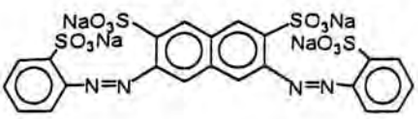
XX. Poncean BS



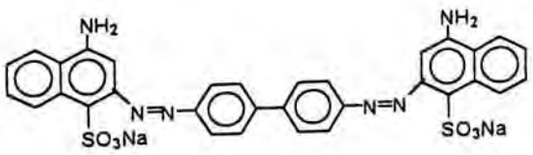
XXI. Nitrosulfonazo III



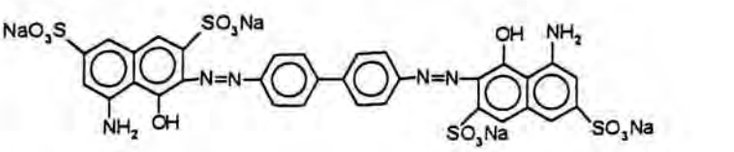
XXII. Sulfonazo III



XXIII. Congo Red



XXIV. Direct Blue 14



XXV. Direct Black 19

Figure 2-1. Chemical structure of azo dyes

2.1.2 Recipes

2.1.2.1 Isolation Medium (I.M.)

Composition per liter:	KH_2PO_4	1.8 g
	$Na_2HPO_4 . 2H_2O$	3.0 g
	$MgSO_4 . 6H_2O$	0.2 g
	Nutrient Broth	5.0 g
	Agar	15.0 g
	Glucose solution	50.0 ml
	Azo dye solution	10.0 ml

Glucose Solution:

Composition per 100 ml:	Glucose	20.0 g

Preparation of Glucose Solution: Add glucose to distilled water and bring volume to 100.0 ml. Mix thoroughly. Sterilized by filtering.

Azo Dye Solution:

Composition per 100 ml:	Azo dye	1.0 g

Preparation of Azo Dye Solution: Add azo dye (Orange II, Reactive Violet 5, and Direct Blue 14) to distilled water and bring volume to 100.0 ml. Mix thoroughly. Sterilized by filtering.

Preparation of medium: Add components, except glucose solution and azo dye solution, to distilled water and bring volume to 940 ml. Mix thoroughly. Adjust pH to 6.8. Autoclave for 15 min at 15 psi pressure-121°C. Cool to 50-55°C. Aseptically add 50 ml sterile glucose solution

and 10 ml azo dye solution. Mix thoroughly. Pour into sterile Petri dishes.

2.1.2.2 Basal Medium (B.M.) (Brilon *et al.*, 1981)

Composition per liter:	KH_2PO_4	1.8 g
	Na_2HPO_4	3.0 g
	$MgSO_4.6H_2O$	0.2 g
	$FeCl_3.6H_2O$	0.02 g
	NH_4Cl	1.0 g
	Glucose solution	25.0 ml
	Azo dye solution	1.0 ml

Glucose Solution:

Composition per 100 ml:	Glucose	20.0 g

Preparation of Glucose Solution: Add glucose to distilled water and bring volume to 100.0 ml. Mix thoroughly. Sterilized by filtering.

Azo Dye Solution:

Composition per 100 ml:	Azo dye	1.0 g

Preparation of Azo Dye Solution: Add azo dyes to distilled water separately and bring volume to 100.0 ml. Mix thoroughly. Sterilized by filtering.

Preparation of medium: Add components, except glucose solution and azo dye solution, to distilled water and bring volume to 965 ml. Mix thoroughly. Adjust pH to 6.8. Autoclave for 15 min at 15 psi pressure-121°C and cooled to room temperature. Aseptically add 25 ml sterile

glucose solution and 10 ml azo dye solution. Mix thoroughly. Distribute into sterile flasks.

2.1.2.3 Luria Broth Medium (LB)

Composition per liter:	Pancreatic digest of casein	10.0 g
	NaCl	5.0 g
	Yeast extract	5.0 g

Preparation of Medium: Add components to distilled water and bring volume to one liter. Mix thoroughly. Adjust to 7.0. Distribute into flasks. Autoclave for 25 min at 15 psi pressure-121°C.

2.1.2.4 Mineral salt medium (M.S.M.) (Zimmermann et al., 1982)

Composition per liter:	KH_2PO_4	1.8 g
	Na_2HPO_4	3.0 g
	$MgSO_4.2H_2O$	0.2 g
	$(NH_4)_2SO_4$	1.0 g
	Trace elements solution	1.0 ml
	sulfanific acid solution	5.0 ml

Trace elements solution:

Composition per liter:	$CaCl_2$	0.1 g
	$EDTA.Na_2$	0.505 g
	$FeSO_4.7H_2O$	0.2 g

$CoCl_2.6H_2O$	0.04 g
H_3BO_3	0.03 g
$ZnSO_4$	0.01 g
$MnSO_4.H_2O$	0.003 g
$CuSO_4.5H_2O$	0.001 g
$Na_2MoO_4.H_2O$	0.002 g

Preparation of trace elements solution: Add components to distilled water and bring volume to 1.0 liter. Mix thoroughly. Sterilized by filtering.

sulfanific acid solution:

Composition per 100 mL: sulfanific acid 1.0 g

Preparation of sulfanific acid solution: Add sulfanific acid to distilled water and bring volume to 100.0 mL. Mix thoroughly. Sterilized by filtering.

Preparation of medium: Add components, except Trace elements solution and sulfanific acid solution to distilled water and bring volume to 994 mL. Adjust PH to 6.8 with 0.5 M NaOH. Autoclave for 15 min at 15 psi pressure-121°C. Ascetically add sulfanific acid solution and Trace elements solution. Distribute into sterile flasks.

2.2 METHODS

2.2.1 Isolation of Azo Dye Decolorization (ADD) Strain.

2.2.1.1 Sample collection.

Both water samples and soil samples were collected from Tuen Mun River in the New territories, and Shing Mun River in the Shatin, Hong Kong. There are several large scale dyeing factories located at Tuen Mun. The large volume of waste water discharged from these factories has polluted heavily the Tuen Mun River at many spots. An unsightly layer of filthy substances has accumulated on the river course. To obtain a microbial flora with a broad azo dye decolorization potential, twenty soil samples of 5 grams each were collected from 20 sites chosen randomly near the side of the water. All of the samples were dug under 5-10 cm from the surface of the soil. They were packed separately in autoclaved paper bags. The fen water samples, each 10 ml volume, were collected from the polluted water and saved in autoclaved 20 ml stopped glass bottles.

2.2.1.2 Preparation of inoculum

To a pooled 2 grams soil samples (0.1 g soil from each collected sample), 100 ml of physiological saline were added.. The mixture was vigorously shaken for 2 hours in a rotary at 250 r.p.m. and allowed to stand

at room temperature for 5 min. 10 millilitres of the supernatant were mixed with a same amount of water samples collected form the rivers. A serial dilutions (10^{-1}-10^{-6}) of this solution were prepared by adding physiological saline in order to obtain an appropriate number of microbial colonies on each plate for easy handling.

2.2.1.3 Selection and isolation strain ADD 16-2

0.2 ml aliquots of the diluted solutions were spread on plates containing Isolation Medium (I.M.) supplemented with either of the azo dyes (Orange II, Reactive Violet 5, and Direct Blue 14) and were incubated at 30°C for 72 hr to allow colony formation. Strains that formed a clear halo around its colony were picked and purified by restreaking on a fresh plates. The strains that showed the largest clear zone was selected for further studies.

2.2.2 Optimal Growth Condition for Strain ADD 16-2

Many factors affect the growth of strain ADD 16-2. Among all factors, the requirement of oxygen seems affecting. It is known that obligate aerobes require oxygen as the terminal electron accepter while obligate anaerobes are unable to tolerate the presence of a trace of oxygen. To test for oxygen requirement, cultures identical containing nutrient were prepared. To each flask containing 50 ml Basal Medium (B.M.), an overnight culture (OD_{650} 1.8) was inoculated (1% final). The cultures were incubated separately either in an

incubator without disturbing or in a rotary shaker at 200 r.p.m.. Both cultures were incubated at 30°C. 2ml aliquots were withdrawn from both experiments at certain time intervals and the growth were monitored by measuring the OD at 650 nm using a UV-1201 spectrophotometer (Shimazn Corporation, Kyoto.Japan).

The temperature effect on growth was studied accordingly by comparing the growth rate of cells suspended in B.M. medium and incubated at various temperature. The temperature chosen were 25°C, 30°C, 33°C, 35°C, 37°C, 40°C. The cultures were incubated individually on a rotary shaker at 200 r.p.m.

To obtain a pH optimal of the strain ADD 16-2, Basal Medium adjusted to various pH values were prepared. The pH of the cultures were adjusted to 6, 6.5, 7, 7.5, and 8.0 individual by using 0.2 M KH_2PO_4/Na_2HPO_4 buffer. Growth of the cells were monitored by the methods as described previously.

The carbon source requirement of strain ADD 16-2 was studied by replacing glucose in the Basal Medium with either one of the following: starch, citrate, acetate and sucrose. The cultures were incubated on a rotary shaker at 30°C and 200 r.p.m. and growth rate of cells were monitored by the methods described above.

2.2.3 Assay of Decolorization Activity

2.2.3.1 Measurement of azo dye concentration

Spectrophotometric measurement was used to determine the concentration of dyes (Pasti-Grigsby *et al.*, 1992). Since different colour dye absorbs radiant energy at different wavelength of absorbance maxima, solution of each dye at its proper concentration was scanned to identify the wavelength of absorbance maxima. These values were reported in results and used here wherever needed. Since the spectra of the dyes were affected by pH, the samples were all adjusted to pH 7.0 before spectrophotometric measurement. To abstain the absorbance maxima of these dyes at pH 7.0, the dyes were dissolved in 0.5 M phosphate buffer (pH 7.0) at the concentration of 25 ppm. These samples were scanned from 200nm to 650 nm using Spectronic 3000 Array spectrophotometer (Milton Roy Company).

For quantifying the dye concentrations, standard curve of each dye was constructed with known weight of the particular dye concerned, and measure at its absorbance maxima chosen. To obtain concentration ranges from 0 to 100 ppm, a serial dilutions were prepared from a stock solution by adding 0.5 M phosphate buffer. The absorbency was measured by UV-1201 spectrophotometer (Shimazn Corporation, Kyoto, Japan), and the values plotted against their concentrations to give a standard curve for that dye.

The wavelength of absorbance maxima and the standard curve of concentration were used in the measurement of concentration. For measurement of the concentration of dye in experimental sample containing suspended cells, cells were was firstly spun down by centrifugation and the supernatant taken for measurement. Whenever a dilution was required, 0.5 M phosphate buffer was employed until the absorbance was in the linear part according to the standard curve.

2.2.3.2 Assay of azo dye decolorizing activity of strain ADD 16-2

The environmental factors can exert a profound effect on the growth rate of bacteria via its influence on the strain itself and by its effect on the substrate. The effect of oxygen, Temperature, pH and the nature of carbon source on the decolorizing activity of strain ADD 16-2 were hence tested.

For the determination of the oxygen effect, 5 ml stock inoculum (OD 2.8) was added to 100 ml B.M. containing 100 ppm Tropaeolin O. The cultures were incubated separately either in an incubator without stirring or in a rotary shaker operating at 200 r.p.m.. The concentration of dye in each culture was monitored by moving the aliquots from the reaction medium at various time intervals and quantified spectrophotometrically ally as described before. For Tropaeolin O, the wavelength of absorption maximum was 430 nm.

The temperature effect on the decolorization ability of strain ADD 16-2 was studied by incubating in B.M. and incubated at 25°C, 30°C, 35°C, 37°C and 40°C respectively. The decolorization of dye was determined by the methods mentioned above.

The effect of pH on decolorization activity was tested in the range of 4.5 to 8.5. The pH of the cultures were adjusted by varying 0.2M Tris-malate buffer. The cultures were incubated at 35°C for 24 hours and the colour of dye determined as before.

For determine the effect of carbon source on the decolorization activity, a one percent of different carbon sources were used to replace glucose in the B.M.. For substitute, sucrose, citric acid, acetic acid and starch were included in this assay. The cultures were incubated at 35°C, pH 6.5 for 24 hours. The decolorization of dye was monitored by the method described above.

The decolorization activity was expressed as the % of dye remained after a fixed assay period for a particular dye. The equation used to calculate the percentage of dye remained was:

$$\text{Percentage of dye remained at } t_x = 100 - \frac{\text{Initial Absorbency} - \text{Absorbency at } t_x}{\text{Initial Absorbency}} \times 100$$

2.2.3.3 Structural speciality of the decolorization reaction.

Azo dye exhibits great structural variety. The decolorization activity of strain ADD 16-2 on different dye was investigated in B.M. medium at a dye concentration of 50 ppm. The cultures were incubated at 35°C, pH 7.0 for 24 hours and the colour intensity was monitored at various time interval by withdrawing aliquots for measurement.

2.2.4 Identification of the ADD Strain Cleavage Product(s)

Dye and its metabolites in the culture fluid were analyzed by reverse-phase high-pressure liquid chromatography in order to understand the product formation of the decolorization reaction (NÖtemann *et al.*, 1986). For HPLC analysis, the samples were taken out from the assay flashs at chosen time intervals and the cells and particles were removal by centrifugation at 10,000 x g for 20 min followed by filtering through a 0.2-μm-pore-size filter (Millepore, GVWP 01300). Fifty microliters of the filtered fluid were injected into an Ultra Pac column (250 by a mm) filled with C-18 (10 μm diameter). The HPLC system was operated with a LKB 2150 pump and 2152 controller connected to a LKB 2141 variable wavelength monitor. The detection wavelengths were 248 nm, 280 nm and 431 nm. The following solvent systems were used as mobile phases: 50% methanol, 48% double distilled water and 2% acetic acid. The flow rate of the mobile phases was 0.5 ml per min.

Retention times and peak areas of both analyses were determined by using a Shimadzu C-R3A computing integrator. Peaks were identified and quantified by comparing with authentic standards.

2.2.5 Degradation of the Intermediates-Sulfanific Acid

The decolorization of azo dyes by strain ADD 16-2 were due to the cleavage of the azo bond and the corresponding aromatic amines accumulated in the medium. In the case of Tropaeolin O, the product is sulfanific acid. In order to totally degrate Tropaeolin O, the sulfanific acid degradation strain had to be isolated.

2.2.5.1 Enrichment and isolation of sulfanific acid degradation (SAD) strains

It is well known the simple aromatic compounds can be degraded under aerobic condition via hydroxylation and ring-opening (Hartmann *et al.*, 1979; Holliger *et al.*, 1992; Vorobjeva *et al.*, 1990; Radehaus *et al.*, 1992). The aerobic condition was employed. To select for a desired microbial organism that can metabolise sulfanilic acid, the standard batch enrichment culture techniques were performed (Lenke *et al.*, 1992) in which 10 g pooled soil samples collected from the course of Tuen Mun and Shing Mun River were added to 100 ml M.S.M. Glass beads (2 mm diameter) were added to the mixture and was shaken at 200 r.p.m. for 2 hours. After precipitating for 5 min, 10 ml of the suspension was removed and added to 100 ml M.S.M. (Liu,

1993). This preparation was incubated at 30°C and stirred at 200 r.p.m.. When growth was observed, as determined by an increase of turbidity, 5 ml of the suspension was transferred to a new flask containing the same M.S.M. and growth was once repeated. The same process was repeated for twenty times in a period of three months. During this period of enrichment, the concentration of sulfanific acid in the medium was stepped up from 50 ppm to 200 ppm. The growth status of the SAD strains (as a mixture) were monitored by measuring the absorbency of the suspension at 650 nm. The degradation of sulfanific acid was also monitored by spectrophotometric method.

As soon as the mixture microorganisms were build that can use sulfanific acid as the sole growth substrate, a serial dilutions (10^{-4}-10^{-9}) of the cell suspension solution were prepared by dilution with physiological saline to give single isolated colonies on LB agar plates. The plates were incubated at 30°C. After 5 days, individual colonies were picked and purified by restreaking on fresh plates. The single colonies were tested for their ability in degradation sulfanific acid.

2.2.5.2 Optimal sulfanific acid degradation condition of strain SAD M-1

The pH and temperature optimum for the reaction were studied in M.S.M. cell suspension. The pH were adjusted by varying H_3PO_3 buffer covering a pH range of 8.0 to 10.0. The temperature ranged from 25 to 40°C.

The degradation of sulfanific acid was monitored by measuring the change of absorbance at 248 nm which is the absorption maximum of sulfanific acid. For most samples, cell concentration were approximately 10^8 cells/ml and sulfanific acid at a final concentration of 100 ppm was used.

2.2.6 Complete Degradation of a Model Azo Dye (Tropaeolin O) by Co-metabolism of Strain ADD 16-2 and Strain SAD M-1

To demonstrate total degradation of Tropeaolin O, the dye was first incubated anaerobically with the strain ADD 16-2 in M.S.M. that 100 ppm Tropeaolin O was used to instead of sulfanific acid. The reaction was operated in a 150 ml flask equipped with screw cap to prevent possible contamination with oxygen during test. The flasks were filled with medium and incubated at 35°C with a pH of 8.0 adjusted by 0.2 M phosphate buffer. The 0.05% glucose was added to enhance the reduction rate of the azo dye decolorization. After complete reduction of Tropaeolin O as monitored by OD, the cells (ADD 16-2) were removed by centrifuging the sample at 10.000 X g for 20 min and filtering through a 0.45 μm millepore filter. Strain SAD M-1 was then added to the treated supernatant in a 250 ml flask. For final transformation to take place, an aerobic environment was required. This was done by incubated on a rotary shaker operating at 200 r.p.m. The temperature of reaction was 33°C.

In the anaerobic-aerobic process, at certain time intervals, aliquots of

culture were taken out. The cells were spun down by microcentrifuge for 5 min. The supernatant were diluted 5 fold with phosphate buffer (pH 7.0) and were scanned from 200 nm to 700 nm. The profiles were used to monitored the degradation of dye.

2.2.7 Assay for the Degradation of the Tropaeolin O by Immobilized Strain ADD 16-2 and Strain SAD M-1

2.2.7.1 Method of immobilization of bacteria in sodium alginate

The immobilization procedure used was based on that of O'reilly *et al.*, (1989); Kearney *et al.*, (1990) and Steenson *et al.*, (1987) method. The microorganism was incubated in the LB medium until stationary phase. The precultured cells were recovered by centrifugation at 10.000 X g for 15 min and the pellet was resuspended in physiological saline (0.85% NaCl). The cell number was properly calculated so that final contained a fixed cell density which ranged from $5x10^8$ to $6.4x10^{10}$ alive cells per ml of gel. This suspension was mixed 1:1 (wt/wt) with cold (5°C), sterile 2x % alginate solution (autoclaved at 121°C for 15 min) to give a final x % alginate concentration. The x varied from 1 to 5 percent. These alginate-cell mixtures was added dropwise into a 0.1 M $CaCl_2$ solution through an 18-gauge needle driven by a peristaltic pump. The drops solidified into beads containing entrapped cells in the $CaCl_2$ solution. By adjusting the flow rate of alginate solution through the needle, the beads size were controlled to having a diameter between 0.5

to 4.0 mm. Routine practice the proper bead size for this study was 2 mm in diameter. The beads were hardened further in $CaCl_2$ solution for 30 min with appropriate stirring and then collected by filtration. They were stored at 4°C in Tris buffer containing 50 mM $CaCl^2$ before used.

2.2.7.2 Optimal reaction condition for immobilization cells

The effects of concentration of alginate gel on the degradation activity of immobilized cells. A serial concentration of alginate containing certain number cells of SAD M-1 and ADD 16-2 were prepared as described above. To test the decolorization activity of the immobilized cells ADD 16-2 in different concentration of gel, the reactions were performed with 150-ml flasks with 50 ml B.M. which Tris buffer (pH 6.5) to replace the phosphate buffer (B.M.T.) 20 ml of beads at different concentration were added to the medium. The culture was incubated at 35°C and aliquots of 0.5 ml were removed at intervals of 1 hour for measurement. As a control, a cell free calcium alginate matrix were used to determined the absorption of dye by the matrix.

In the degradation of sulfanific acid, 10 ml of beads containing SAD M-1 cells were incubated in 50 ml M.S.M.in which phosphate buffer was replaced by pH 8.0 MOPS buffer (M.S.M.M.). 100 ppm of sulfanific acid was added as sole carbon source. The culture was incubated at 35°C with shaking at 200 rpm. The degradation of sulfanific acid were monitored by the method described above.

The effects of number of cells entrapped in alginate beads on degradation activity. In immobilization samples, the reaction rate increase with the cell load. However, diffusion hindrance can arise at a high cell load. The behaviour of immobilized cells at different cell load was tested. In the experiment the bacterial cells ADD 16-2 and SAD M-1 were entrapped in 2% calcium alginate gel at a serial concentration of cell number. For the cell ADD 16-2, 10 ml of each concentration beads were added to 50 ml B.M.T. containing 100 ppm Tropaeolin O and incubated at 35°C, pH7.0 under anaerobic condition. For the cell SAD M-1 10 ml of each concentration beads were added to 50 ml M.S.M.M. containing 100 ppm sulfanific acid and incubated at 30°C in a rotary shaker operating at 200 r.p.m.. The degradation of the tested compound was monitored by the method described above.

The number of cells in gel was determined by standard plate counting on I.M. agar. Before counting, the alginate beads were dissolved in 0.2 M potassium phosphate buffer (pH 6.8).

The effect of pH and temperature on the degradation activity of the immobilized strain ADD 16-2 and strain SAD M-1. Since changes in the local cellular environment often accompany immobilization, immobilized microbial behaviour may thus be expected to be different with the free-living cells under the solution condition. So the change of degradation activity of immobilized cells at different pH value and temperature were investigated and compare with free cells.

10 ml of the beads containing ADD 16-2 mention above were added in 50 ml B.M.T. The pH and temperature of cultures were adjusted according to the parameter in free cell experiments. The cultures were incubated at 35°C. Similarly, the immobilized cells SAD M-1 were incubated in M.S.M.M. at different pH and temperature used in free cell experiment. At certain time intervals, the aliquots were taken out. The degradation of the tested compounds was monitored by the method described above.

2.2.7.3 The decolorization activity of free and immobilized cells at different dye concentration

In order to evaluate the effect of different concentration of Tropeaolin O on the activity of decolorization, the same number of free and immobilized cells were incubated in B.M.L. containing various amount of dye. The reaction were tested at complete similar condition (35°C, pH 7.0). At certain time intervals, the aliquots were taken out. The degradation of the tested compounds was monitored by the method described above.

2.2.8 Construction of a Continuous Column Systems for Complete Dye Degradation

The density of immobilized cells in their carriers were higher than that of freely suspended cells (6.4×10^{10} cells/ml gel). This situation enables a faster reaction rate to be achieved by the immobilized cells. At the same time, the

used of immobilized cells provide an opportunity to degrade continuously the model compound Tropaeolin O in column system.

2.2.8.1 A continuous anaerobic/aerobic pack-bed column system

Two glass column (2.5 by 30 cm) each made available to house the microbial strain were constructed (Figure 2-2). Both columns have inlet opened at the bottom to allow upward medium flow. The reaction columns have a water jacket attached on its outside so that the content in the column was maintained at a constant temperature close to its optimal one.

In operation, fifty grams of calcium alginate-immobilized ADD 16-2 cell beads were added with enough MOPS buffer to immerse the beads was contained in the first column. The second column was packed with SAD M-1 cell beads. To obtain a good distribution of the liquid phase over the entire reactor, the medium containing 100 ppm Troaeolin O were pumped from the bottom and removed from the top by peristaltic pump. The flow rate was adjusted from 3 ml/min to 1 ml/min to confirm the dye were decolorized completely in the outlet medium. Since the presence of an additional carbon source stimulates the dye decolorization by ADD 16-2, the medium used in this study contained 0.05% glucose. The pH of the medium was adjusted to 7.5 by 0.05 M MOPS buffer. The test was carried out at 30°C.

The decolorized culture removed from the first column was aerated in a

reservoir before pumping to the second packed-bed column. The aerated culture containing the intermediates of decolorization was pumped to the second column then. The effluent concentrations of the dye and the degradation intermediates were monitored by spectrophotometric method described above.

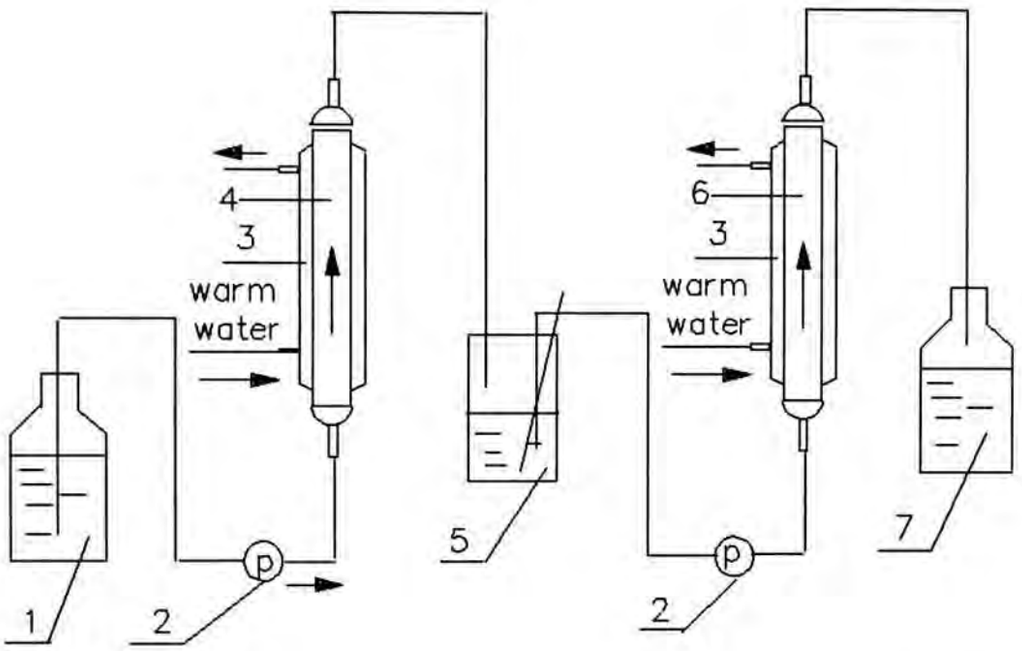


Figure 2-2. A sketch of the continuous anaerobic/aerobic packed-bad column system. (1) dye solution reservoir, (2) peristaltic pump, (3) heat exchanger, (4) anaerobic packed-bed column, (5) aerator, (6) aerobic packed-bed column, (7) treated fluid reservoir.

2.2.8.2 A continuous anaerobic packed-bed column and aerobic airlift-loop reactor

This set up utilized an airlift-loop reactor as the second column in which outer loop and integrated sedimentation section was constructed (Wagner *et al.*, 1988). The reactor consisted of four main parts: a rising section, a gas and liquid separator, a sedimentation part, and an outer loop (Figure 2-3). The rising section was surrounded by a heat exchanger to control the reaction temperature. The volume of the reactor was large enough to hold 50 ml beads. The air introduced into the draft tube at 4 cm/s from bottom of the reactor cause a recirculation of liquid in which the beads were suspended. The effluent concentration of the dye and the intermediates were monitored by spectrophotometric method.

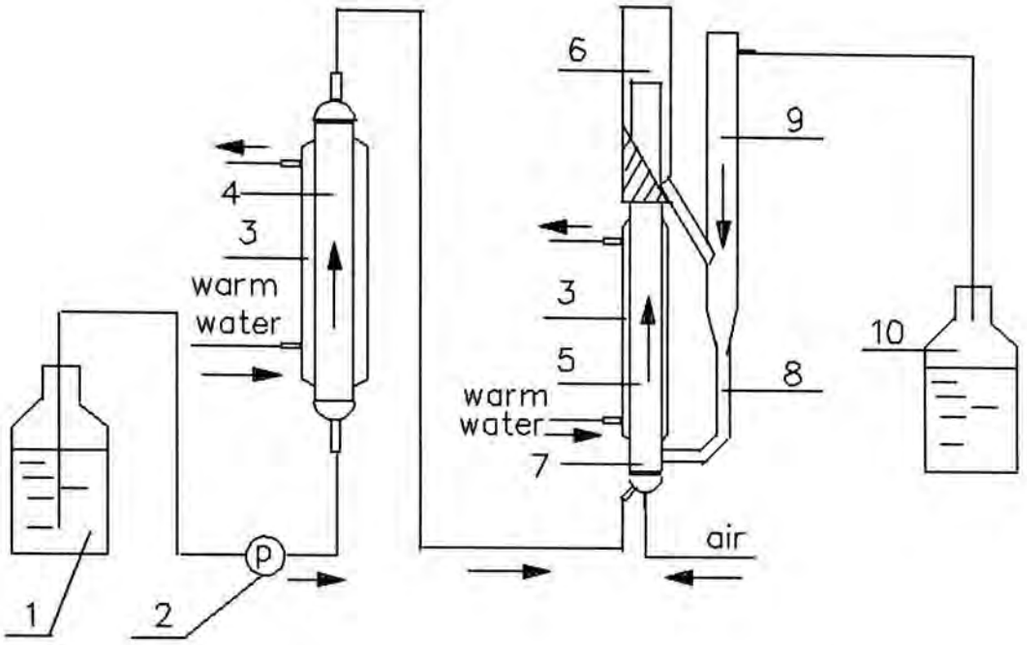


Figure 2-3. A sketch of the continuous anaerobic packed-bed column and aerobic airlift-loop reactor system. (1) dye solution reservoir, (2) peristaltic pump, (3) heat exchanger, (4) anaerobic packed-bed column, (5) riser of airlift-loop reactor, (6) gas-liquid separator, (7) aeration sprayer, (8) out loop, (9) sedimentation tube, (10) treated fluid reservoir.

CHAPTER THREE

RESULTS

3.1 DECOLORIZATION OF AZO DYES

3.1.1 Isolation of ADD Strain

Among a thousand or so microbial colonies formed on I.M. plates, four isolates exhibited an ability to decolorize azo dye resulting in clear halos surrounding their colonies. All four of them exhibited the shape of short rod. The decolorization ability of these strains were tested by comparing the size of the halo formed. Strain ADD 16-2 that held the largest clear zone was selected for further studies. A typical culture of strain ADD 16-2 was depicted in Figure 3-1 and Figure 3-2.

3.1.2 Growth Condition of Strain ADD 16-2

3.1.2.1 The effect of aeration on the growth of strain ADD 16-2

Although the method used for testing the effect of aeration on growth for strain ADD 16-2 was not very precise, the growth curve nevertheless indicated that with sufficient shaking, a typical growth curve was observed with lag period of approximate 5 hours. The static sample exhibited a longer lag period (22 hours) and a reduced cell density at stationary phase (Figure 3-

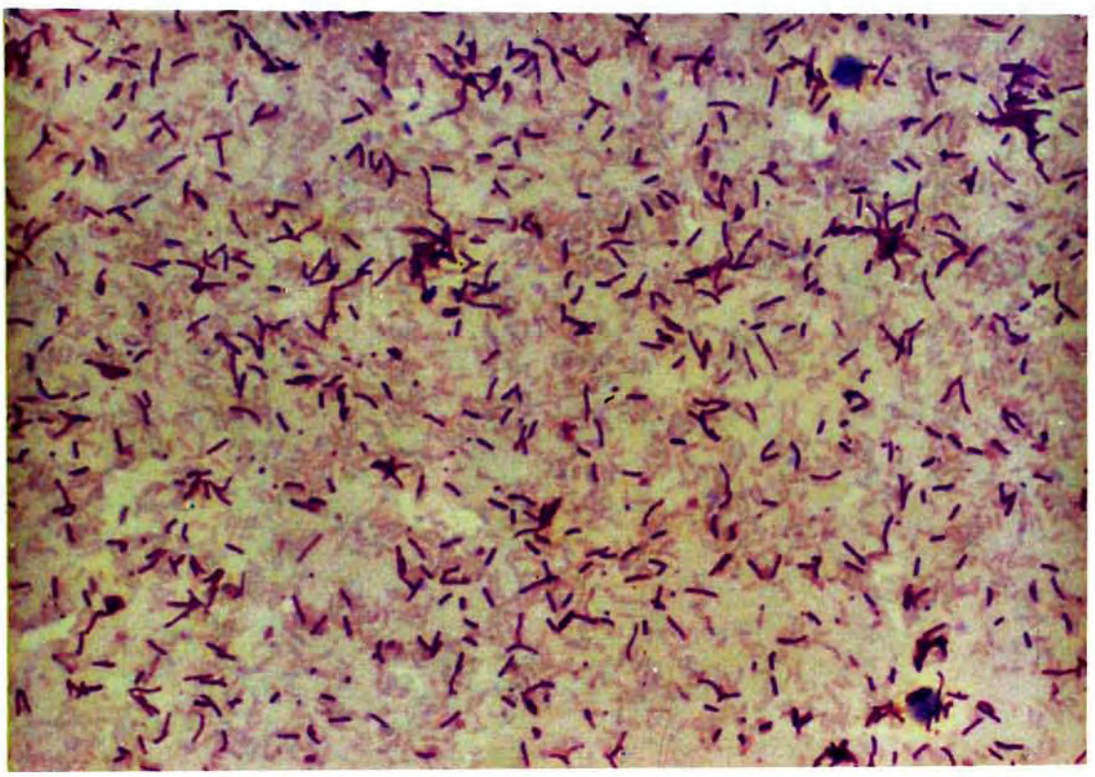


Figure 3-1. Strain 16-2 under microscope (magnification: 1,000X). The cell was cultured in M.B. and harvested at log phase. The strain stained with crystal violet.

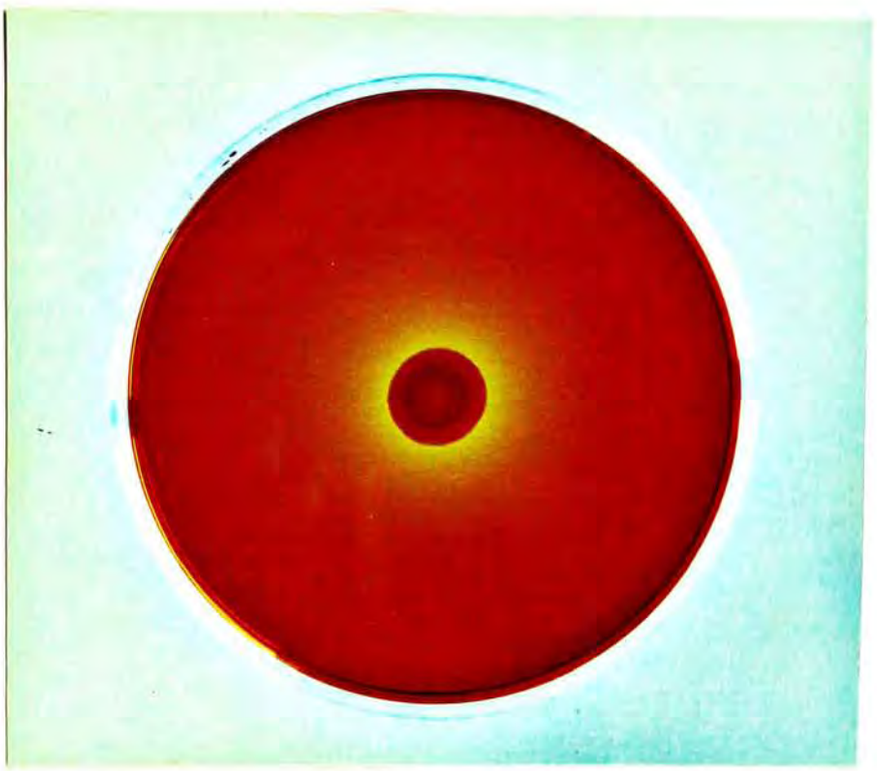


Figure 3-2. A decolorized halo produced by strain ADD 16-2 colony. A single colony of strain ADD 16-2 was inoculated on an I.M. plate and incubated at 30°C for 72 hours. The orange background was the colour of Orange II at 50 ppm. A clear halo was developed around the colony.

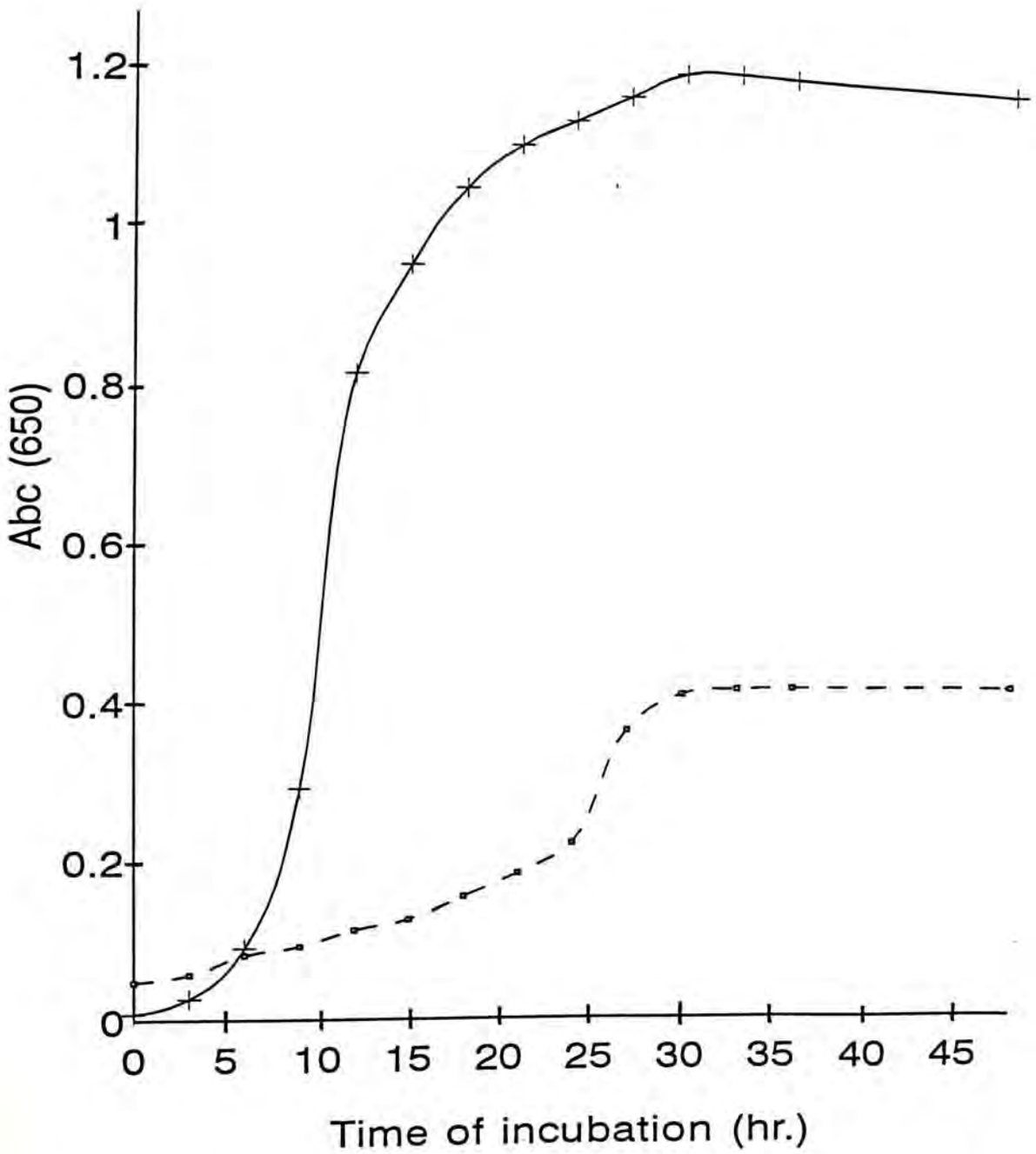


Figure 3-3. The effect of aeration on the growth of strain ADD 16-2. The strain ADD 16-2 was grown in B.M.. The cultures were incubated either anaerobically in an incubator or aerobically in a shaker operated at 200 r.p.m.. The temperature of both cultures was set at 30°C. Samples were taken out at 3 hours intervals and the cell densities were measured spectrophotometrically at 650 nm.

Symbols: —▫— static, —+— shaking.

3). This observation suggests a positive demand of oxygen for cell growth. The strain ADD 16-2 is hence, one of araban.

3.1.2.2 Other factors affecting the growth of strain ADD 16-2

Most bacteria can live within a pH range of 4-9, and they usually display a pH optimum for growth around neutrality. A study of the effect of pH on strain ADD 16-2 growth indicates that the strain ADD 16-2 had the fastest growth rate at pH 6.5 (specific growth rate : 0.297 hr^{-1}). Within 16 hours, the density of cells reached the maximum. pH 6.5 is the optimum for the bacterial growth (Figure 3-4, Table 3-1).

The environmental temperature can exert a profound effect on the growth rate of bacteria. The growth rate of strain ADD 16-2 was determined within a temperature range from 30°C to 40°C. 35°C was found to be the optimum of growth (Figure 3-5, Table 3-2). At this temperature, the strain ADD 16-2 obtained the highest specific growth rate (0.427 hr^{-1}) and the greatest final cell concentration (2.351 $O.D._{650}$). When the temperature reached 40°C, the growth of the strain almost stopped. Apparently, the strain ADD 16-2 is a mesophile.

3.1.2.3 Effect of carbon source on growth

The growth rate of strain ADD 16-2 varied with the carbon sources

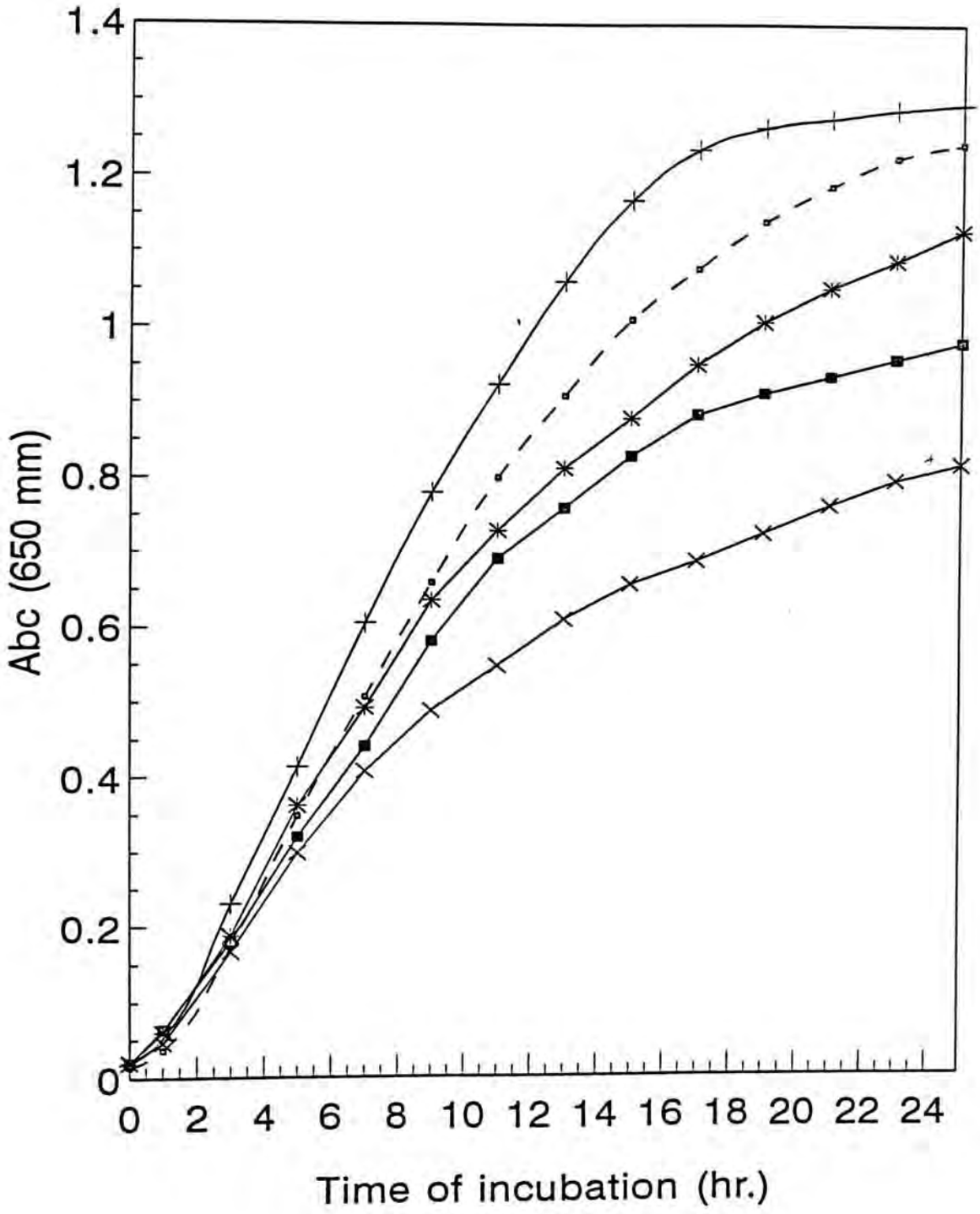


Figure 3-4. The effect of pH on the growth of strain ADD 16-2. Stock strain ADD 16-2 was grown aerobically in B.M.. 1 ml of this culture, pregrown aerobically in B.M. to OD_{650} of 1.8., was inoculated to a 100 ml fresh B.M. for growth. The pH of individual culture was adjusted by using phosphate buffer at 0.2 M and monitored with a pH meter. The cell was grown at 30°C. OD measurements were taken at 650 nm.

Symbols: —•— pH 6.0, —+— pH 6.5, —✳— pH 7.0, —■— pH 7.5, —×— pH 8.0.

Table 3-1: Effect of pH on the growth of strain ADD 16-2

pH	μ	D
6.0	0.273	1.249
6.5	0.296	1.285
7.0	0.257	1.135
7.5	0.232	0.889
8.0	0.221	0.815

Growth rate of the strain 16-2 at log phase was evaluated with the following equation:

μ (specific growth rate) = $(\ln a_1 - \ln a_2)/(t_1 - t_2).\ln 2$

a_1 : cell density ($O.D._{650}$) at time t_1

a_2 : cell density ($O.D._{650}$) at time t_2

D : cell density at end of experiment (25 hours)

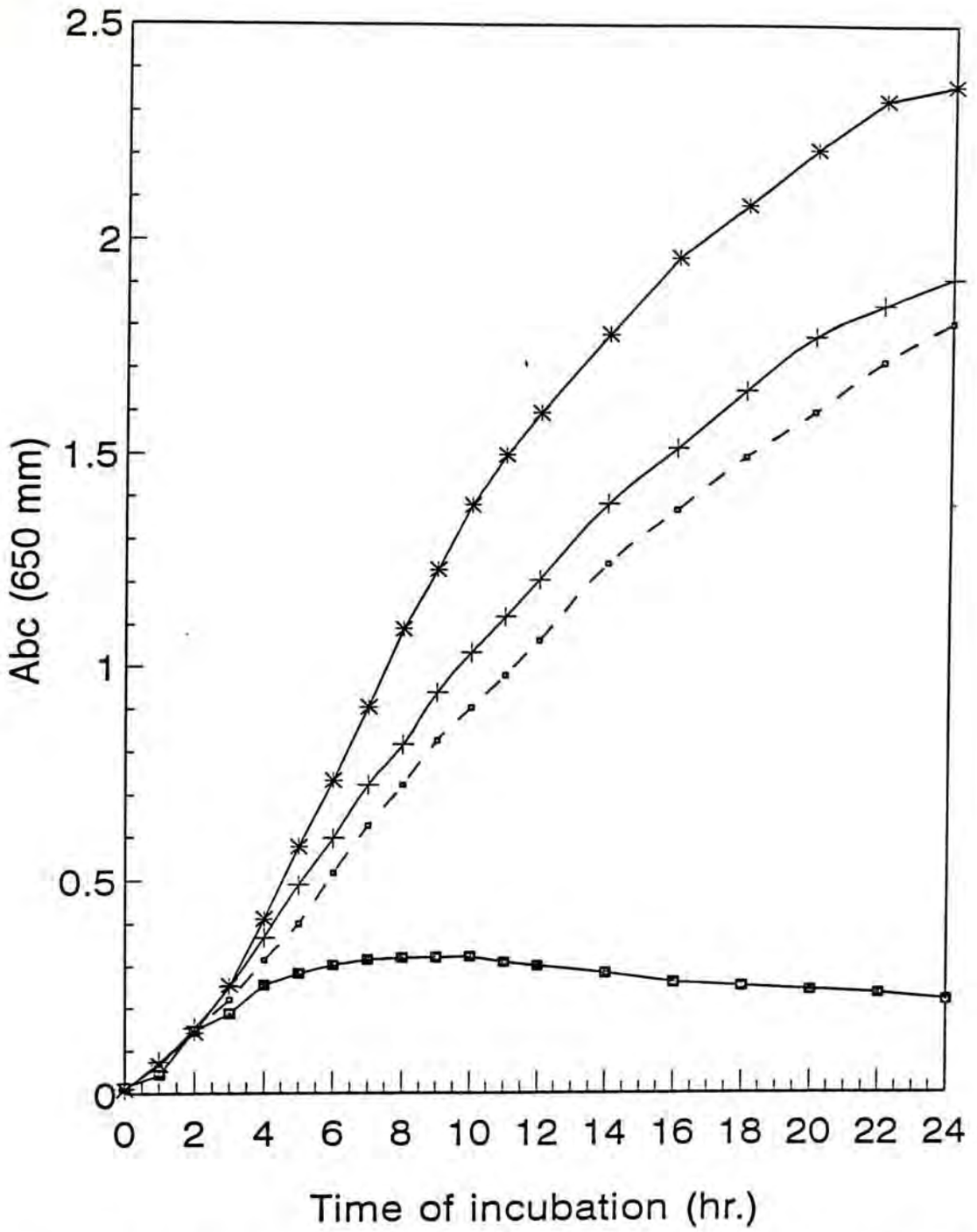


Figure 3-5. The effect of temperature on the growth of strain ADD 16-2. The condition for the growth of strain ADD 16-2 was similar to that described in Figure 3-4. The chosen pH was 6.5. Individual cultures were incubated at 30°C, 33°C, 35°C, and 40°C for 24 hours. At certain time intervals, samples were taken out and cell densities were measured by spectrophotometric method at 650 nm.
Symbols: —▫— 30°C, —+— 33°C, —✳— 35°C, —▫— 40°C.

Table 3-2: Effect of temperature on the growth of strain ADD 16-2

temp.	μ	D
30°C	0.338	1.801
33°C	0.341	1.903
35°C	0.427	2.351
40°C	0.318	0.215

Growth rate of the strain 16-2 at log phase was evaluated with the following equation:

μ (specific growth rate) = $(\ln a_1 - \ln a_2)/(t_1 - t_2).\ln 2$

a_1 : cell density ($O.D._{650}$) at time t_1

a_2 : cell density ($O.D._{650}$) at time t_2

D : cell density at end of experiment (25 hours)

added to the Basal Medium. Table 3-3 shows that among the five carbon sources used, glucose is the best carbon source giving a specific growth rate of 0.49 hr^{-1}. Sucrose is equally good in supporting strain ADD 16-2 growth. Both starch and citrate can also support cell growth but at a slower rate. Acetate cannot be utilized by strain ADD 16-2 as a carbon source.

3.1.3 Decolorization of Azo Dyes

3.1.3.1 Determination of dye concentration

3.1.3.1.A. Determination of the wavelengths of the absorption maxima of azo dyes

All 25 azo dyes show absorption maxima in the range of 354-647 nm. Some of the scanning profiles of aqueous solution of dyes were shown in Figure 3-6, 3-7, 3-8. A list of wavelength of absorption maxima for each azo dye was reported in Table 3-4. For experiments mentioned in this investigation, the wavelengths of the absorption maxima listed here were used when the corresponding dyes were used.

3.1.3.1.B Standard concentration curve of azo dyes

A standard curve for each azo dye was constructed by measuring its absorbency of a chosen range of concentrations of the dye at the absorption

Table 3-3: Effect of carbon sources on the growth of strain ADD 16-2

carbon sources	μ	D
glucose	0.492	1.168
sucrose	0.499	1.020
citrate	0.250	2.478
starch	0.381	2.101
acetate	N.D.	0.030

Growth rate of the strain 16-2 at log phase was evaluated with the following equation:

μ (specific growth rate) = $(\ln a_1 - \ln a_2)/(t_1 - t_2).\ln 2$

a_1 : cell density ($O.D._{650}$) at time t_1

a_2 : cell density ($O.D._{650}$) at time t_2

D : cell density at stationary phase

N.D.: not determined

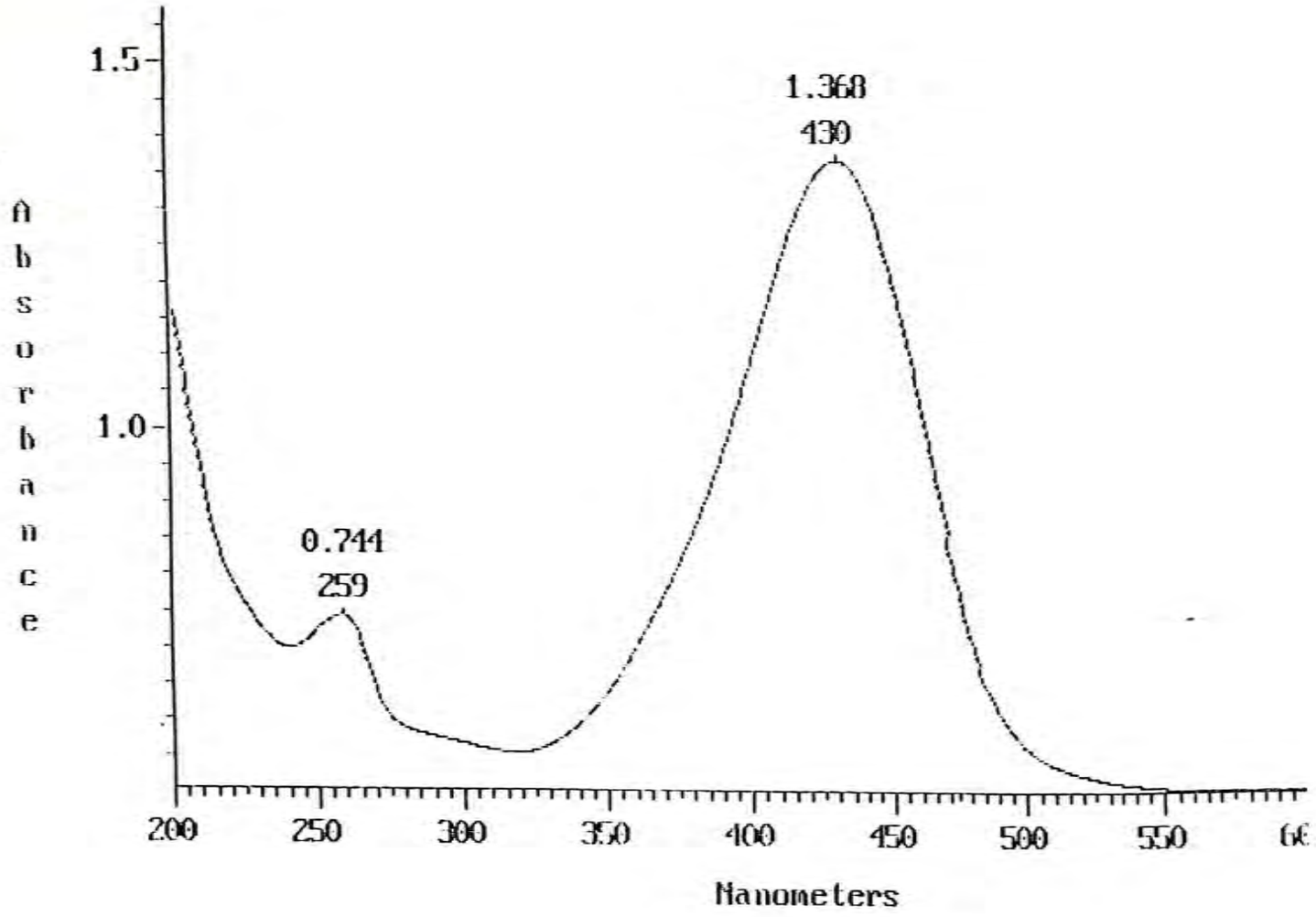


Figure 3-6. Absorption curve of Tropaeolin O in phosphate buffer solution. The dye was dissolved in 0.5 M pH 7.0 phosphate buffer to a final concentration of 25 ppm. The absorbency was scanned from 200-600 nm using a Beckmen Du-7 spectrophotometer.

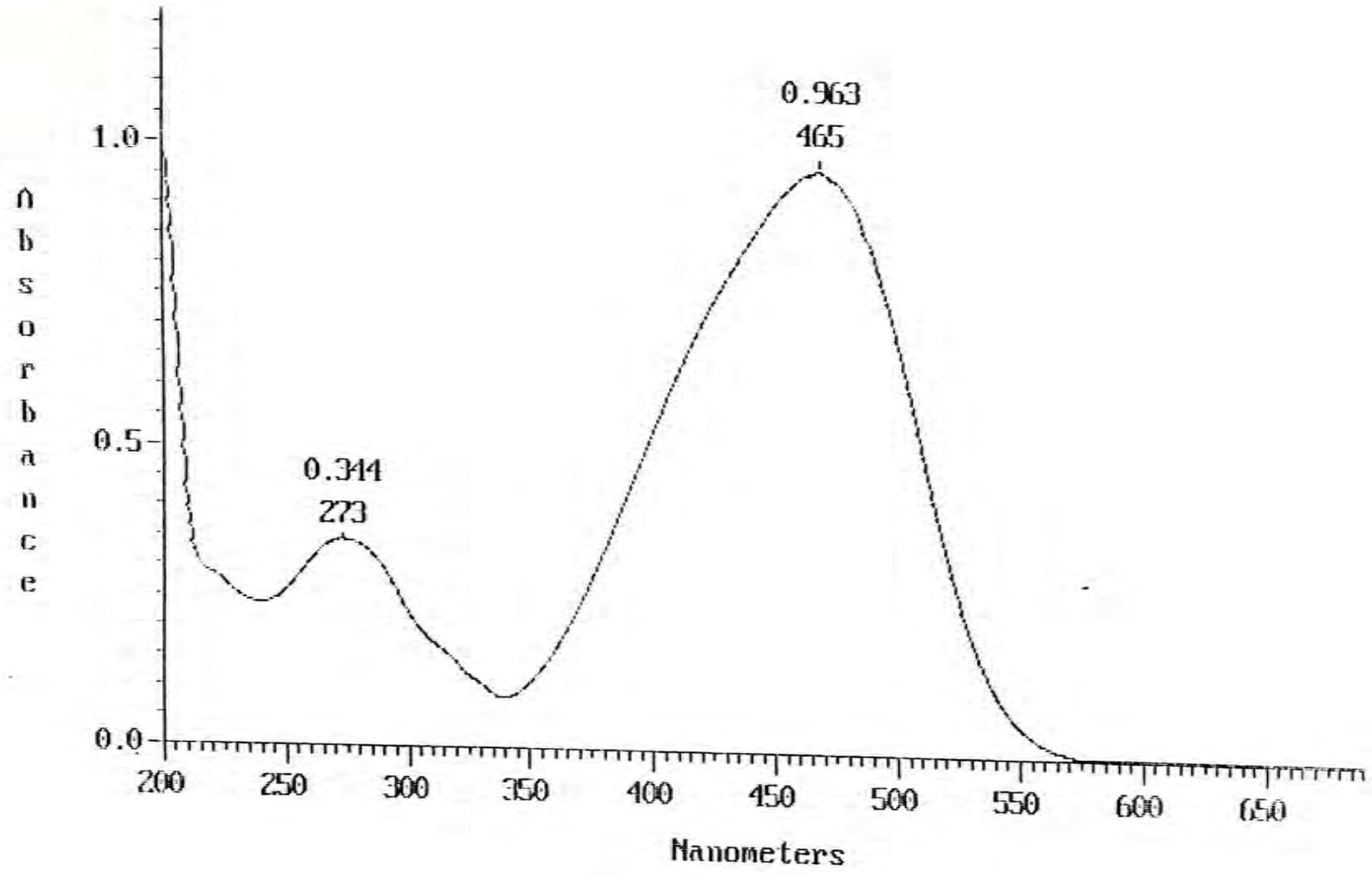


Figure 3-7. Absorption curve of Methyl Orange in phosphate buffer solution. The dye was dissolved in 0.5 M pH 7.0 phosphate buffer to a final concentration of 25 ppm. The absorbency was scanned from 200-700 nm using a Beckmen Du-7 spectrophotometer.

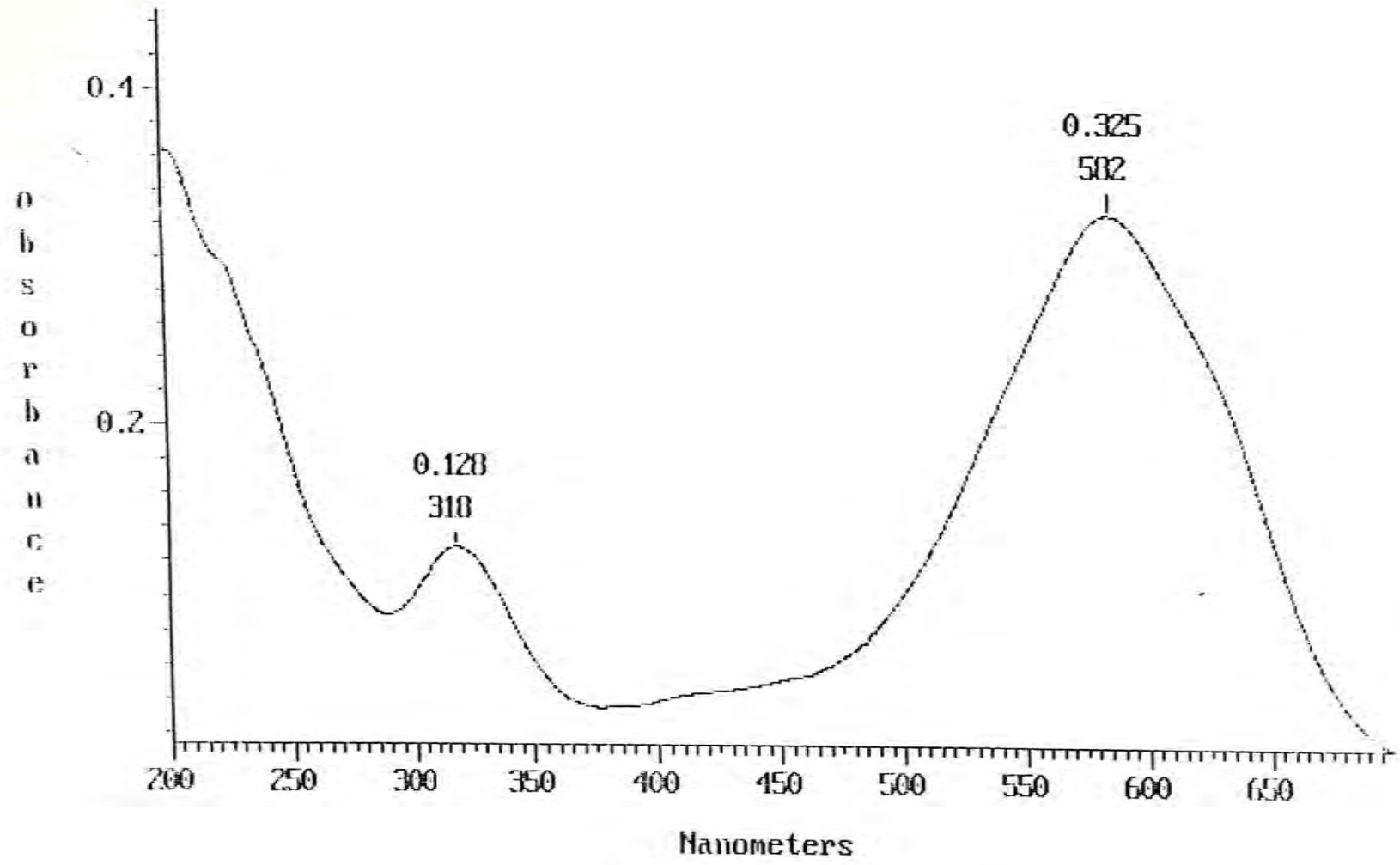


Figure 3-8. Absorption curve of Direct Blue 14 in phosphate buffer solution. The dye was dissolved in 0.5 M pH 7.0 phosphate buffer to a final concentration of 25 ppm. The absorbency was scanned from 200-700 nm using a Beckmen Du-7 spectrophotometer.

Table 3-4. The wavelengths of the absorption maxima

Name of dye	CAS-NO*	Absorption maximum (nm)
Alizarin Yellow 2G	584-42-9	354
Acid Yellow 9	2706-28-7	388
Tropaeolin O	547-57-9	430
Orange G	1936-15-8	432
Metanie Yellow	587-98-4	437
Tropaeolin OO	554-73-4	445
Methyl Orange	547-58-0	465
Orange I	523-44-4	479
Crocein Orange G	1934-20-9	483
Orange II	633-96-5	487
Acid Orange 8	5850-86-2	490
Congo Red	573-58-0	490
Poncean Xylidine	3761-53-3	506
Poncean BS	4196-66-0	506
Chromotrop 2R	4197-07-3	508
Acid Red 88	1658-56-6	509
Sulfanilic Acid Azochromotrop	23647-14-5	511
Chromotrop 2B	548-80-1	512
Crocein Scarlet Moo	5413-75-2	511
Reactive Violet 5	12226-38-9	560
Sulfonazo III	1738-02-9	572
Nitrosulfanazo III	1964-89-2	579
Direct Blue 14	72-57-1	582
Nitrazine Yellow	5423-07-4	589
Direct Black 19		644
Hydroxynaphthol Blue	6345-35-4	647

* Chemical Abstract Registry Number (Sigma Chemical Co.and Fluka Chemika-Biochemika).

maxima. Three typical spectra were depicted here (Figure 3-9, 3-10, 3-11). The concentration and the absorbance exhibited a linear relationship when the value of absorbency was lower than 1.0 for each dye. In this case, the degree of absorbance change could reflect directly the extent of reduction in the dyes concentration. In all future studies, the supernatants containing dye solutions were all diluted to give an absorbance lower than 1.0 before measurement. These curves were referred to in the calculations of dye content when necessary.

3.1.3.2 Optimal condition for dye decolorization

3.1.3.2.A Effect of aeration

Unlike its growth condition, the ability of azo dye decolorization by strain ADD 16-2 worked better in an anaerobic condition (Figure 3-12). For a 100 ppm Tropeaolin O, the decolorization process occurred efficiently and a close to complete decolorization was noticed at hour 25. Shaking not only slowed the rate, but also inhibited the reaction at 50% decolorization.

3.1.3.2.B Effect of temperature

The temperature effects on the decolorization of azo dye were determined at 25°C, 30°C, 35°C, 37°C, and 40°C (Figure 3-13). It was established that the optimum was 35°C. When the temperatures were lower than the optimum, a

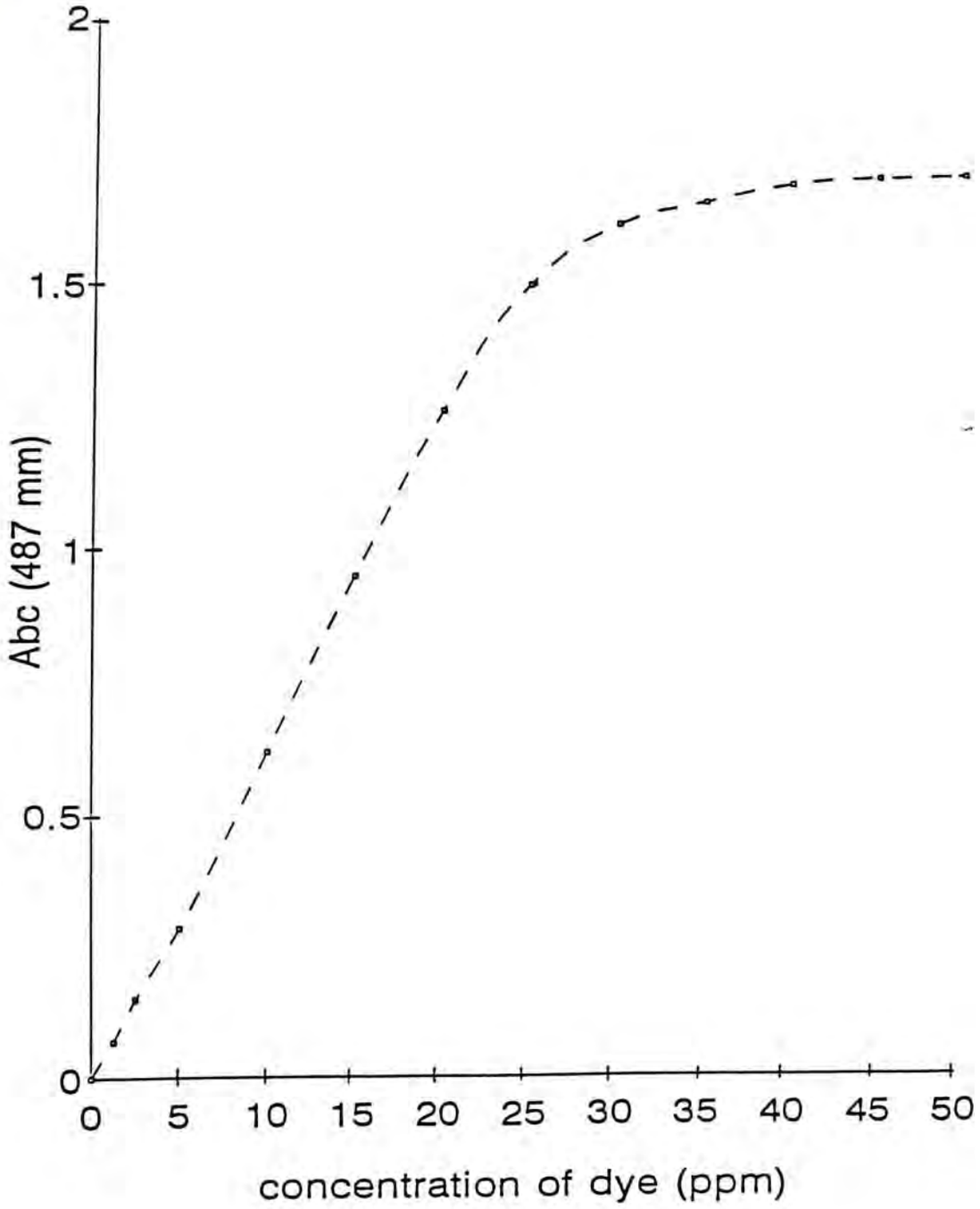


Figure 3-9. The standard curve of Tropaeolin O. A serial dilutions were prepared from a 1% stock solution of Tropaeolin O by diluting with 0.5 M phosphate buffer. 0.5 M phosphate buffer was chosen as blank. The absorbency of these dilutions were measured at absorption maxima (430 nm) by Hitachi U-1100 spectrophotometer.

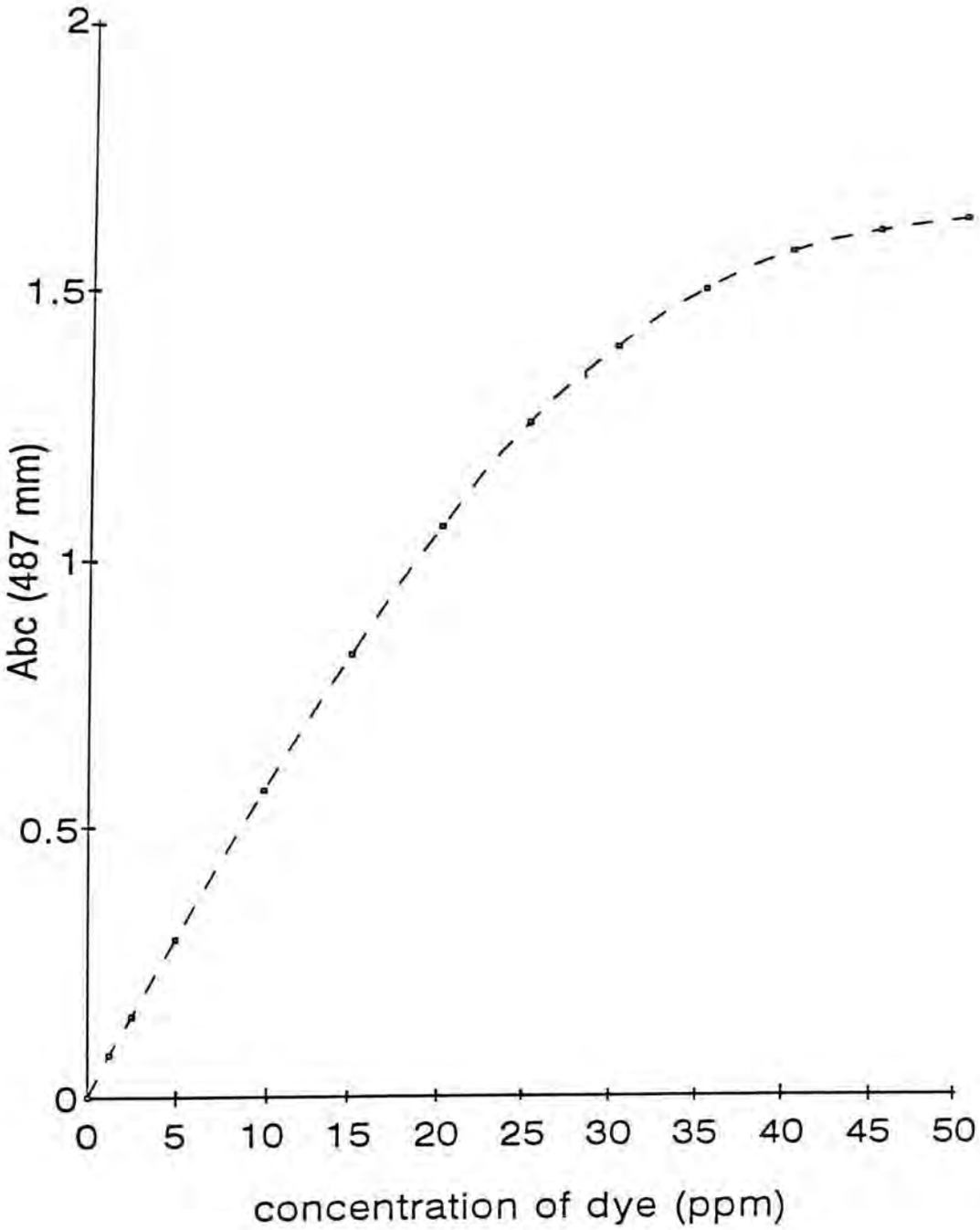


Figure 3-10. The standard curve of Orange II. A serial dilutions were prepared from a 1% stock solution of Tropaeolin O by diluting with 0.5 M phosphate buffer. 0.5 M phosphate buffer was chosen as blank. The absorbency of these dilutions were measured at absorption maxima (487 nm) by Hitachi U-1100 spectrophotometer.

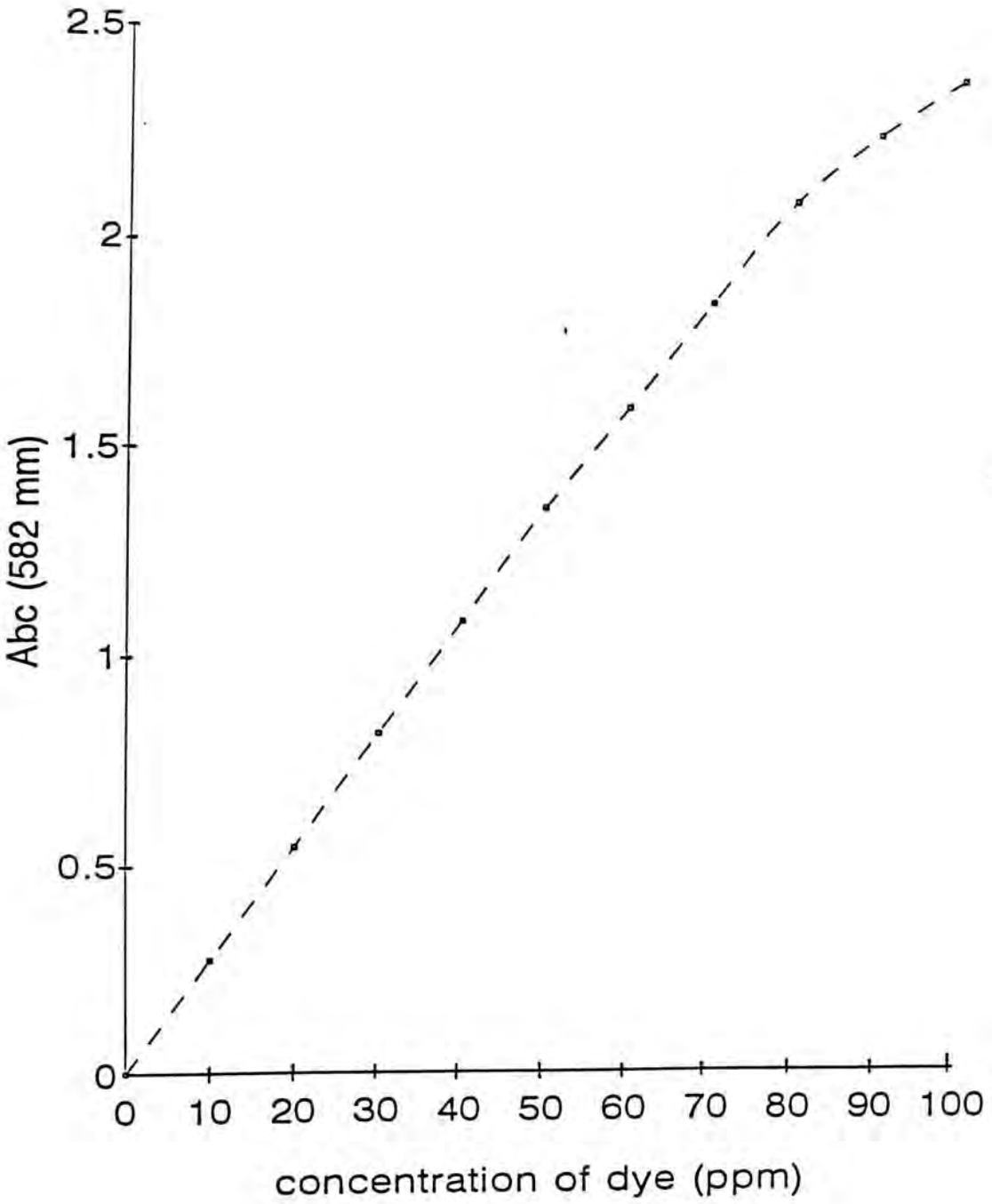


Figure 3-11. The standard curve of Direct Blue 14. A serial dilutions were prepared from a 1% stock solution of Tropaeolin O by diluting with 0.5 M phosphate buffer. 0.5 M phosphate buffer was chosen as blank. The absorbency of these dilutions were measured at absorption maxima (582 nm) by Hitachi U-1100 spectrophotometer.

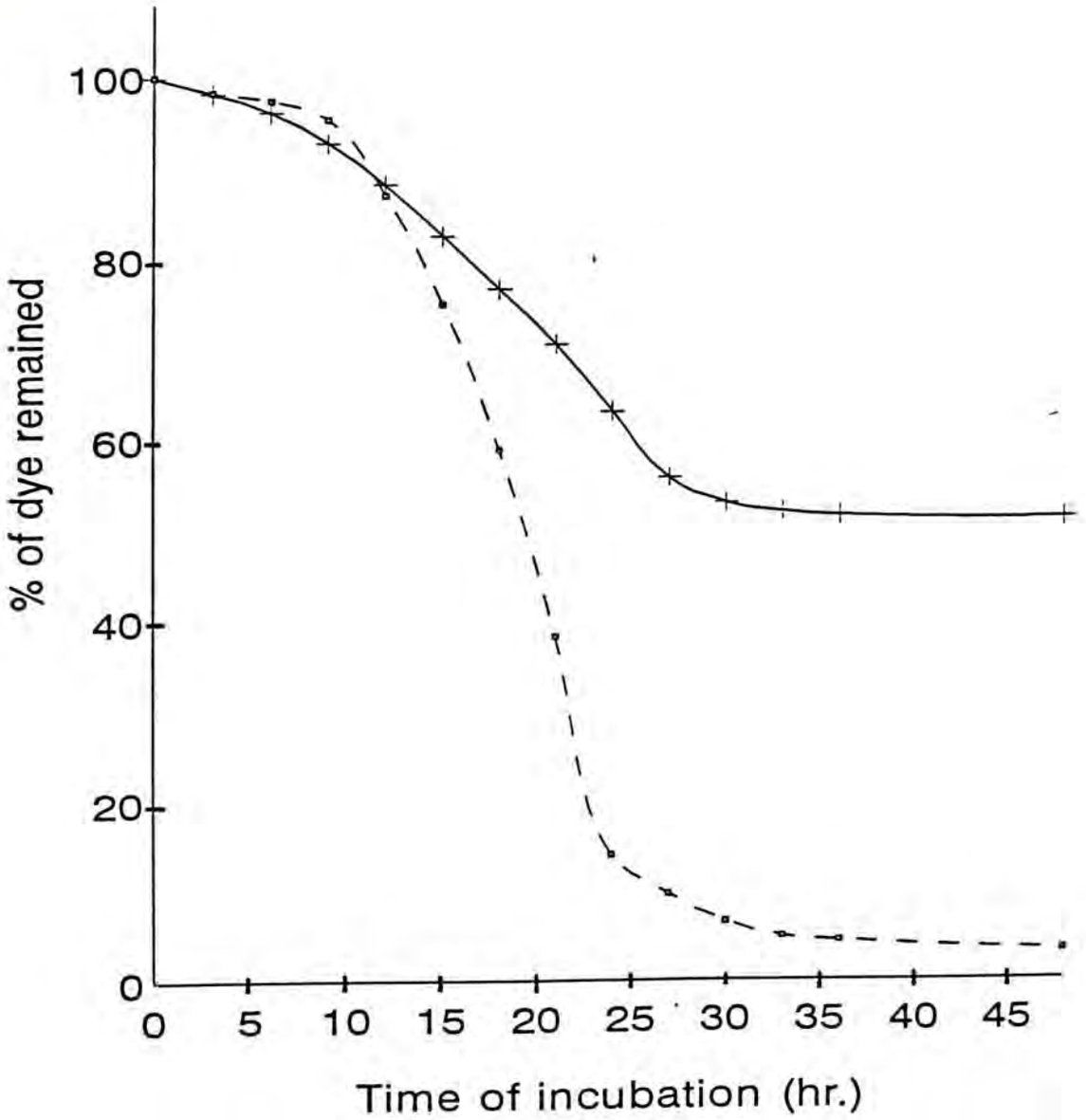


Figure 3-12. The effect of aeration on the decolorization of Tropaeolin O by strain ADD 16-2. 5 ml of stock inoculum (OD 2.8) was added to 100 ml B.M. containing 100 ppm of Tropaeolin O at its final concentration. The cultures were incubated under anaerobic and aerobic condition conrrespondingly. For anaerobic condition, the culture was incubated statically in a 150 ml screw capped flask stood at 30°C for 48 hours. For aerobic condition, the culture was incubated in a 250 ml flask placed in a rotary shaker operated at 200 r.p.m. for 48 hours. 1 ml samples were removed and centrifuged. The supernatants were used for OD measurement (at 430 nm) after proper dilution (usually 5-fold) with 0.5 M phosphate buffer. The extent of decolorization of dye was expressed in percentage of dye remained in culture.
Symbols: —▪— static, —+— shaking.

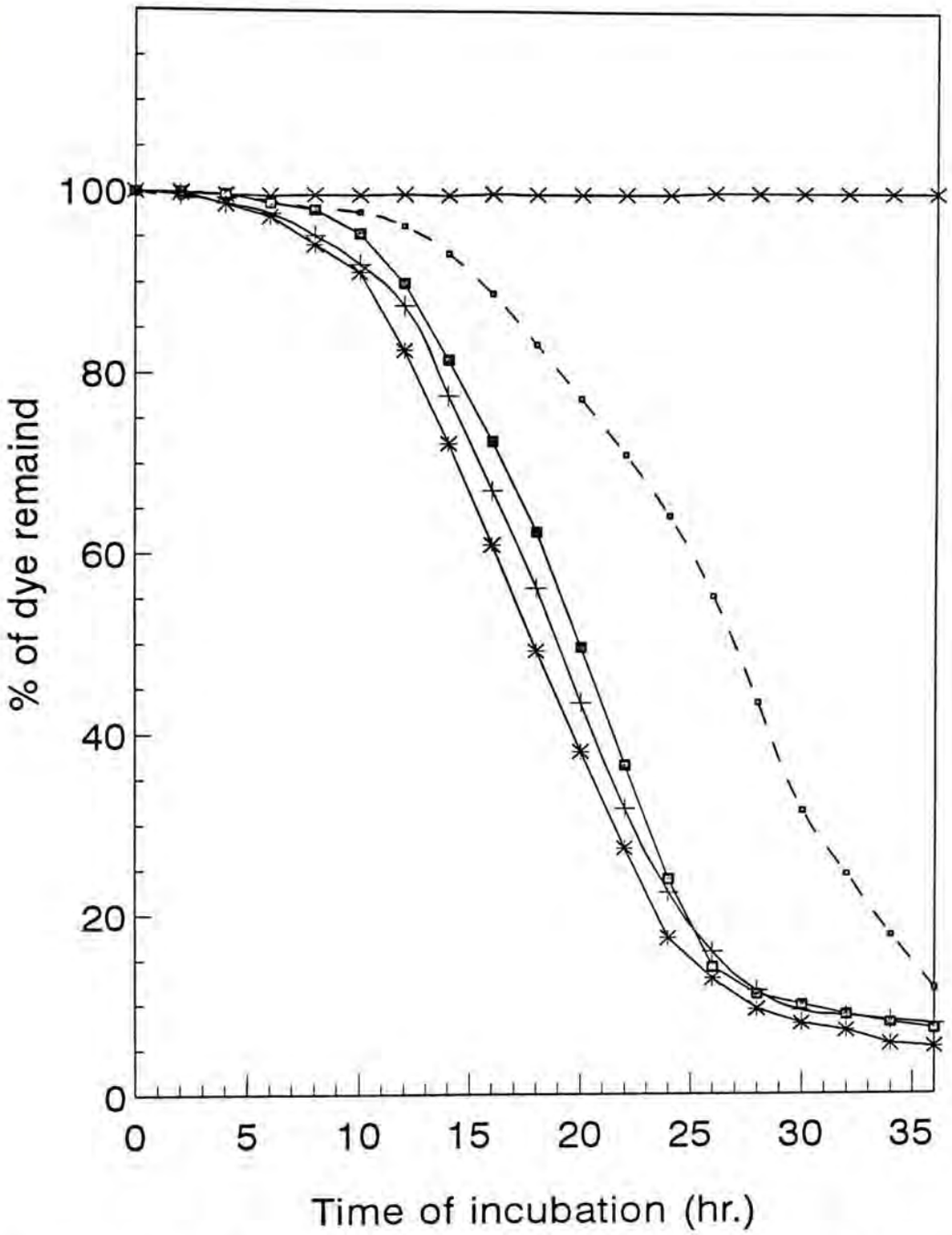


Figure 3-13. The effect of temperature on the decolorization of Tropaeolin O by strain ADD 16-2. The strain ADD 16-2 was added to B.M. as mentioned in Figure 3-12. The cultures were incubated anaerobically at 25°C, 30°C, 35°C, 37°C, and 40°C correspondingly for 36 hours. 1 ml samples were taken out at proper time intervals and the cells in the medium was spun down by microcentrifugation. The clear supernatants were used for OD measurement (at 430 nm) after proper dilutions (usually 5-fold) with 0.5 M phosphate buffer. The extent of decolorization of dye was expressed in percentage of dye remained in culture.

Symbols: —▫— 25°C, —+— 30°C, —✳— 35°C, —▫— 37°C, —×— 40°C.

change of temperature did not influence the activity of decolorization strongly. However, the strain was sensitive to high temperatures. When the temperature reached 40°C, the strain lost its ability of decolorization completely.

3.1.3.2.C Effects of pH

The strain activity plot as a function of pH for strain ADD 16-2 was shown in Figure 3-14. The initial pH of the medium was adjusted to 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 with Tris-maleate buffer. Figure 3-14 shown that the optimal initial pH of the medium for decolorization was 7.0.

3.1.3.2.D Effect of different carbon sources

As the presence of a carbon source stimulates azo dyes decolorization by the strain ADD 16-2, the effect of different kinds of carbon sources on the ability of decolorization was studied (Figure 3-15). Comparing Table 3-3 with Figure 3-15, decolorization activity of the dye followed a similar trend as that of the organism growth by the carbon sources. A relative similar efficiency of Tropaeolin O decolorization was found when the medium contained sucrose instead of glucose. There was a 12-hour lag period when starch was used as carbon source. When citrate and acetate was used, the strain has practically lost all its ability of decolorization.

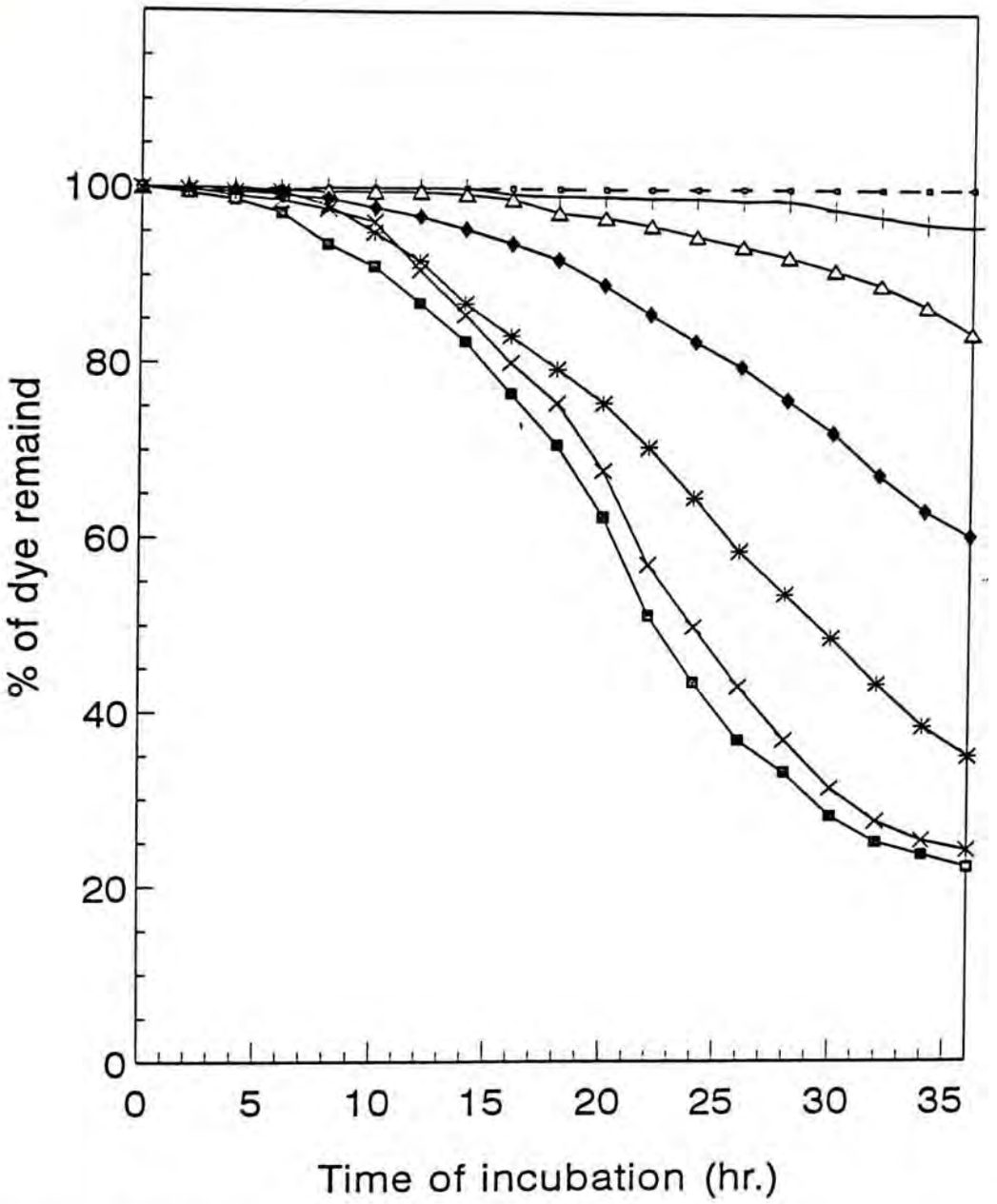


Figure 3-14. The effect of pH on the decolorization of Tropaeolin O by strain ADD 16-2. The strain ADD 16-2 was added to B.M. as mentioned in Figure 3-12. The initial pH of each medium was adjusted to 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 with Tris-maleate buffer respectively. At proper time intervals (2 hours), a 1 ml sample of medium was taken out from each culture and the cells was spun down by microcentrifugation. The clear supernatants were used for OD measurement (at 430 nm) after proper dilution (usually 5-fold) with 0.5 M phosphate buffer. The decolorization of dye was expressed in percentage of dye remained in culture.

Symbols: → pH5.5, —+— pH 6.0, —✳— pH 6.5, —▫— pH 7.0, —✳— pH 7.5, —◆— pH 8.0, —△— pH8.5.

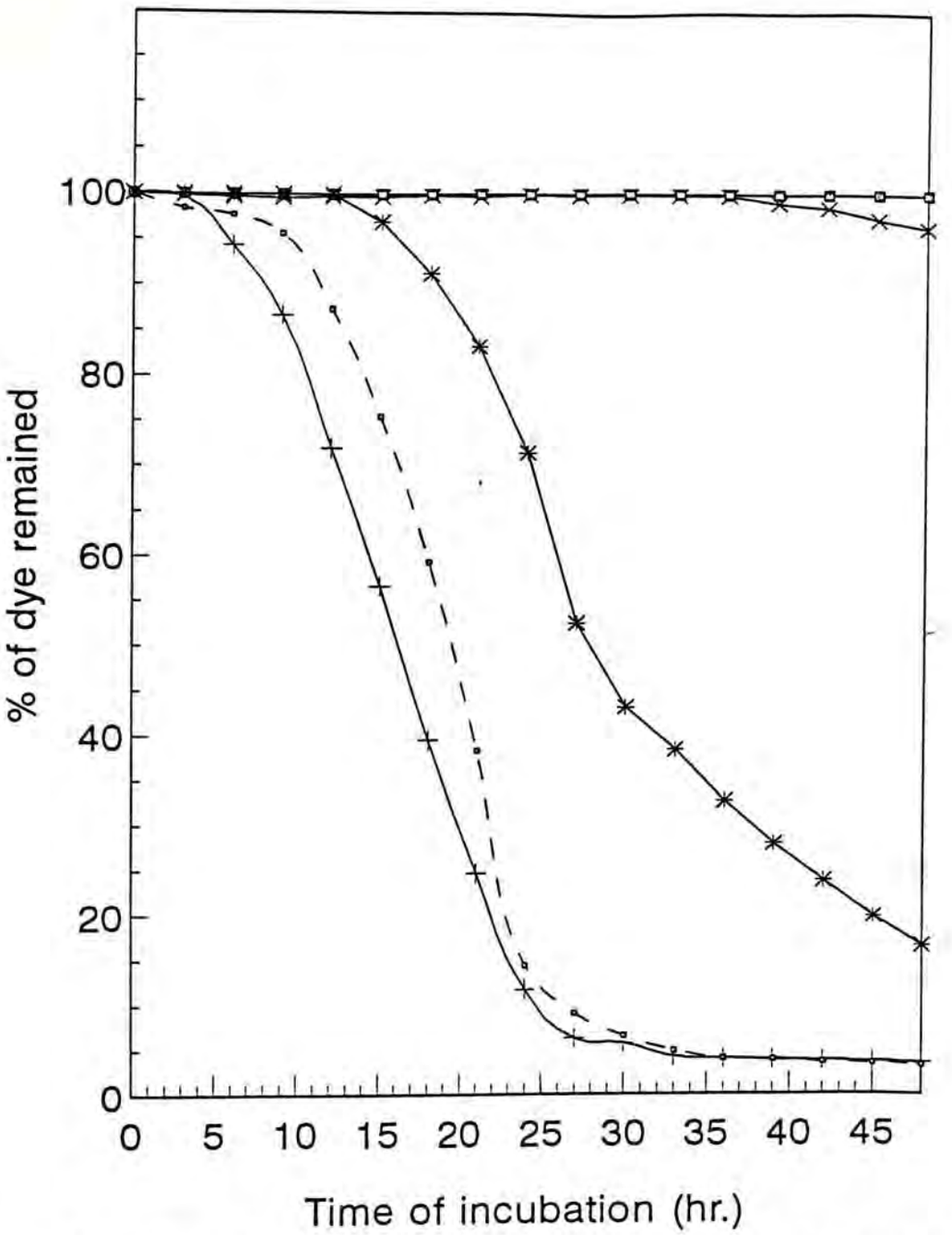


Figure 3-15. The effect of different carbon sources on the decolorization of Tropaeolin O by strain ADD 16-2. The strain ADD 16-2 was added to modified Basal Medium as described in Figure 3-12. The glucose in the original medium was substituted by same amount of sucrose, citric acid, acetic acid, or starch respectively. The cultures were incubated at 35°C. The pH of medium was 7.0. At proper time intervals (2 hours), a 1 ml sample of medium was taken out from each culture and the cells was spun down by microcentrifugation. The clear supernatants were used for OD measurement (at 430 nm) after proper dilution (usually 5-fold) with 0.5 M phosphate buffer. The decolorization of dye was expressed in percentage of dye remained in culture.

Symbols: —• Glucose, —+— Sucrose, —▫— Citrate, —✳— Starch, —×— Acetate.

3.1.3.3 Structural specificity of the azo dye decolorization reaction.

The kinetics of dye decolorization of 25 azo dyes were compared under the same optimal assay condition with strain ADD 16-2. The profiles of all these reactions were presented in Figure 3-16, 3-17 and 3-18. For easy comparison, a t_{50} value was used to indicate the time required to reduce the half the dye in assay solution. Thus, a smaller t_{50} means a quicker reaction. A second value used was the residue %. This percentage designated the amount of dye left at the end of the reaction. In most cases, because of lack of sensitivity of the colour measurements at the end of the reaction, a 5 to 10 % would mean a close to complete decolorization reaction.

As shown in Table 3-5, most of these dyes were quickly decolorized. They are Methyl Orange (I), Tropaeolin OO (IV), Acid Yellow 9 (V), Orange I (VI), Chromotrop 2B (X), Nitrazine Yellow (XI), Reaction Violet 5 (XIV), Sulfanic Acid Azochromotrop (XV), Acid Red 88 (XVI), Hydroxynaphthol Blue (XVII), Metanil Yellow (XVIII), Crocein Scarlet Moo (XIX), and Sulfonazo III (XXII). All of the above dyes gave a t_{50} smaller than one hour. A second group of dyes exhibited a slower reaction rate. They are Acid Orange 8 (II), Tropaeolin O (IV), Crocein Orange G (VIII), Chromotrop 2R (IX), Poncean Xylidine (XIII), Poncean BS (XX), Nitrosulfonazo III (XXI), Congo Red (XXI), and Direct Black 19 (XXV). A third group showed a much slower reaction rate. It contained Orange II and Direct Blue 14 (XXIV). Since the 25 dyes purchased from the market are not sufficient to give a full picture

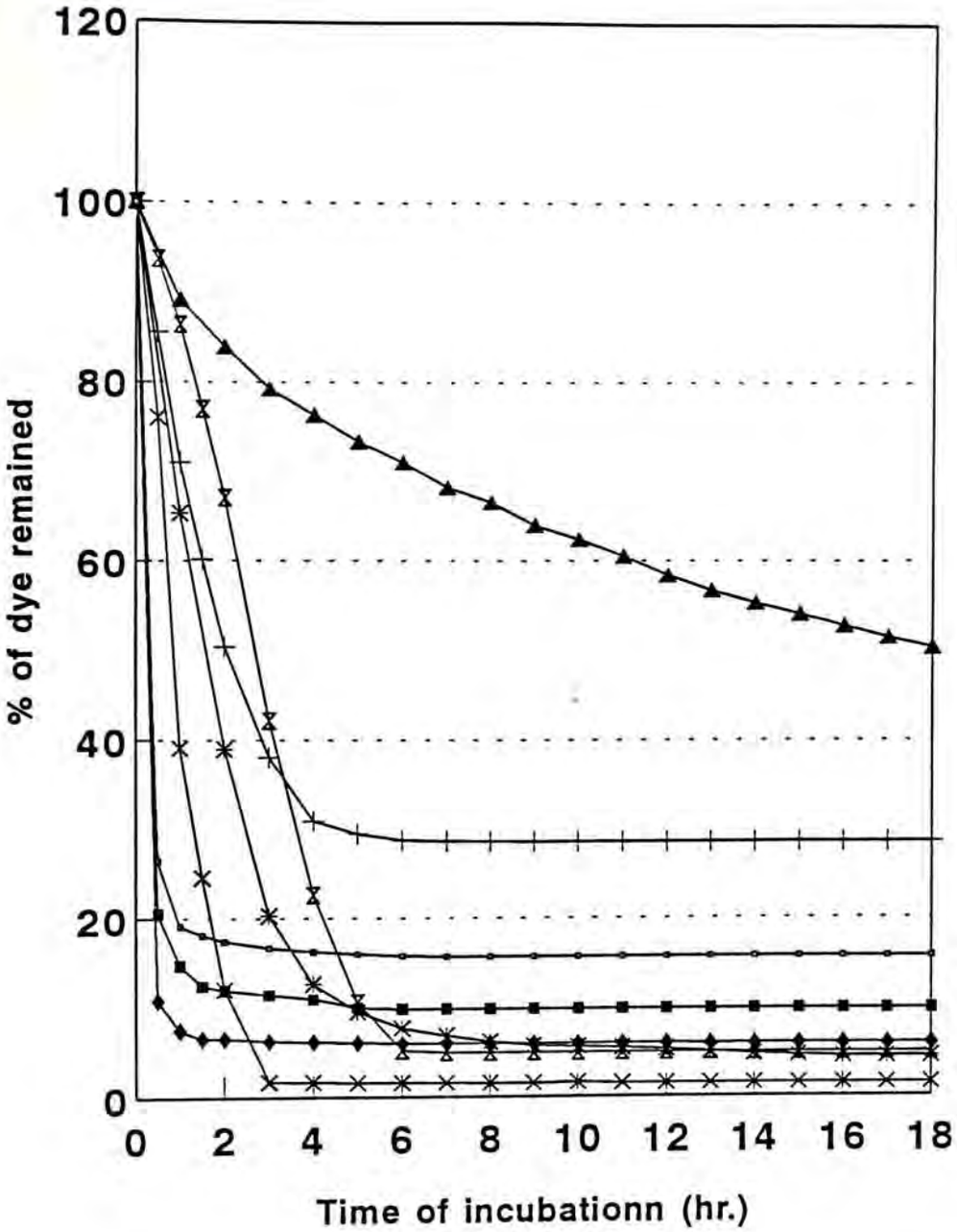


Figure 3-16. The decolorization curves of various azo dyes by strain ADD 16-2. The stock cell ADD 16-2 was added to 100 ml B.M. (final concentration of cells: 2.5×10^9 cells/ml) containing 50 ppm dye content. The cultures were incubated at 35°C, pH 7.0 for 24 hours. 1 ml samples of these culture media were taken out at fixed time intervals and the cells in the medium was spun down by microcentrifugation. The clear supernatants were diluted 2.5-fold with 0.5 M phosphate buffer and were used then for spectrophotometric measurements at maximum absorbance of each dye. The decolorization of each dye was expressed by percentage of compound remained in culture.

symbols: —○— Methyl Orange (I), —+— Acid Orange 8 (II),
—✳— Tropeaolin O (III), —■— Tropaeolin OO (IV),
—✕— Acid Yellow (V), —◆— Orange I (VI),
—▲— Orange II (VII), —⧖— Crocein Orange G (VIII).

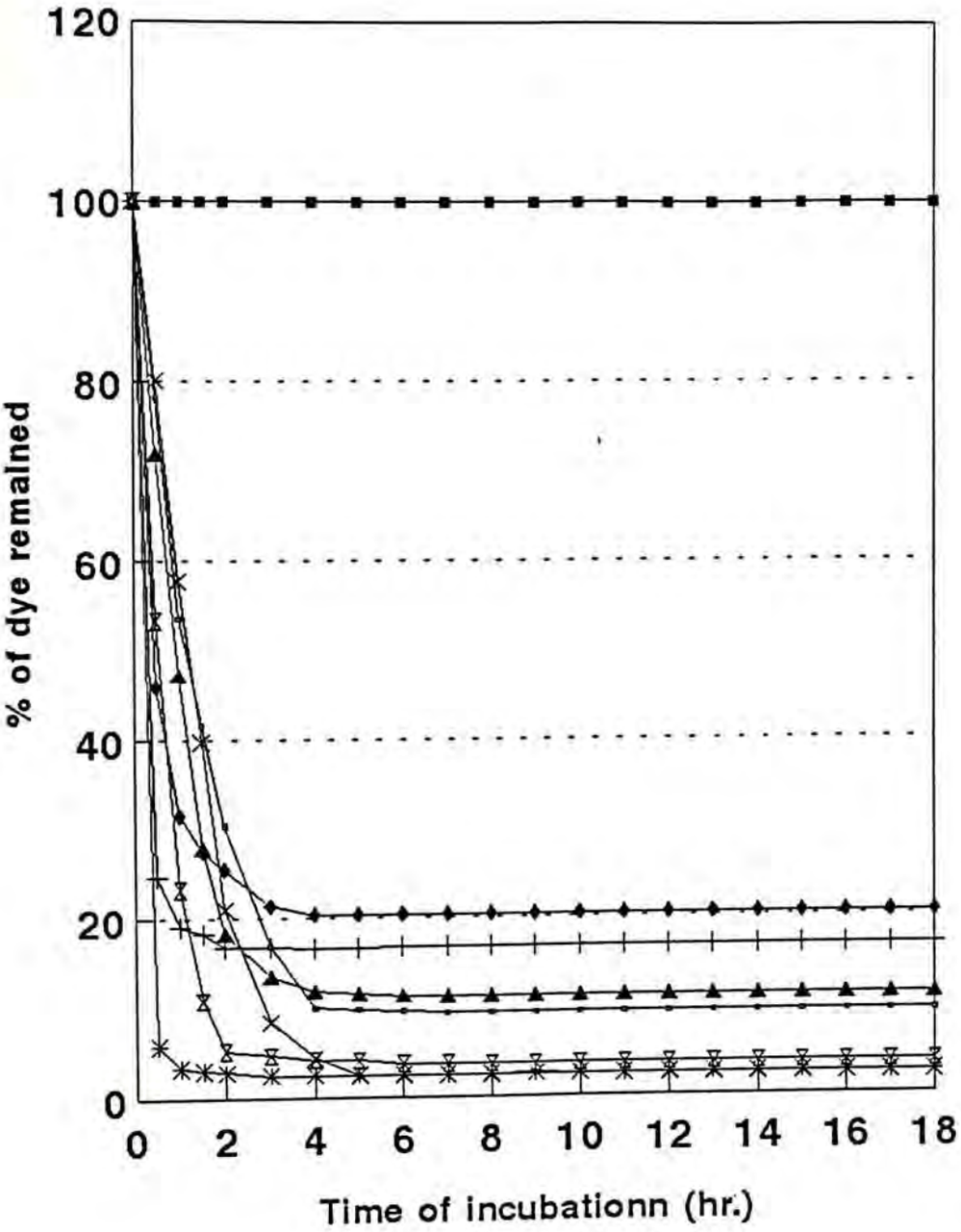


Figure 3-17. The decolorization curves of various azo dyes by strain ADD 16-2. The stock cell ADD 16-2 was added to 100 ml B.M. (final concentration of cells: 2.5×10^9 cells/ml) containing 50 ppm dye content. The cultures were incubated at 35°C, pH 7.0 for 24 hours. 1 ml samples of these culture media were taken out at fixed time intervals and the cells in the medium was spun down by microcentrifugation. The clear supernatants were diluted 2.5-fold with 0.5 M phosphate buffer and were used then for spectrophotometric measurements at maximum absorbance of each dye. The decolorization of each dye was expressed by percentage of compound remained in culture.

symbols: —○— Chromotrop 2R (IX), —+— Chromotrop 2B (X),
—✳— Nitrazine Yellow (XI), —■— Orange G (XII),
—✕— Poncean Xylidine (XIII), —▲— Reactive Violet 5 (XIV),
—◆— Sulfanilic Acid Azochromotrop (XV),
—⧖— Acid Red 88 (XVI).

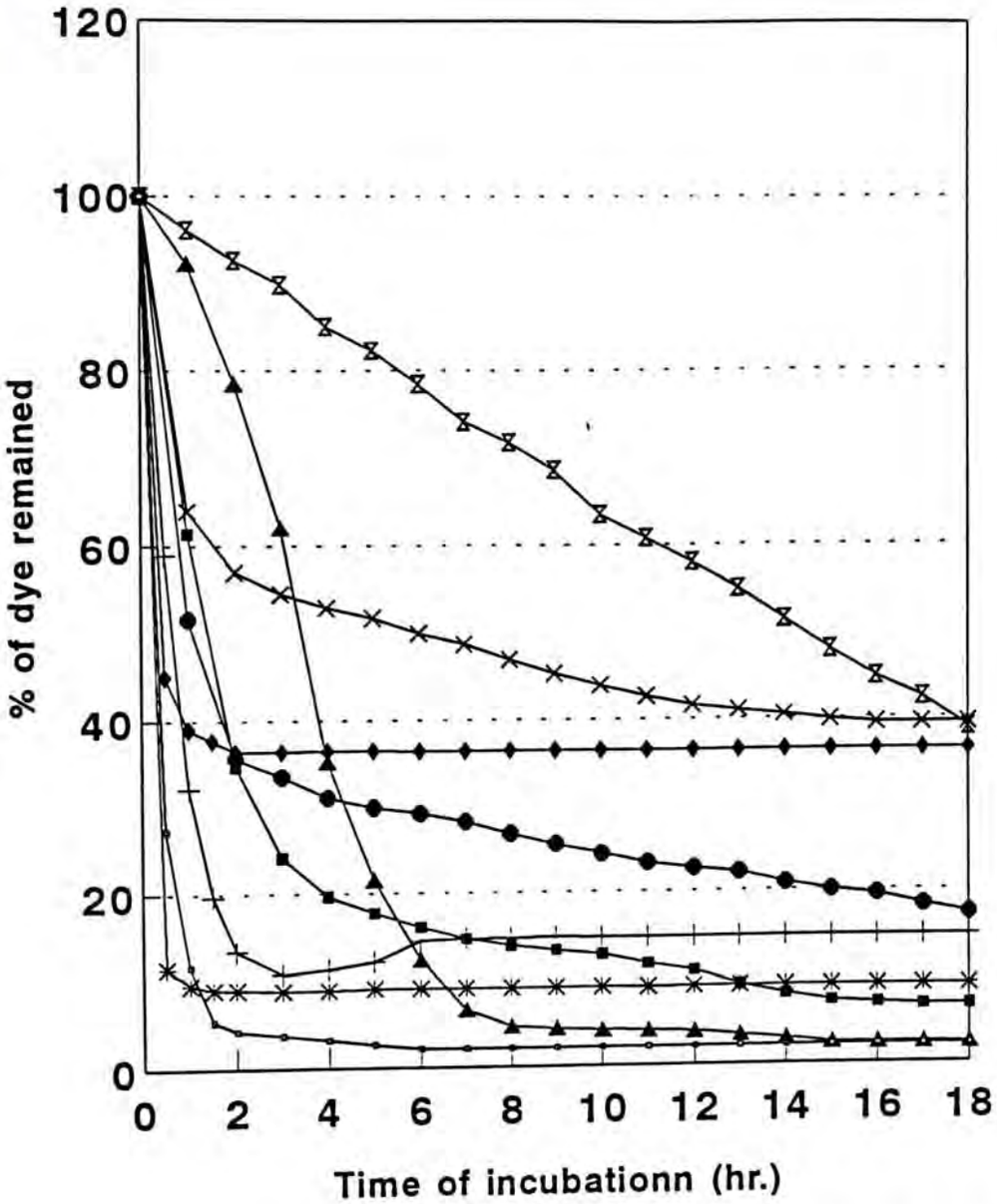


Figure 3-18. The decolorization curves of various azo dyes by strain ADD 16-2. The stock cell ADD 16-2 was added to 100 ml B.M. (final concentration of cells: 2.5×10^9 cells/ml) containing 50 ppm dye content. The cultures were incubated at 35°C, pH 7.0 for 24 hours. 1 ml samples of these culture media were taken out at fixed time intervals and the cells in the medium was spun down by microcentrifugation. The clear supernatants were diluted 2.5-fold with 0.5 M phosphate buffer and were used then for spectrophotometric measurements at maximum absorbance of each dye. The decolorization of each dye was expressed by percentage of compound remained in culture.

symbols: —○— Hydroxynaphthol Blue (XVII), —+— Metanol Yellow (XVIII),
—✳— Crocein Scarlet Moo (XIX), —■— Poncean BS (XX),
—✳— Nitrosulfonazo III (XXI), —◆— Sulfonazo III (XXII),
—▲— Congo Red (XXIII), —⧖— Direct Blue 14 (XXIV),
—●— Direct Black 19 (XXV).

Table 3-5. Azo dyes decolorization by strain ADD 16-2 over a period of 18 huors

Azo dye no.	Name of azo dye	t_{50}*	%left**
I.	Methyl Orange	0.37	15.70
II.	Acid Orange 8	2.00	28.70
III.	Tropaeolin O	1.57	4.41
IV.	Tropaeolin OO	0.32	9.84
V.	Acid Yellow 9	0.80	1.51
VI.	Orange I	0.29	5.92
VII.	Orange II	18.00	50.07
VIII.	Crocein Orange G	2.63	4.92
IX.	Chromotrop 2R	1.40	9.32
X.	Chromotrop 2B	0.33	16.85
XI.	Nitrazine Yellow	0.28	2.42
XII.	Orange G		96.70
XIII.	Poncean Xylidine	1.34	2.39
XIV.	Reactive Violet 5	0.45	20.41
XV.	Sulfanilic Acid Azochromotrop	0.95	11.20
XVI.	Acid Red 88	0.55	3.63
XVII.	Hydroxynaphthol Blue	0.45	2.14
XVIII.	Metanil Yellow	0.62	14.74
XIX.	Crocein Scarlet Moo	0.31	9.04
XX.	Poncean BS	1.74	6.67
XXI.	Nitrosulfonazo III	6.00	39.40
XXII.	Sulfonazo III	0.47	36.42
XXIII.	Congo Red	3.50	2.36
XXIV.	Direct Blue 14	14.10	38.82
XXV.	Direct Black 19	1.05	17.35

* t_{50} = time in hours for 50% decolorization of dye.

** % left = percentage of dye remained in medium at the end of

of the positional effect of any substituted group on any side of the azo bond, it is not too profitable to draw any simple conclusion for the structural specificity of the decolorization reaction. Even though a direct comparison chosen between dye pairs such as Orange I and Orange II, which differ only in a minor side group position, may show some insights about the catalysis. Shifting a hydroxyl group from the para position to the ortho position nearly rendered the reaction to a standstill. Such a kind of reaction may also be observed in Orange G. But in reality, Acid Red 88 with structure similar to Orange II except one additional ring to give two naphthalene groups on both side of the azo bond exhibited a much unexpected fast decolorization reaction. Apparently, we need more information before any detail analysis could be made.

3.1.3.4 Analysis of the biodegradation products from Tropaeolin O

Figure 3-19 shows a gradual development of the spectrum of Tropaeolin O degradation products in the course of decolorization by strain ADD 16-2 at indicated time intervals. At the beginning, two peaks were observed on the profile at 431 nm and 248 nm respectively. The peak at 431 nm disappeared slowly in approximately 16 hours which coincided with the disappearance of the yellow colour in the medium. Nevertheless the peak at 248 nm remained fairly constant up to 48 hours. The compound(s) with a maximum A_{248} accumulated in the medium. Similar results were obtained in the decolorization of other dyes (Figure 3-20 and 3-21). The residue absorbency

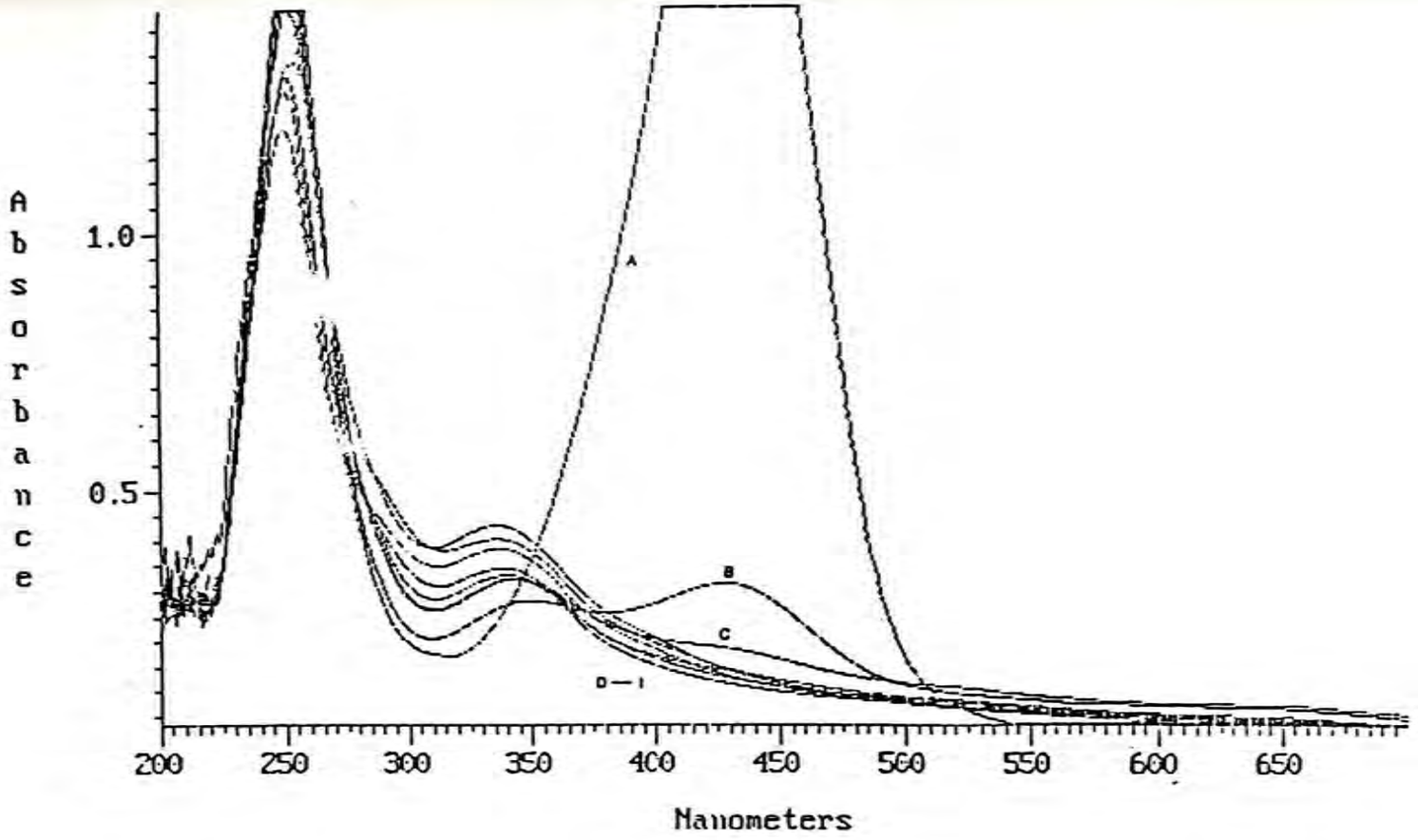


Figure 3-19. Spectroscopic profiles of culture media taken at different time intervals during the decolorization of Tropaeolin O by strain ADD 16-2. The reaction was initiated by inoculating 5 ml stock culture of strain ADD 16-2 at OD=2.8 to 100 ml B.M. with a final concentration of 100 ppm Tropaeolin O (final concentration of cell: $3.26X10^8$ cells/ml). The culture was incubated at 35°C anaerobically for 48 hours. A 1 ml sample of medium was taken out from culture and the cells was spun down by microcentrifugation. The clear supernatants were used for scanning measurement with Spectronic 3000 Array spectrophotometer after proper dilution (usually 2.5-fold) with 0.5 M phosphate buffer. (A, 0 hr; B, 6 hr; C, 12 hr; D, 18 hr; E, 24 hr; F, 30 hr; G, 36 hr; H, 42 hr; I, 48 hr).

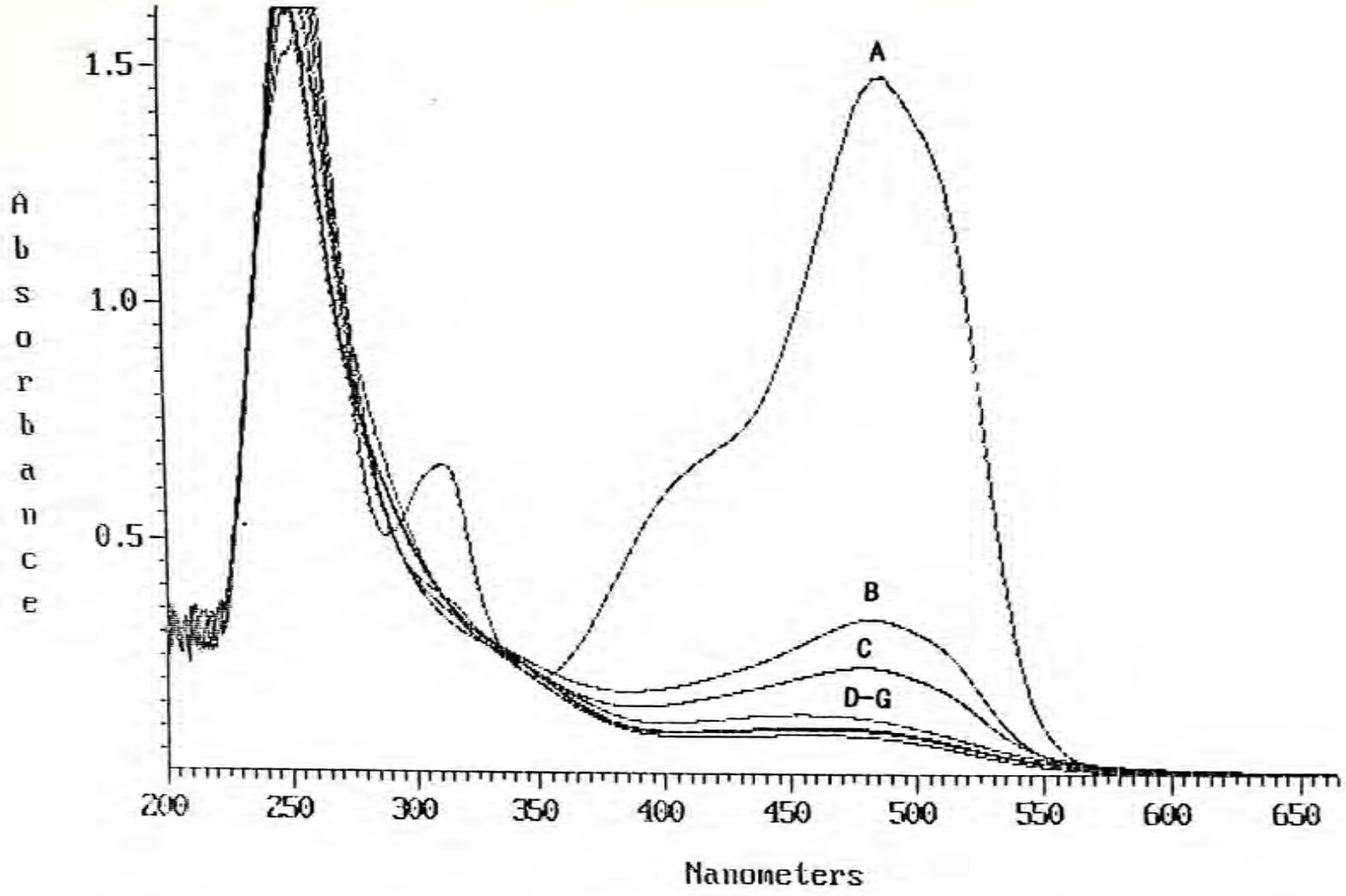


Figure 3-20. Spectroscopic profiles of culture media taken at different time intervals during the decolorization of Orange II by strain ADD 16-2. The reaction was initiated by inoculating 5 ml stock culture of strain ADD 16-2 at OD=2.8 to 100 ml B.M. with a final concentration of 100 ppm Tropaeolin O (final concentration of cell: $3.26X10^8$ cells/ml). The culture was incubated at 35°C anaerobically for 48 hours. A 1 ml sample of medium was taken out from culture and the cells was spun down by microcentrifugation. The clear supernatants were used for scanning measurement with Spectronic 3000 Array spectrophotometer after proper dilution (usually 2.5-fold) with 0.5 M phosphate buffer. (A, 0 hr; B, 18 hr; C, 24 hr; D, 30 hr; E, 36 hr; F, 42 hr; G, 48 hr).

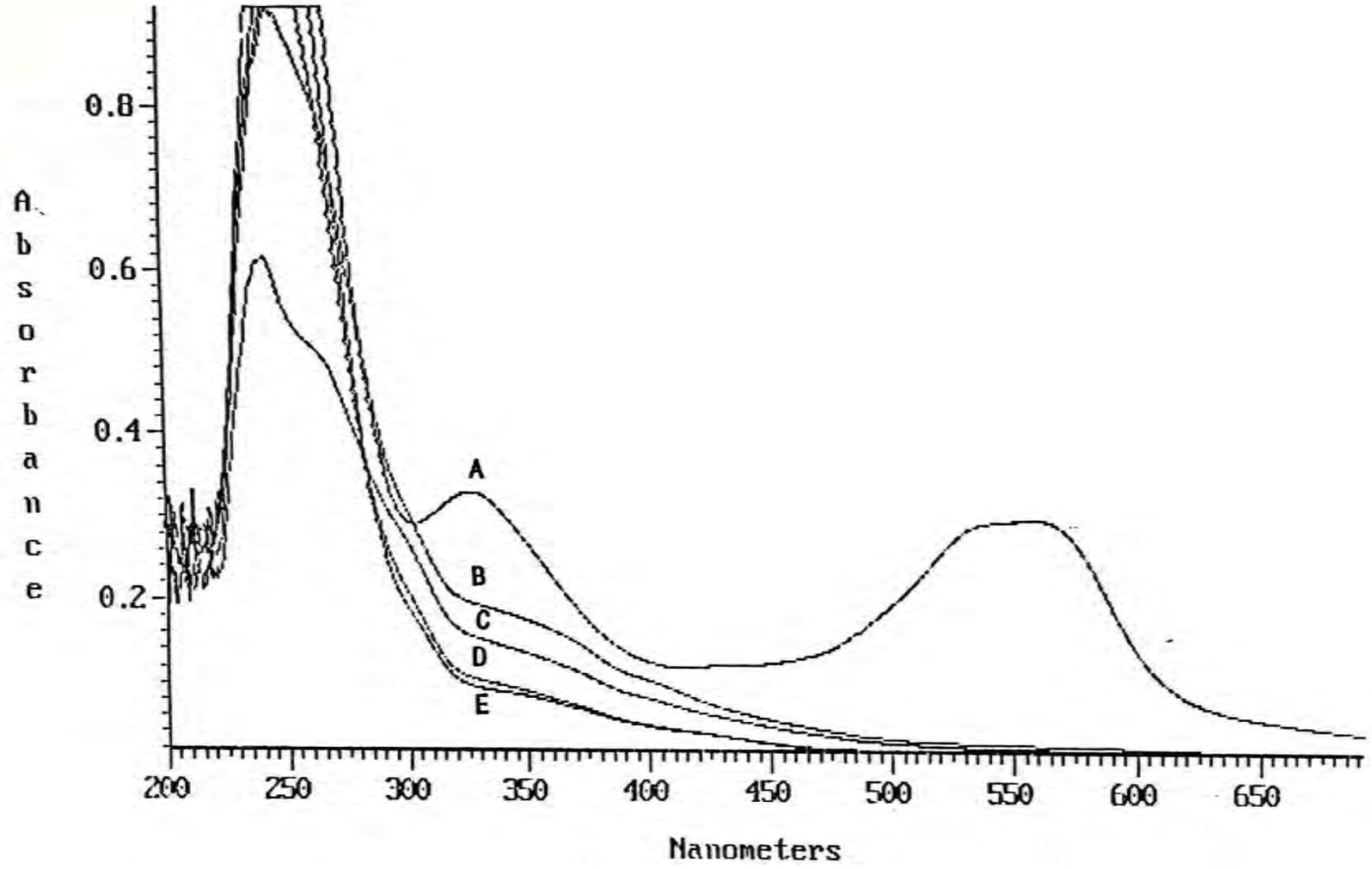


Figure 3-21. Spectroscopic profiles of culture media taken at different time intervals during the decolorization of Direct Violet 5 by strain ADD 16-2. The reaction was initiated by inoculating 5 ml stock culture of strain 16-2 at OD=2.8 to 100 ml B.M. with a final concentration of 100 ppm Reactive Violet 5 (final concentration of cell: $3.26X10^8$ cells/ml). The culture was incubated at 35°C anaerobically for 48 hours. A 1 ml sample of medium was taken out from culture and the cells was spun down by microcentrifugation. The clear supernatants were used for scanning measurement with a Spectronic 3000 Array spectrophotometer after proper dilution (usually 2.5-fold) with 0.5 M phosphate buffer. (A, 0 hr; B, 6 hr:; C, 12 hr; D, 18 hr; E, 24 hr).

in the UV range was probably resulted from a ring compound accumulated in the medium. This implies that the ring structure of the original dye molecule was intact.

The reaction medium were separated by HPLC as shown in Figure 3-19. In order to identify this reaction "intermediate(s)", the dye reaction mixtures were collected at various time intervals and compared with a putative authentic standard. In the condition chosen with HPLC, the retention time of Tropaeolin O was 6.2 min (Figure 3-22a). After 5 hours of incubation, the peak at 6.2 min retention time reduced about half and an extra new peak emerged at a retention time of 5.3 min (Figure 3-22b). After 24 hours of incubation, essentially 100% of the colour had disappeared that corresponded to a total disappearance of the 6.2 min retention time peak (Figure 3-22c). On the other hand, the new 5.3 min peak was fully developed. Since an authentic sample of sulfanific acid gave a peak with retention time 5.3 min, it is obvious that the product of the decolorization of Tropaeolic O is sulfanific acid. The HPLC profiles hence also indicate that strain ADD 16-2 decolorizes Tropaeolin O by a cleavage of the azo bond to produce aromatic amines as its product.

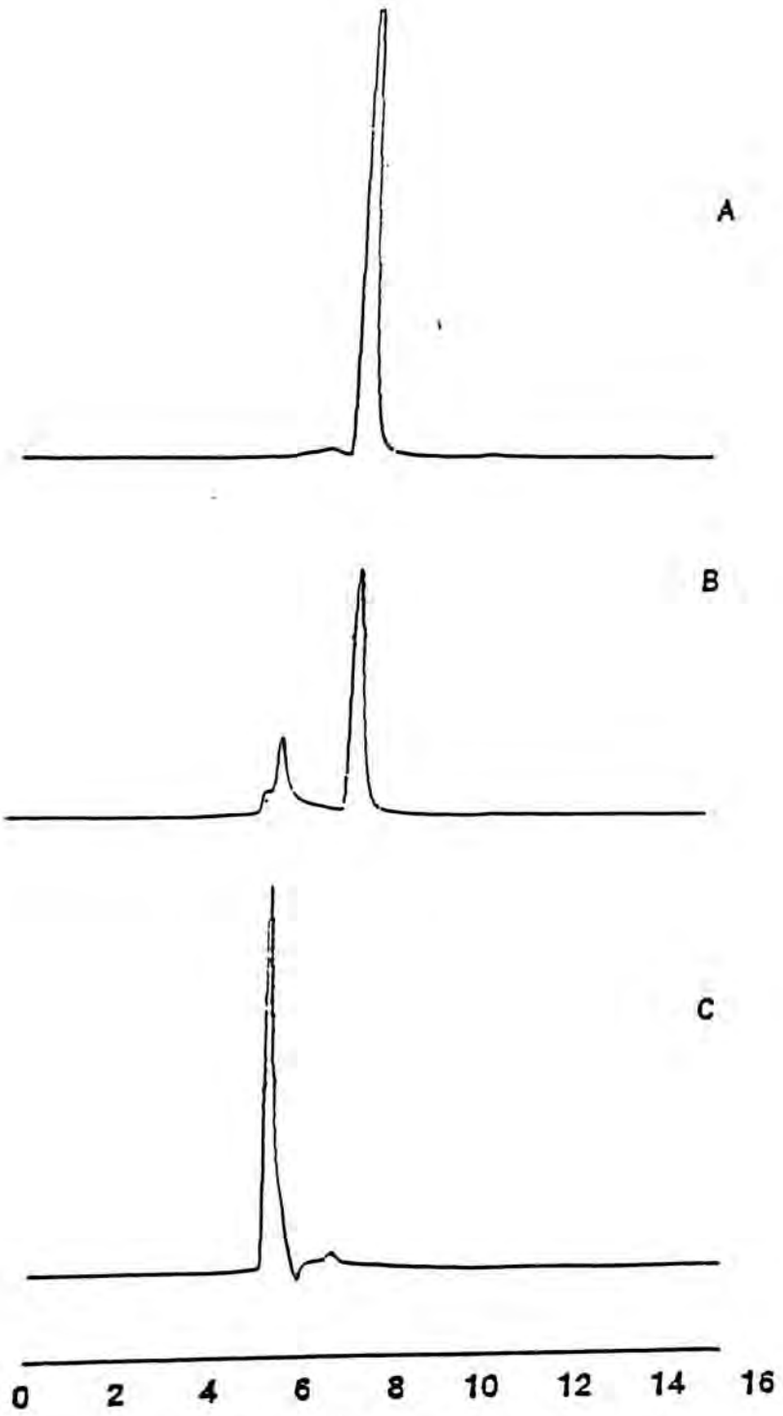


Figure 3-22. HPLC analysis of decolorization products of Tropaeolin O at various time intervals. A, 0 hr; B, 5 hr; C, 18 hr. The condition of the incubation was the same as described in Figure 3-16. The samples were centrifugated at 10.000 X g for 20 min and filtered through a 0.2-μm-pore-size filter. 50% methanol, 48% double distil water and 2% acetic acid were used as mobile phase. The flow rate of the mobile phase was 0.5 ml per min. Peaks were identified and quantified by comparing with 100 ppm authentic standards (Tropaeolin O and sulfanific acid).

3.2 DEGRADATION OF THE INTERMEDIATE SULFANIFIC ACID

3.2.1 Enrichment and Isolation of Strains that Can Degrade The Azo Dye Decolorization Product(s).

Enrichment of microorganisms on a xenobiotic compound can be defined either as a decrease in the lag period or functionally as an increase in the degradation rate on a particular compound. Figure 3-23 shows the shape of the growth curves of a series of continuous subcultures in the enrichment cultures. The total enrichment period was approximately 3 months before a stable consortium growth rate on a medium containing sulfanilic acid was reached. The concentration of sulfanilic acid in medium increased from 50 ppm to 200 ppm progressively during the whole enrichment period. Figure 3-24 shows the changing scanning profiles of the compound concentration on the culture medium at different time intervals during an enrichment subculture. The results indicated that the enriched microorganisms have achieved the ability to degrade sulfanific acid effectively. Hence a microbial community was enriched to use sulfanilic acid as a sole carbon source.

A diluted culture taken from the latest phase of the enrichment was plated on LB agar plates and single colonies were isolated and purified. Five strains were isolated from the enrichment culture. Although enrichment media containing one single compound as the source of carbon and energy, only two strains, M-1 and M-2 had the ability of degrading the compound. Perhaps the

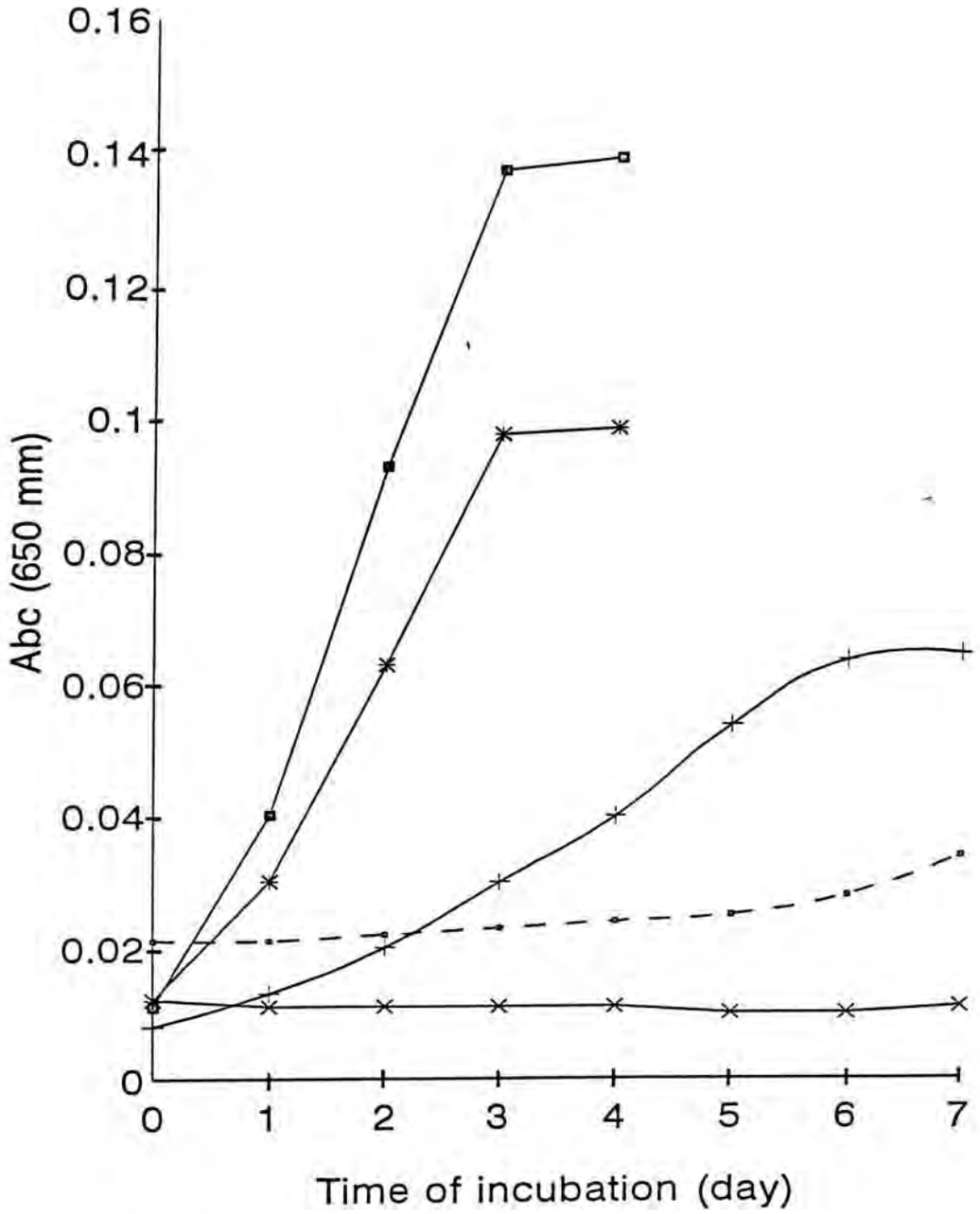


Figure 3-23. The growth curves of subcultures in the enrichment cultures. The mixed microorganisms collected from the polluted sites were incubated in 100 ml M.S.M. which sulfanific acid was used as the sole carbon source. The concentration of Sulfanifi Acid in the medium was increased from 50 ppm to 200 ppm (1th-4th: 50 ppm, 5th-19th: 100 ppm, after 20th: 200 ppm). The culture was incubated at 30°C and stirred at 200 r.p.m.. At the end of subcultures, 5 ml of the suspension was transferred to a fresh M.S.M.. Growth status of strains was monitored by measuring the absorbency of the suspension at 650 nm.

Symbols: —•— 1th, —+— 4th, —*— 10th, —□— 20th, —×— control.

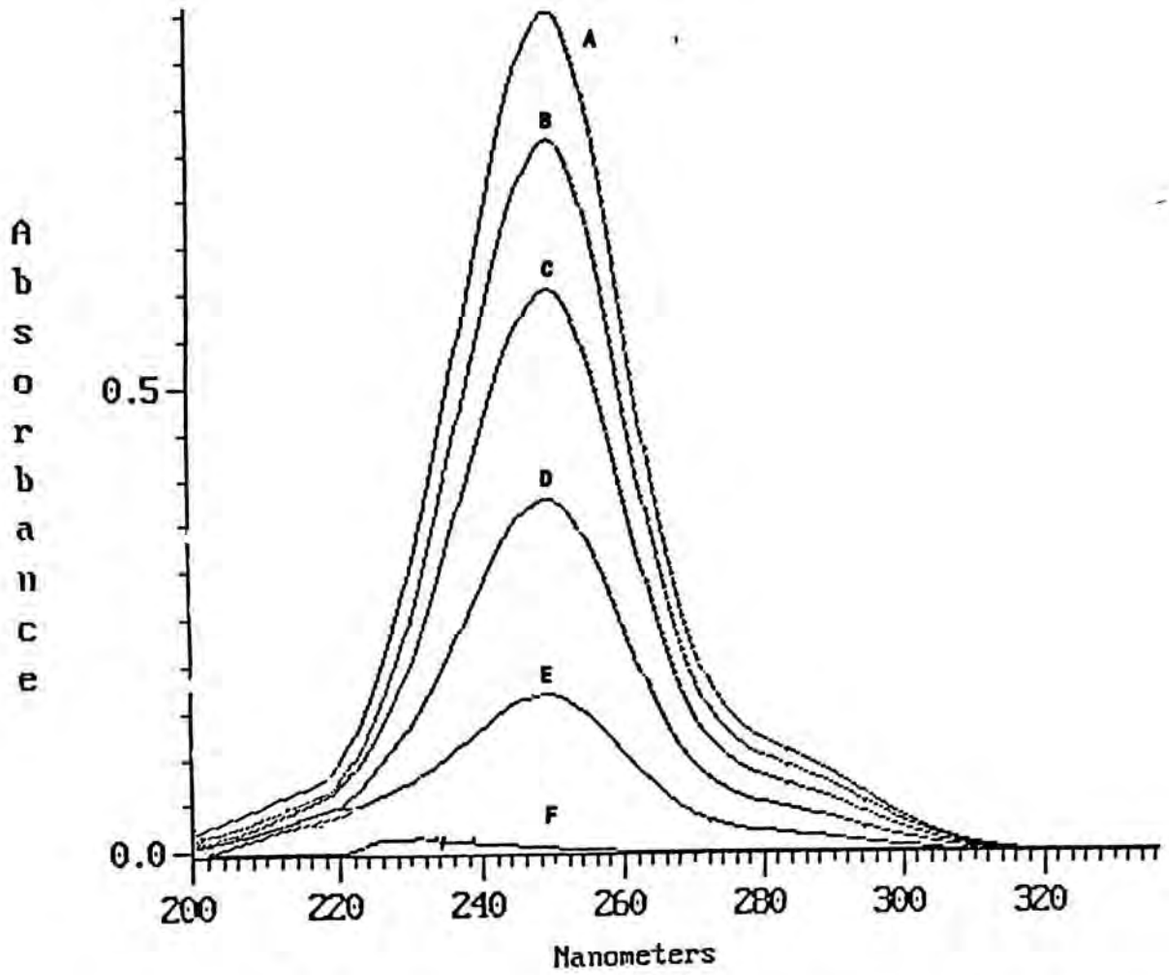


Figure 3-24. Spectroscopic profiles of culture media taken at different time intervals during enrichment period. 5 ml suspension (19th subculture) was transferred to a fresh M.S.M. that containing 200 ppm sulfanific acid. The culture was incubated at 30°C and stirred at 200 r.p.m. for 5 dyes. A 1 ml sample of medium was taken out from culture during the enrichment period each day in 20th subculture and the cells was spun down by microcentrifugation. The clear supernatants were used for scanning measurement with a Spectronic 3000 Arrau spectrophotometer after proper dilution (usually 2.5-fold) with 0.5 M phosphate buffer. A, 0 day; B, 1 day; C, 2 day; D, 3 day; E, 4 day; F, 5 day.

other strains are opportunistic strains that growing on excretion or lysis products from the primary degrading organisms. The strain SAD M-1 which exhibits higher activity of degradation was selected for future studies (Figure 3-25).

3.2.2 Condition of Sulfanific Acid Degradation

3.2.2.1 The effect of pH

A study of the effect of pH on strain SAD M-1 degrading sulfanific acid indicates that the optimal initial pH of the medium was 8.5 (Figure 3-26). In this pH, the strain shown the fastest degradation rate (time for 50% degradation was approximately 13 hours). Although strain SAD M-1 can exhibit more or less equal degradation activity of sulfanific acid in the pH range of 8.0 to 9.0. At higher extreme alkaline environment (pH > 9.5), the cells lost all activity suddenly.

3.2.2.2. The effect of temperature

A studying of the degradation activity in the range of 25-40°C, the strain SAD M-1 showed an optimum at 33°C (Figure 3-27). When the temperatures were in the range of 25-35°C, the strain SAD M-1 could degrade sulfanific acid efficiently in an narrow scale of activity change. When the temperature increased to 37°C, the activity decreased abruptly. At 40°C, the strain lost all

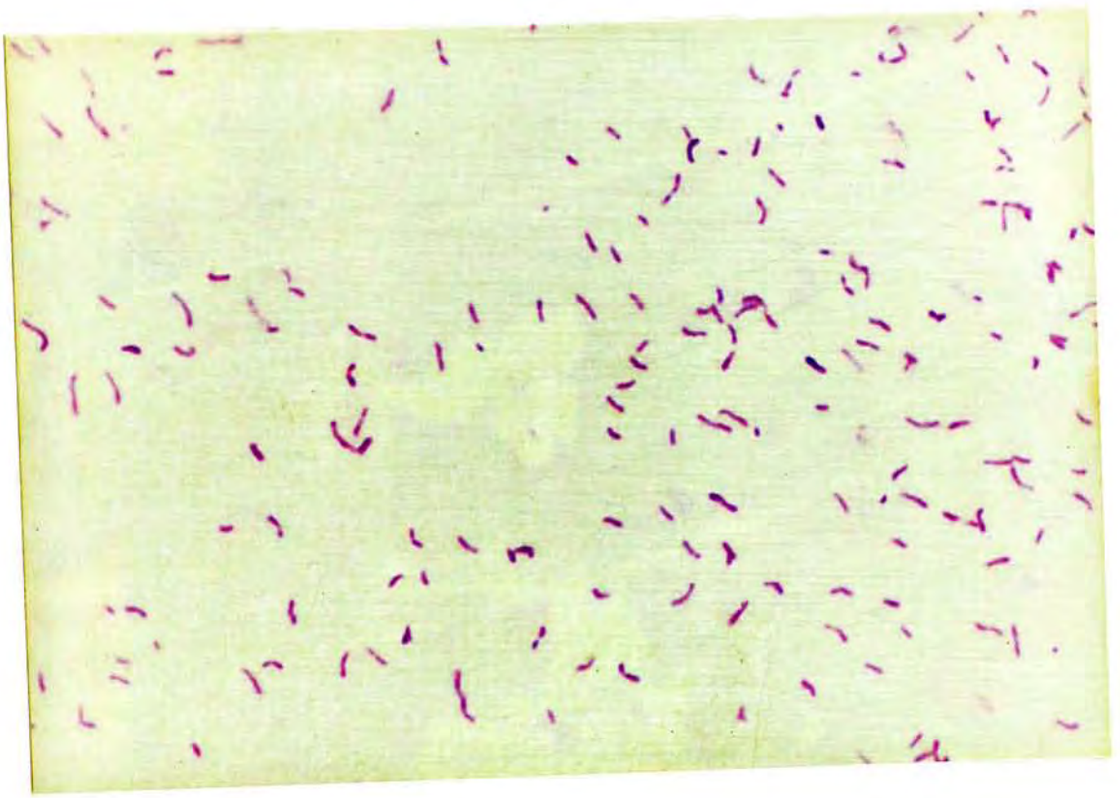


Figure 3-25. Strain SAD M-1 under microscope (magnification: 1,000X). The cell was cultured in M.S.M. and harvested at log phase. The strain stained with crystal violet.

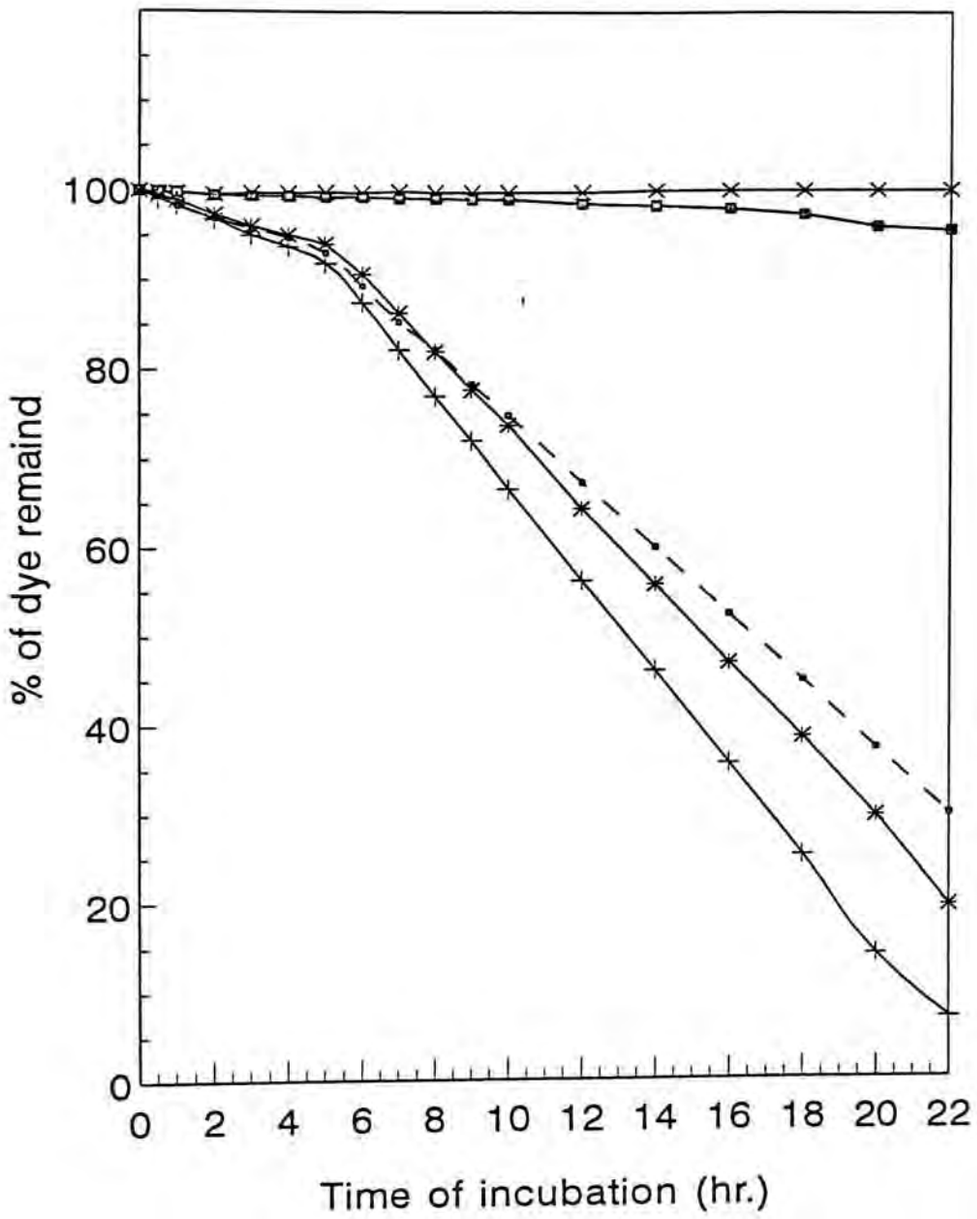


Figure 3-26. The effect of pH on the degradation of sulfanific acid by strain SAD M-1. 5 ml of stock inoculum (OD 2.2) was added to 100 ml M.S.M. containing 100 ppm of Sulfanific Acid at its final concentration. The initial pH of the medium was adjusted to 8.0, 8.5, 9.0, 9.5, and 10.0 with H_3PO_3 buffer. The cultures were incubated at 30°C on a rotary shaker. At proper time intervals, a 1 ml sample of medium was taken out from each culture and the cells was spun down by microcentrifugation. The clear supernatants were used for OD measurement (at 248 nm) after proper dilution (usually 5-fold) with 0.5 M phosphate buffer. The decolorization of dye was expressed in percentage of dye remained in culture.
Symbols: -•- pH8.0, -+- pH 8.5, -*- pH 9.0, -▪- pH 9.5, -×- pH 10.0.

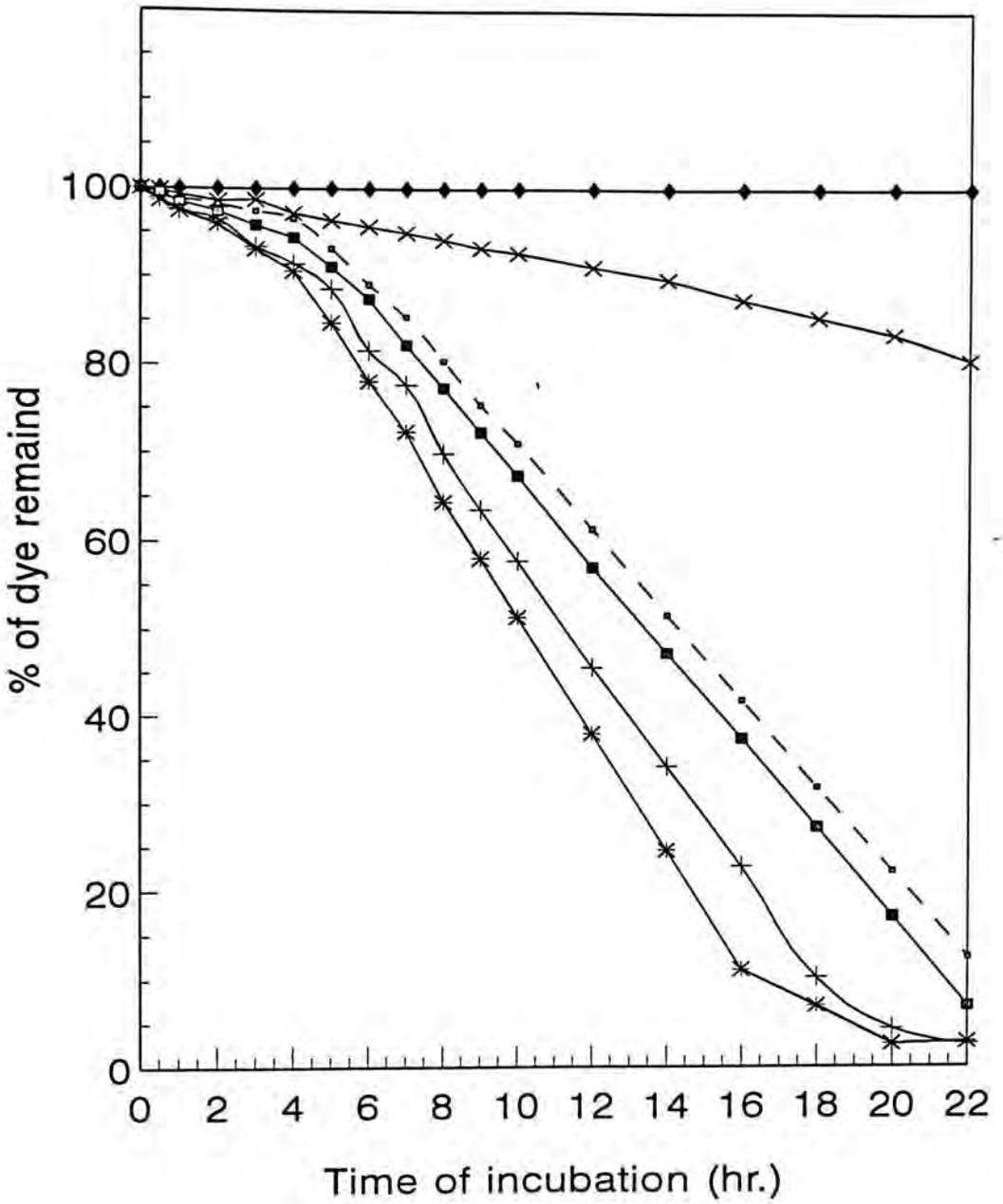


Figure 3-27. The effect of temperature on the degradation of sulfanific acid by strain SAD M-1. The strain SAD M-1 was added to M.S.M. as mentioned in Figure 3-26. The cultures were incubated aerobically at 25°C, 30°C, 33°C, 35°C, 37°C, and 40°C separately for 22 hours. At proper time intervals, a 1 ml sample of medium was taken out from each culture and the cells was spun down by microcentrifugation. The clear supernatants were used for OD measurement (at 248 nm) after proper dilution (usually 5-fold) with 0.5 M phosphate buffer. The decolorization of dye was expressed in percentage of dye remained in culture.

Symbols: —•— 25°C, —+— 30°C, —✳— 33°C, —■— 35°C, —×— 37°C, —◆— 40°C.

its ability of degradation. It suggests that strain M-1 is a mesophile similar to the strain ADD 16-2.

3.3 AN ATTEMPTED OF COMPLETE DEGRADATION OF TROPAEOLIN O BY STRAINS ADD 16-2 AND SAD M-1 WITH A COMBINED ANAEROBIC-AEROBIC PROCESS

To demonstrate a total degradation of Tropaeolin O by microorganisms, the dye was first incubated anaerobically with the strain ADD 16-2. Glucose was added to enhance the degradation rate of the azo dye. When complete decolorization of the dye was achieved, the strain ADD 16-2 was replaced by strain SAD M-1. Air was reintroduced into the culture and aerobic degradation of sulfanific acid started immediately. Figure 3-28 shows the scanning profiles of the culture medium at different steps. It is shown that the ring compounds disappeared eventually from the medium.

3.4 A STUDY OF THE DECOLORIZATION POTENTIAL OF STRAIN ADD 16-2 UNDER IMMOBILIZED CONDITION

The aim of the present work was to observe the behavioral responses of strain ADD 16-2 in an immobilized matrix. Calcium alginate immobilization was chosen because it entraps whole cells under mild conditions and causes only minimal cell damage if there is any.

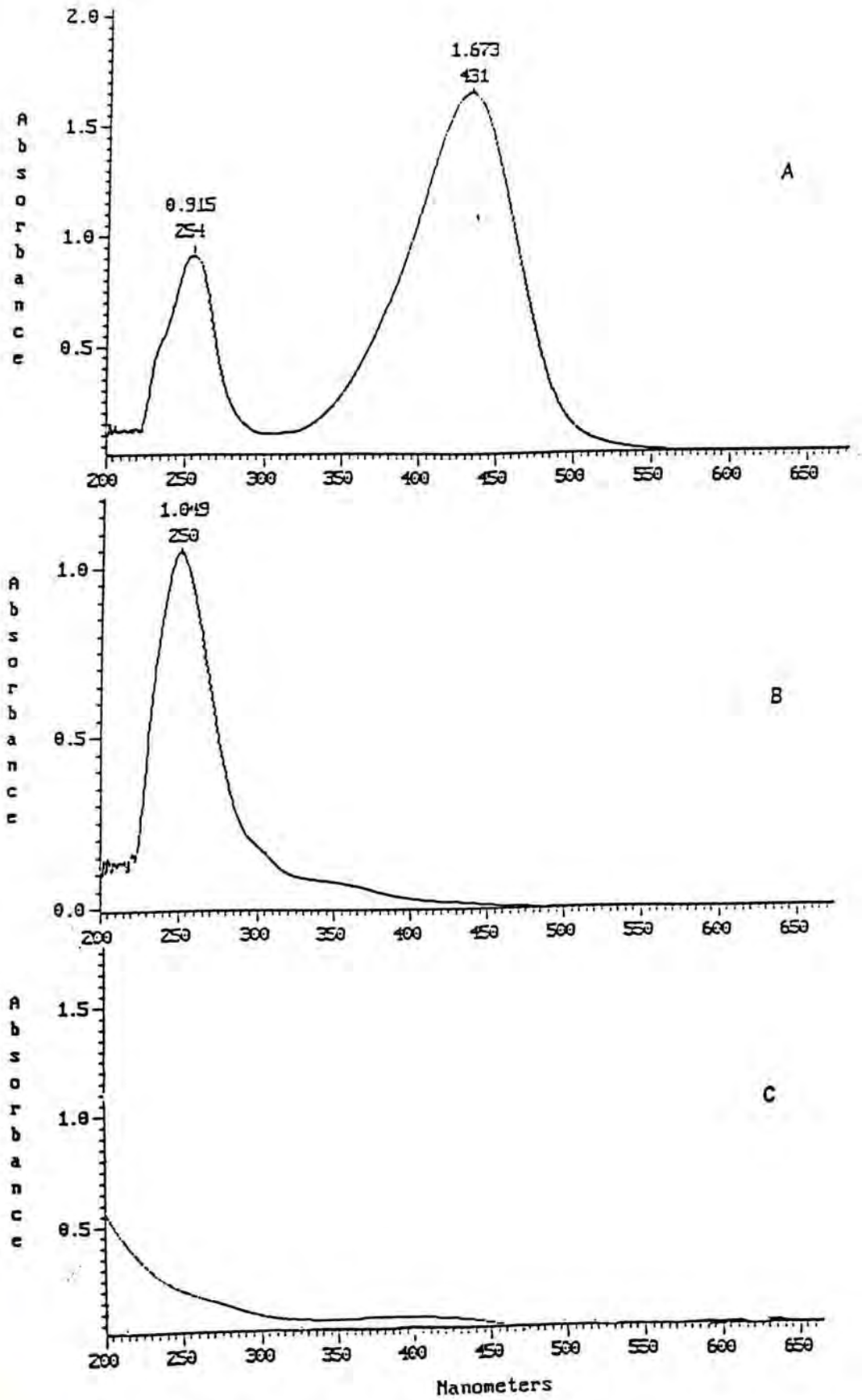

2.0
1.5
1.0
0.5
Absorbance
1.673
431
0.915
254
A
200
250
300
350
400
450
500
550
600
650
1.049
250
1.0
0.5
0.0
B
1.5
1.0
0.5
C
Nanometers

Figure 3-28. Spectroscopic profiles of culture media at different steps that degradated completely Tropaeolin O by strain ADD 16-2 and strain SAD M-1 through an anaerobic-aerobic process. 100 ppm of Tropaeolin O was first incubated anaerobic with the strain ADD 16-2 in M.S.M. at 35°C (cell concentration 3.65×10^8). 0.05% glucose was added to enhance the reduction rate of the dye. When complete decolorization of the dye was reached (about 18 hour), the cell ADD 16-2 was totally removed by filtration and the strain SAD M-1 was added (final concentration of cell 1.25×10^8). Then the culture was incubated aerobically on a shaker at 200 r.p.m. for 24 hours. At each stage, the medium was taken out and scanned by a spectrophotometer after cells were spun down.

A, original medium; B, after complete decolorization by ADD 16-2; C, after degradation of the intermediate by SAD M-1.

3.4.1. Condition of Decolorization of Tropaeolin O by the Immobilized Cell ADD 16-2.

3.4.1.1 The effect of alginate concentration on the decolorization potential of strain ADD 16-2

Figure 3-29 shows the effect of alginate concentration on the decolorization potential of bacteria ADD 16-2. The nature of alginate beads with various concentrations influenced significantly the activity of decolorization. The maximum activity was observed at an alginate concentration between 1 and 2% (w/v). At concentrations greater than 3%, a slight increase in the alginate gel concentration decreased the activity of decolorization rapidly. Since the strength of gel was better with 2% alginate beads, this gel concentration was used for subsequent studies.

3.4.1.2 The effect of cell number entrapped in different size beads on the decolorization ability of the cell ADD 16-2

Various initial cell concentrations were immobilized in 4 mm and 0.5 mm diameter gel beads. They were then used to assay the effect of the microbial content in different bead size on the activity of dye decolorization. Figure 3-30 shows that in the case of 4.0 mm beads, the maximum rate of decolorization was about 20 μg of dye per ml gel per hour when the concentration of cell increase to $1.6X10^{10}$. At a cell content greater than $1.6X10^{10}$, increasing the

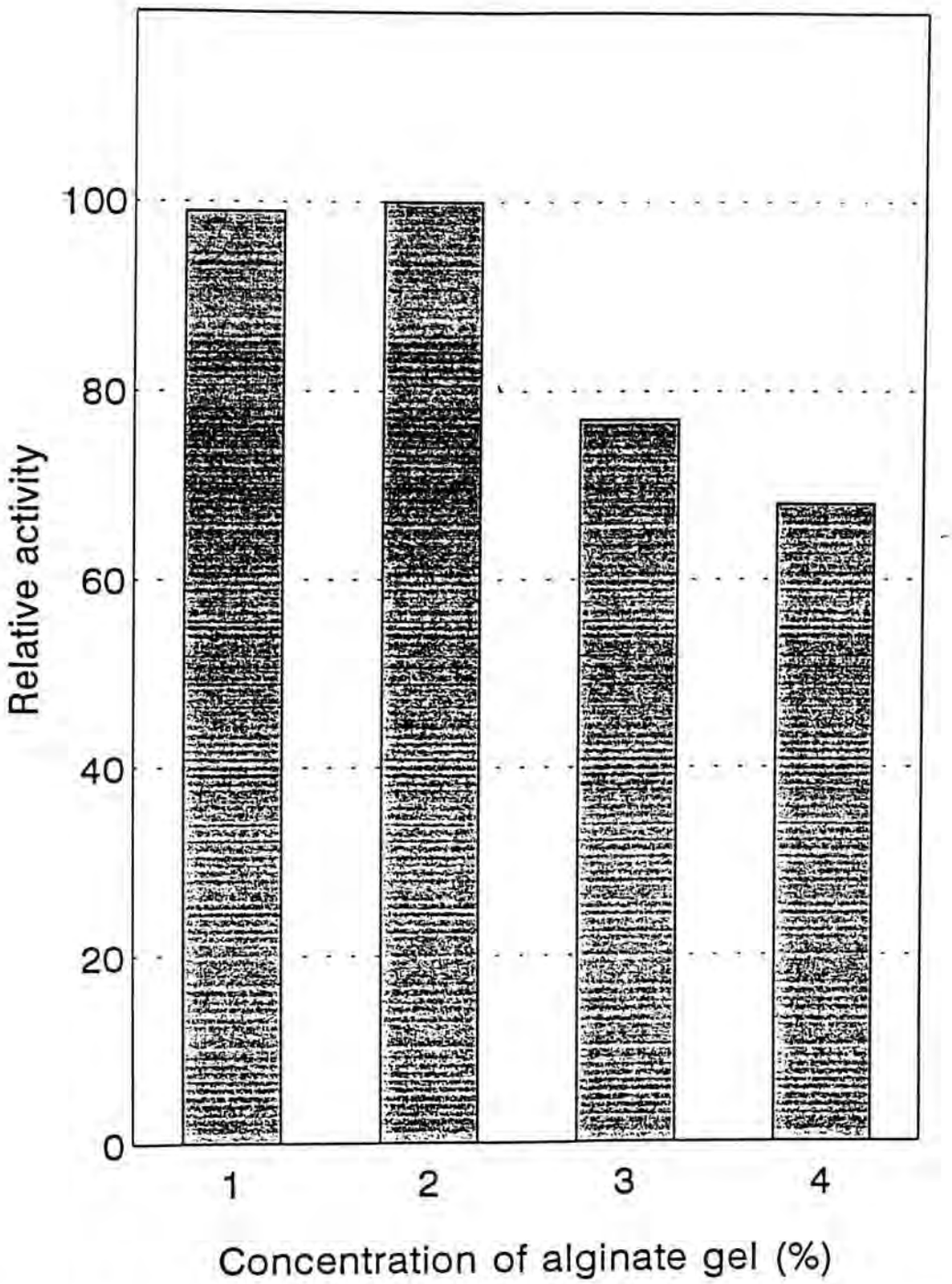


Figure 3-29. The effect of alginate concentration on the degradation of Tropaeolin O. The cells ADD 16-2 were entrapped in a series of concentration of alginate gel (1%, 2%, 3%, and 4%). 10 ml beads (2 mm diameter, 8.7 X 10^8 cells per ml gel) were added into 50 ml B.M. containing 100 ppm Tropaeolin O. The cultures were incubated anaerobically at 35°C separately for 36 hours. At proper time intervals, a 1 ml sample of medium was taken out from each culture and the cells was spun down by microcentrifugation. The clear supernatants were used for OD measurement (at 430 nm) after proper dilution (usually 5-fold) with 0.5 M phosphate buffer. The fastest decolorization rate at different concentration of gel was taken as 100%.

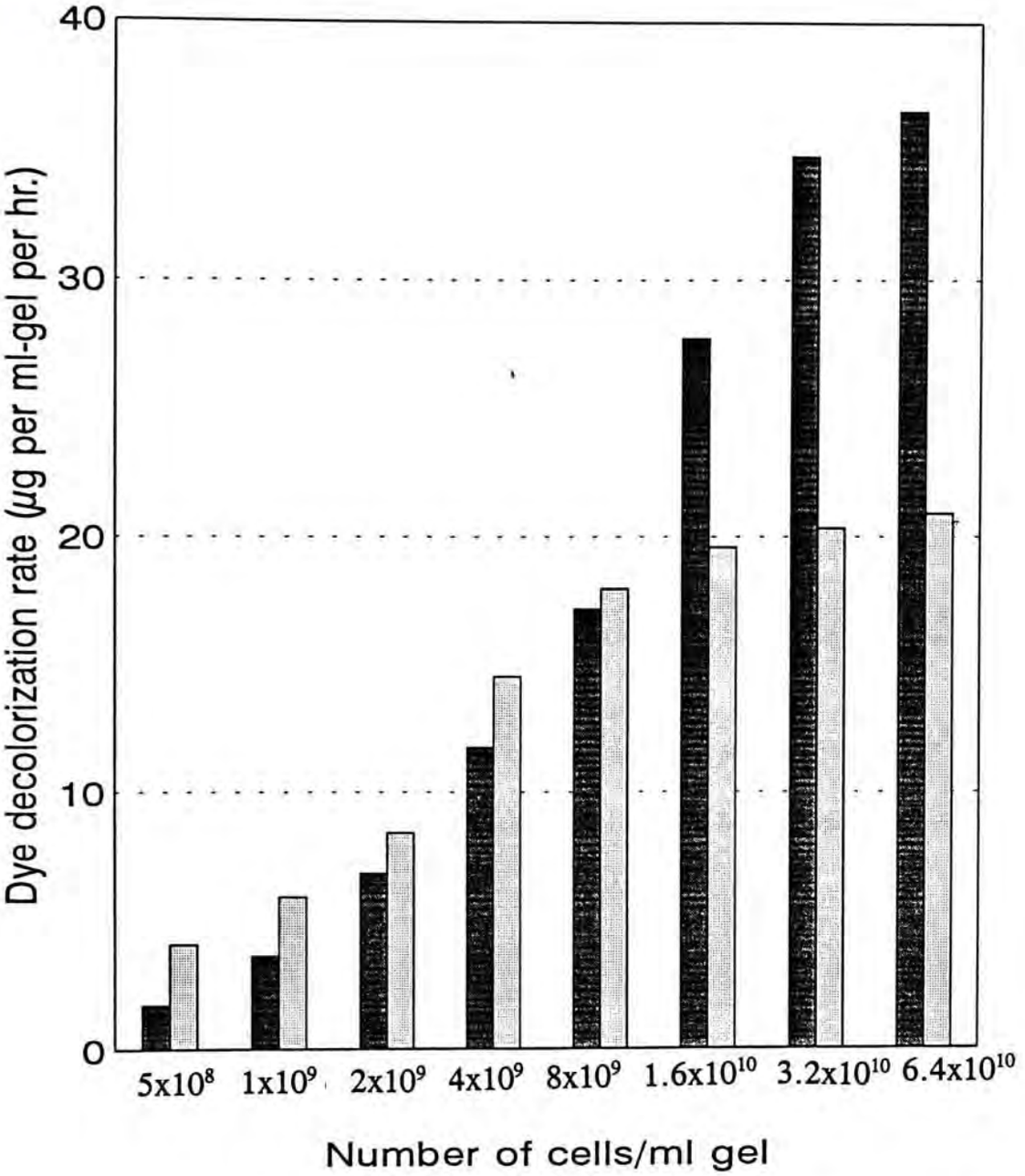


Figure 3-30. Comparison of the decolorization activity of different bead size (4 mm and 0.5 mm in diameter) with different amount of cells entrapped in the gel. 10 ml of each beads type were added to B.M. and incubated at 35°C for 24 fours. 1 ml samples of these culture media were taken out at fixed time interval and the cells in the medium was spun down by microcentrifuge. The clear supernatants were diluted 5-fold with 0.5 M phosphate buffer and were used then for spectrophotometric measurements at 430 nm.

Symbols: ■ 0.5 mm ▨ 4 mm

concentration of cells did not significantly influence the activity of decolorization by immobilized gel. In 0.5 mm beads, the rate of decolorization could reached 36.6 μg of dye per ml gel per hour when the concentration of cell increase to $6.4X10^{10}$. The optimum microbial content was $1.6X10^{10}$ cells per ml gel in a 4 mm diameter gel beads, while the optimum cell concentration was $6.4X10^{10}$ per ml gel in 0.5 mm diameter gel beads instead.

3.4.1.3 The effect of pH on the decolorization potential of immobilized strain 16-2

A study of the effect of pH on the decolorization potential of immobilized cells ADD 16-2 indicates that the immobilized cells had almost the same decolorization activity at pH 6.5, 7.0, and 7.5. The pH effect on the decolorization activity was not so affected as compared with those of the free cell. Even at extreme pHs of 5.5 or 8.5, cells entrapped in gel still exhibited a considerable ability of decolorization (Figure 3-31). On a close examination of the pH activity curves of the both free cells and entrapped ones, it is interesting to note that the curve for the immobilized cells flattened around the optimum pH. From a practical standpoint, the entrapped cells maintain a constant activity in a wide range of pH values, especially at the acidic pH side (Figure 3-32).

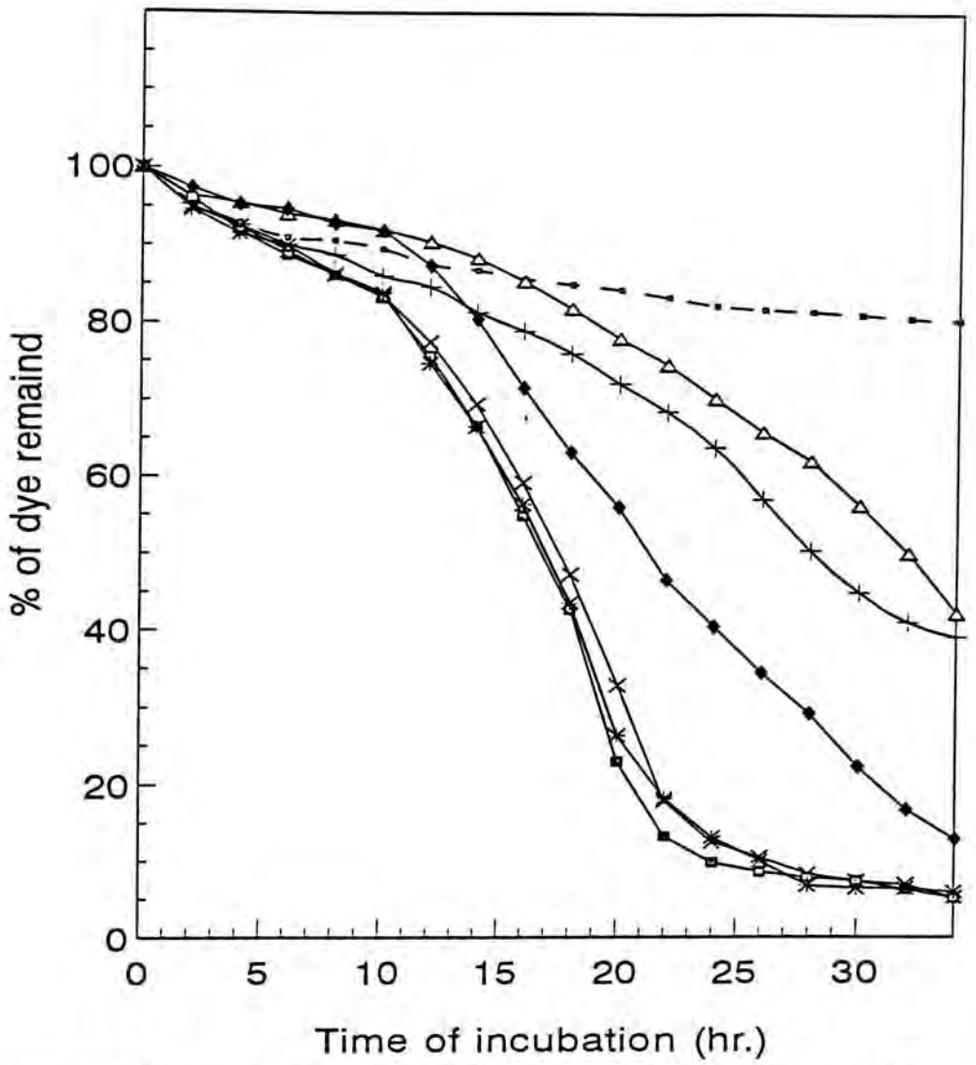


Figure 3-31. The effect of pH on the decolorization of Tropaeolin O by immobilized strain ADD 16-2 cells. 10 ml beads (0.5 mm diameter, 8.7 X 10^8 cells per ml gel) were added into 50 ml B.M. containing 100 ppm Tropaeolin O. The initial pH of the medium was adjusted to 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 with Tris-maleate buffer. The cultures were incubated at 35°C for 34 hours. At proper time intervals, a 1 ml sample of medium was taken out from each culture and the cells was spun down by microcentrifugation. The clear supernatants were used for OD measurement (at 430 nm) after proper dilution (usually 5-fold) with 0.5 M phosphate buffer. The decolorization of dye was expressed in percentage of dye remained in culture.

Symbols: —·— pH5.5, —+— pH 6.0, —*— pH 6.5, —□— pH 7.0, —×— pH 7.5, —◆— pH 8.0, —△— pH8.5.

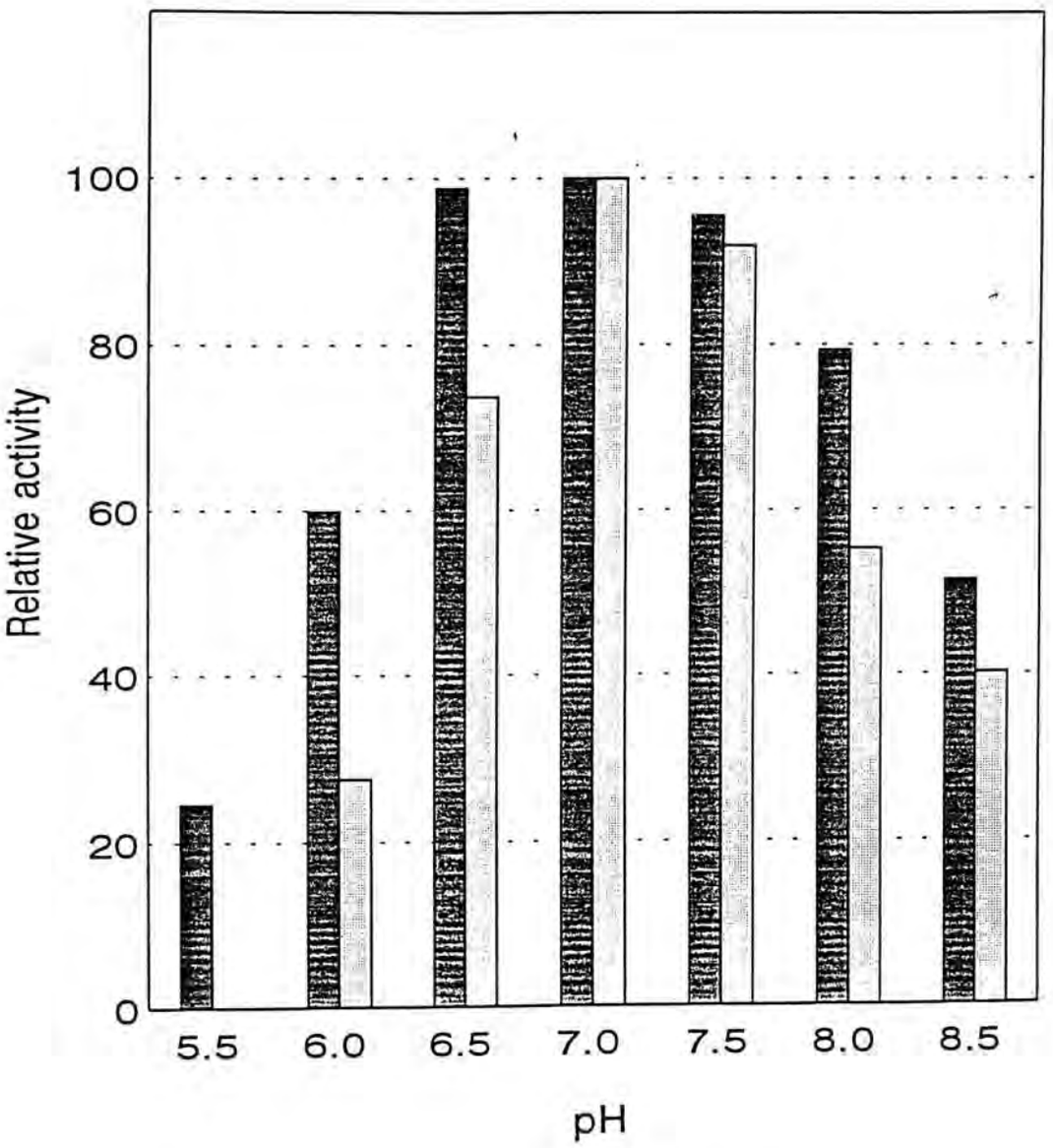


Figure 3-32. Comparison of the decolorization activity of free and immobilized cells ADD 16-2 at different pHs. Both free and immobilized cells were incubated at the same condition (Figure 3-14 and 3-28). The fastest decolorization rate at different pH was taken as 100%. At proper time intervals, a 1 ml sample of medium was taken out from each culture and the cells was spun down by microcentrifugation. The clear supernatants were used for OD measurement (at 430 nm) after proper dilution (usually 5-fold) with 0.5 M phosphate buffer. The decolorization of dye was expressed in percentage of dye remained in culture.
Symbols: ▤ immobilized cell, ☐ free cell.

3.4.1.4 The effect of temperature on the decolorization potential of the immobilized cell ADD 16-2

The temperature dependence of the immobilized cells reaction is shown in Figure 3-30. Similar to the free cell, the optimal temperature for decolorization dye was 35°C. On comparison of the temperature-activity profiles, the entrapped and the free cells did not differ significantly when the temperature was lower than 35°C (Figure 3-13 and Figure 3-33). In lower temperatures, the relative activity of free cells were even higher than the immobilized counterpart. However at temperatures higher than the optimum, the immobilized cells showed a higher relative activity as compared with those of the free cells cultures. At 40°C, the free cells lost the ability of dye decolorization completely, while the immobilized counterpart had 78.3% relative activity yet remained (Figure 3-34).

3.4.1.5 The effect of Tropaeolin O concentration on the decolorization activity of strain ADD 16-2.

The azo dye Tropaeolin O was found to be toxic to microbial populations in fresh water (Michaels *et al.*, 1985). The inhibitory effect of different concentrations of Tropaeolin O on the decolorization ability of the free and the immobilized strain ADD 16-2 were tested. Table 3-6 shows the results. When the concentration of dye was lower than 500 ppm, both free cells and the immobilized cells exhibited similar activity. However when the concentration

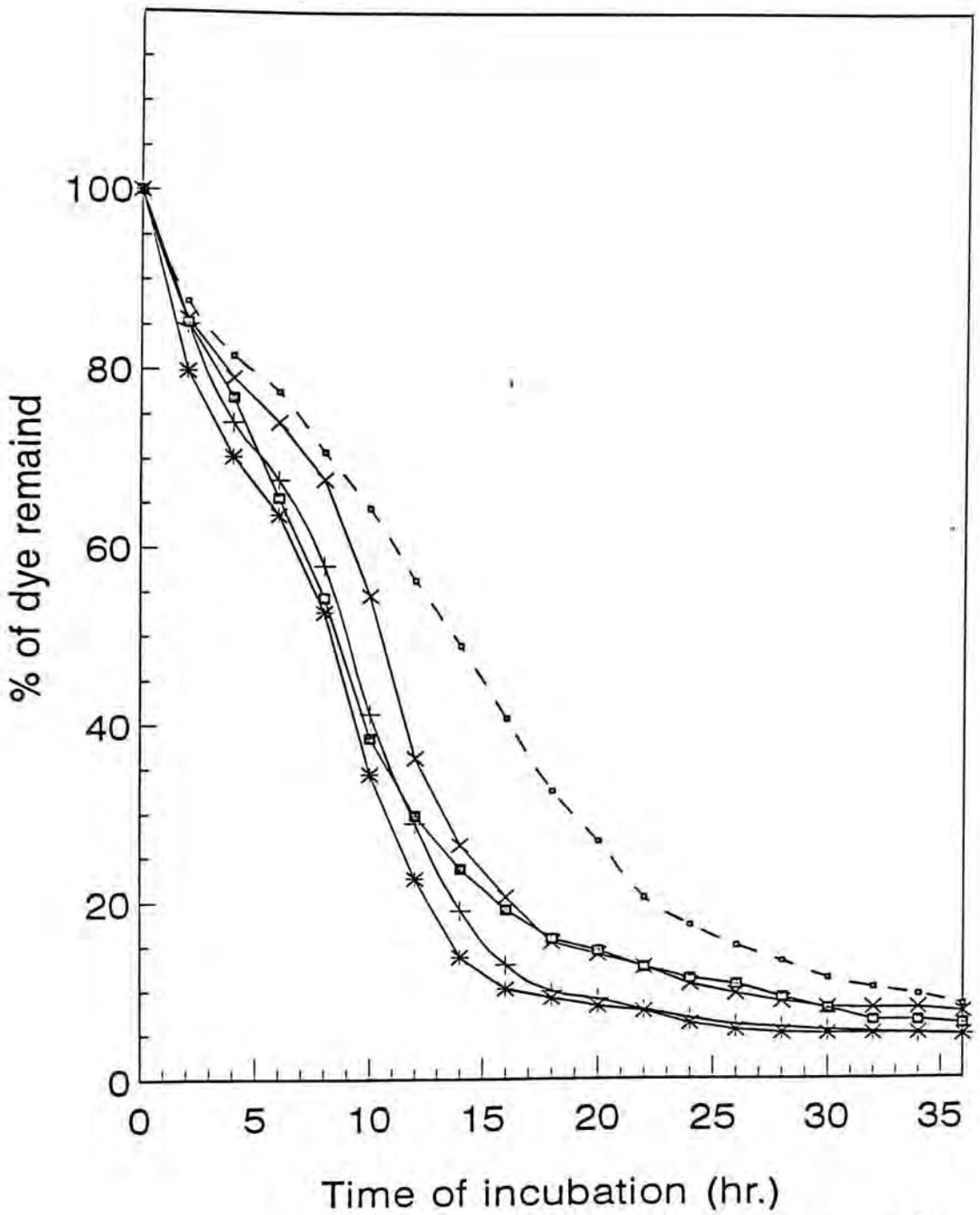


Figure 3-33. The effect of temperature on the decolorization of Tropaeolin O by immobilized strain ADD 16-2 cell. 10 ml beads (0.5 mm diameter, 8.7 X 10^8 cells per ml gel) were added into 50 ml B.M. containing 100 ppm Tropaeolin O. The cultures were incubated anaerobically at 25°C, 30°C, 35°C, 37°C, and 40°C separately for 36 hours. At proper time intervals, a 1 ml sample of medium was taken out from each culture and the cells was spun down by microcentrifugation. The clear supernatants were used for OD measurement (at 430 nm) after proper dilution (usually 5-fold) with 0.5 M phosphate buffer. The decolorization of dye was expressed in percentage of dye remained in culture..
Symbols: —▫— 25°C, —+— 30°C, —✳— 35°C, —▫— 37°C, —✳— 40°C.

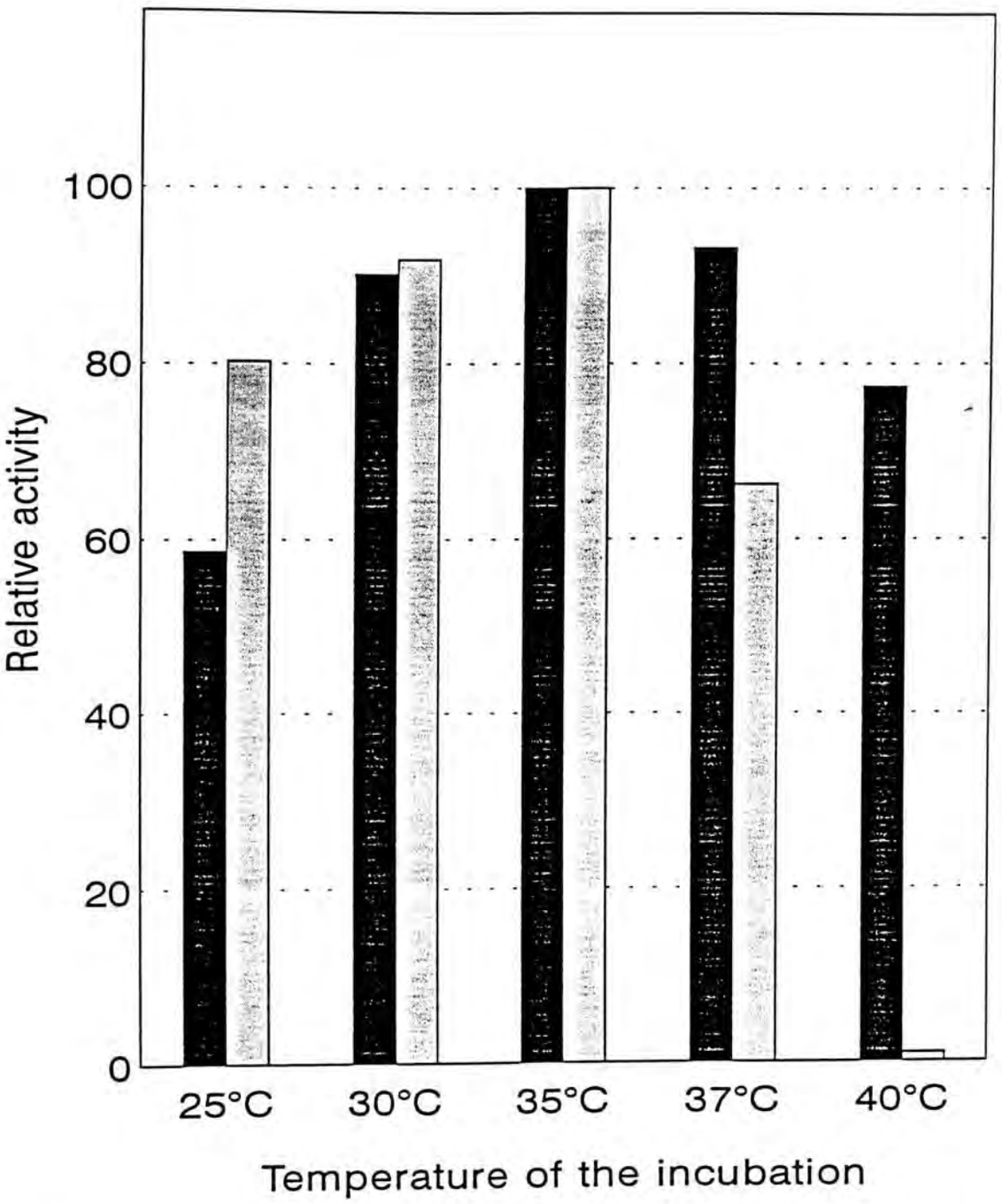


Figure 3-34. Comparison of the decolorization activity of the free and the immobilized cells ADD 16-2 at different temperatures. The free and the immobilized cells were both incubated at same condition (Figure 3-13 and 3-30). The maximnm decolorization rate was taken as 100%.
Symbols: ▬ immobilized cell, ☐ free cell.

Table 3-6: Comparison of decolorization activity of the free (6.25×10^8 cells/ml) and the immobilized cells ADD 16-2 in different dye concentrations

Tropaeolin O conc. (ppm)	t_{50}* (hr)		% left** (%)	
	free	immob.	free	immob.
50	5.6	8.2	3.15	3.45
100	6.7	8.0	2.86	3.11
200	7.8	8.0	3.28	2.78
500	8.1	7.3	3.03	1.76
1000	9.6	7.9	18.4	2.07
1500	-	8.0	56.1	2.03
2000	-	8.5	59.1	3.47
2500	-	9.2	90	3.55
3000	-	11.2	95	5.56
3500	-	29.6	98	47.5

* t_{50} = time in hours for 50% decolorization of Tropaeolin O.
** % left = percentage of Tropaeolin O remained in medium at the end of experiment.

of dye was increased to 1500 ppm, the free cells lost most of its ability. Whereas for the immobilized cells, the effect was not observed until the concentration reached 3500 ppm. It is apparent that cells immobilized achieved an extra protective effect that have not been observed in free cells.

3.5 ASSAY FOR THE DEGRADATION OF SULFANIFIC ACID BY THE IMMOBILIZED CELLS SAD M-1

One potential problem of cell immobilization is the increased resistance of substrates diffusion through the immobilization matrices. In an aerobic reaction, owing to the low solubility of oxygen in water and the high local cell density, oxygen transfer often becomes the bottle-neck in determining if an immobilization method is successful or not. A batch reactor experiment was performed in 250 ml flasks to determine the degradation of the sulfanific acid by immobilized cells SAD M-1. When shaking was adopted, it was found that sulfanific acid was degraded efficiently. It shows that oxygen limitation was not observed in this alginate-immobilized-cell systems.

3.5.1 Optimizing the Condition of Degradation of Sulfanific Acid by Immobilized Cells SAD M-1

3.5.1.1 The effects of alginate gel concentration on the degradation potential of immobilized cells SAD M-1

To study the effects of concentration of alginate gel on the activity of strain SAD M-1, the final gel concentrations were prepared at 1, 2, 3, 4, and 5%. From results shown in Figure 3-35, the maximum activity was observed at an alginate concentration between 1 and 2% (w/v). When the strength of gel is taken into consideration, the 2% of the gel was chosen for future studies.

3.5.1.2 The effect of entrapped cell number in alginate beads on the degradation activity of sulfanilic acid

Various initial cell concentrations were immobilized in alginate gel beads (0.5 mm in diameter). They were then used to degrade sulfanilic acid. The kinetics of the degradation of the compound were monitored by the spectrophotometric method. Figure 3-36 shows the results of the assay. When the concentration of the cells used was below $1.26X10^9$, an increase in cell concentration accompanied an increase in the degradation rate. At cell content greater than $1.26X10^9$, the increase of cell did not influence the activity of the immobilized gel significantly. An initial cell population of about $1.26X10^9$

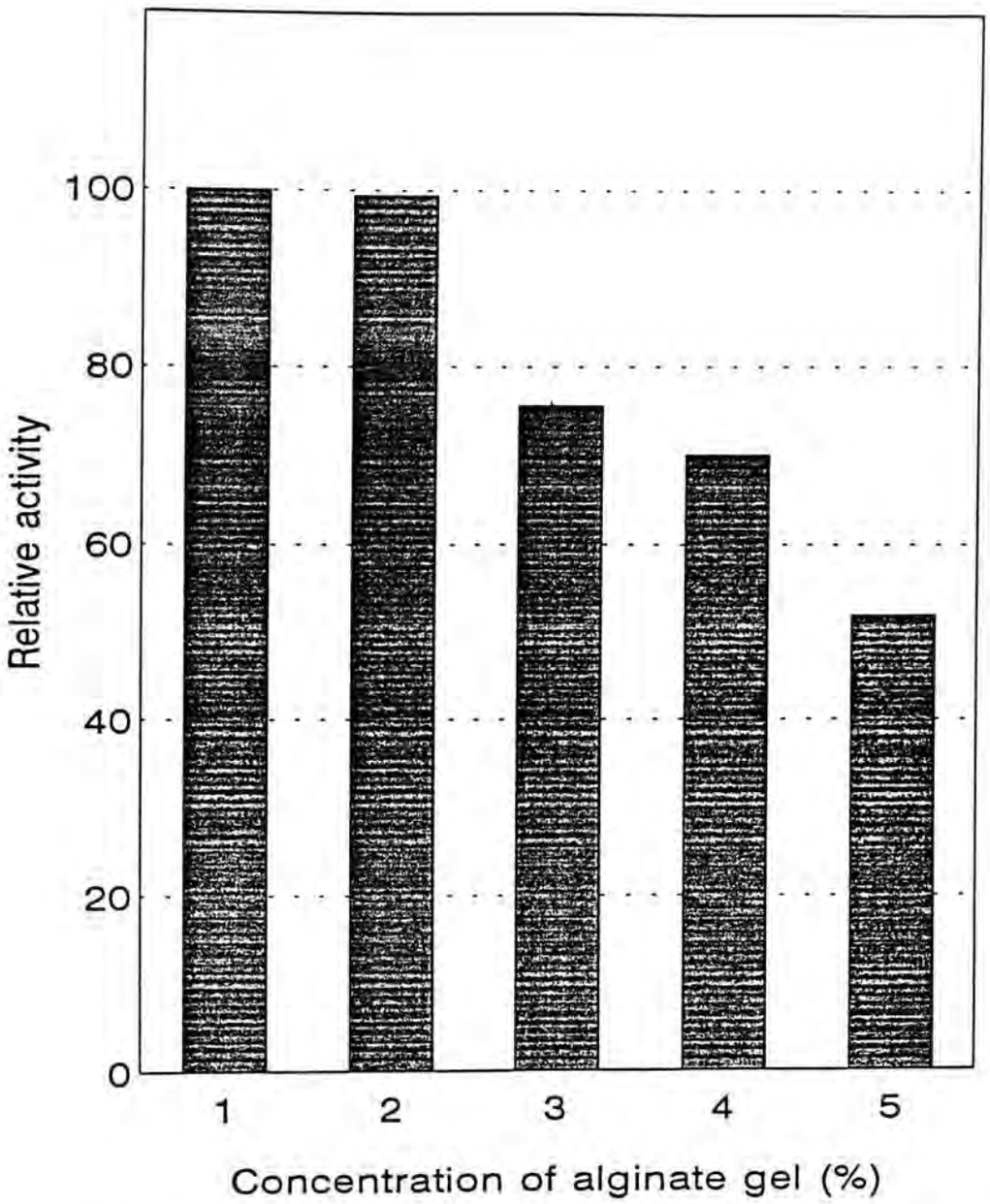


Figure 3-35. The effect of alginate concentration on the degradation of sulfinatic acid by immobilized strain SAD M-1 cell. The cells SAD M-1 were entrapped in a series of alginate gel concentrations (1%, 2%, 3%, 4%, and 5%). 10 ml beads (2 mm diameter, 1.25 X 10^8 cells per ml gel) were added into 50 ml M.S.M.. The cultures were incubated aerobically at 35°C separately for 36 hours. 1 ml samples of these culture media were taken out at two hours intervals and the cells in the medium was spun down by microcentrifugation. The clear supernatants were diluted 5-fold with 0.5 M phosphate buffer and were used then for spectrophotometric measurements at 248 nm. The maximum decolorization rate gel was taken as 100%.

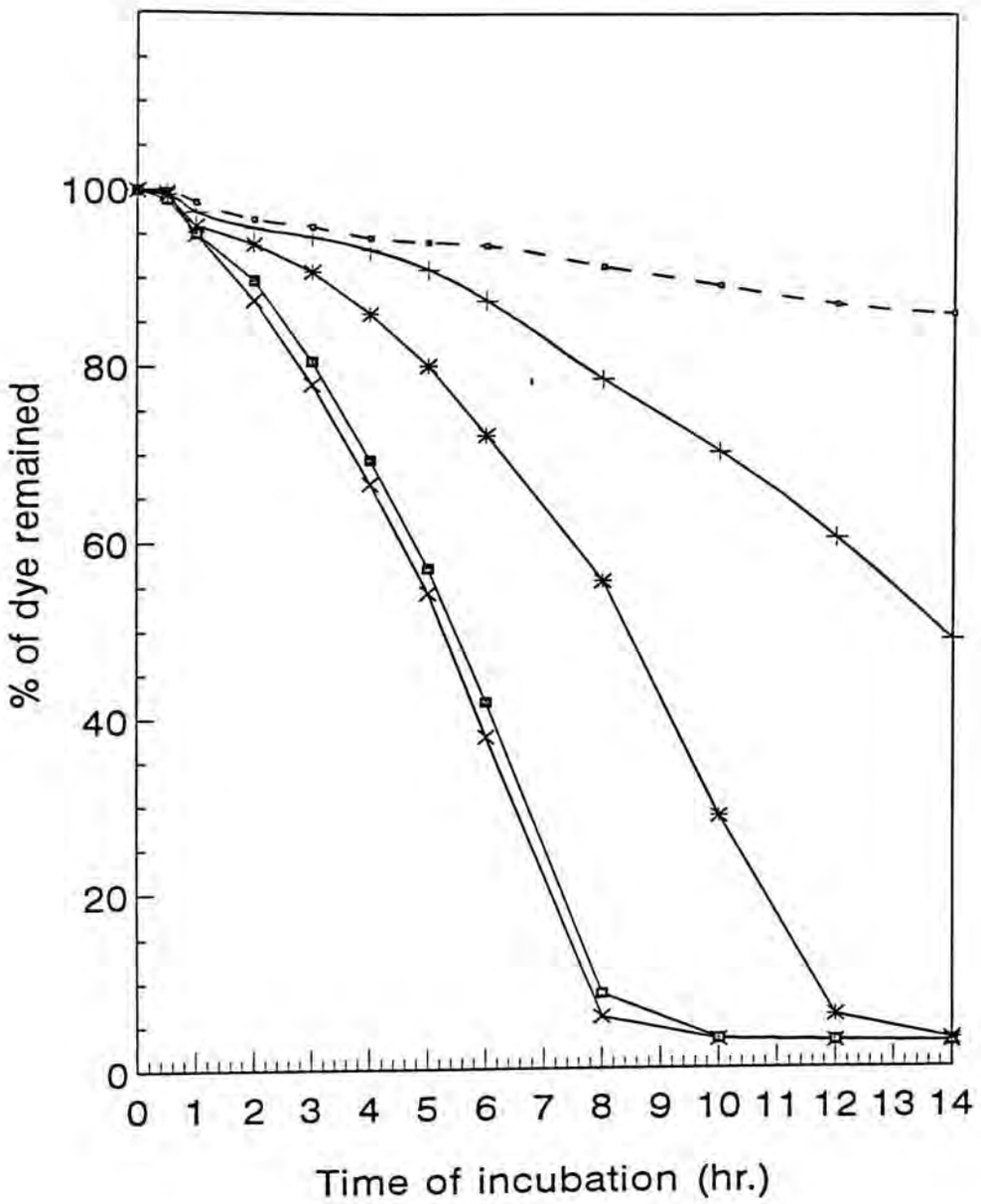


Figure 3-36. The effect of the cells number entrapped in gel on the degradation ability of SAD M-1. The SAD M-1 cells were entrapped in alginate beads (0.5 mm in diameter) at $1.27x10^7$, $1.27x18^8$, $6.35x10^8$, $1.27x10^9$,and $2.54x10^9$ cells per ml gel. 10 ml beads were added into 50 ml M.S.M. The cultures were incubated at 35°C on a shaker (200 r.p.m.) for 14 hour. 1 ml samples were taken out at fixed time interval and the cells in the medium was spun down by microcentrifugation. The clear supernatants were diluted 5-fold with 0.5 M phosphate buffer and used then for spectrophotometric measurements at 248 nm.

Symbols: $1.27x10^7$, $1.27x18^8$, $6.35x10^8$, $1.27x10^9$, $2.54x10^9$

cells/ml of gel is therefore chosen for most of the subsequent studies.

3.5.1.3 The effect of pH on sulfanific acid degradation by the immobilized bacterial SAD M-1 cells

The pH effect on the activity of immobilized SAD M-1 cells were examined. Figure 3-37 shows the degradation patterns throughout a pH range from 8.0 to 10.0 adjusted with H_3PO_3 buffer. As a result, the strain SAD M-1 activity appeared to be highest at pH 9.0. The immobilized cells only lost their activity of degradation at an extreme alkaline (pH 10) environment. A comparison of the relative activity for the free and the immobilized bacteria was given in Figure 3-38. A slight shift of the pH optimum was observed for both cell conditions. It is found that immobilized cells showed a somewhat higher relative activity than free cells at an alkaline pH medium.

3.5.1.4 The effect of temperature on degradation potential of the immobilized bacterial cells SAD M-1

The temperature dependence of the degradation activity was shown in Figure 3-39. The optimal temperature for the immobilized cells was 35°C and within the temperature range of 25-35°C, the immobilized cells had an almost stable degradation activity. At 40°C, the immobilization cells lost all activity of degradation.

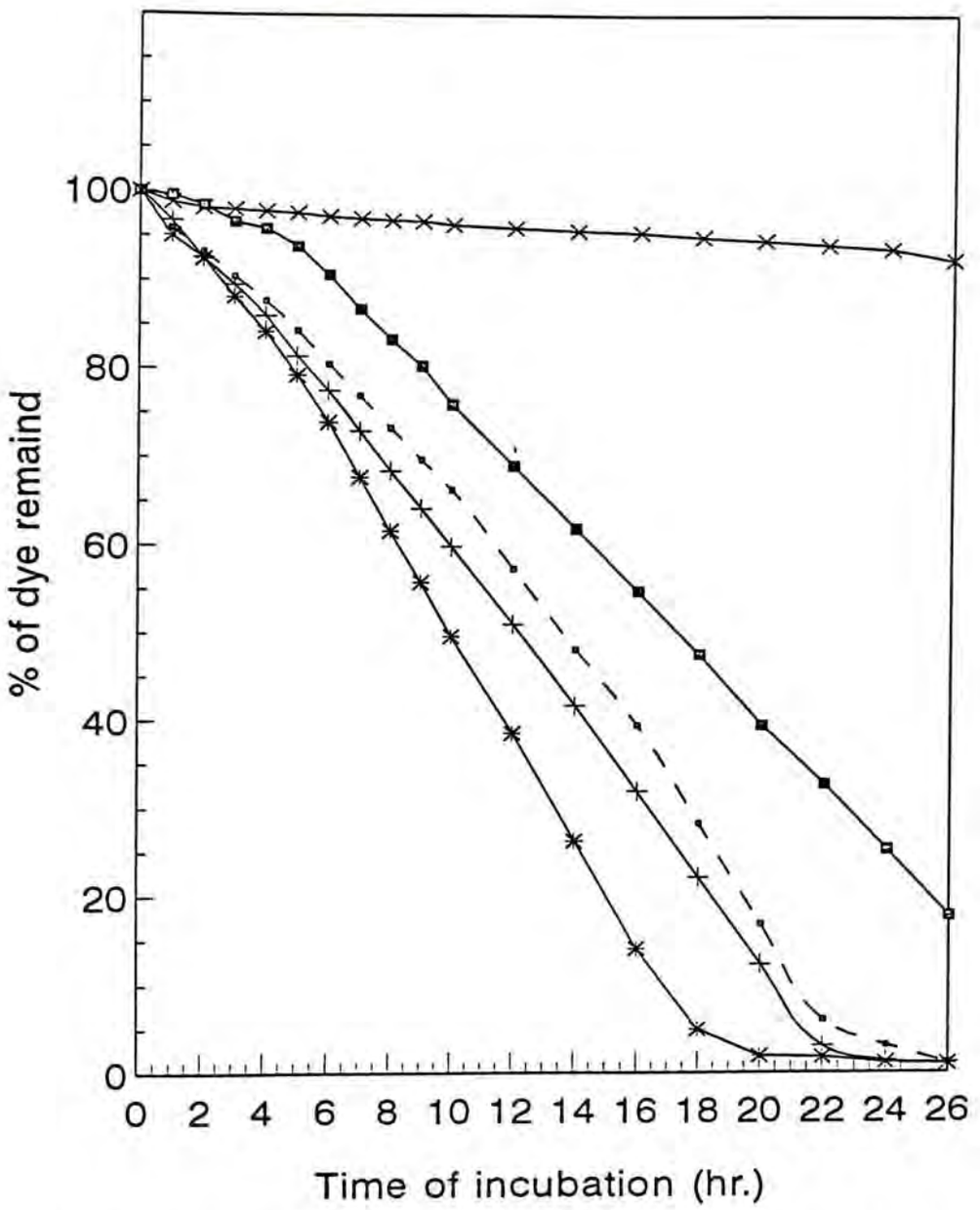


Figure 3-37. The effect of pH on the degradation of sulfanific acid by immobilized strain SAD M-1. 10 ml beads (0.5 mm diameter, 1.25 X 10^8 cells per ml gel) were added into 50 ml M.S.M. The initial pH of the medium was adjusted to 8.0, 8.5, 9.0, 9.5, and 10.0 with 0.05 M H_3PO_3 buffer. The cultures were incubated at 35°C for 26 hours. 1 ml samples of the media were taken out at fixed time intervals and were centrifuged and then diluted five folds with 0.5 M phosphate buffer. Sulfanific acid present in the medium was then measured spectrophotometrically at 248 nm.
Symbols: -•- pH8.0, -+- pH 8.5, -*- pH 9.0, -▫- pH 9.5, -×- pH 10.0.

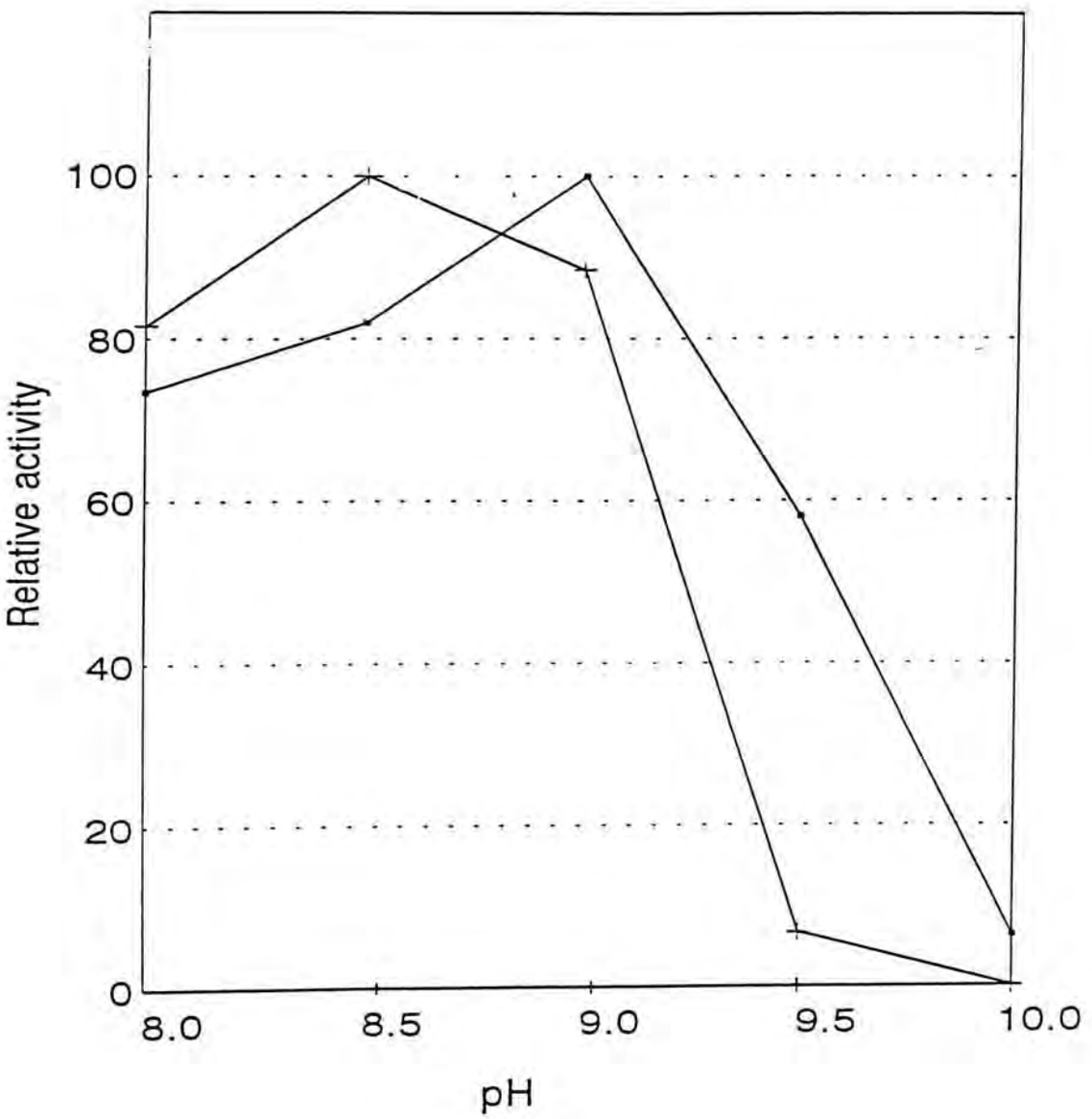


Figure 3-38. Comparison of the degradation activity of the free and the immobilized cells SAD M-1 at different pHs. The free and the immobilized cells were incubated at the same condition (Figure 3-23 and 3-38). The maximum decolorization rate was taken as 100%.
Symbols: —•— immobilized cell, —+— free cell.

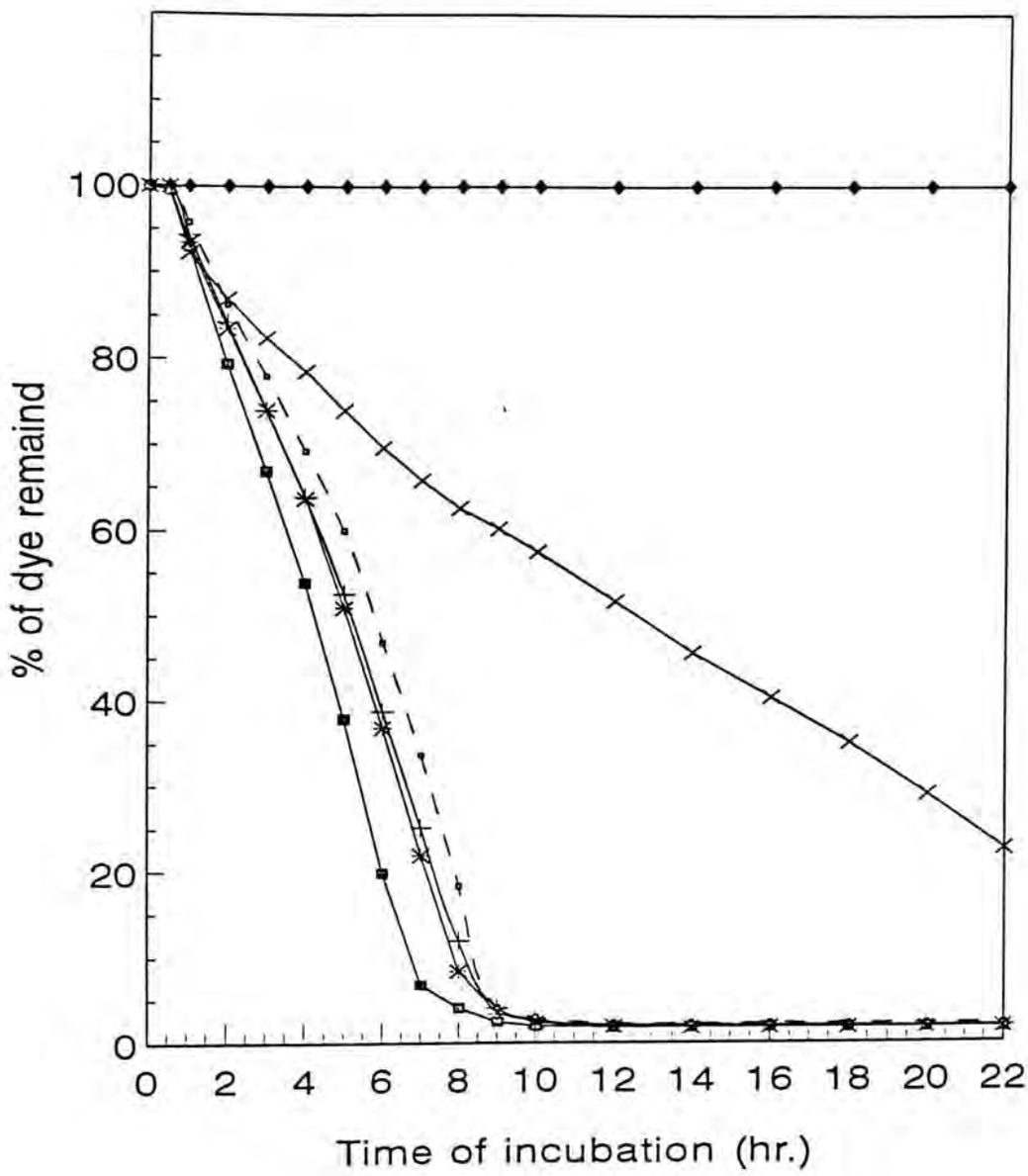


Figure 3-39. The effect of temperature on the degradation of sulfanific acid by immobilized strain SAD M-1 cells. 10 ml beads (0.5 mm diameter, 1.25 X 10^8 cells per ml gel) were added into 50 ml M.S.M.. The cultures were incubated aerobically at 25°C, 30°C, 33°C, 35°C, 37°C, and 40°C separately for 22 hours. 1 ml samples of these culture media were taken out at certain time interval and the cells in the medium was spun down by microcentrifugation. The clear supernatants were diluted 5-fold with 0.5 M phosphate buffer and were used then for spectrophotometric measurements at 248 nm. The degradation of sulfanific acid was expressed as percentage of compound remained in culture.

Symbols: —•— 25°C, —+— 30°C, —*— 33°C, —▫— 35°C,

—×— 37°C, —◆— 40°C.

As illustrated in Figure 3-40, a comparison of the temperature-activity of the free and the immobilized cells reveals that the temperature optimum shifted from 33°C to 35°C and the temperature dependence are roughly similar.

3.6 DEGRADATION OF TROPAEOLIN O BY IMMOBILIZED STRAINS IN A CONTINUOUS ANAEROBIC/AEROBIC COLUMN SYSTEM

In order to degrade Tropaeolin O completely by a continuous method, a two packed-bed column system (Figure 2-2) was constructed. In the first column, the azo dye was decolorized anaerobically by the immobilized strain ADD 16-2. The flow rate of the medium through the column was adjusted from 3 to 1 ml/min to assure the colour was decolorized completely in the effluent. For the first 10 days, the flow rate of the medium through the column was operated at 3 ml/min. Between day 11 and 18, the flow rate was lowered to 2.5 ml/min indicating a drop in degradation activity. Then at day 19 to 24, the flow rate was set at 2.0 ml/min. From dye 25 to 30, the flow rate was again reduced to 1.5 ml/min. Finally, in the last seven days, the rate was further reduced to 1ml/min. During these 37 days, approximately, a total of 5.75 g of Tropaeolin O was decolorized in the continuous column reactor. The results shown in Figure 3-41 demonstrate that strain ADD 16-2 can be successfully immobilized by entrapment and used in a continuous flow-through column system to decolorise azo dyes.

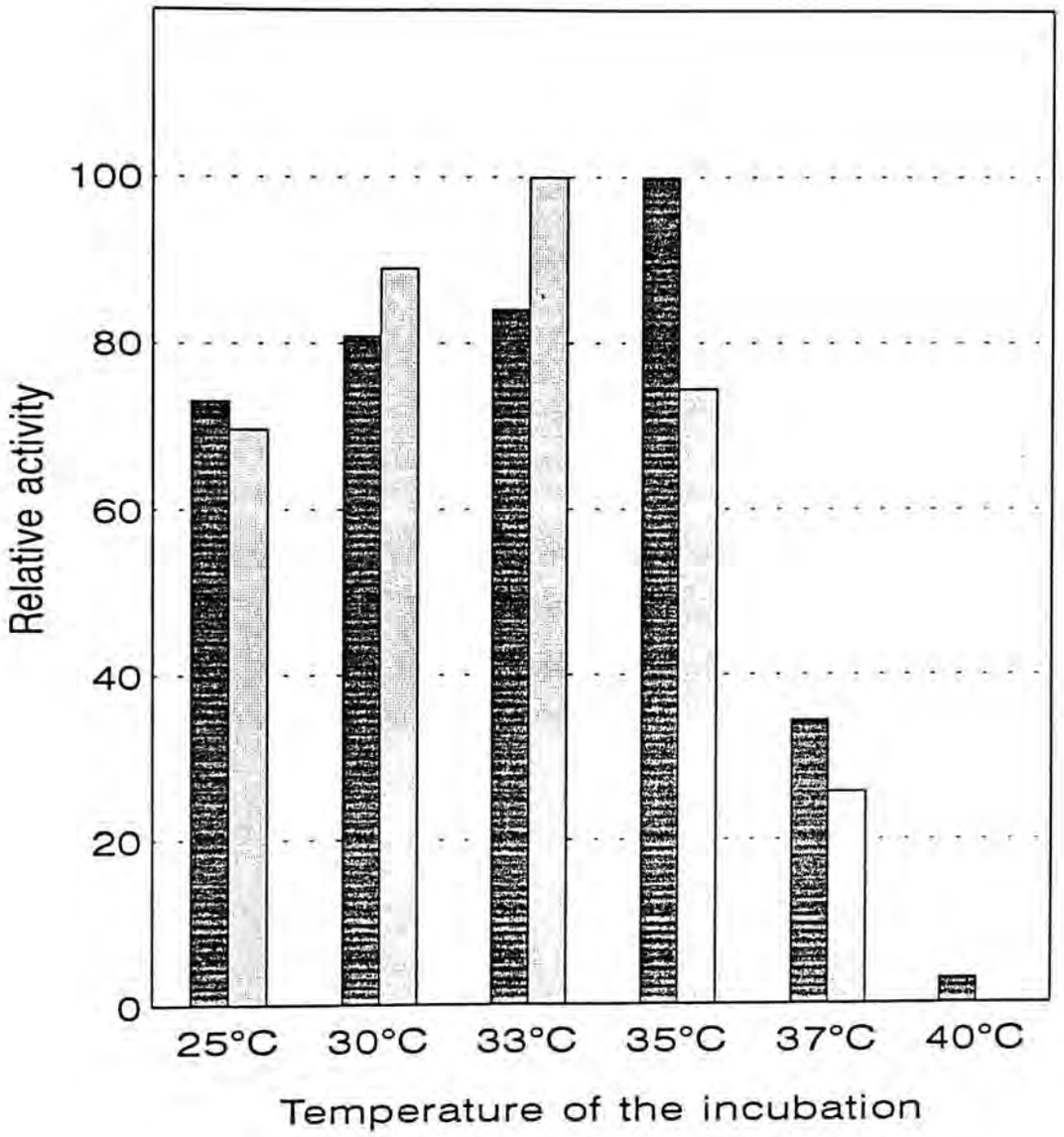


Figure 3-40. Comparison of the degradation activity for free and immobilized SAD M-1 cells at different temperatures. The free and the immobilized cells were incubated in the same condition (Figure 28 and 40). The maximum decolorization rate was taken as 100%.
Symbols: ▬ immobilized cell, ▭ free cell.

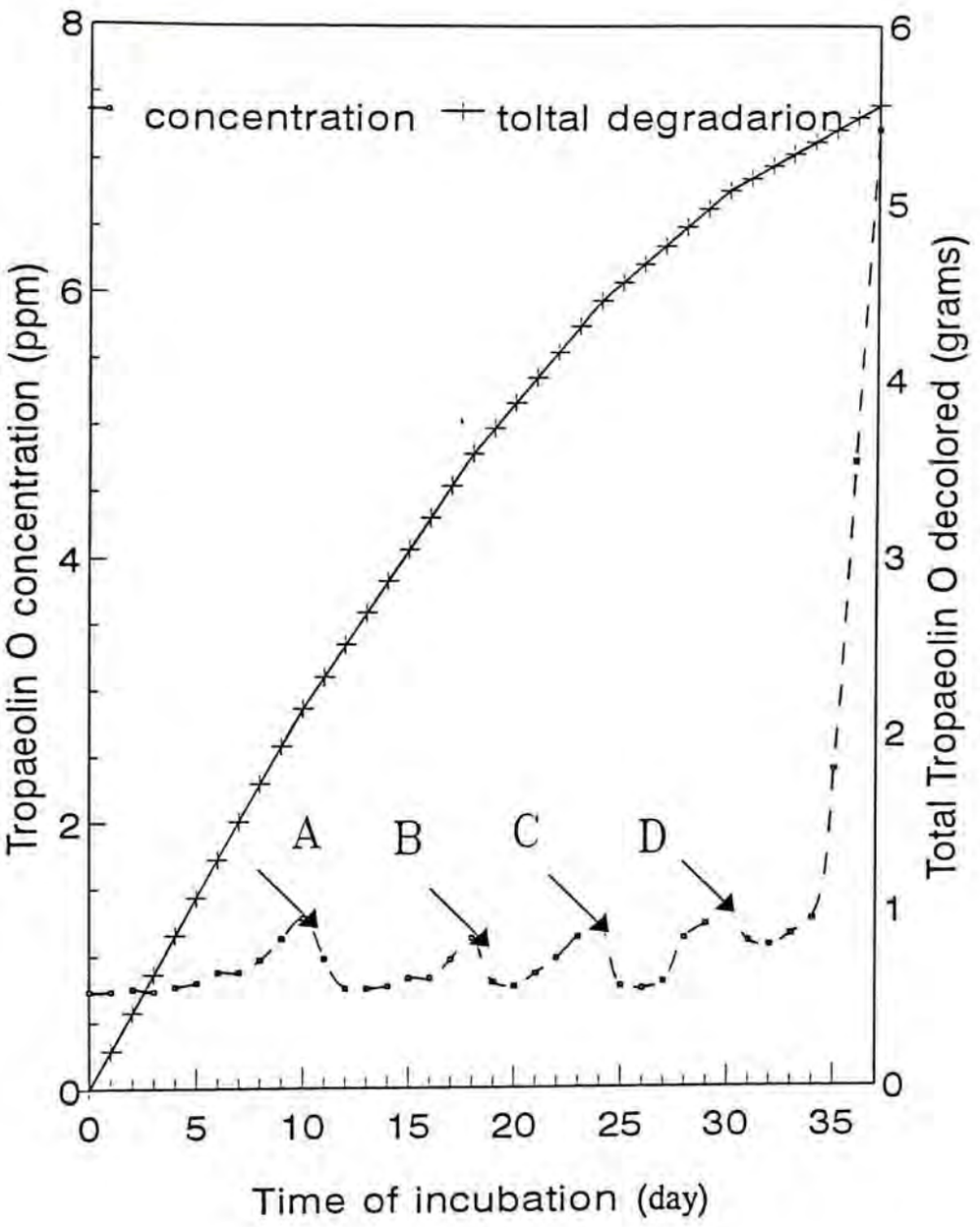


Figure 3-41. Total amount of Tropaeolin O decolorized by immobilized ADD 16-2 cells within the first anaerobic pack-bed column reactor. 50 g beads ($6.4x10^{10}$ cells per ml gel) was added to fill the column. The medium (M.S.M.) containing 100 ppm Troaeolin O was pumped in from the bottom of the column. The flow rate of the column was regulated to be slow enough to ensure the effluent to be completely decolourized. The pH of medium was adjusted to 7.5 by 0.05 M MOPS buffer. The temperature of reaction was 30°C. The Tropaeolin O concentration in the effluent was measured each day by the method mentioned above. The total amount of dye decoloured was calculated on the basis of mass balance. The column underwent a series of changes in flow rate as controlled by the ability of decolorization. Flow rate decreased from: A, 3.0 to 2.5 ml/min; B, 2.0 ml/min; C, 1.5 ml/min and D, 1 ml/min.

The medium flowed out from the first column was introduced into the second packed-bed column. In order to obtain an aerobic condition for aromatic amines to be degraded in the second degradation step, the medium was aerated in a reservoir prior to its entry into the following column. The result of such an operation showed that 80% of the aromatic amines still remained in the medium (Figure 3-42 A). This indicates that the degradation of the aromatic amines has not achieved perfectly by a simple aeration the medium in the reservoir before pumping into the packed-bed column.

A modified setup was thus designed, in which an airlift-loop reactor was involved (Figure 2-3). This reactor even enhanced the problem that beads were densely packed together in previous setup resulted from a retardation of the aeration process to be solved. The airlift-loop design gives direct aeration to the column (Smart *et al.*, 1984). Figure 3-42 B shows a scanning profile of the culture medium at the various steps of the reaction. It depicts that a total degradation of azo dye has been achieved by this continuous system.

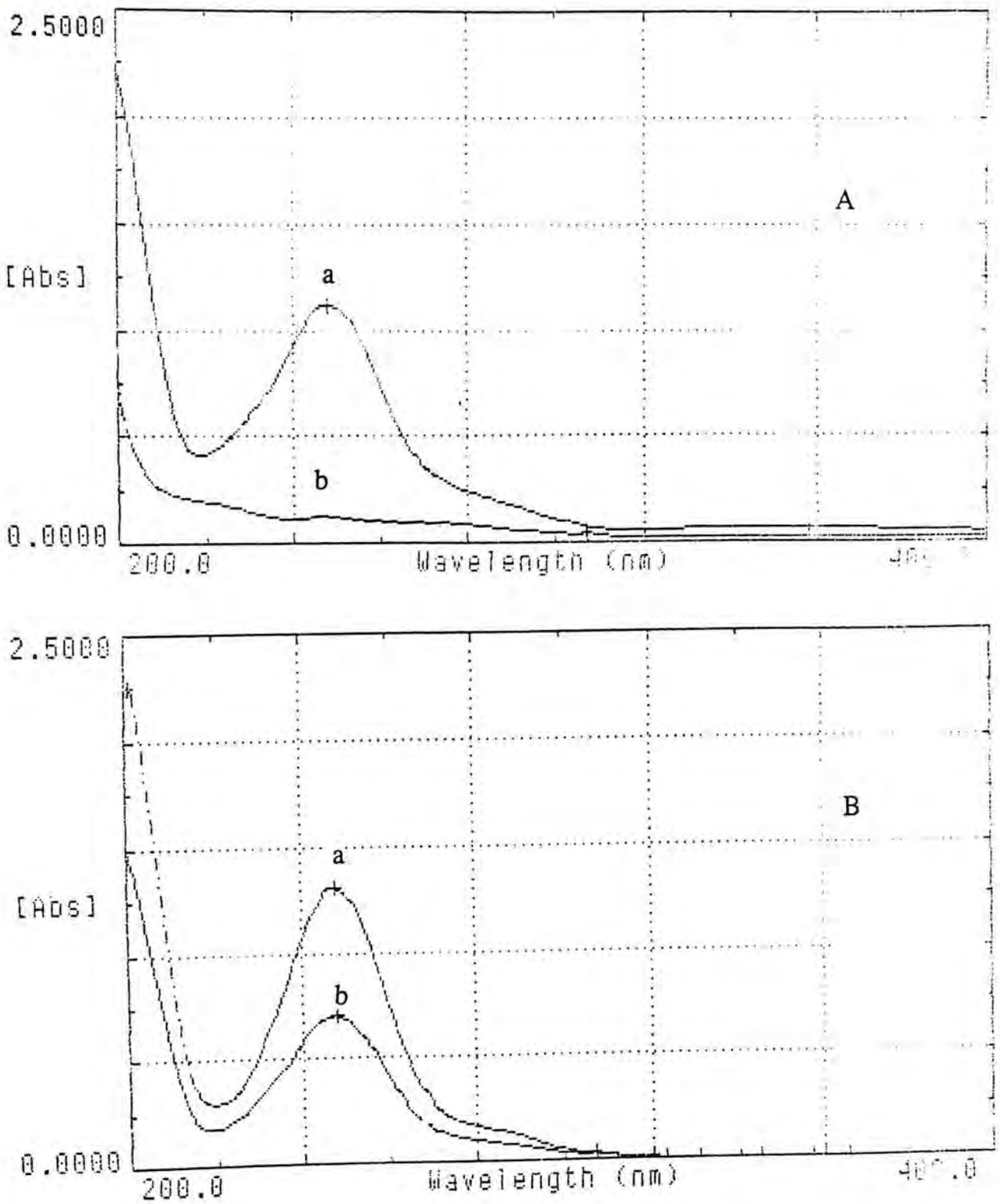


Figure 3-42. The degradation of intermediate formed in the first decolorization column by an aerobic packed-bed and airlift-loop reactor. The decolorized culture discharged from the anaerobic column (mentioned in Figure 42) was introduced either into an airlift-loop system (A) or an aerobic packed-bed reactor (B) separately. Both compartments contained an equal content: eash packed with 50 ml beads (0.5 mm in diameter, containing strain SAD M-1 at a concentration of 1.25 x 10^{10} cells/ml gel). The effluent samples from both compared after proper cell removal and dilution (0.5 M phosphate Buffer).

a, culture discharged from the first anaerobic column;

b, effluents from the second aerobic column.

CHAPTER FOUR

DISCUSSIONS

4.1 DECOLORIZATION OF AZO DYE

The earliest organism on earth emerged about three billion years ago. Three billion years of evolution have gone into building a complex biosphere system. It has the ability to recycle and regenerate itself. Microbial degradation plays a most significant role in this system. The wastes of humans and other animals, their bodies and the tissues of plants are dumped onto or buried in the soil. Somehow they all disappear. It is the microbes that mediate these changes---the conversion of complex organic matter into substances that provide the nutrient for the plant world. The process is essential for the maintenance of life on our planet.

But in recent years this system has met with challenges because human activities caused widespreading environmental interventions. The demented introduction to the environment unnatural substances or natural substances at unnatural levels that exceeds its capacity to recover. Some of these substances are pollutants. They are compounds whose permanent or temporal accumulation in the environment causes adverse effects on man. These effects may be due to a direct action on man or an indirect damage inflicted on terrestrial or aquatic ecosystems which eventually leaks to a lowering of the quality of human life.

As described in the introduction part, the dye is a kind of these compounds. During its manufactural as well as in the dyeing process, a lot of wastewater containing dyes would be discharged into the environment causing a serious pollution. The cumulative effect of dyes to human health and to the environment have led to an increased public concern (Brown *et al.*, 1988; El-Geundi, 1991; Ogawa *et al.*, 1990).

Wastewater is polluted water containing unnatural substances or natural substances at an unnatural high level. An additional concern with the textile wastewater is the presence of colour. High levels of colour are common in textile wastewater. The discharge of coloured water generates an environmental concern due to a loss of aesthetic value of the receiving water and a decrease of light transmittance which endanger normal aquatic life. Recently, many countries have passed laws that control the effluent colour intensity. For instance, American state and federal agencies called for the effluent colour to be less than 250 ADMI by 1991 and less than 200 ADMI by 1992 (McCurdy, 1992). The removal of colour is therefore an additional challenge to the waster treatment facilities in processing wastewater.

Azo dyes represent a significant portion of dyes in use today. Colour development in dye is mostly associated with the presence of a delocalized π-electron system. In azo dyes, the aromatic rings and the conjugated double bond constitute an extended chromophorous system for colour production. Hence to destroy the colour of dye molecule requires a chemical reaction that

intercepts the conjugation on the corresponding system. Unfortunately as a class, azo dyes are difficult for municipal biological treatment systems to degrade. Shaul *et al.* (1991) found that of 18 azo dyes tested on activated sludge systems, only three were degraded to a detectable extent.

To solve this problem, waste treatment facilities should probably become more specific and better targeted to the defined effluents. This goal can be reached by a separate treatment for such defined wastes or by inoculating classical sewage treatment facilities with specialized strains having the ability to degrade the specific compounds (Ghisalba, 1983).

The purpose of the present research is to isolate suitable bacterial strains that can degrade azo dyes successfully. In this study, azo dye Tropaeolin O was chosen as the model compound. Tropaeolin O is an acidic azo dye which is characterized by the presence of a sulphonic acid group. Since neither sulphonic group nor azo bond is natural existing, this compound is recalcitrant to oxidative biodegradation. Tropaeolin O was found also to be toxic to microbial populations in water by Michails *et al* (1985).

A practical approach to isolate the required strain at the percent time begins with a careful selection of indigenous microorganisms from nature. Usually, the first place to search for the microorganisms is in sites where the wastes have been in contact with soil for some time so that the microorganisms have had an opportunity to develop an ability for of

biodegrading the waste.

Strain ADD 16-2 was isolated from polluted site which possesses an excellent ability to decolorize azo dyes. The strain ADD 16-2 grew only under aerobic condition. However the decolorization of azo dyes by ADD 16-2 occurred under anaerobic condition. This shows that the presence of oxygen inhibited the decolorization process by ADD 16-2. The inhibiting effect of oxygen on the decolorization activity may due to one or all the following mechanisms: 1). Under anaerobic conditions, azo dyes can interact non-specifically with compounds of the electron transport chain and thereby serve as artificial electron acceptors. When oxygen exist, due to the oxidation of reduced electron carriers by the natural acceptor of oxygen, the electrons may not reach the azo dyes. As a result the azo bond cannot be reduced in the oxidation of reduced electron carriers (Chung *et al.*, 1993). 2). The azo reductase is sensitive to oxygen. The enzyme may be irreversibly inactivated in the presence of oxygen. This kind of enzyme had been obtained from different isolates (Rafii *et al.*, 1990).

Dyes absorbing light in the range of wavelength between 250 to 650 nm which is in most cases associated with the presence of a delocalized π-electron system. Aromatic rings and conjugated double bond in particular may form a so-called chromophore, a structural moiety that exhibits a characteristic uv/vis absorption spectrum. In such systems, the most probable electron transitions are promotions of π electrons from bonding to antibonding π

orbitals. Such transitions are commonly referred to as π-π^* transitions, and they give, in general, the most intense absorption bands in the spectrum. If a π-electron system contains atoms with nonbonding electrons (hetero atoms), such as nitrogen atoms in azo dyes, a so-called n-π^* transitions may also be observed. This transition occurs at longer wavelengths (lower energy) as compared with π-π^* transition, which exists on the visible wavelength range (Schwarzenbach et al., 1993). When the azo bond is broken, the absorption in the visible range band disappears. As described in results, the peaks in the visible range disappeared at the end of the experiment parallel to the colour disappearance. This was probably due to the cleavage of the azo group that made the absorption peak shifts to a visible wavelength range caused by the n-π^* transition. The peak at the uv range remained constant in the whole process. It is suggested that this peak stems from the residue ring compounds accumulated in the medium. The ring compound is sulfinafic acid Tropaeolin O serves as dye for the decolorization reaction. Hence the degradation of azo dye by ADD 16-2 appears to involve the breakdown of the azo linkage only. Apparently, this is a one step chemical reaction with one enzyme dictating the bond cleavage.

The product of the decolorization reaction did not promote bacterial growth. Consequently, without the addition of extra carbon sources, the rate of Tropaeolin O decolorization reaction was very slow. The addition of glucose enhances the reduction rate. Perhaps, the regeneration of some cofactors in the enzymatic reaction are necessary.

Strain ADD 16-2 decolorized twenty four out of a randomly purchased twenty five azo dyes. The result indicated that the strain ADD 16-2 possessed an extraordinary ability of decolorizing azo dyes. Structural specificity of the decolorization rate was inconclusive since the list of compounds under studied was neither complete nor exhaustive to give a full picture of the structural modification. Meanwhile, as the whole cell was used in this study, the rate of any particular biodegradation could be affected by several very different steps. First, the rate of dye uptake by the cells may be rate limiting. Since dyes are foreign to the microorganism, it is expected that the cells may not have transport systems associated with their exterior membranes to pick up the dye molecule actively and carry it in to the interior of the cell. The permeation of dyes into the cells was probably elicited by a passive uptake. If permeation is the bottleneck, the structure of the organic dye will affect this uptake rate most prominently (Schwarzenbach *et al*, 1993). After all, the transport of dye through membrane might be an important factor governing the rate of azo reduction. Once the dye molecule arrives at the cellular site where it can be processed by enzyme, the reduction rate would be effected by any electronic substituents in dye molecule. It may alter the energy required to break the critical bonds in the dye or may change the affinity of the enzyme-dye association(Goronszy *et al.*, 1992). The electron density on the azo group region determines the reduction rate of azo dyes. The evidence obtained from the result does not seem to be sufficient to prove this hypothesis. More work has to be done in the future.

4.2 MINERALIZATION OF THE DECOLORIZATION INTERMEDIATE

As mention in the HPLC and spectrophotometric results, the azo dye was reduced. But the strain ADD 16-2 can not degrade azo dye completely. The aromatic amines was accumulated in the medium. These products can be highly toxic and behaves as carcinogens to human beings (Ames *et al.*,1975; Chung *et al.*, 1981; Hahon, 1985; Milvy *et al.*, 1978; Nesnow *et al.*, 1988; Ogawa *et al.*, 1988). For counteracting the dye pollution problem, this remaining pollutant has to be further taken care of.

Ordinary aromatic compounds are readily degraded and utilized by bacteria. The breakdown of aromatic compounds is a vital biochemical step in the natural carbon cycle (Wallnöfer, 1984). Aromatic compounds such as those found in lignin are among the most common units found in nature.

On the other hand, artificially synthesized azo dyes contain benzennoid moieties, including phenyl and naphthyl in addition to its unique azo group.. Consequently, one would predict the existence of microorganisms having the ability to degrade these dyes that contain similar structure with natural existing substance. However since most dyes have to be chemically insert for their wide application (for example to provide water solubility), many dyes contain auxochrome. The auxochrome normally contains sulphonic acid, nitre and chlore. As a mater of fact, aromatic sulphonic acid are rare among natural products. This nature confers a xenobiotic character in most azo dyes. In

short, xenobiotic dyes are difficult to be mineralized and hence an additional catabolic potential is required (Beller *et al.*, 1992; Brilon *et al.*, 1981; Nörtemann *et al.*,1991; Nörtemann *et al.*, 1986).

The isolation of adapted microorganisms with the required degradation capacities is usually preceded by an acclimation period. There are a number of possibilities for enrichment of such vanguard organisms. Batch culture is probably the simplest way. This is achieved by inoculating a suspension of the contaminated soil into a growth medium with the pollutants as sole carbon source (NÖrtemann and Hempel, 1991; Leisinger *er al.*, 1984). It is well known that the simple aromatic compounds can be degraded under aerobic condition via hydroxylation and ring-opening (Hartmann *et al.*, 1979; Holliger *et al.*, 1992; Vorobjeva *et al.*, 1990; Radehaus *et al.*, 1992). The same technique of enrichment in batch culture under aerobic condition was employed in this study. It is based on the following strategy: in an inorganic aqueous growth medium containing a recalcitrant compound as the sole and growth-limiting source of an essential nutrient, only those organisms that possess the necessary degradative ability will grow. It creates a selective advantage for the organisms having the existing potential or ability to degrade the compound which may be present in an inoculum obtained from nature.

In response to the new stimulus, which in our case, is sulfanific acid, the target organisms needed a long period to hold on the ability for a degradation or mineralization to be detected. As a result, a lag period had been seen. The

enrichment of microorganism on the xenobiotic compound perhaps is the result of several mechanisms: 1) The microbial population increase its proportion of species capable of degrading the compound. Here the strategy is simple. Sufficient time is allowed so that organisms have ample opportunity to multiply, and become a significant fraction of the total microbial population. It can be called population shifts; 2) An interval may be necessary to allow mutations in genetic codes enabling the development of enzymes capable of performing new transformation; 3) Plasmid transfers may be required to allow existing microorganisms to develop or combine suitable enzymatic tools. Perhaps this capability from one species which is not thriving in the system has passed over to another species having more competitive advantages but hitherto unable to degrade the test compound before a successful degradation function can be observed; 4) Already available enzymes are "repressed" and a considerable long period must pass or some special conditions must change before they begin to act. According to the possible mechanisms described above, microbial selection on xenobiotic compounds often requires an extremely long acclimation period. The selection of degradation microbial strain on sulfanific acid lasted for three months by subculturing until the microbial community growing on the compound has built up. Strain SAD M-1 exhibited a maximum sulfanific acid degradation rate among three or more so collected strains. It is this strain that can satisfy our goal to serve as a tool in an azo dye complete degradation process.

4.3 TWO-STEP AZO DYE MINERALIZATION

Biodegradation of azo dye can occur in an aerobic or in an anaerobic condition. It is important to differentiate clearly between these two conditions because anaerobic decolorization is relatively easy to achieve (Athanasopoulos, 1992; Chung *et al.*, 1978; Chung *et al.*, 1992; Manning *et al.*, 1985; Rafii *et al.*, 1990). Anaerobic transformation by all of these microorganisms begins with reductive fission of the azo linkage, resulting in the formation and accumulation of colourless aromatic amines. Further anaerobic degradation of these aromatic intermediates has not been reported.

Since this aromatic amine products from the decolorization reaction can be toxic and carcinogenic (Nesnow *et al.*, 1988; Ogawa *et al.*, 1988). A lot of attempts have been tried by scientists over the globe to obtain suitable microorganisms that would degrade azo dye completely under aerobic condition. At the beginning, efforts to isolate such microorganisms have largely been unsuccessful. Zimmermann *et al* however, in 1982, did develop two aerobic bacterial *Pseudomonas* sp., strain KF 46 and KF 44 by chemostat cultures. The two organisms, being too specific towards one single azo dye, are not considered to be of any practical value for degrading the mixtures of azo dyes that occur in wastewater. More recently, biodegradation researchers have become increasingly interested in the versatile lignin-degrading white-rot fungus *Phanerochaete chrysosporium.* Cripps *et al.* (1990) found that the fungus aerobically degrades three azo dyes: Tropaelin O, Congo Red, and

Orange II. The authors showed that the ligninolytic system of this fungus was involved in the degradation of the three azo dyes. Paszczynski *et al* (1992), using ^{14}C-radiolabeled Acid Yellow 9, Orange II, Orange I and sulfanific acid as substrate, found that *Phanerochaete chrysosporium* can mineralize sulfonated azo dye, including naphthol derivatives. Unfortunately, the rate of degradation was very low. The process of dyes degradation lasted 21 days. Notwithstanding it is not efficient enough for cleaning up the pollutants in the textile effluents.

It should be emphasized here that not all elements of a complete catabolic pathway have to be housed and expressed by one single organism. In natural environments, biodegradation may be carried out by several members of the consortium present, this is known as co-metabolism. Co-metabolism, may be an important ecological mechanism in the degradation of many environmental pollutants (Matlosz, 1990).

It is well known that simple aromatic compounds can be degraded under aerobic condition via hydroxylation and ring-opening (Brilon *et al.*, 1981; Nishino *et al.*, 1993; Reineke *et al.*, 1984; Schwien *et al.*, 1982). Brown *et al.* (1983) and Idaka *et al.* (1987) found that some of the aromatic amines formed from the degraded azo dyes under anaerobic condition were in turn biodegradable by sludge under aerobic conditions. These results suggest that azo dyes may be partially degraded under anaerobic conditions, producing products that may then by further metabolized under aerobic conditions. This

implies that an aerobic process following the anaerobic process should be able to biodegrade the azo dye completely(Haug *et al.*, 1991)

Such a combination of anaerobic/aerobic co-metabolism processes appears to be a very promising concept. In this investigation, this metabolic trait was used for total degradation of the sulfonated azo dye Tropaerolin O by two different consortiums strain ADD 16-2 and SAD M-1. Firstly, the azo dye was reduced to correlative aromatic intermediates by strain ADD 16-2 under anaerobic condition. It was followed by mineralization of the aromatic intermediates using microorganisms SAD M-1 under aerobic condition. The process was shown to require only two days in a batch process with laboratory glassware and scale. Although the process is efficient to degrade the model compound, the alternation of two employed strain by filtering do not appear to be a suitable industrial operation. It requires a more effective cooperation between the two steps. This leads to having the cells immobilized.

4.4 FUNCTIONAL ASPECTS OF IMMOBILIZED CELLS

Immobilized cells have several potential advantages for the waste treatment over free cells. These include: the retention of catalytic activity, the operation of bioreactors at flow rates that are independent of the growth rate of the microorganisms employed, more efficient substrate mineralization through retention of intermediary products, and the protection of cells from the effects of inhibitory substrates. This last advantage is important to be

considered especially in this process of biodegradation of azo dyes as we know that a lot of azo dyes were toxic.

Immobilization methods were used in the present study to improve the behaviour of azo dyes degradative strains and simplify the process of two-step azo dye mineralization. The immobilizing agent must be a material that can immobilize microorganisms at normal temperature and under normal pressure. It has to be hydrophillic so that dyes can diffuse into the pellets. The most popular cell entrapment matrix used in recent years is calcium alginate (Akin, 1987; Fumi *et al.*, 1991; Champagme *et al.*, 1992; Tsay *et al.*, 1986; Nawaz *et al.*, 1992; Kutuey *et al.*, 1985). Calcium alginate met these requirements and was chosen in our study as immobilizing agent and found to be satisfactory. The two cell strains entrapped can still function equally well if not better.

The effects of dye concentration on the activity of decolorization were tested for immobilized cells and free cells. Since immobilized cells can act at 3500 ppm dye concentration while their free counterpart seize to act at 1500 ppm, either the alginate or the immobilization process help to protect the cell or its metabolic activities. This protective nature of the alginate may be due to the formation of a dense 'membrane' of very high alginate concentrations at the Ca^{2+}-alginate interface, which slowed down the diffusion of dye into the beads, particularly in high cell concentration. It has been reported that concentration gradients arising from diffusion-limited mass transport would

allow the exposure of dye to the centrally-located cells to be more gradual (Dwyer *et al.*, 1986). This means that the dye was degraded at relatively lower concentration in the central part of the gel so that substrate inhibition effect is less severe.

The immobilization of cells by gel entrapment holds many advantages. However, one disadvantage of immobilization is the increased resistance of substrates to diffusion through matrices. As a effect of diffusion limitation, the ability of catabolism is low in immobilized cells compared to their free counterparts (Westrin *et al.*,1991 ; Muhr *et al.*, 1982; Stewart *et al.*, 1988). But in our study, the decolorization activity was more effective in immobilized counterparts (Figure 3-13 and 3-33). One explanation for the phenomenon is the limitation on oxygen transfer into the interior region of the immobilization matrix causing an anaerobic microenvriomeent to exist in the interior of the matrix (O'reilly *et al.*, 1989). As decolorization occurs faster under anaerobic condition, the microenvironment exist in the gel was beneficial to dye decolorization.

In conclusion, immobilization results in high decolorization activity and increased stability of the decolorization reaction especially at higher dye concentrations. These are advantageous to be used in tackling environmental toxicological problems.

Another beneficial feature of the immobilized cells is their heat stability

at higher temperatures. This was a result of the protection against the change of environmental changes due to immobilization (Omata *et al.*, 1979). This is an obvious advantage when applied to decolorize textile wastewater because the effluent in most cases is hot.

Immobilization may increase the cell loading capacity and increase rates of degradation of dyes. This is, however, partly offset by the dense matrix created by the cells themselves and by the polymeric net work of the gel. Of cause, diffusion hindrance can arise in gel, thus decreasing the apparent reaction velocity. The effect is obvious at a high cell load. However such a limitation can be overcome by decreasing the diameter of the beads. A determination of the optimal amount of cell entrapped in alginate beads and the bead size on the decolorization activity shows that: in a growth condition, when the amount of cells reach $1.6X10^{10}$ per ml gel, the activity of decolorization increased consequently to about 20 μg dye per hr per ml gel in 4 mm diameter beads. The activity would not increase even more cells were added to the gel. But for smaller beads (0.5 mm diameter), the highest rate of decolorization of dye reached 36 μg dye per hr per ml gel and the cell concentration topped to $6.4X10^{10}$ cells per ml gel.

With rapidly catabolizing immobilized cells, active cells were found only to a depth of 50-200 μm into a bead (Stormo *et al.*, 1992; Ogbonna *et al.*, 1988; Burrill *et al.*, 1983), so that as much as 80% of the volume of a 2-mm bead may contain mainly inactive cells. Obviously, preparing beads that

contain more active cells by decreasing the diameter of the beads offers great advantages in terms of the speed of reaction.

Beside the size of the beads, the concentration of the gel also effects the activity of cells in the gel. When the concentration of alginate gel is higher than 2%, the rate of decolorization decreased obviously. This may be due to the fact that the porosity of the gel beads decreases with increasing alginate concentration that is important element for diffusion.

Most importantly the degradation activity of the immobilized strain ADD 16-2 was very stable. The half-life of the system was 15 days. Over a period of 30 days more than 95% of dye added to the reactor could be decolorized. This ensures that enzymatic "hardwares" in the form of immobilized cells can be made available for a continuous but complete dye "mineralization system to be constructed.

4.5 A CONTINUOUS AZO DYE MINERALIZATION PROCESS COMBINING TWO MICROBES WITH IMMOBILIZATION TECHNOLOGY

We have built in the previous sections two pieces of equipments required to generate a complete mineralization system for azo dye wastewater. These two equipments are: 1). The two functional strains, ADD 16-2 and SAD M-1 have been captured. It has been shown that by combining these two strains in

a batch process. Azo dye with Tropaeolin O as a typical example was indeed mineralized. 2). A convenient physical confinement for easy microbial handling has been mastered. This is the calcium alginate immobilized cells beads of both strains. Results showed that the process of immobilization does absolutely no damage to the function of both microbial strains. Now, the main challenge is to bring the two equipments together and ask them to operate cooperatively and in total harmony. This is not an easy job since the strain ADD 16-2 works anaerobically while its follower, the strain SAD M-1 works aerobically. The first attempt with an aerating reservoir is not a total failure. It works but sluggishly and it needs sufficient oxygen. To meet the requirement of high rates of oxygenation, a rather simple airlift reactor with an external circulation loop was designed. At the laboratory scale, it enables the system to operate by mineralization sulfinafic acid completely.

The modified airlift reactor does not need a mechanical device for dispersing the gas phase. A circulating flow of liquid is produced by aerating it at the riser section. The main advantages of the system are: 1). High rate of oxygenation. It solved the oxygen limitation problem that occurs frequently to entrapped cells in the alginate immobilization system (O'reilly *et al.*, 1989). For an airlift reactor with a large height-to-diameter ratio, the bubble residence time increases, it would have a higher oxygen transfer coefficient. It is suitable to be used in our experiment; 2). Low shear. Since airlift reactor does not require a mechanical stirrer, it shows a low shear characteristics (Träger *et al.*, 1989; Smart *et al.*, 1983). This lengthens the half life of

alginate beads. It was found by the end of our experiment (30 days), most of the alginate beads were intact while a total of 5 gram or more Tropaeoln O dye has been degraded completely.

Even though immobilized microorganisms has been employed by other investigation to degrade aromatic compounds, we believe that this is the first instance in which immobilized cells have been used to degrade azo dye and its intermediates in an continue column fashion.

REFERENCES

Akin, C. 1987. Biocatalysis with immobilized cells. *In*: Biotechnology & genetic engineeting reviews. Russell, G.E. (ed) pp: 319-367. Intercept. Pontelamd, Newcastle Upon Tyne.

Ames, B.N., J. Mccann and E. Yamasaki. 1975. Methods for detecting carcinogens and mutagens with the salmonella/mammalian-microsome mutagenicity test. *Mutation Res.* 31:347-364.

Andrianova, M.M. 1970. carcinogenic properties of the red food dyes, amaranth, SX Purple and 4R Purple. *Vopr. Pitan.* 29:61-65.

Athanasopoulos, N. 1992. Biodegeadation of textile wastrwaters. *In*: Biologecal degradation of wastes. Martin, A.M. (ed) pp: 388-411. Elsevier Applied Science, London and New Youk.

Beller, H.R., D. Grbic-Galic and M. Reinhard. 1992. Microbial degradation of toluene under sulfate-reducing conditions and the influence of iron on the process. *Appl. Environ. Microbiol.* 58:786-793.

Beszedits, S., A. Lugowski and H.K. Miyamoto. 1980. Color removal fron textile mill effluents. B & LInform. Services, Toronto, Canada.

Bhattachcharya, S.K. 1991. Treatment of textile wastes. *In*: Handbook of industrial waste traetment. Wang, L.K., and M.H.S. Wang. (ed) pp307-321. Marcel Dekker, Inc., New York. Basel. Hong Kong.

Bonser, J.W., L. Bradshaw, D.B. Clayson and J.W. Jull. 1956. A further study of the carcinogenic properties of ortho-hydroxyamines and related compounds by bladder implantation on the mouse. *Br. J. Cancer.* 10:539-546.

Boyle, R. 1973. Dyestuffs. *In*: Fuels, Explosives and Dyestuffs. Tooley, P. (ed) pp.192-240. John Murray Albemarle Street London.

Brilon, C., W. Beckmann and H.J. Kanckmuss. 1981. Enrichment and isolation of naphthalenesulfonic acid-utilizing *Pseudomonads*. *Appl. Environ. Microbiol.* 42:39-43.

Brilon, C., W. Beckmann and H.J. Kanckmuss. 1981. Catabolism of naphthalenesulfonic acids by *Pseudomonas* sp. A3 and *Pseudomonas* sp. C22. *Appl. Environ. Microbiol.* 42:44-55.

Brown, A.B. and S.D. Devito. 1993. Predicting azo dye toxicity. *Environ. Sci.Technol.* 23(3):249-324.

Brown, D. and P. Laboureur. 1983. The aerobic biodegradability of primary aromatic amines. *Chemosphere* 12:405-414.

Brown, D. and R. Anliker. 1988. Dyestuffs and the environment-a risk assessment. Richardson, M. L. (ed) pp. 398-413. The Royal Society of Chemistry.

Burrill, H.N., L.E. Bell, P.F. Greengield and D.D.DO. 1983. Analysis of distributed growth of *saccharomyces cerevisiae* cells immobilized in polyacrylamide gel. *Appl.Environ. Microbiol.* 46:716-721.

Cao, W.G., B. Mahadevan., K.L. Crawford and R. L. Crawford. 1993. Characterization of an extracellular azo dye-oxidizing peroxidase from *Flavobacterium* sp. ATCC 39723. *Enzyme Microb. Technol.* 15:810-817.

Case, R.A.M., M.E. Hosker, D.B. McDonald and J.T. Pearsin. 1954. Tumours of the urinary bladder in workmen engaged in the manufacture and use of certain dyestuff intermediates in the British chemical industry. I. The role of aniline, benzidine, alpha naphthylamine and beta naphthylamine. Br. J. Ind. Med. 11:75-79.

Champagne, C.P., C. Gaudy, D. Poncelet and R.J. Neufels. 1992. *Lactococcus lactis* release from calcium alginate beads. *Appl.Environ. Microbiol.* 58:1429-1434.

Chung, K.T., G.E. Fulk and A.W. Andrews. 1981. Mutagenicity testing of some commonly used dyes. *Appl. Environ. Microbiol.* 42:641-648.

Chung, K.T., G.E. Fulk and M. Egan. 1978. Reduction of azo dyes by intestinal anaerobes. *Appl. Environ. Microbiol.* 35:588-562.

Chung, K.T and S.E. Stevens, Jr. 1992. The reduction of azo dyes by the intestinal microflora. *Crit. Rev. Microbiol.* 18:175-190.

Chung, K.T and S.E. stevens, Jr. 1993. Degradetion of azo dyes by environmental microorganisms and helminths. *Environ. Toxicol. Chem.* 12:2121-2132.

Cripps, C., J.A. Bumpus and S.D. Aust. 1990. Biodegradation of azo and heterocyclic dyes by *Phanerochaete chrysosporium. Apll. Environ. Microbiol.* 56:1114-1118.

Douch, P.G.C. and S.S.B. Blair. 1975. The metabolism of foreign compounds in the cestode, *Moniezia expansa*, and the nematode, *Ascaris lumbricoides* var *suum. Xenobiotica* 5: 279-292.

Dwyer, D.F., M.L. Krumme., S.A. Boyd and J. M. Tiedje. 1986. Kinetics of phenol biodegradation by an immobilized Methanogenic consortium. *Appl. Environ. Microbiol.* 1986:345-351.

El-Geundi, M.S. 1991. Colour removal from textile effluents by adsorption techniques. *Wat. Res.* 25:271-273.

Fowler, J.D. and C.R. Robertson. 1991. Hydraulic Permeability of immobilized bacterial cell aggregates. *Appl. Environ. Microbiol.* 57:102-113

Freeman, H.S. and J.F. Esancy. 1991. An approach to the design of nonmutagenic azo dyes: Analogues of the mutagen 4-amino-3-methoxyazobenzene. *In*: Colour Chemistry. Peters, A.T., and H.S. Freeman. (ed.) pp.85-114. Elsevier Science Publishers Led. England.

Fumi, M.D., A. Silva, G. battistotti and O. Colagrande. 1991. Living immobilized acetobacter in ca-alginate in vinegar production: preliminary study on optimum conditions for immobilization. *Biotechnol. Lett.* 55:17-20.

Fung, D.Y.C. and R.D. Miller. 1973. Effect of dyes on bacterial growth. *Appl. Microbiol. 25:794-799.*

Ghisalba, O. 1983. Microbial degradation of chemical waste, an alternative to physical methods of waste disposal. *Experientia*. 39:1247-1257.

Godrej, A.N. and J.H. Sherrard. 1988. Kinetics nad stoichiometry of activated sludge treatment of a toxic organic wastewater. *Journal WPCF*. 60:221-225.

Goronszy, M.C. and H. Tomas. 1992. Characterization and biological treatability of a textile dyehouse wastewater. *In*: Proceesings of the 47th industrial waste conference. pp.743-764. Lewis Publishers. London, Tokyo.

Gray, L.E. Jr., U.S. Ostby., R.J. Kavlock and R. Marshall. 1992. Gonadal effects of fetal exposure to the azo dye Congo Red in mice: infertility in female but not male offspring. *Appl. Toxicol.* 19:411-422.

Gregory, P. 1991. Colorants for high technology. *In*: Colour Chemistry. Peters, A.T. and H.S. Freeman. (ed.) pp.193-224. Elsevier Science Publishers Led. England.

Griflly, M., M. Casellas., J.M. Bayona and A.M. Solanas. 1992. Isolation and characterization of a fluorene-degrading bacterium: Identification of ring oxidation and ring fission products. *Appl. Environ. Microbiol.* 58:2910-2917.

Groff, K.A. and B.R. Kim. 1988. Textile wastes. *Journal WPCF*. 60:884-886.

Groff, K.A. and B.R. Kim. 1989. Textile wastes. *Journal WPCF*. 61:872-874.

Hahon, N. 1985. Inhibition of viral interferon induction in mammalian cell cultures by azo dyes and derivatives activated with rat liver S9 fraction. *Environ. Res.* 37:228-238.

Hartman, C.P., G.E. Fulk and A.W. Andrews. 1978. Azo reduction of Trypan Blue to a known carcinogen by a cell-free extract of a human intestinal anaerobe. *Mutation Res.* 58:125-132.

Hartmann, J., W. Reineke and H.J. Knackmuss. 1979. Metabolism of 3-chloro-, and 3,5-dichlorobenzoate by a *Pseudomonad*. *Appl. Environ. Microbiol.* 37:421-428.

Haug, W., A. Schmidt., B. Nortemann., D.C. Hempel., A. Stolz and H.-J. Knackmuss. 1991. Mineralization of the sulfonated azo dye Mordant Yellow 3 by a 6-aminonaphthalene-2-sulfonate-degrading bacterial consortium. *Appl. Environ. Microbiol.* 57:3144-3149.

Heitkamp, M.A. and W.J. Adams. 1992. Glyphosate degradation by immobilized bacteria: laboratory studies showing feasibility for glyphosate removal fron waste water. *Can. J. Microbiol.* 38:921-928.

Holliger, C., G. Schraa. and A.J.M. Stams. 1992. Enrichment and properties of an anaerobic mixed culture reductively dechlorinating 1,2,3-trichlorobenzene to 1,3-dichlorobenzene. *Appl. Environ. Microbiol.* 58:1636-1644.

Holme, I. 1984. Ecological aspects of colour chemistry. *In*: Developments in the chemistry and technology of organic dyes. Griffiths, J. (ed.) pp.111-128. Blackwell Scientific Publications, Oxford.

Horitsu, H., M. takeda, E. Idaka, M. Tomoyeda and T. Ogawa. 1977. Degradation of *p-aminoazobenzine* by *Bacillus subtilis*. *Eur.J. Appl. Microbiol.* 4:217-224.

Houk, J., M.J. Doa, M. Dezube and J.M. Rovinski. 1991. Evaluation of dyes submitted under the toxic substances control act new chemicals programmn. *In*: Colour Chemistry. Peters, A.T., and H.S. Freeman. (eds) PP. 135-150. Elsevier Applied Science, London and New Youk.

Hwang, M.C. and K.M. Chen. 1993. The removal of color from effluents using Polyamide-Epichlorihydrin-Cellulose polymer.I. preparation and use in direct dye removal. *J. Appl. Polym. Sci.* 48:299-311.

Idaka, E., T. Ogawa, H. Horitsu and M. Tomoyeda. 1978. Degradation of azo compounds by *Aeromonas Hydrophila* var. 24B. *J. Soc. Dyers Colourists*. 94:91-94.

Idaka, E., H. Horitsu., and T. Ogawa. 1987. Some properties of azoreductase produced by *Pseudomonas cepacia. Bull. Environ. Contam. Toxicol.* 39:982-989.

Idaka, E., T. Ogawa., C. Yatome and H. Horitsu. 1985. Behavior of activated sludge with dyes. *Bull. Environ. Contam. Toxicol.* 35:729-734.

Idaka, E., T. Ogawa and H. Horitsu. 1987. Reductive metabolism of aminoazobenzenes by *Pseudomonas cepacia. Bull. Environ. Contam. Toxicol.* 39:100-107.

Idaka, E., T. Ogawa and H. Horitsu. 1987. Oxidative pathway after reduction of p-aminoazobenzene by *Pseudomonas cepacia. Bull. Environ. Contam. Toxicol.* 39:108-113.

Kanekar, P. and S. Sarnaik. 1991. An activated sludge process to reduce the pollution load of a dye-industry waste. *Environ. Pollut.* 70:27-33.

Kearney, L., M. Upton and A. Mc Loughlin. 1990. Enhancing the viability of *Lactobacillus plantarum* inoculum by immobilizing the cells in calcium alginate beads. *Appl. Environ. Microbiol.* 56:3112-3116.

Kennedy, M.T., J.M. Morgan, L.K. Benefiels and A.F. McFadden. 1992. Color removal from textile dye wastewater: a case study. *In*: Proceesings of the 47th industrial waste conference. pp.727-741.

Khan, A.k.A., G. guthrie., H.H. Johnston., S.C. Truelove and D.H. Williamson. 1983. Tissue and baterial splitting of sulfasalazine. *Clin. Sci.* 64:349-353.

Kimberly, A.G. and R.K. Byung. 1988. Textile wastes. *Journal WPCF.* 60:884-886.

Kimberly, A.G. and R.K. Byung. 1989. Textile wastes. *Journal WPCF.* 61:872-876.

Koizumi, J.I., M. Zhang., T. Imanaka and S. Aiba. 1990. Does single-amino-acid replacement work in favor of or against improvement of the thermostability of immobilized enzyme? *Appl. Environ. Micribiol.* 56:3612-3614.

Kulla, H.G. 1981. Aerobic bacteriol degradation of azo dye. *In*: Microbial degradatio of xenobiotics and recalcitrant compounds. Leisinger, T., A.M. cook, R. Hutter and J. Nuesch. (eds) pp.387-399. Academic, Londin, UK.

Kutney, J.P., L.S.L. Choi, G.M. Hewitt, P.J. Salisbury and M. Singh. 1985. Biotransformation of dehydroabietic acid with resting cell suspensions and calcium alginate-immobilized cells of *Mortierella isabellina. Appl. Environ. Microbiol.* 49:96-100.

Leaist, D.G. 1988. Diffusion with adsorption and partial immobilization of solute. *J. Phys. Chem.* 92:5833-5836.

Leisinger, T. and Brrunner, W. 1984. Poorly degradable subatances. *In*: Biotechnology Volume 6_a Biotransformations. Rehm, H.-J. and Reed, G. (ed.) pp 477-513. Verlag Vhemie, Weinheim.

Lenke, H., D.H. Pieper and C. Bruhn. 1992. Degradation of 2,4-dinitrophenol by two *Rhodococcus erythropolis* strains, HL 24-1 and HL 24-2. *Appl. Environ. Microbiol.* 58:2928-2932.

Liu, D. 1993. Technical methods section: Determination of the primary biodegradability of water-soluble and water-insoluble chemicals. *Environ. Toxicol. Water Qual.* 8: 231-237.

Lui, Z. and H. Yang. 1989. The decolorization and biodegrading metabolism of azo dyes by *Pseudomonas* S-42. *Acta Microbiol. Sinica.* 29:418-426.

Lobos, J.H., T.K. Leib and T.M. Su. 1992. Biodegradation of bisphenol A and other bisphenols by a gram-negative aerobic bacterium. *Appl. Environ. Microbiol.* 58:1823-1831.

Manning, B.W., C.E. cerniglia and T.W. Federle. 1985. Metabolism of the benzidine-based azo dye direct black 38 by human intestinal microbiota. *Appl. Environ. Microbiol.* 50:10-15.

Manukovski, N.S., M.I. Teremova., Yu.L. Gurevich and I.M. Pan'kova. 1990. Phenol and naphthalene degradation by mixed culture of microorganisms. *In*: Proceedings of the International Symposium on Biotechnology, Bratislava, Czecho-Slovakia. Blazej, A., and V. Privarova. (eds) pp.155-163. Elsevier Science Publishing Company Inc. U.S.A.

Matsuoka, M. 1991. Molecular orbital design, synthesis and characteristics of functional dyes for electro-optical applications. *In*: Colour Chemistry. Peters, A.T., and H.S. Freeman. (eds) pp.61-84. Elsevier Science Publishers Led. England.

Matlosz, M.M. and R.B. Trattner. 1990. Biological treatment of hazardous wastes. *In*: Encyclopedia environmental control technology Volume 4 Hazardous waste containment and treatment. Cheremisinoff, P.N. (ed) pp.411-445. Gulf Pubkishing Compamy. Houston, London, Paris, Zurich, Tokyo.

McCurdy, M.W., G.D. Boardman, D.L. Michelsen and B.M. Woodby. (1992). Chemical reduction and oxidation combined with biodegradation for the treatment of a textile dye wastewater. *In*: Proceeding of the 46th indudtrial waste conference. pp.735-738. Lewis Publishers. Houston, London, Paris, Tokoy.

Meyer, U. 1981. Biodegradation of synthetic organic colorants. *In*: Microbial degradation of xenobiotics and recalcitrant compounds. Leisinger, T., A.M. Cook., R. Hutter and J. Nuesch. (ed.) pp.371-385. Academic Press, London.

Michails, G.B. and D.L. Lewis. 1985. Sorption and toxicity of azo and triphenylmethane dyes to aquatic microbial populations. *Envieon. Toxicol. Chem.* 4:45-50

Miguel, A.C. and J.L. Lebeault. 1993. Selection of xenobiotic-degradation microorganisms in a biphasic aqueous-organic system. *Appl. Environ. Microbiol.* 59:1717-1724

Milvy, P. and K. Kay. 1978. Mutagenicity of 19 major graphic arts and printing dyes. *J. Toxicol. Environ. Health.* 4:31-36.

Muhr, A.H. and J.M.V. Blanshard. 1982. Diffusion in gels. *Polymer.* 23:1012-1026.

Nawaz, M.S., W. Franklin and C.E. Cerniglia. 1992. Degradation of acrylamide by immobilized cells of a *Pseudomonas* sp. and *Xanthomonas maltophilia*. *Can. J. Microbiol.* 39:207-212.

Nesnow, S. and H. Bergman. 1988. A case study of mammalian hepatic azoreduction. *J. Toxicol. Environ. Health.* 24:499-513.

Nikolov, L. and D. Karamanev. 1990. The inverse fluidization - A new approach to biofilm reactor design, to aerobic wastewater treatment. *In:* Proceedings of the International Symposium on Biotechnology, Bratislava, Czecho-Slovakia. Blazej, A. and V. Privarova. (eds) pp.177-182. Elsevier Science Publishing Company Inc. U.S.A.

Nishino, S.F. and J.C. Spain. 1993. Degradation of nitrobenzene by a *Pseudomonas pseudoalcaligenes*. *Appl. Environ. Microbiol.* 59:2520-2525.

Nörtemann, B. and D.C. Hempel. 1991. Application of adapted bacterial cultures for the degradation of xenobiotic compounds in industrial waste-waters. *In*: Biological degeradation of wastes. Martin, A.M. (ed.) pp.261-321. Elsevier Science Publishers Ltd. England.

Nörtemann, B., J. Baumgarten., H.G. Rast and H.J. Knackmuss. 1986. Bacterial communities degrading amino- and hydroxynaphthalene-2-sulfonates. *Appl. Environ. Microbiol.* 52:1195-1102.

Ogawa, T. and C. Yatome. 1990. Biodegradation of azo dyes in multistage rotating biologecal contactor immobilized by assimilating bacteris. *Bull. Environ. Contam. Toxicol. 44:561-566.*

Ogawa, T., E. Idaka and C. Yatome. 1981. Influence of azo dye on synchronized cells of *Bacillus subtilis*. *Bull. Environm. Contam. Toxicol.* 26:38-41.

Ogawa, T., M. Shibata., C. Yatome and E. Idaka. 1988. Growth inhibition of *Bacillus subtilis* by basic dyes. *Bull. Environ. Contam. Toxicol.* 40:545-552.

Ogbonna, J.C., Y. Amano and K. nakamura. 1988. Elucidation of optimum conditions for immobilization of viablecells by using calcium alginate. *J. Ferment. Bioeng.* 67: 92-96.

Omata, T., A. Tanaka, T. Yamane and S. Fukui. 1979. Immobilization of microbial cells and enzymes with hydrophobic photo-crosslinkable resin prepolymers. *Eur. J. Appl. Microbiol. biotechnol.* 6: 207-215.

O'reilly K.T. and R.L. Crawford. 1989. Degradation of pentachlorophenol by polyurethane-immobilized *Flavobacterium* cells. *Appl. Environ. Microbiol.* 55:2113-2118.

O'reilly, K.T. and R.L. Crawford. 1989. Kinetics of p-cresol degradation by an immobilized *Pseudomonas* sp. *Appl. Environ. Microbiol.* 55:866-870.

Pasti-Grigsby, M.B., A. Paszczynski, S. Goszczynski, D.L. Crawford and R.L. Crawford. 1992. Influence of aromatic substitution patterns in azo dye degradability by *Streptomyces* spp. and *Phanerochaete chrysosporium. Appl. Environ. Micrebiol.* 11:3605-3613.

Paszczynski, A., M.B. Pasti., S. Goszczynski., D.L. Crawford and R.L. Crawford. 1991. New approach to improve degradation of recalcitrant azo dyes by *streptomyces* spp. and *Phanerochaete chrysosporium. Enzyme. Microb. Technol.* 13:378-384

Paszczynski, A., M.B. Pasti., S, Goszczynski., D.L. Crawford and R.L. Crawford. 1992. Mineralization of sulfonated azo dyes and aulfanilic acid by *Phanerochaete chrysosporium* and *Streptomyces chromofuscus. Appl. Environ. Micrebiol.* 58:3598-3605.

Peasvento, M., C. Riolo and M. Achilli. 1988. Extraction-atomic absorption spectrophotometric determination of palladium as its anionic complex with a sulfonated azo dye. *Anal. Chem.* 60:332-335.

Peters, A.T. and H.S. Freeman. 1991. Colour chemistry. Elsevier Applied Science , London and New Youk.

Radehaus, P.M. and S.K. Schmidt. 1992. Characterization of a novel *Pseudomonas* sp. that mineralizes high concentrations of pentachlorophenol. *Appl. Environ. Microbiol.* 58:2879-2885.

Rafii, F., W. Franklin.k and C.E. Cerniglia. 1990. Azoreductase acticity of anaerobic bacxteria isolated from human interstinal microflora. *Appl. Enciron. Microbiol.* 56:2146-2151.

Reineke, W. and H.J. Knackmuss. 1984. Microbial metabolism of haloaromatics: isolation and properties of a chlorobenzene-degrading bacterium. *Appl. Environ. Microbiol.* 47:395-402.

Roxon, J.J., A.J. Ryan and S.E. Wright. 1966. Reduction of tartrazine by a *Proteus* species isolated from rats. *Food Cosmet. Toxicol.* 4:419-426.

Sakaki, K., T. Nozawa and S. Furusaki. 1988. Effect of intraparticle diffusion in ethanol fermentation by immobilized *Zymomonas mobilis*. *Biotechnol. Bioeng.* 31:603-606.

Samiullah, Y. 1990. The aquatic environment. *In*: Prediction of the environmental fate of chemicals. p166-183. Elsevier Science Publishers Ltd. England.

Saucedo, J.E.N., J. Barbotin., M. Velut and D. Thomas. 1989. Ultrastructural examination of *Gibberella fujikuroi* mycelia: effect of immobilization in calcium alginate beads. *Can. J. Microbiol.* 35:1118-1131.

Scheline, R.R., R.T. Nygaard and B. Longberg. 1970. Enzymatic reduction of the azo dye, Acid Yellow, by extracts of *Streptococcus faecalis* isolated from rat intestine. *Food Cosmet. Toxicol.* 8:55-58.

Schmidt, E., M. Hellwig and H.J. Knackmuss. 1983. Degradation of chlorophenols by a defined mixed microbial community. *Appl. Environ. Microbiol.* 46:1038-1044.

Schwarzenbach, R.P., P.M. Gschwend and D.M. Imboden. 1993. Biological transformation reactions. *In*: Environmental organic chemistry. Schwarzenbach, R.P., P.M. Gschwend., and D.M. Imboden. (ed) pp.485-546. John Wiley & Sons, Inc., New York, Chichester, Brisbane, Toronto, Singapore.

Schwien, U., and E. Schmidt. 1982. Improved degradation of monochlorophenols by a constructed strain. *Appl. Environ. Microbiol.* 44:33-39.

Shaul, G.M., T.J. Holdsworth., C.R. Dempsey and K.A. Dostal. 1991. Fate of water soluble azo dyes in the activated sludge process. *Chemosphere*. 22:107-119.

Smart, N.J. and M.W. Fowler. 1984. An airlift column bioreactor suitable for large-scale cultivation of plant cell suspensions. *J. Exp. Botany*. 35: 531-537

Spadaro, J.T., M.H. Gold and V. Renganathan. 1992. Degradation of azo dyes by the Lignin-degrading fungus *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* 58:2397-2401.

Steenson, L.R. and T.R. Klaenhammer. 1987. Conjugal transfer of plasmid DNA between streptococci immobilized in calcium alginate gel beads. *Appl. Environ. Microbiol.* 53:898-900.

Steiner, R.I. and J.D. Miskie. (1992) Dye application, manufacture of dye intermediates and dyes. *In*: Handbook of industrial chemistry. Kent, J.K. (ed) pp.863-915. Van Nostrand Reinhold, New York.

Stewart, P.S. and C.R. Robertson. (1988). Product inhibition of immobilized *Escherichia coli* arising from mass transfer limitation. *Appl. Environ. Microbiol.* 54: 2464-2471

Stormo, K.E. and R.L. Crawford. 1992. Preparation of encapsulated microbial cells for environmental applications. *Appl. Environ. Microbiol.* 58:727-730.

Träger, M., G.N. Qazi, U. onken and C.L. Chopra. 1989. Comparison of airlift and stirred reactors for fermentation with *Aspergillus niger*. *J. Ferment. Bioeng*. 68: 112-116.

Tsay, S.S. and K.T. To. 1986. Citric acid production using immobilized conekia of *aspergillus niger* TMB 2022. *Biotechnol. Bioeng*. 29:297-304.

Vorobjeva, L.I., L. V. Modyanova., P.B. Terentijev., F.M. Chasaeva and E. V. Davgilevich. 1990. Microbial treatment of industrial wastes. *In:* Proceedings of the International Symposium on Biotechnology, Bratislava, Czecho-Slovakia. Blazej, A., and V. Privarova. (eds) pp.221-228. Elsevier Science Publishing Company Inc. U.S.A.

Wagner, K. and D.C. Hempel. 1988. Biodegradation by immobilized bacteria in and airlift-loop reactor-Influence of biofilm diffusion limitation. *Biotechnol. Bioeng.* 31:559-566.

Wallnöfer, P.R. and G. Entelhardt. 1984. Aromatic and heterocyckic structures. *In*: Biotechnology Volume 6_a Biotransformations. Rehm, H.-J. and Reed, G. (ed.) pp 477-513. Verlag Vhemie, Weinheim.

Watabe, T., N. Ozawa and F. Kobayashi. 1980. Reduction of sulphonated water-soluble azo dyes by microorganisms from human facess. *Food Cosmet. Toxicil.* 18:349-353.

Westrin, B.A. and A. Axelsson. 1991. Diffusion in gels containing immobilized cells: A critical review. *Biotechnol. Bioeng.* 38:439-446.

Yang, X.K. 1990. Treatment of dyeing wastewater in China. *Text. Asia.* 21:178-183.

Zimmermann, T., H.G. Kulla and T. Leisinger. 1982. Propertiesof purified Orange II azoreductase, the enzyme initiating azo dye degradation by *Pseudomonas* KF46. *Eur. J. Biochem.* 129:197-203.