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MICROBIAL DECOLORIZATION OF REACTIVE DYES IN AN ANAEROBIC-AEROBIC TREATMENT SYSTEM

Mr. Nuttapun Supaka

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By	Mister Nuttapun Supaka
Field of Study	Biotechnology
Thesis Advisor	Professor Somsak Damronglerd, Dr. Ing.
Thesis Co-advisor	Professor Pierre Strehaiano, Dr. de l'INPT
Thesis Co-advisor	Associate Professor Kanchana Juntongjin, Ph.D.

Accept by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctor's Degree

……Dean of Faculty of Science
(Professor Piamsak Menasveta, Ph.D.)

THESIS COMMITTEE

……Chairman
(Mr. Supat Chareonpornwattana, Ph.D.)

……Thesis Advisor
(Professor Somsak Damronglerd, Dr. Ing)

……Thesis Co-adsisor
(Professor Pierre Strehaiano, Dr. de l'INPT)

……Thesis Co-advisor
(Associate Professor Kanchana Juntongjin, Ph.D.)

……Member
(Mrs. Marie-Line Delia, Dr. de l'INPT)

……Member
(Associate Professor Pairoh Pinphanichakarn, Ph.D.)

……Member
(Assistant Professor Suthep Thaniyavarn, Ph.D.)

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ในการวิจัยนี้ได้ทำการคัดแยกแบคทีเรียจากระบบกำจัดน้ำเสียของโรงงานฟอกย้อมเพื่อนำมาใช้ในการกำจัดสีย้อมรีแอกทีฟ พบว่าเชื้อผสมระหว่างแบคทีเรียสายพันธุ์ S1 กับ A5 มีความสามารถสูงที่สุดในการกำจัดสีย้อมชนิดโมโนเอโซ โมโนเอโซที่เป็นสารประกอบเชิงซ้อนกับโลหะหนัก ไดเอโซ และ แอนทรา- ควิโนน ผลจากการจำแนกชนิดทางอนุกรมวิธานด้วยการวิเคราะห์ลำดับเบสของจีน 16 เอสไรโบโซมัลอาร์เอ็นเอ พบว่าทั้งสองสายพันธุ์เป็นแบคทีเรียในสกุล *Paenibacillus* ในงานวิจัยนี้ได้ศึกษาสภาวะที่เหมาะสมในการกำจัดสีโดยแบคทีเรียทั้งสองสายพันธุ์ และพบว่าไนเตรทและ Cu^{2+} มีผลในการยับยั้งการกำจัดสีของแบคทีเรีย สารแอนทราควิโนน-2,6-ไดซัลโฟเนตสามารถเพิ่มอัตราการกำจัดสีย้อมชนิดเอโซของแบคทีเรียสายพันธุ์ A5 ได้โดยสารชนิดนี้จะถูกรีดิวซ์โดยเอนไซม์ของสายพันธุ์ A5 ไปเป็นแอนไฮโดรควิโนนที่สามารถรีดิวซ์สีเอโซภายนอกเซลล์ได้โดยปฏิกิริยาเคมี จากการศึกษาพบว่า เอ็นเอดีเอช : ควิโนนออกซิโดรีดักเตส ในระบบการหายใจภายในเยื่อหุ้มเซลล์แบคทีเรียมีบทบาทสำคัญในการรีดิวซ์ควิโนนชนิดนี้ ในกรณีของการกำจัดสีย้อมชนิดแอนทราควิโนนพบว่าการกำจัดสีชนิดนี้เกิดจากการดูดซึมสีไว้กับเซลล์แบคทีเรีย การพิสูจน์ความสามารถในการกำจัดสีของแบคทีเรียสายพันธุ์ A5 และ S1 ที่ถูกตรึงในเม็ดแอลจิเนตและบรรจุในเครื่องปฏิกรณ์ฟลูอิไดซ์เบดที่ดำเนินการกำจัดสีแบบกะ พบว่าสีย้อมเอโซถูกกำจัดในขั้นแอนแอโรบิก ในขณะที่ค่า ซีโอดีถูกกำจัดในขั้นแอโรบิก

ภาควิชา……..ลายมือชื่อนิสิต……...

สาขาวิชา……ลายมือชื่ออาจารย์ที่ปรึกษา……

ปีการศึกษา……ลายมือชื่ออาจารย์ที่ปรึกษาร่วม……

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม……

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NUTTAPUN SUPAKA: MICROBIAL DECOLORIZATION OF REACTIVE DYES IN AN ANAEROBIC-AEROBIC TREATMENT SYSTEM. THESIS ADVISOR: PROFESSOR SOMSAK DAMRONGLERD, Dr.Ing., THESIS CO-ADVISOR: PROFESSOR PIERRE STREHAIANO, Dr.de l'INPT, ASSOCIATE PROFESSOR KANCHANA JUNTONGJIN, Ph.D. 231 pp. ISBN 974-17-3969-9

In this work, bacterial strains isolated from a textile wastewater treatment facility were used to decolorize reactive dyes. Mixed culture of strain S1 and A5 performed the highest decolorization for monoazo, metal complex monoazo, diazo and anthraquinone dyes. Base on 16S rRNA gene sequence analysis, the strains were shown to belong to the genus *Paenibacillus*. Optimal decolorizing conditions were determined. Nitrate and Cu^{2+} were found to cause inhibition of azo dyes removal. Anthraquinone-2,6-disulfonate increased the reduction rate of azo dyes by strain A5. This quinone is enzymatically reduced, the resulting hydroquinone then reduces the azo dye outside the cells by chemical redox reaction. It was suggested that the membrane-bound NADH:quinone oxidoreductase of respiratory chain is responsible for this activity. In the case of anthraquinone dye, the decolorization was found to result from biosorption of the dye onto the surface of biomass. The feasibility of using a fluidized bed reactor operated as sequencing batch reactor to decolorize azo dyes by alginate-immobilized cells of strain S1 and A5 was proved: color was reduced by the anaerobic phase, meanwhile, chemical oxygen demand was mainly removed in the subsequent aerobic phase.

Department……..Student's Signature……
Field of study……...Advisor's Signature……
Academic Year…… Co-Advisor's Signature……
Co-Advisor's Signature……

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Abbreviations

A = frequency factor
b = constant of the affinity of the binding sites (l/mg)
C_t = concentration of dyes at any particular time (mg/l)
C_0 = initial concentration of dye (mg/l)
C_{eq} = equilibrium concentration (mg/l)
COD = chemical oxygen demand (mg/l)
DO = dissolved oxygen (mg/l)
[Dye] = concentration of reactive azo dye (mg/l).
E_a = activation energy (cal/mol)
E_h = redox potential
K_d = first order kinetic of decolorization
k_f = Freundlich constants (related to adsorption capacity)
K_m = Michaelis constant (mg/l)
m = biomass dose used (g)
n = constant related to intensity of adsorption
q_{eq} = amount of adsorbed dye per unit weight of biomass (mg/g)
Q_o = Langmuir constants (mg/g)
r_{dye} = specific decolorization rate
$r_{dye,max}$ = maximum specific decolorization rate (mg/h/g cell)
R = ideal gas law constant (1.987 cal/mol/ K)
S = smoothing constant
T = absolute temperature (K)
t_i = sampling times
μ_I = specific growth rate
x = amount adsorbed dye (mg)
x_i = experimental dye concentration at time t_i

Chapter 1

Introduction

Large amounts of dyes are annually produced and applied in many different industries, including the textile, cosmetic, paper, leather, pharmaceutical and food industries (Zollinger, 1991; Meyer et al., 1981). The textile industry is the major part of the total dyestuff market, consuming a large proportion of reactive azo dyes due to the actual high demand for cotton fabrics with brilliant color (Phillips, 1996). The reactive dyes bind to the cotton fibers by addition or substitution mechanisms under alkaline conditions and high temperature. Under these conditions a significant fraction of the dye is hydrolyzed and released into the environment with the rejected dyebaths or wash waters (Ganesh et al., 1994). The effluents from textile industry contains not only many various unfixed dyes with high intensity of color but also other residual chemical reagents used for processing, and impurities from the raw materials and other hazardous materials in the finishing process, including starch, polyvinyl alcohol, surfactants and other toxic compounds (Uygur, 1997). In addition, to high organic content, the textile wastewater exhibits low BOD/COD ratios due to the difficult biodegradable nature of dyes (Pagga and Brown, 1986). The final effluent from textile wastewater treatment plants always exhibits a certain degree of color intensity. Color present in dye effluent gives a straightforward indication of water being polluted, and discharge of this highly colored effluent can damage directly the receiving water (Zollinger, 1987).

The decolorization of textile wastewater is still a major environmental concern because the synthetic dyes used are difficult to remove by the conventional wastewater treatment systems based on adsorption and aerobic biodegradation (O'Neill et al., 1999). In spite of the low toxic effect on receiving water bodies, the dye constitute an aesthetic problem with great impact in the public opinion and color restricts the downstream use of the wastewater (O'Neill et al., 1999). In some cases, traditional biological procedures were combined with physical or chemical treatment processes to achieve better decolorization (Vandevivere et al., 1998), but chemical or physico-chemical methods are generally high cost, less efficient and of limited applicability, and produce wastes, which difficult to dispose of. As a viable alternative, biological processes have received increasing interest owing to their cost effectiveness, ability to produce less sludge, and environmental friendly (Banat et al., 1996). Therefore, to develop a practical bioprocess for treating dye-containing wastewater is of great significance.

Furthermore, a great number of the reported examples of reactive dyes, especially azo dyes, decolorization by microorganisms starts by reductive cleavage of the azo bond, under anaerobic conditions, with the formation of corresponding aromatic amines (Wuhrmann et al., 1980: Carliell et al., 1995; Nigam et al., 1996). This step is responsible for color removal but it does not remove the dye-related hazard from the wastewater since anaerobic mineralization of aromatic amines has not been reported except, for a few hydroxy- and carboxy-substituted amines, and some of these aromatic amines can be toxic and carcinogenic (Pasti-Grisby et al., 1992).

Some aromatic amines can be aerobically degraded (Nortemann et al., 1986). Recent studies have used the combination of anaerobic and aerobic steps in attempt to achieve not only decolorization but also mineralization of reactive azo dyes (Seshadri and Bishop, 1994; Field et al., 1995; FitzGerald and Bishop, 1994). However, dyes of different structures not only azo structures are often used in the textile processing industry, and, therefore, the effluents from the

industry are markedly variable in composition day to day. To gain a widespread reception, the azo-decolorizing bacteria should exhibit decolorizing ability for a wide range of textile dyes. Moreover, textile dye wastewater is well known to contain strong color, high pH, temperature, high COD and low biodegradability, especially the effluent from the dyeing stages and finishing process.

Lacking of necessary information for microbial decolorization as mentioned above, thus, the main objective of this study is to provide informations of the bacterial decolorization of monoazo, diazo, metal-complex, anthraquinone reactive dyes containing vinylsulfone as reactive group, which have a wide spectrum of substantivity by anerobic/aerobic treatment system. The working steps included: isolation and identification of active dye-decolorizing microorganisms, determination on the decolorization kinetic and mechanisms of various chemical structure reactive dyes and investigation the effects of environmental parameters on bacterial decolorization efficiency. The decolorization of mixed dyes was also determined. Finally, sequential anaerobic/aerobic bioreactor was used in this study for colorant biodegradation in a synthetic wastewater containing sulfonated reactive dyes to provide the information used for building-up the high performance pilot and full-scale sequential anaerobic/aerobic treatment plants, which becoming increasingly popular in wastewater treatment of textile industry as a way of complete removal of dyestuffs.

Chapter 2

2. Bibliography

2.1 Dyes

2.1.1 Background

Dyes are natural or synthetic substances that add color to fibers, yarns, or fabrics. Dye may chemically combined with fiber molecules, attach themselves to the fiber's surface, or be absorbed into fiber without chemical action.

Dyes can be classified by the hue produced, by chemical classes, by the types of fibers to which they can be successfully applied, and by the method of application. Most dyes used today are synthetic, categorized according to their chemical compositions. The most widely system for classification of dyes is that developed by the Society of Dyers and Colorists (SDC) and the American Association of Textile Chemists and Colorists (AATCC) for their publication provides a *generic name*, which denotes the recognized usage class, and a *Color Index constitution number* (CI number), which denotes the chemical structure, or constitution.

The ten generic names used in the Color Index are acid, azoic, basic, direct, disperse, pigments, reactive, solvent, sulfur and vat.

2.1.2 Chromophore

The ability of an organic compound to create a desired color derives from the presence of chemical groups called chromophores. Substances that contain chromophores in varied arrangements produce sensation of different color hues. Every chromophore is composed of atoms joined in a sequence composed of alternating single or double bonds carbon-to-carbon. To obtain the deep colors, the number of chromophores is increased and weak chromophore systems are replaced by strong ones (Hudson et al., 1993)

The major chromophores are the azo group, the thio group, the nitroso group, the nitro group, the azoxy group, the anthraquinoid group, the quinoid nuclease, the pyrazine ring, the quinoiphthalone group, the lactone ring, the carbonyl group, and the tetrabenzoporphyrazine nucleus. These chromophores and others are shown in Figure. 2.1

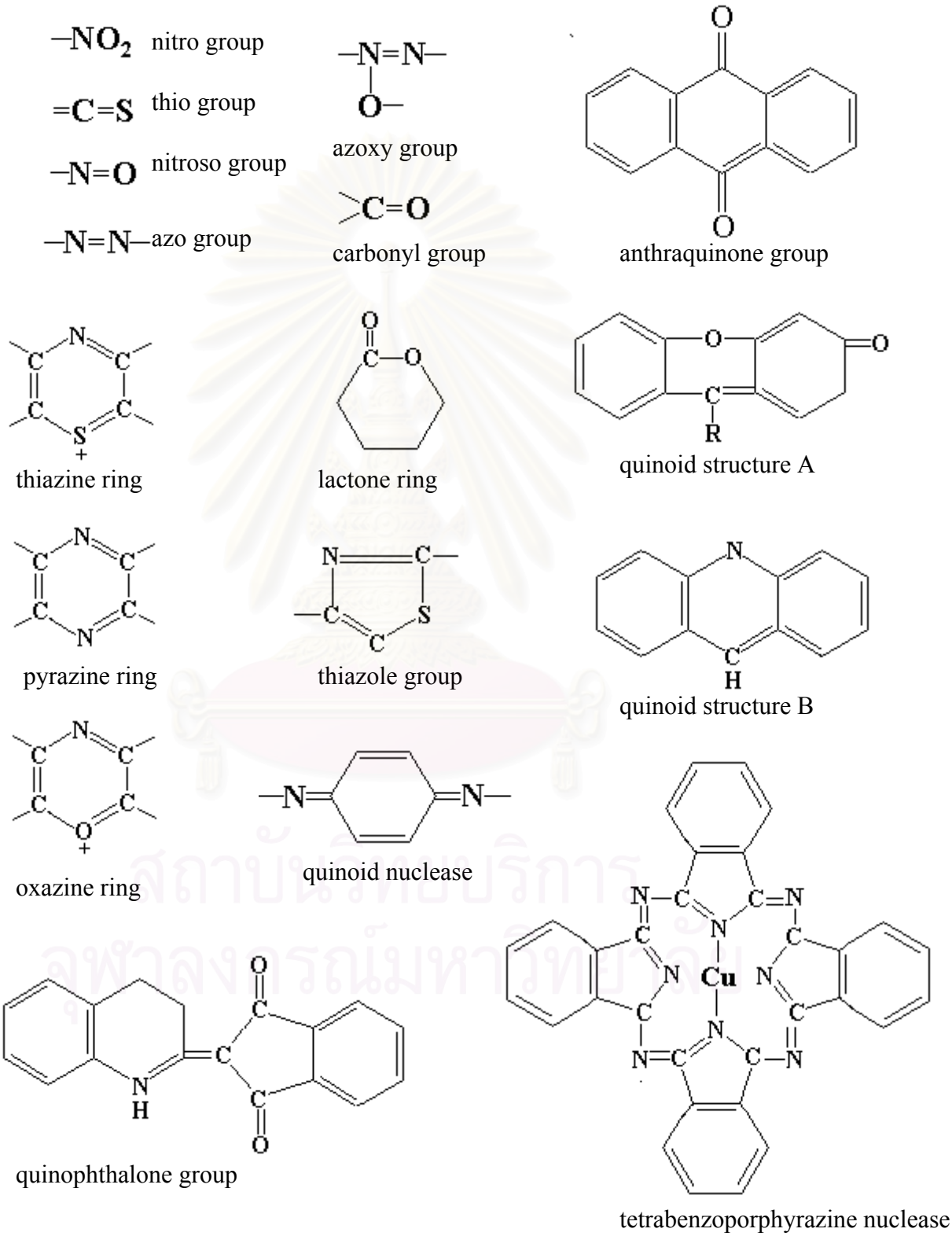


Figure. 2.1 Major chromophores presented in textile dyes

Among a number of different chromophores, azo (phenylazobenzene) and anthraquinone are the major units. Azo dyes, characterized by nitrogen to nitrogen double bonds (-N=N-), account for up to 70 % of all textile dyestuffs produced, and bond the most common chromophore of the reactive dyes. Another important chromophore group is the anthraquinone series, which is the basis of many reactive, disperse and vat dyes (Carliell et al, 1995).

2.1.3 Auxochrome

Although chromophores confer color on a substance, the intensity, of brightness, of the color depends on the presence of one or more substance called auxochromes. The auxochromes may also give water solubility to a dye to provide the groups that form associative bonds with the fiber. They include as follow;

- - SO_3H,
- - $N(CH_2)_2$,
- - $NHCH_3$,
- - NH_2,
- - NaO_3S,
- - NC_2H_5,
- - OH and
- - OCH_3.

A dye is technically defined as a compound that can be fixed on a substance in a more or less permanent state and that stimulates the visual sensation of a specific hue. Dyes have a particular affinity for a specific hue. Dyes that have a particular affinity to specific fiber are said to be substantive to that fiber. Dyes themselves, or combinations of other chemicals with dye in the dye bath, contribute both chromophores and auxochromes to the dyeing process (Hudson et al., 1993).

2.2 Reactive dyes

Cotton is currently responsible for approximately 50% of the world's fiber consumption and is expected to retain its dominant position into the next century (Phillips et al., 1999). Cotton is dyed with five major classes of dyes; the non-ionic vat, sulfur and azoic classes and the anionic direct and reactive classes. Reactive dyes, which form a covalent bond between the dye and the cellulosic substrate as a result of an alkaline fixation step during dyeing, are the fastest growing class of cellulosic dyestuffs. (Phillips et al., 1999).

The first reactive dyes for cellulose, Procion M dyes, were introduced in 1956 by ICI (Taylor et al., 2001). These dyes contain a highly reactive dichlorotriazinyl group (Figure. 2.2), which reacts with cellulose in the presence of alkali at room temperature. In subsequent years, ICI and Ciba marketed dyes containing the lower reactivity (hot dyeing) monochlorotriazinyl (MCT) reactive group, whilst Hoechst (DyStar, in the present) marketed Remazol (warm-dyeing) dyes bearing the vinyl sulfonyl (VS) reactive group. Both of these reactive groups are still used widely today, approximately 40 years after their first exploitation in reactive dyes for cotton. Among a large number of different reactive groups, three major reactive groups have been commercially exploited as follow:

2.2.1 Halotriazine reactive groups

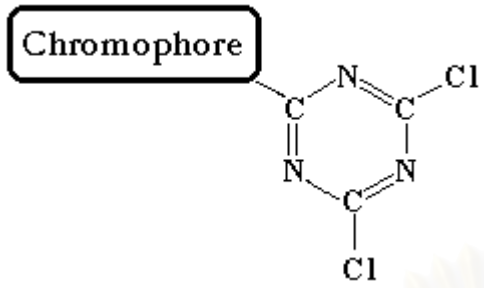
Reactive dyes for direct printing are traditionally monochlorotriazinyl (MCT) dyes where the second leg is usually a substituted arylamino residue. Such products appear under the Procion H and Cibacron P trade names. Cibacron from Ciba-Geigy and Procion H from ICI , shown in Figure. 2.2, are two examples of monochlorotriazinyl dyes. The only difference between Cibacron and Procion H series are the position of sulfonate group on the aniline ring, which is in *ortho*-position on Cibacron, but in *meta*- or *para*-position in Procion H series (Denizli and Piskin, 2001).

The corresponding monofluorotriazinyl (MFT) dyes are marketed by Ciba-Geigy as Cibacron F dyes and DyStar as Levafix E-N dyes. They are used for warm exhaust dyeing (60 ^{0}C) and, to a lesser degree in (semi-)continuous dyeing. If two chromophores are twinned together using a linking diamine as second leg (e.g. *p*-phenylenediamine, ethylenediamine), then the resulting homo bi-functional dyestuff. is particularly suited to exhaust dyeing: e.g.

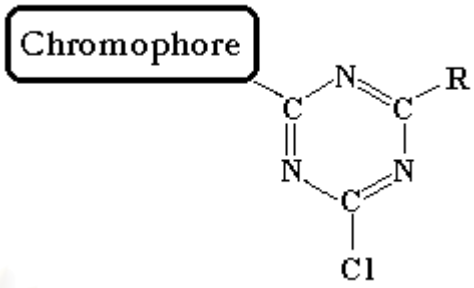
Procion H-E/H-EXL (BASF); bis MCT (substituted) arylene as second leg

Drimaren XN (Clariant); bis MCT (substituted) alkylene as second leg

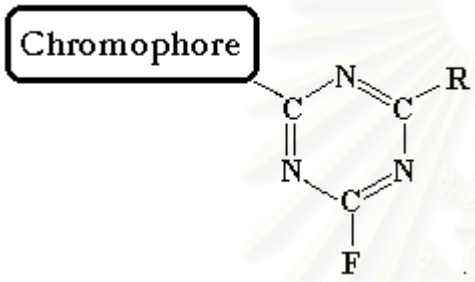
Cibacron LS (Ciba); bis MFT (substituted) alkylene as second leg



a.) Procion MX series (ICI)



b.) Cibacron (Ciba-Geigy) and Procion H (ICI)

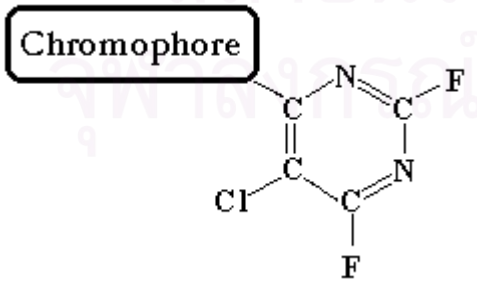


c.) Cibacron F series (Ciba-Geigy) and Levafix E-N (DyStar)

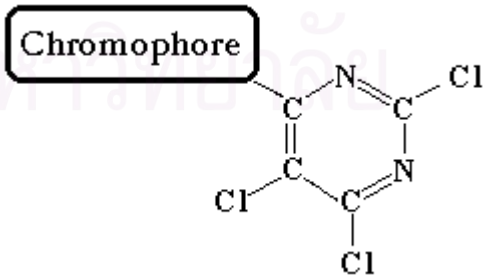
Figure. 2.2 Structures of some reactive dye molecules with halotriazine as reactive group

2.2.2. Difluorochloropyrimidine reactive group

Nowadays this reactive system, marketed by both DyStar (as Levafix E-A dyes, which contain difluorochloro-pyrimidnyl as a reactive group) and Sandoz (Drimarene dyes, which contain trichloro-pyrimidnyl as reactive group) is probably the most important of the remaining pyrimidinyl reactive systems.



a.) Levafix E-A , Bayer

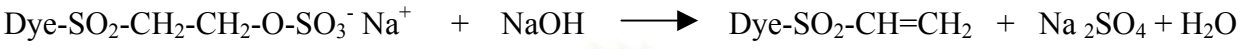


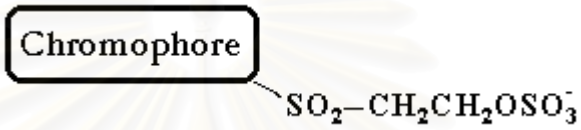
b.) Drimarene, Sandoz

Figure. 2.3 Structures of some of the reactive dye molecules with difluorochloropyrimidine as reactive group

2.2.3. Sulfoethylsulfone (SES)/Vinylsulfone (VS) reactive group

This reactive system has been exploited by DyStar in their Remazol range and is well suited to both (warm) exhaust dyeing and (semi-)continuous dyeing. Vinylsulfone reactive dyes may be sold in the vinyl sulfone form or as sulfoto-precursors. In dye bath under alkaline conditions, the sulfoto group is readily released to form the reactive vinylsulfone group which then undergoes nucleophillic addition with ionized hydroxyl groups of cellulose.





Remazol series (Hoechst, DyStar)

Figure. 2.4 Structure of some reactive dye molecules with vinylsulfone as reactive group.

In the dying process, there are significant amounts of unused dyes left in wastewater. This especially true for reactive dyes, which contained reactive group as vinylsulfone, because the hydrolyzed form has no affinity for textile substrates. Under alkaline conditions, reactive dyes form a reactive vinylsulfone ($-SO_2-CH=CH_2$) group which bonds fabrics (Figure. 2.5). Unfortunately, the vinylsulfone group further undergoes hydrolysis and the hydrolyzed byproduct will not form a bond with hydroxy-group of cotton fabrics. Thus, an average of 30 % of applied reactive dyes present in the hydrolyzed form may enter wastewater (Gahr et al., 1994). After the dyeing process, a concentration as high as 800 mg/l hydrolyzed dyes may remain in the dye bath (Steenken and Kermer, 1992). Typically, 90 % of the reactive dyes will pass though the wastewater treatment plants. These dyes may be stable and remain in the environment for a longer time (Pierce, 1994).

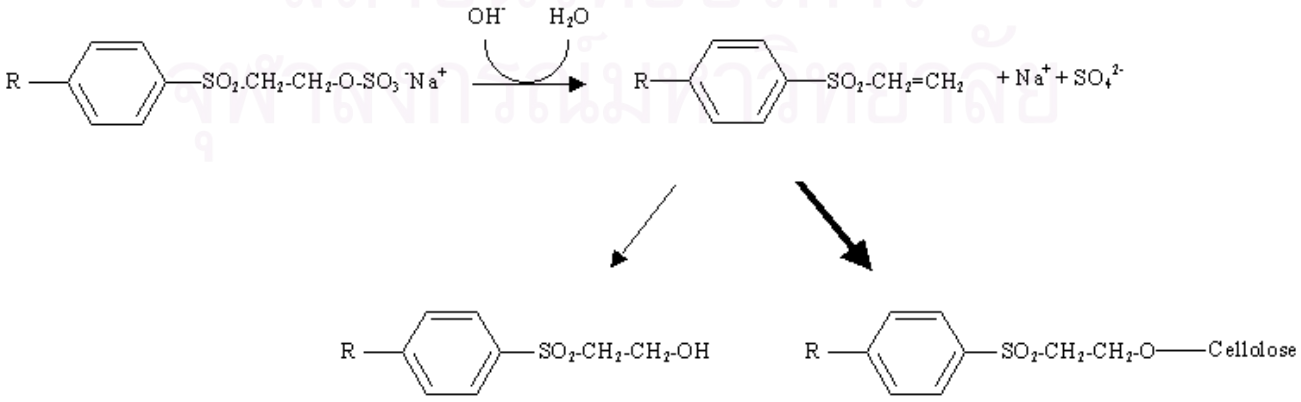


Figure. 2.5 Reaction of vinylsulfone reactive dyes under dyeing process. (Webber and Stickney, 1992)

2.3 Toxicity of textile dyes

Interest in the pollution potential of textile dyes has been primarily prompted by concern over their possible toxicity and carcinogenicity. This is mainly due to the fact that many dyes are made from known carcinogens, such as benzidine and other aromatic compounds, all of which might be reformed as a result of microbial metabolism (Clarke and Anliker, 1980) It has been shown that azo- and nitro-compounds are reduced in sediments (Weber and Wolfe, 1987) and intestinal environment (Chung et al., 1978), resulting of generation of the parent toxic amines. Anthraquinone-based dyes are very resistant to biodegradation due to their fused aromatic structures, which remain colored for long period of time. Some disperse dyes have also been shown to have a tendency to bioaccumulate (Anliker et al., 1981) and heavy metal ions from textile effluents have also been reported at high concentrations in both algae and higher plants exposed to such effluents (Srivastava and Prakash, 1991).

The toxicity of some dyes to the single cell green algae, *Selenastrum capricornutum*, and fathead minnow, *Pimephales promelas*, is shown in Table 2.1. In general, cationic dyes were toxic (e.g., LC_{50} for Basic Violet 1 is only 0.05 mg/l to *P. promelas*), anionic acid dyes the next, and direct dyes are the least toxic (LC_{50} > 180 mg/l). For *Ceriodaphnia dubia*, lower LC_{50} values were reported for Solvent Yellow 1(4-phenylazoaniline) at 0.07 mg/l and Solvent Yellow 7 (4-phynylazophenol) at 0.9 mg/l, two dyes identified from the wastewater of a dye manufacturing plant. The toxicity of the acid orange 7 dye to nitrifiers is between those highly toxic compounds (phenolic and metals) and other organic compounds (e.g., formalin) (Digiano et al., 1992). The activated sludge treat textile effluent exhibits a similar toxicity toward *Daphnia magna* ($LC_{50,\ 48\ h}$ = 4 to8 %), when compared with the raw textile waste (Moran. The fact that the activated sludge treatment does not reduced the toxicity of the textile waste is probably due to the nonbiodegradable compounds. On the other hand, the toxicity of another textile effluent from the activated sludge unit is not detectable using the Microtox technique when compared with the influent toxicity up to 9 toxicity unit (Hao et al., 1996). Thus, the tested species and experimental conditions are important parameters for determining the toxicity of industrial colored wastewater.

Table 2.1 Toxicity of dyes at T = 15 - 18 ^{0}C (Little, et al., 1974; ADMI, 1974)

Dye	Class	*Selebastrum capricirnutum* Concentration producing at least 50 % inhibition (mg/l)	Fathead minnow *Pimephales promelas* $LC_{50,\ 96\ h}$, mg/l)
Acid Orange 7	monoazo		165
Acid Orange 24	diazo		130
Acid Yellow 17	monoazo		>180
Acid Yellow 38	diazo		3
Acid Yellow 151	monoazo		29
Acid Black 1	diazo	10	>180
Acid Black 52	monoazo		7
Acid Blue 25	anthraquinone	10	2
Acid Blue 45	anthraquinone		>180
Acid Blue 113	diazo	10	4
Acid Green 25	anthraquinone	10	6.2
Basic Blue 3	Oxazin		4
Basic Blue 39		1	
Basic Blue 9		1	
Basic Blue 21		1	
Basic Violet 1	triphenylmethane	1	0.05
Basic Brown 4	diazo	10	5.6
Basic Red 2			1
Basic Red 18		1	
Basic Yellow 11	polymethane	1	3.2
Basic Yellow 37			1
Basic Green 4	triphenylmethane	1	0.12
Direct Yellow 4	diazo		>180
Direct Yellw 12	diazo		125
Direct Yellow 28	monoazo		>180
Direct Yellow 50	diazo		>180
Direct Blue 6	diazo	10	>180
Direct Blue 218	diazo	10	>180
Direct Red 81	diazo		>180
Direct Brown 95	polyazo	10	>180
Direct Black 38	polyazo	10	>180
Direct Black 80	polyazo	10	>180
Disperse Yellow 3	monoazo		>180
Disperse Yellow 54	quinoline		>180

Some azo dyes have been linked to bladder cancer in humans, to spleenic sarcomas, hepatocarcinomas, and nuclear anomalies in experimental animals, and to chromosomal aberrations in mammalian cells (Medvedev et al., 1988; Patterson and Butler, 1982; Percy et al., 1989) Following the consumption of an azo dye, the dye is reduced and the azo bond is cleaved by azoreductase enzymes to produce aromatic amines. The toxicity and carcinogenicity of certain azo dyes in mammalian systems may result either from interactions of the intact molecules with cytosoloic receptors (Collier et al., 1993) or from the formation of free radicals and arylamines during azoreduction.

Reduction of azo dyes in mammalian systems is catalyzed by hepatic enzymes in the liver (Martin and Kennelly, 1981 and by bacteria with azoreductase activity in intestinal tract. The bacterial azoreductases are much more active than hepatic azoreductase (Cerniglia et al., 1982; Collier et al., 1993). Reduction of azo dyes may produce compounds that are more or less toxic than the parent molecule so that azo reduction may decrease or increase any toxic or carcinogenic effects of the dyes (Collier et al., 1993). For example, the azo dye Direct Black 38 is metabolized to mutagen benzidine by human intestinal microflora (Cerniglia et al., 1986).

The German government has declared a second amendment to ‘German Regulations on Consumer Goods’ (ETAD, 1997), that any azo dye, which could release one or more of listed 20 carcinogenic aromatic amine (see below), should not be used in the manufacture of consumer goods.

2.3.1 Twenty carcinogenic aromatic amines

2-Aminoazotoluene
2-Amino-4-nitrotoluene
4-Chloroaniline
4-Cresidine
4,4-Diaminodiphenylmethane
3,3’-Dichlorobenzidine
3,3’-Dimethyl-4,4’-diaminodiphenylmethane
2-Naphthylamine
4,4’-Thiodianiline
o-Tolidine
4-Aminodiphenyl
Benzidine
4-chloro-2-toluidine
2,4-Diaminoanisole
o-Dianisidine
3,3’-Dimethylbenzidine
4,4’-Methylene-bis-(2-Chloroaniline)
4,4’-Oxydianiline
2,4-toluenediamine
2,4,5-trimethylaniline

As intact azo dye molecules, free radical generated during azo reduction, and reduced products may all contribute to the toxicity and carcinogenicity of azo dyes (Collier et al., 1993), it was essential to evaluate the safety of these dyes before and after metabolism by bacterial azoreductases. The majority of the azo dyes known to be mutagenic are benzidine based (Cerniglia et al., 1982) and are not approved by the US Food and Drug Administration (FDA) for used in foods, drugs, and cosmetics. Garner and Nutman (1977), evaluating the mutagenicity of reduction products of different dyes, observed that at least two amino groups, two nitro groups or one of each are necessary for mutagenic activity. Furthermore, these investigators noted that none of the sulfonate of hydroxylated derivatives were mutagenic and they concluded that ring substitution by these groups prevents the activation of the amino group by liver enzymes.

Although azo dyes can be decolorized under anaerobic conditions due to reduction of the azo bond, the resultant aromatic amines resist further degradation and may be toxic or genotoxic. Toxicity could be eliminated through bacterial fission of the aromatic ring structure, a process that requires oxygen. Therefore, aerobic treatment can be used to detoxify azo dyes (Seshadri et al., 1994).

Gottlieb et al. (2003) determined the toxicity of C.I. Reactive Black 5 and three Procion dyes found in textile effluents by using the bioluminescent bacterium *Vibrio fischeri*. The results showed that the toxicity of Reactive Black 5 increased after anaerobic decolorization of the dye (EC_{50} 0.2 mg/l). However, the toxicity was not detectable when decolorized Reactive Black 5 was metabolized under subsequent aerobic conditions.

2.4 Textile finishing wet processes

From the perspective of cotton process, the processes were grouped as dry and wet processes. Dry processes, which are not applied in the selected enterprise, include processing of fibers into yarn. Wet processes are implemented in the production of all fabrics. Wet processing stages were taken into account from the point of view of resource conservation. In general, wet treatment processes such as bleaching, dyeing, and finishing are very important in terms of the environmental aspects of textile production.

2.4.1. Desizing process

In the desizing process, sizes (e.g., starch and polyvinyl alcohol) and other ingredients added during slashing in the weaving mill are removed by washing them in a detergent solution at high temperatures or using amylase and then rinsing them with fresh water. The process are major contributers to organic load in textile effluents.

2.4.2. Scouring process

Scouring is another washing process using steam and detergents (e.g., sodium hydroxide or surface active agent) to remove oils and mineral material. The scouring can be done by batch in pressurized vessels known as kiers or on a continuous basis. In either case, high temperatures and long retention times, up to 12 hours, are used to ensure thorough saturation and cleaning. Finally, the fabric is rinsed. A waste stream of warm, contaminated water is produced.

2.4.3. Bleaching process

Bleaching removes natural pigments from raw cloth using sodium hypochlorite or oxidizer, such as hydrogen peroxide solution.

2.4.4. Mercering process

Mercerization is frequently invoked as a pretreatment technique for cotton fabrics. This process is mainly used to ‘‘pad’’ the fabrics through exposure to 25% caustic soda followed by a complete washout of the caustic. It is that this treatment changes the crystalline structure of the cotton and swells the fibers so as to create more sites for chemical and physical binding of dye molecules.

2.4.5. Dyeing process

The dyeing process varies considerably, depending on the type of dyestuff, type of fabric, and desired final characteristics (for example, solid color or prints.) Dyeing is a wet process and may be done in batches or continuously. After the dye is set by aging or heating, the fabric may be washed again and dried and/or heat cured. Large quantities of warm, colored wastewater are produced.

2.5 Characteristics of textile effluent

In textile industry, color is applied to finished products though dyeing or printing, resulting in the generation of different wastewaters. The effluent (including sizing, desizing, scouring, bleaching, dyeing, rinsing and finishing wastes) contains many unfixed dyes with a high intensity of color. Although, one sees the colored waste in the textile effluent, the waste, in fact, may consist of hundreds of different shades or hues, as they inhibit light absorbance in the wavelength range to 350 to 500 nm. A trace concentration of these dyes as low as 0.1 mg/l will yield a color index of A465 nm. (absorbance at 465 nm.) = 50 (Matsui et al., 1981).

The dyes and colors present in wastewaters, along with other residual chemical reagents used for processing, and impurities from the raw materials and other hazardous materials in the finishing process, including polyvinyl alcohol, starches, surfactants, pesticides, and biocides in textile industries (Uygur, 1997), have presented significant problems for wastewater treatment. In addition to high organic content, the difficult biodegradable nature of the dyes. The final effluent from textile wastewater treatment plants always exhibits a certain degree of color intensity.

The wet process part of the textile industry is a large consumer of process water, chemicals, textile auxiliary agents and dyes. It produces a lot of effluents that is mostly released into the environment. The quantity and toxicity of wastewater produced in textile wet processing depend on the type of textile processed, dyes used, type of equipment, etc. There are four types of waste produced by textile wet processing as follow (Smith,1986):

1. Hard to treat wastes (dyes, metals, phenols, toxic organic compounds);
2. Dispersible wastes (print paste, waste from coating operations, waste solvents, unused finished mixes);
3. Hazardous wastes or toxics (metals, chlororinated solvents, non-degradable surfactants);
4. High volume wastes (wastewater, alkaline wastes from preparation, bath dye wastes).

Table 2.2 Dye concentrations of different classes in textile effluents (Uygur, 1997)

Dye classes	Waste Dye (%)
Basic	2
Acid	5
Disperse	5
Direct	10
Reactive	30

Table 2.3 Major pollutant types in textile wastewaters (Correia et al., 1994)

Pollutants	Major chemical types	Main Processes of origin	Impact on biological treatment
Organic load	Starch, enzymes, fats, greases, waxes, surfactants	Desizing Scouring Washing Dyeing	High demand on aeration systems Activated sludge bulking problems
Color	Dyes, scoured wool impurities	Dyeing Scouring	Insufficient removal in bioreactor
Nitrogen and Phosphorus	Ammonium salts, Urea, phosphate-based buffers and sequestants	Dyeing	Not removed in anaerobic processes Increased complexity and sensitivity of aerobic processes
pH and salt	NaOH, mineral/organic acids, NaCl, sulfate, carbonate	Scouring Desizing Bleaching Mercerizing Dyeing Neutralizing	Inhibition/collapse of bioreactors
Sulfur	Sulfate, sulfide and hyposulfite, sulfuric acid	Dyeing	Sulfate-reduction in anaerobic reactors
Toxicants	Heavy metals, reducing and oxidizing agents	Desizing Bleaching Dyeing Finishing	Inhibition of sensitive microbial groups in bioreactors
Refractory organics	Surfactants, dyes, resins, synthetic sizes, carrier organic solvents	Scouring Desizing Bleaching Dyeing Washing Finishing	Insufficient removal in bioreactors and accumulation in biomass

2.6 Color measurement

Currently, there are five methods for determining the color of samples in the lastest edition of Standard Method (APHA, 1995).

2.6.1. Visual comparison (Standard Method 2120 B)

The visual method is based on the sample comparison with standard color solution of K_2PtCl_6. The platinum-cobalt method of measuring color is the standard method, the unit of color being that produced by 1 mg platinum/l in the form of the chloroplatinate ion. The ratio of cobalt to platinum may be varied to match the color of natural waters. This method is useful for naturally occurring materials, but is inadequate for quantify the color intensity in dye wastes.

2.6.2. Spectrophotometric (Standard Method 2120 C)

The spectrophotometric method is used to determine color characteristics, that is, hue (e.g., yellow, blue, red, etc.), dominant wavelength, luminance (degree of brightness), and the purity (degree of saturation). Chromaticity coordinates, x and y, are calculated from the three stimuli values. X, Y and Z related to red, green and blue light rays. Y is the percent luminance. Located point (x, y) on a chromaticity diagram allows the determination of the dominant wavelength and the purity. There is no single index value with this method thus, the results are impractical to compare with other samples.

2.6.3. Tristimulus (Standard Method 2120 D)

The tristimulus filter method uses three tristimulus light filters to obtain color data from the information (e.g., hue, wavelength, etc.) obtained in the spectrophotometric method. The percentage of tristimulus light transmitted by the solution is determined for each of three filters. The transmittance values then are converted to trichromatic coefficients and color characteristic values. This method is applicable to potable and surface waters and both domestic and industrial wastewaters.

2.6.4. ADMI (American Dye Manufacturer Institute) (Standard Method 2120 E)

The ADMI tristimulus filter method is an extension of the tristimulus method and a single ADMI color index is calculated. By the method a measure of the sample color, independent of hue, may be obtained. It is based on the use of Adams-Nickerson chromatic value, i.e., uniform color differences. For example, if two colors, A and B, are judged visually to differ from colorless to the same degree, their ADMI color values will be the same. This method is applicable to colored waters and wastewater having color characteristics significantly different from platinum-cobalt standards.

2.6.5. Alternate ADMI method

The ADMI color value can be determined spectrophotometrically using a spectrophotometer with narrow (10 nm. or less) spectral band and an effective operating range of 400 to 700 nm. Tristimulus values may be calculated from transmittance measurements. There is

a good correlation between the transparency and the ADMI values, a transparency of about 15 cm. corresponds to approximately the ADMI color value of 570 (Chang et al., 1994).

Qualitatively, the type of the color and its intensity are easily visualized though our eyes. Quantitatively, the concentration of dyes can be measured by thin-layer chromatography, high performance liquid chromatography (HPLC) and high performance capillary electrophoresis. The trace concentrations of the reactive dyes and their derivatives (vinylsulfone and hydroxylate) can be quantified with a swept-potential electrochemicaldetector and HPLC or cathodic stripping voltammetry. However, the peak absorbance of given wavelength (λ_{max}) for a particular color is easy to measure in a visible spectroscopy to quantify the magnitude of absorbance to the color intensity. The range of the wavelength corresponding to a particular color is shown in Table 2.4.

Table 2.4 Relationship between wavelength and color (Ingamells, 1993)

Wavelength range (nm)	Hue	Perceived hue
400-465	Violet	Greenish-yellow
465-482	Blue	Yellow
482-497	Blue-green	Orange
497-530	Green	Red
530-575	Greenish yellow	Purple
575-580	Yellow	Blue
580-587	Yellowish orange	Violet
587-598	Orange	Greenish blue
598-620	Orange-red	Greenish blue
620-700	Red	Blue-green

In the transparency method, the sample is directly analyzed for its apparent color (Kang and Kao, 1999). Because the hue, concentration of color, and SS in lakes are stable, the transparency method is widely used as an indicator for evaluation of eutrophication. However, industrial effluents are highly variable in both the composition and concentration of SS and color, The color characteristics of industrial wastewater depended on the wastewater type and the performance of wastewater treatment plants; a significant derivation from natural water. The transparency method is a subjective visual comparison analysis. Therefore, it is an unsuitable method for colored industrial regulation.

2.7 Color standards

Color in water may result from the presence of naturally occurring color substances or the colored industrial wastewaters. In general, color in water is divided in to true color and apparent color. The apparent color is determined on the original sample without pretreatment of removal of suspended solid (SS) or turbidity. On the other hand, the SS or turbidity must be removed before measuring the true color (APHA, 1995). The characteristics of colored industrial effluents are highly erratic in both hues and concentrations of color. It is more complicated to quantify colored industrial effluents than naturally occurring color substances.

Many methods both visual comparison and spectrophotometric methods are employed as standard analytical methods (APHA, 1995). In addition, colored industrial effluents may become visual eyesores, and colored pollutants have been found to be toxic and carcinogenic to aquatic environments. Most countries have enforced SS, biochemical oxygen demand (BOD) and chemical oxygen demand (COD) regulations in the effluent standard. However, the color is still not taken as an effluent standard in some countries.

In order to reduce colored effluents discharged into natural water bodies, enforcement of effluent color standard has become a concern of legislators in many countries. As shown in Table 2.5, several countries or areas have settled different standards including different analytical methods and corresponding limits to control color in the effluent. The correlation among those color indicators will also provide important information for designing a wastewater treatment plant.

Table 2.5 The effluent color limits and analytical methods

Countries or areas	Analytical methods	Limits	References
Austria	3-WL (DFZ) values	28, 24 and 20 m^{-1}	
China	Dilution ratio	50-160 times	Kang and Kuo (1999)
Germany	3-WL (DFZ) values	7, 5 and 3 m^{-1}	Gahr et al.(1994)
Hong Kong	Lavibond unit	0-20 units	Chang et al.(1994)
Korea	ADMI	200-400 units	Kang and Kuo (1999)
Philippine	Platinum-cobalt	100-200 units	Chang et al.(1994)
Italy	Dilution ratio	1:2 undetectable	Rozzi et al. (1999)
Singapore	Lavibond unit	7 units	Chang et al.(1994)
Taiwan	ADMI	400 units	Kao et al. (2001)
USA;			
Alabama	ADMI	50 units	Kennedy et al. (1992)
Virginia	ADMI	2000 units	Ganash et al. (1994)
Rhode Island	ADMI	200 units	Kang and Kuo (1999)
Wisconsin	ADMI	225-600 units	Wisconsin State (1997)
Vietnam	Lavibond unit	7 units	Hochiming City (1993)

In USA, the color standards in different states may vary from locality to locality, presumably based on the receiving water quality. For example, the downstream color in a receiving river should not be greater than the upstream value by more than 50 ADMI unit for and Alabama dye waste discharge permit (Kennedy et al., 1992), or the color should be less than 2000 ADMI unit for the wastewater discharge into a municipal wastewater treatment plant in Virginia (Ganash et al., 1994).

The color standards in Germany for textile wastewater are based on the absorbance measurements on three wavelength at 436 nm. (yellow), 525nm. (red) and 620 nm. (blue), with the respective standard (DFZ) value of 7, 5 and 3 m^{-1} (Gahr et al., 1994). These values are determined as:

$$DFZ = A(1000)/d$$

where A = absorbance and d = length of cell in mm. For a typical dye waste (100-200 mg/l), the DFZ value is about 200 to 600 m^{-1}; thus, color removal of more than two orders of magnitude (99 %) is required to meet the standards in Germany.

The color limitation standards in Austria are based on the spectrum absorption coefficient (DFZ) at 436, 525 and 620 nm. as 28, 24 and 20 m^{-1}, respectively.

In Thailand, there are no numerical color standards issued to any industrial wastes; only qualitative description for prohibiting waste discharge with significant color is provided.

2.8 Treatments of textile wastewater

2.8.1 Chemical methods

This is the most commonly used method of decolorization by chemical means. This is mainly due to its simplicity of application. The main oxidizing agent is usually hydrogen peroxide (H_2O_2). This agent needs to be activated by some means, for example, ultra violet light. Many methods of chemical decolorization vary depending on the way in which the H_2O_2 is activated (Slokar and Le Marechal, 1997). Chemical oxidation removes the dye from the dye-containing effluent by oxidation resulting in aromatic ring cleavage of the dye molecules (Raghavacharya, 1997).

2.8.1.1 H_2O_2.Fe(II) salts (Fentons reagent)

Fentons reagent is a suitable chemical means of treating wastewaters which are resistant to biological treatment or are poisonous to live biomass (Slokar and LeMarechal, 1997). Chemical separation uses the action of sorption or bonding to remove dissolved dyes from wastewater and has been shown to be effective in decolorizing both soluble and insoluble dyes (Pak and Chang, 1999). One major disadvantage of this method is sludge generation through the flocculation of the reagent and the dye molecules. The sludge, which contains the concentrated impurities, still requires disposal. It has conventionally been incinerated to produce power, but such disposal is seen by some to be far from environmentally friendly. The performance is dependent on the final floc formation and its settling quality, although cationic dyes do not coagulate at all. Acid, direct, vat, mordant and reactive dyes usually coagulate, but the resulting floc is of poor quality and does not settle well, yielding mediocre results (Raghavacharya, 1997).

2.8.1.2 Ozonation

The use of ozone was first pioneered in the early 1970s, and it is a very good oxidizing agent due to its high instability (oxidation potential, 2.07) compared to chlorine, another oxidizing agent (1.36), and H_2O_2 (1.78). Oxidation by ozone is capable of degrading chlorinated hydrocarbons, phenols, pesticides and aromatic hydrocarbons (Lin and Lin, 1993; Xu and Lebrun, 1999). The dosage applied to the dye-containing effuent is dependent on the total color and residual COD to be removed with no residue or sludge formation (Ince and Gonenc, 1997) and no toxic metabolites (Gahr et al., 1994). Ozonation leaves the effluent with no color and low COD suitable for discharge into environmental waterways (Xu and Lebrun, 1999). This method shows a preference for double-bonded dye molecules (Slokar and Le Marechal, 1997).

One major advantage is that ozone can be applied in its gaseous state and therefore does not increase the volume of wastewater and sludge. Chromophore groups in the dyes are generally organic compounds with conjugated double bonds that can be broken down forming smaller molecules, resulting in reduced coloration (Peralto-Zamora et al., 1999). These smaller molecules may have increased carcinogenic or toxic properties, and so ozonation may be used alongside a physical method to prevent this. Decolorization occurs in a relatively short time. A disadvantage of ozonation is its short half-life, typically being 20 min. This time can be further shortened if dyes are present, with stability being affected by the presence of salts, pH, and temperature.

2.8.1.3 Photochemical

This method degrades dye molecules to CO_2 and H_2O (Yang et al., 1998; Peralto-Zamora et al., 1999) by UV treatment in the presence of H_2O_2. Degradation is caused by the production of high concentrations of hydroxyl radicals. UV light may be used to activate chemicals, such as H_2O_2, and the rate of dye removal is influenced by the intensity of the UV radiation, pH, dye structure and the dye bath composition (Slokar and Le Marechal, 1997). This may be set-up in a batch or continuous column unit (Namboodri and Walsh, 1996). Depending on initial materials and the extent of the decolorization treatment, additional by-products, such as, halides, metals, inorganic acids, organic aldehydes and organic acids, may be produced (Yang et al., 1998). There are advantages of photochemical treatment of dye-containing effluent; no sludge is produced and foul odors are greatly reduced.

2.8.1.4 Sodium hypochloride (NaOCl)

This method attacks at the amino group of the dye molecule by the Cl^-. It initiates and accelerates azo bond cleavage. An increase in decolorization is seen with an increase in Cl^- concentration. The use of Cl^- for dye removal is becoming less frequent due to the negative effects it has when released into waterways (Slokar and Le Marechal, 1997) and the release of aromatic amines which are carcinogenic, or otherwise toxic molecules (Banat et al., 1999).

2.8.1.5 Electrochemical destruction

It has some significant advantages for use as an effective method for dye removal. There is little or no consumption of chemicals and no sludge build up. The breakdown metabolites are generally not hazardous leaving it safe for treated wastewaters to be released back into waterways. It shows efficient and economical removal of dyes and a high efficiency for color removal and degradation of recalcitrant pollutants (Ogutveren and Kaparal, 1994; Pelegrini et al., 1999).

2.8.2 Physical treatments

2.8.2.1 Adsorption

Adsorption techniques have gained favor recently due to their efficiency in the removal of pollutants too stable for conventional methods. Adsorption produces a high quality product, and is a process which is economically feasible (Choy et al., 1999). Decolorization is a result of two mechanisms: adsorption and ion exchange (Slokar and Le Marechal, 1997), and is influenced by many physio-chemical factors, such as, dye/sorbent interaction, sorbent surface area, particle size, temperature, pH, and contact time (Kumar et al., 1998).

2.8.2.2 Activated carbon

This is the most commonly used method of dye removal by adsorption (Nasser and El-Geundi, 1991) and is very effective for adsorbing cationic, mordant, and acid dyes and to a slightly lesser extent, dispersed, direct, vat, pigment and reactive dyes (Raghavacharya, 1997; Rao et al., 1994). Performance is dependent on the type of carbon used and the characteristics of the wastewater. Removal rates can be improved by using massive doses, although regeneration or re-use results in a steep reduction in performance, and efficiency of dye removal becomes unpredictable and dependent on massive doses of carbon. Activated carbon, like many other dye-removal treatments, is well suited for one particular waste system and ineffective in another. Activated carbon is expensive. The carbon also has to be reactivated otherwise disposal of the concentrates has to be considered. Reactivation results in 10.15% loss of the sorbent.

2.8.2.3 Peat

The cellular structure of peat makes it an ideal choice as an adsorbent. It has the ability to adsorb transition metals and polar organic compounds from dye-containing effluents. Peat requires no activation, unlike activated carbon, and also costs much less (Poots and McKay, 1976a). Due to activated carbon's powdered nature, it has a much larger surface area, and hence has a better capacity for adsorption

2.8.2.4 Wood chips

They show a good adsorption capacity for acid dyes although due to their hardness, it is not as good as other available sorbents (Nigam et al., 2000) and longer contact times are required (Poots and McKay, 1976). Adsorbed wood is conventionally burnt to generate power although there is potential for solid state fermentation of the dye adsorbed wood chips.

2.8.2.5 Fly ash and coal (mixture)

A high fly ash concentration increases the adsorption rates of the mixture due to increasing the surface area available for adsorption. This combination may be substituted for activated carbon, with a ratio of fly ash:coal, 1:1 (Gupta et al., 1990).

2.8.2.6 Other materials

The use of these substrates such as natural clay, corn cobs, rice hulls etc., for dye removal is advantageous mainly due to their widespread availability and cheapness. They are economically attractive for dye removal, compared to activated charcoal, with many comparing well in certain situations (Nawar and Doma, 1989; Nasser and El-Geundi, 1991; Nigam et al., 2000).

2.8.2.7 Membrane filtration

This method has the ability to clarify, concentrate and, most importantly, to separate dye continuously from effluent (Mishra and Tripathy, 1993; Xu and Lebrun, 1999). It has some special features unrivalled by other methods; resistance to temperature, an adverse chemical environment, and microbial attack. The concentrated residue left after separation poses disposal problems, and high capital cost and the possibility of clogging, and membrane replacement are its disadvantages.

2.8.2.8 Ion exchange

Ion exchange has not been widely used for the treatment of dye-containing effluents, mainly due to the opinion that ion exchangers cannot accommodate a wide range of dyes (Slokar and Le Marechal, 1997). Wastewater is passed over the ion exchange resin until the available exchange sites are saturated. Both cation and anion dyes can be removed from dye-containing effluent this way. Advantages of this method include no loss of adsorbent on regeneration, reclamation of solvent after use and the removal of soluble dyes. A major disadvantage is cost. Organic solvents are expensive (Mishra and Tripathy, 1993).

2.8.2.9 Electrokinetic coagulation

This is an economically feasible method of dye removal. It involves the addition of ferroussulfate and ferricchloride, allowing excellent removal of direct dyes from wastewaters. Unfortunately, poor results with acid dyes, with the high cost of the ferroussulfate and ferricchloride, means that it is not a widely used method (Mishra and Tripathy, 1993). Production of large amounts of sludge occurs, and this results in high disposal costs (Gahr et al., 1994).

Table 2.6 Advantages and disadvantages of the current methods of dye removal from textile effluents (Robinson et al., 2001)

Physical/chemical methods	Advantages	Disadvantages
Fentons reagent	Effective decolorization of both soluble and insoluble dyes	Sludge generation
Ozonation	Applied in gaseous state: no alteration of volume	Short half-life (20 min)
Photochemical	No sludge production	Formation of by-products
NaOCl	Initiates and accelerates azo-bond cleavage	Release of aromatic amines
Electrochemical destruction	Breakdown compounds are non-hazardous	High cost of electricity
Activated carbon	Good removal of wide variety of dyes	Very expensive
Peat	Good adsorbent due to cellular structure	Specific surface areas for adsorption are lower than activated carbon
Wood chips	Good sorption capacity for acid dyes	Requires long retention times
Silica gel	Effective for basic dye removal	Side reactions prevent commercial application
Membrane filtration	Removes all dye types	Concentrated sludge production
Ion exchange	Regeneration: no adsorbent loss	Not effective for all dyes
Irradiation	Effective oxidation at lab scale	Requires a lot of dissolved O_2
Electrokinetic coagulation	Economically feasible	High sludge production

Table 2.7 Summary of the effectiveness of major treatment processes for various dye classes (Richardson, 1983)

Dye class	Coagulation alum	Activated carbon	Conventional biological	Physico-chemical and biological	Ozone	Sludge
Azoic	-	+	-	+	+	N
Reactive	-	+	-	+	+(S)	-
Acid	-	+	-	+	+	-
Basic	-	+ (S)	+	+	+	+
Disperse	+	-	-	+	-	-
Vat	+	-	-	+	+	N
Sulfur	+	-	-	+	+	N
Direct	+	N	N	+	N	+

Color removal: - = unsatisfactory; + = good; S = specially suitable; N = No report.

2.9 Biological methods

2.9.1 Decolorization by white-rot fungi

White-rot fungi are those organisms that are able to degrade lignin, the structural polymer found in woody plants (Barr and Aust, 1994). The most widely studied white-rot fungus, in regards to xenobiotic degradation, is *Phanerochaete chrysosporium*. This fungus is capable of degrading dioxins, polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs) and other chloro-organics (Chao and Lee, 1994; Reddy, 1995).

Azo dyes, the largest class of commercially produced dyes, are not readily degraded by other microorganisms but these can be degraded by *P. chrysosporium* (Paszczynski and Crawford, 1995). Other fungi such as, *Hirschioporus larincinus*, *Inonotus hispidus*, *Phlebia tremellosa* and *Coriolus versicolor* have also been shown to decolorize dye-containing effluent (Banat et al., 1996; Kirby, 1999). Although white-rot fungi have been shown to decolorize dyes in liquid fermentations, enzyme production has also been shown to be unreliable. This is mainly due to the unfamiliar environment of liquid fermentations.

Kirby (1999) has shown that *P. chrysosporium* had the ability to decolorize artificial textile effluent by up to 99% within 7 days. White-rot fungi are able to degrade dyes using enzymes, such as lignin peroxidases (LiP), manganese dependent peroxidases (MnP). Other enzymes used for this purpose include H_2O_2-producing enzymes, such as, glucose-1-oxidase and glucose-2-oxidase, along with laccase, and a phenoloxidase enzyme (Archibald and Roy, 1992; Thurston, 1994; Schliephake and Lonergan, 1996; Kirby, 1999). These are the same enzymes used for the lignin degradation (Lonergan, 1992; Barr and Aust, 1994; Reddy, 1995).

Lignin and manganese peroxidases show a similar reaction mechanism and are oxidized during their catalytic cycle by H_2O_2 to an oxidized state which is reduced by the substrates (e.g. azo dyes) in two subsequent one electron transfer steps to the native form of the enzyme. While lignin peroxidases are able to oxidize nonphenolic aromatic compounds, manganese peroxidases preferentially oxidize Mn^{2+} to Mn^{3+}, and the Mn^{3+} is responsible for the oxidation of many phenolic compounds. Laccases are copper-containing enzymes produced by a number of plants and fungi which oxidize phenols and anilines in the presence of oxygen (Barr and Aust 1994; Glenn et al., 1986).

The oxidation of the non-sulfonated azo dye 1-(4.-acetamidophenylazo)-2-naphthol (a structural analogue of the industrially relevant azo dye Disperse Yellow 3) by the lignin peroxidase from *P. chrysosporium* resulted in the formation of 1,2-naphtoquinone and acetanilide (Figure. 2.6).

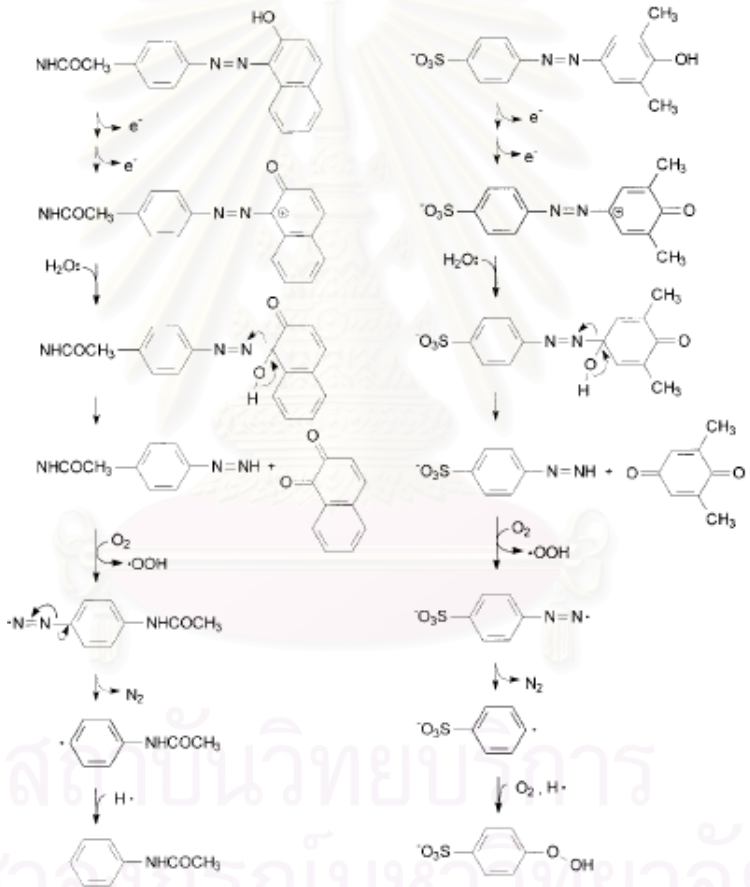


Figure. 2.6 Proposed reaction mechanisms for the oxidation of 1-(4-acetamidophenylazo)-2-naphthol (*left*) and 3,5-dimethyl-4-hydroxyazobenzene-4.-sulfonate (*right*) by the lignin peroxidase from *Phanerochaete chrysosporium* (Chivukula et al., 1995; Goszczynski et al., 1994)

2.9.2 Decolorization by non-white-rot fungi

Whilst degradation pathways utilized by these fungi are not described in the literature, it is expected that they would be similar to those reported to be involved in the metabolism of other aromatic hydrocarbons (Wunderwald et al., 1997). Detailed investigations have been carried out on one such isolate, a strain of *Geotrichum candidum* Dec1 isolated from soil and capable of decolorizing a number of anthraquinone dyes (Kim et al., 1995). The broad substrate specificity exhibited by this isolate led Kim and Shoda (1999) to propose the existence of an extracellular peroxidase-type enzyme.

2.9.3 Decolorization by actinomycetes

Actinomycetes, particularly *Streptomyces* species, are known to produce extracellular peroxidases that have a role in the biodegradation of lignin. These prokaryotic peroxidases are involved in the initial oxidation of lignin to produce various water-soluble polymeric compounds. The ability of actinomycetes to decolorize and mineralize textile dyes was initially investigated by three groups.

In 1989, Ball et al. screened 20 strains of actinomycetes, representing a wide range of genera, for their ability to decolorize the polymeric dye Poly R (Ball et al. 1989). Only three of the 20 strains were observed to significantly decolorize the dye: *Streptomyces badius* 252, *Streptomyces* sp. strain EC22, and *Thermomonospora fusca* MT800.

Subsequently, Zhou and Zimmermann (1993) embarked on an even larger screening process in which the decolorizing capabilities of 159 actinomycetes were investigated. Of particular interest in this study was the investigators use of actual textile effluents in the screening process. Five separate effluents each containing a single dye of known concentration were used. Each dye was structurally distinct, ranging from the azo compound Reactive Red 147 to the phthalocyanine Reactive Blue 116. The widespread ability of actinomycetes to bring about dye decolorization was demonstrated by positive results being obtained for 83 of the isolates. The finding that actinomycetes are capable of the aerobic decolorization and degradation of azo dyes was significant given the recalcitrance of the compounds to degradation by other bacteria under such conditions.

Finally, a group based at the University of Idaho initiated an investigation into the ability of ligninolytic microbes, both white-rot fungi and *Streptomycetes*, to mineralize and decolorize textile dyes. Initially, 14 streptomycetes were investigated for their ability to decolorize two polymeric dyes, Poly B-411 and Poly R-478, as well as the azo dye Remazol Brilliant Blue R (RBBR) (Pasti and Crawford 1991). With two of the dyes, RBBR and Poly B-411, identical results were essentially obtained, with a strong correlation between the ability of the isolate to decolorize dyes and its ligninolytic capability. This observation, coupled with their finding that enhanced dye decolorization could be achieved as a result of extracellular H_2O_2 production when strains were grown in the presence of glucose, suggested the involvement of peroxidases in the decolorization process.

Recently, Burke and Crawford (1998) partially purified a novel extracellular peroxidase from *S. viridosporus* T7A in an effort to identify the class of peroxidase involved in dye decolorization by *Streptomyces* species. The peroxidase was found to have a substrate specificity similar to that of fungal Mn-peroxidases (Magnuson 1996).

2.9.4 Decolorization by yeast

Dye removal by the yeast was physically biosorption of the dye non-specific manner to cell peripheries and than followed by specific accumulation into the cell. Yeast biomass is an inexpensive, ready available source of biomass that has potential for dye bioaccumulation at lower pH values (Banat et al., 1999).

Yeasts, such as *Klyveromyces marxianus*, are capable of decolorizing dyes. Banat et al. (1999) showed that *K. marxianus* was capable of decolorizing Remazol Black B by 78.98%. In general, the increase in dye concentration inhibited the growth of yeast and caused a long lag period. Remazol Blue dye gave a considerably higher dye bioaccumulation percentage by *Candida tropicalis* than Reactive Black and Reactive Red (Donmez, 2002).

2.9.5 Decolorization by living/dead microbial biomass adsorption

The uptake or accumulation of chemicals by microbial mass has been termed biosorption (Hu, 1996; Tsezos and Bell, 1989; Kumar et al., 1998). Dead bacteria, yeast and fungi have all been used for the purpose of decolorizing dye-containing effluents. Textile dyes vary greatly in their chemistries, and therefore their interactions with microorganisms depend on the chemistry of a particular dye and the specific chemistry of the microbial biomass (Polman and Brekenridge, 1996). Depending on the dye and the species of microorganism used different binding rates and capacities will be observed. It can be said that certain dyes have a particular affinity for binding with microbial species. Biosorption capacities showed that this type of biomass had a significantly high affinity for dye removal, and so widened the spectrum of use for biomass (Bustard et al.,1998). The use of biomass has its advantages, especially if the dye-containing effluent is very toxic. Biomass adsorption is effective when conditions are not always favorable for the growth and maintenance of the microbial population (Modak and Natarajan, 1995). Adsorption by biomass occurs by ion exchange.

Hu (1992) demonstrated the ability of bacterial cells to adsorb reactive dyes. Biosorption tends to occur reasonably quickly: a few minutes in algae to a few hours in bacteria (Hu, 1996). This is likely to be due to an increase in surface area caused by cell rupture during autoclaving (Polman and Brekenridge, 1996).

2.9.6 Decolorization by aerobic bacteria

A number of reports exist suggesting the aerobic conversion of specific azo dyes. Certain carboxylated analogues of sulfonated azo compounds are also utilized aerobically as sole carbon and energy source by preadapted bacteria. Indeed, oxygen-insensitive azoreductases, Orange I azoreductase[NAD(P)H:1-(4-sulfophenylazo)-4-naphthol oxidoreductase] and Orange II azoreductase [NAD(P)H:1-(4̃-sulfophenylazo)-2-naphthol oxidoreductase], were purified and characterized from a *Pseudomonas* strain K24 (Recently, Blhmel et al. (1998) reported the isolation of an unidentified bacterial strain, S5, capable of utilizing the model sulfonated azo compound 4-carboxy-4.-sulfoazobenzene (CSAB) as sole carbon and energy source. Elucidation of the degradation pathway demonstrated that the azo linkage of CSAB undergoes an initial reductive cleavage to form 4-aminobenzoate and 4-aminobenzenesulfonate, which are subsequently metabolized by conventional aromatic catabolic pathways (Blhmel et al. 1998).

Attempts to characterize the enzyme responsible for the azo-bond cleavage in crude cell extracts have so far proven unsuccessful. In addition to azo dyes, the ability of bacteria to aerobically metabolize other dye classes has also attracted interest but yielded little success.

2.9.7 Decolorization by anaerobic textile-dye bioremediation systems

In contrast to the few reports of aerobic decolorization of azo dyes, a wide range of organisms are able to reduce azo compounds under anaerobic conditions. This has been shown for purely anaerobic (e.g. *Bacteroides* sp., *Eubacterium* sp; *Clostridium* sp.), facultatively anaerobic (e.g. *Proteus vulgaris*, *Streptococcus faecalis*), and aerobic (e.g. *Bacillus* sp., *Sphingomonas* sp.) bacteria, yeasts, and even tissues from higher organisms (Adamson et al., 1965; Bragger et al., 1997; Dieckhues 1960; Dubin and Wright 1975; Rafii et al., 1990; Scheline et al., 1970; Walker 1970; Wuhrmann et al., 1980). The main interest in this field has been focused on bacteria from the human intestine that are involved in the metabolism of azo dyes ingested as food additives (Chung et al. 1992).

Azo compounds constitute the largest and the most diverse of dye and pigments used in commercial applications (Zollinger, 1991). Over 3,000 different azo dyes are used to satisfy the consumer demand for color appeal in food, textile and printing applications (Meyer, 1981). In 1991, the world production of dyes was estimated at 668,000 tonnes (Ollgaard et al., 1999) of which an estimated 70% were azo dyes (ETAD, 1997). A mass balance of azo dye production and consumption revealed that about 4% of azo dye production is lost to domestic and industrial wastewater (Ollgaard et al., 1999). Azo dyes are soluble in solution, and are not removed via conventional biological treatments. Reactive dyes have been identified as the most problematic compounds in textile dye effluents (Carliell et al., 1996, 1994). Anaerobic bioremediation allows azo and other water-soluble dyes to be decolorized. This decolorization involves an oxidation-reduction reaction with hydrogen rather than free molecular oxygen in aerobic systems.

Typically, anaerobic breakdown yields methane and hydrogen sulfide (Carliell et al., 1996). Azo dye acts as an oxidizing agent for the reduced flavin nucleotides of the microbial electron chain and is reduced and decolorized concurrently with reoxidation of the reduced flavin nucleotides. In order for this to occur, additional labile carbon source is required in order for decolorization to proceed at a viable rate. This additional carbon is converted to methane and carbon dioxide, releasing electrons. These electrons cascade down the electron transport chain to a final electron acceptor, in this case, the reactive azo dye. The electrons react with the dye reducing the azo bonds, and ultimately causing decolorization (Carliell et al., 1996). This process is catalyzed by a variety of soluble cytoplasmic enzymes with low-substrate specificity which are known as azoreductases. Under anoxic conditions, these enzymes facilitate the transfer of electrons via soluble flavins to the azo dye, which is then reduced.

A mechanism for azo reduction was proposed by Gingell and Walker (1971) involving a two stage reduction of the azo bond as given in reactions (1) and (2). The intermediate product of reaction (1) is an unstable colorless compound and the azo bond can reformed upon oxidation regaining the color.

$$2e^- + 2H^+ + (R–N=N–R^1) \longrightarrow (R–NH–NH–R^1) \qquad (1)$$

$$2e^- + 2H^+ + (R–NH–NH–R^1) \longrightarrow (R–NH_2) + (R^1–NH_2) \qquad (2)$$

where R and R^1 are variously substituted phenyl and naphthol residues.

Carriers in the electron transport chain, utilize azo compounds as terminal electron acceptors, thus regenerating themselves and fortuitously reducing azo bond, breaking the dyes chromophore. This process however, is inhibited by the presence of oxygen, which is more energetically favorable oxidizing agent, and also a readily degradable carbon source is required for metabolism to produce the electron carriers (Carliell et al., 1995).

Baughmann and Weber (1994) demonstrated that in anoxic sediment environments uncharged azo dyes readily undergo biologically (and presumably microbial)-mediated reduction to the corresponding amines.

Donlon et al. (1997) reported the partial mineralization of the azo dye Mordant Orange 1 by a methanogenic granular sludge in a continuous-upflow anaerobic sludge blanket. In this study, however, complete mineralization of only one of the azo cleavage products, 5-aminosalicylic acid, was possible, with the other product (1,4-phenylenediamine) accumulating in the reactor.

The role that such cytoplasmic enzymes have in vivo is, however, uncertain. Cell extracts show generally much higher rates for the anaerobic reduction of azo dyes than do preparations of resting cells (Mechsner and Wuhrmann 1982; Walker 1970; Wuhrmann et al., 1980).Reports indicate that intestinal bacteria decolorize certain azo dyes and their polymeric derivatives at roughly equivalent rates (Brown 1981). As it is unlikely that these polymeric dye molecules, or highly charged sulfonated azo dyes, can actually pass through the bacterial cell membrane, then the possibility of non-cytoplasmic azoreductase capabilities exists (Keck et al. 1997). Therefore, it appears reasonable that, in vivo, intracellular enzymes like flavin reductases are of little importance for the reduction of sulfonated azo compounds (Russ et al. 2000).

A different model for the unspecific reduction of azo dyes by bacteria which does not require transport of the azo dyes or reduced flavins through the cell membranes was recently suggested for *Sphingomonas xenophaga* BN6. The anaerobic reduction of azo compounds by this strain was significantly increased after the addition of different quinones, such as anthraquinone-2-sulfonate or 2-hydroxy-1,4-naphthoquinone. It was suggested that in this system the quinones acted as redox mediators which were enzymatically reduced by the cells of *S. xenophaga* BN6 and that the hydroquinones formed reduced the azo dyes in the culture supernatants in a purely chemical redox reaction (Figure 2.7). Cell fractioning experiments demonstrated that the quinone reductase activity was located in the cell membranes of *S. xenophaga* BN6 and that therefore no transport of the sulfonated azo compounds or of the hydroquinone/quinone redox mediator via the cell membrane was necessary (Kudlich et al. 1997).

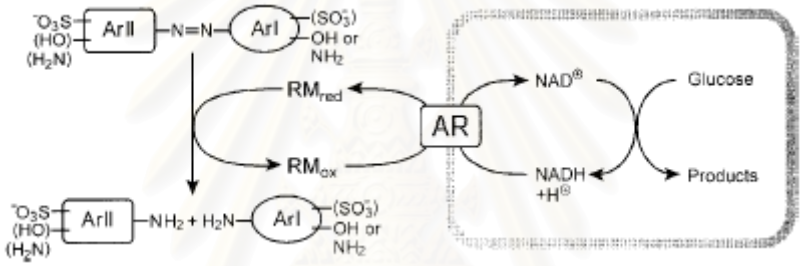


Figure. 2.7 Proposed mechanism for the redox-mediator-dependent reduction of azo dyes by *Sphingomonas xenophaga* BN6. *AR* Azoreductase, *RM* redox mediator (Keck et al. 1997)

Anaerobic degradation of textile dyes yields only azo reduction. Mineralization does not occur. It has been shown that azo- and nitro-components are reduced in the sediments and in the intestinal environment, resulting in the regeneration of the parent toxic amines (Banat et al., 1996). Therefore, careful monitoring is required before treated wastewater is released into waterways. A major advantage of this anaerobic system, apart from the decolorization of soluble dyes, is the production of biogas.

In addition to azo dyes, the bacterial metabolism of other dye molecules under anoxic conditions has also been studied. Henderson et al. (1997) demonstrated that a range of axenic bacterial cultures which are commonly found in the human gastrointestinal tract, as well as consortia of microbes from human, mouse, and rat intestines, all readily reduced the triphenylmethane dye malachite green. The metabolite produced, leucomalachite green, is a suspected carcinogen and raises concerns over the continued use of malachite green in aquaculture in various regions worldwide.

Based on current knowledge, anaerobic reduction of azo bond by bacteria is suited for the decolorization of azo dyes in textile effluents treatment systems (Delee et al., 1998). The putative advantages and disadvantages of anaerobic treatment are:

Advantages

1. High BOD levels can be efficiently and cheaply removed.
2. Dyes can be reductively decolorized by anaerobic, facultatively anaerobic and aerobic bacteria.
3. Heavy metals retained through sulfate reduction.
4. No foaming problems with surfactants.
5. High effluent temperatures can be favorable.
6. High pH effluents can be acidified.
7. Degradation of refractory organics can be initiated.
8. The dye reduction rates generally increase in the presence of other carbon sources. The reduction equivalents that are formed during anaerobic oxidation of these carbon sources are finally used for the reduction of azo bonds.
9 The reaction take place at neutal pH values and are expected to extremely unspecific when low-molecular redox mediators are involved.

Disadvantages

1. BOD removal can be insufficient
2. Dyes and other refractory organic are not mineralized
3. Nutrients (N, P) are not removed
4. Sulfate give rise to sulfide

2.9.8 Decolorization by anaerobic-aerobic biodegradation of dyes

To overcome the problem of the relative recalcitrance of azo dye breakdown products under anoxic conditions, a number of groups have used a two-stage treatment process (Oxspring et al., 1996; O'Neill et al., 2000). In the first anaerobic stage, the azo dye is readily reduced to the corresponding colorless aromatic amines, which are then metabolized relatively easily under aerobic conditions. A detailed review on the anaerobic treatment of textile effluents can be found elsewhere (Delee et al., 1998).

In 1997, Kudlich et al. described the complete mineralization of the sulfonated azo dye Mordant Yellow 3 by the bacterium *Sphingomonas* sp. BN6 co-immobilized with an uncharacterized 5-aminosalicylate degrading isolate in alginate beads. Although the beads were maintained under aerobic conditions, their centers maintained anoxic conditions and it was here that cells of *Sphingomonas* sp. BN6 reductively cleaved Mordant Yellow 3 to produce 5-aminosalicylate and 6-aminonaphthalene-2-sulfonate. At the aerobic surface of the beads, 6A2NS was also converted to 5AS by *Sphingomonas* sp. BN6 before being mineralized by cells of the isolate 5AS1. These observations suggest that it may be possible to develop biofilm-based reactors for the complete mineralization of industrial-dye-contaminated wastewaters.

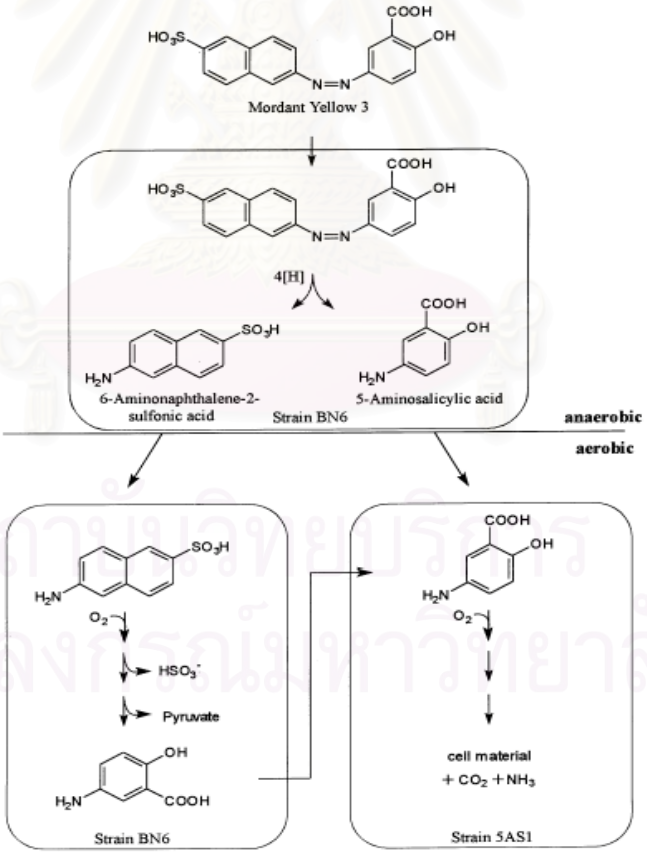


Figure.2.8 Schematic metabolic pathway for the degradation of the azo dye Mordant Yellow 3, by an anaerobic/aerobic treatment using a mixed bacterial community (Nortemann et al., 1986; Haug et al., 1991; Stolz et al., 1992)

During the last few years, different reactor designs have been proposed in order to obtain an effective continuous anaerobic/aerobic treatment of azo dyes: an anaerobic and an aerobic rotating biological contactor (Zaoyan et al. 1992), an anaerobic fixed-film fluidized bed reactor followed by an aerobic suspended-bed activated sludge reactor (Fitzgerald and Bishop 1995; Seshadri et al., 1994), a combination of anaerobic and aerobic rotating-drum reactors (Harmer and Bishop 1992; Sosath and Libra 1997), and an anaerobic upflow fixed bed column together with an aerobic agitated tank (An et al. 1996; O'Neill et al. 2000; Rajaguru et al., 2000).

In general, it may be concluded that, in continuous anaerobic/aerobic systems which are fed with substrate mixtures with a high biological and chemical oxygen demand (BOD, COD) and low dye concentrations to the anaerobic stage, a complete decolorization of the dyes and a significant BOD and COD removal can be achieved in the anaerobic stage. In the subsequent aerobic step, the remaining BOD from the auxiliary substrates may be mineralized. There are several examples demonstrating COD removal in the anaerobic/aerobic processes of 70–95% (e.g. for the treatment of Reactive Red 141 (Procion Red H-E7B) in a simulated textile effluent containing modified starch, O'Neill et al., 2000).

Similar results have also been described for the treatment of wastewater from a dyeing factory on a laboratory scale (Zaoyan et al. 1992). Because the concentrations of the azo dyes are generally much lower than those of the auxiliary substrates, the fate of the aromatic amines formed (especially if they are auto-oxidizable) in the aerobic treatment process is still unclear and some contradicting results have been published. For the treatment of the copper-containing dye Reactive Violet 5 in an anaerobic/aerobic system with three rotating-disc reactors, no indications for a mineralization of the amines in the aerobic stage were detected by Sosath and Libra (1997). In contrast, the analysis of the fate of nitrogen-containing compounds (presumed amines) in the aerobic step of Reactive Red 141 treatment suggested a decrease in the concentration of nitrogen containing metabolites (O'Neill et al., 2000).

In a study performed by Luangdilok and Panswad (2000) the color and chemical oxygen demand (COD) removal efficiencies were investigated using anaerobic/aerobic SBR reactor for treatment of 20 mg l^{-1} of anthraquinone and diazo dyes. A total of 90% COD and 45% color removal efficiencies were obtained while acetic acid and glucose were used as carbon source.

O'Neill et al. (2000) demonstrated 86% color and 66% COD removal efficiencies, respectively, in a upflow anaerobic sludge blanket (UASB)/activated sludge sequential reactor system treating reactive azo dyestuffs.

Tan et al. (1999), observed 69% COD removal efficiency at an organic loading of 210 mg/l/day with ethanol–COD concentration of 2,000 mg/l in an anaerobic-aerobic sequential reactor system removing Mordant Green dyestuff.

In a study performed by An et al. (1996), 83% color and 69% COD removal efficiencies were obtained in anaerobic (hydraulic retention times, HRT = 6–10 h) and aerobic (HRT = 6.5 h) sequential reactor degrading Acid Yellow and Basic Red dyes at a organic loading rate of 5.3 kgCOD m^{-3} per day and a COD concentration of 1,200 mg/l.

Kalyuzhnyi and Sklyar (2000) obtained 56% decolorization efficiency in an anaerobic/aerobic hybrid reactor at a organic loading rate of 0.3 gCOD/l per day using ethanol as carbon source. It was observed that 400 mg/l of Procion Red (H-E7B) azo dye was degraded with 72% and 11% COD removal efficiencies in anaerobic and aerobic reactors, respectively at an organic loading rate of 3.34 kgCOD m^{-3} per day (O'Niell et al., 2000).

Zaoyan et al. (1992), observed 65% color and 74% COD removal efficiencies in an anaerobic/aerobic rotating biodisc system treating azo dyestuffs containing textile wastewaters at a HRT about 8 h and organic loading rate of 45 gCODm^{-3} per day.

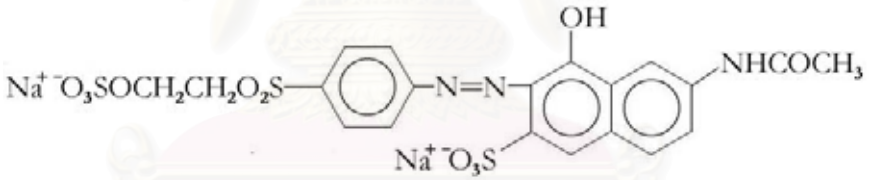
Chapter 3

Materials and Methods

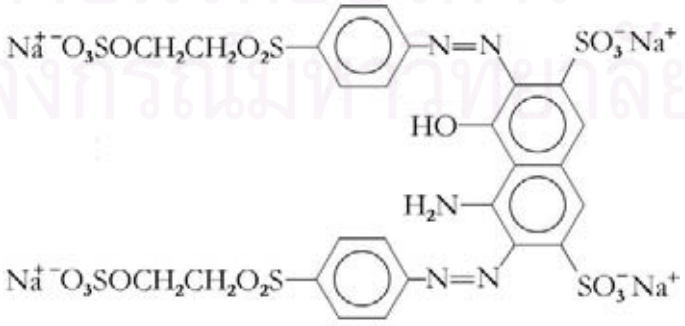
3.1 Chemicals

Commercially important and commonly used reactive dyes for cotton dyeing Remazol Brilliant Orange 3R (C.I. reactive orange 16, Figure. 3.1A), Remazol Black B (C.I. reactive black 5, Figure. 3.1B), Remazol Brilliant Violet 5R (C.I. reactive violet 5, Figure. 3.1C) and Remazol Brilliant Blue R (C.I. reactive blue 19, Figure. 3.1D) were obtained from Dystar Thai Ltd., Bangkok, Thailand. The dyes were used at a quality identical to that being used in the textile industry. Dye stock solutions were prepared and used in all experiments.

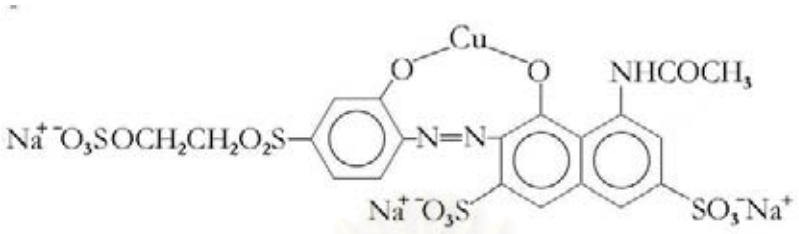
A. Remazol Brilliant Orange 3R (Monoazo-vinylsulfone structure)



B. Remazol Black B (Diazo-vinylsulfone structure)



C. Remazol Brilliant Violet 3R (Cupper complex-monoazo-vinylsulfone structure)



D. Remazol Brilliant Blue R (Anthraquinone-vinylsulfone)

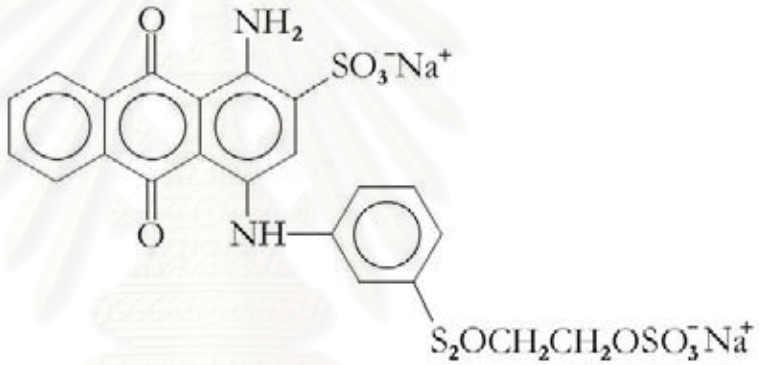


Figure. 3.1. Chemical structure of reactive dyes used in this study

Table 3.1 General properties of reactive evaluated in this work

Trade name	Generic name	CI number	Chromophore	λ_{max} (nm)	Molecular weight
Remazol Brilliant Orange 3R	reactive orange, 16	17757	Monoazo	495	617
Remazol Brilliant Violet 5R	reactive violet, 5	18097	monoazo (metal complex)	557	735
Remazol Black B	reactive black, 5	20505	diazo	595	991
Remazol Brilliant Blue R	reactive blue, 19	61200	anthraquinone	587	626

3.2 Synthetic textile wastewater (modified from O'Neill et al., 2000)

The basic composition of synthetic textile wastewater was (in g/l):

reactive dye	0.1
soluble starch	1.0
acetic acid	0.15
$(NH_4)_2SO_4$	0.28
NH_4Cl	0.23
KH_2PO_4	0.067
$MgSO_4 \bullet 7H_2O$	0.04
$CaCl_2 \bullet 2H_2O$	0.022
$FeCl_3 \bullet 6H_2O$	0.005
yeast extract	0.2
NaCl	0.15
$NaHCO_3$	1.0

and 1 ml/l of a trace element solution containing (in g/l)

$ZnSO_4 \bullet 7H_2O$	0.01
$MnCl_2 \bullet 4H_2O$	0.1
$CuSO_4 \bullet 5H_2O$	0.392
$CoCl_2 \bullet 6H_2O$	0.248
$NaB_4O_7 \bullet 10H_2O$	0.177
$NiCl_2 \bullet 6H_2O$	0.02

3.3 Bacterial isolation and cultivation

Wastewater and activated sludge samples were collected from the wastewater treatment facility of a local dyeing house and cultivated in a synthetic wastewater.under the static conditions. Three azo dyes including Remazol Brilliant Orange 3R, Remazol Brilliant Violet 5R and Remazol Black B were used as the indicators of microbial decolorization activity. Mixed culture that showed quick and stable decolorization activity was transferred to newly prepared synthetic wastewater. After five success transfers, it was plated on synthetic wastewater agar containing 100 mg/l of each dye. The plate was incubated at 30 ^{o}C in an anaerobic jar with Gaspak ($CO_2/N_2/H_2$, 80:15:5) sachets (Oxoid Ltd., Basingstoke, UK). Bacterial colonies around which clear zones expanded quickly were collected for further physiological identification.

3.4. Identification of selected reactive dye-degrading bacteria

Bacterial isolates with the greatest decolorization abilities were first examined by Gram staining, and further identifications were performed by the procedures in Bergey's manual of systematic bacteriology (Krieg and Holt, 1984).

3.5 Bacterial Inoculation

Paenibacillus sp. strain A5 and S1 were used as the indicator strains to indicate decolorization performance. To obtain synchrony in the division of cultures for study, a loopful of each strain seed taken from storage slants was precultured in 500 ml flasks containing 100 ml of synthetic wastewater without dyes at 30 ^{o}C, 200 rpm using a shaker water bath for 24 h. Total each precultured broth was then inoculated into 1 l fresh synthetic wastewater without dyes and cultivated at the same conditions. After 24 h of cultivation, the cells mass were harvested by centrifugation (8,000 rpm, 10 min) and resuspended in 0.85% NaCl solution. Equal volume of each bacterial suspension solutions were mixed together to make a defined mixed bacterial culture used as an inoculum in an anaerobic-aerobic decolorization.

3.6 Anaerobic-aerobic batch decolorization operations

Each single bacterial culture or mixed bacterial cultures was aseptically transferred into dye-containing synthetic wastewaters to reach an initial dry weight cell concentration of 0.2-0.5 g/l for color removal experiments. The anaerobic batch decolorization experiments were performed in serum bottles [300 ml total volume], seal with rubber stopper, containing 250 ml synthetic wastewater under an anaerobic-incubation condition and incubated at 30 ^{o}C by circulated thermostated water. (Figure. 3.2). To maintain anaerobic conditions during sampling bottles were stored in constant temperature cabinet flushed with nitrogen.

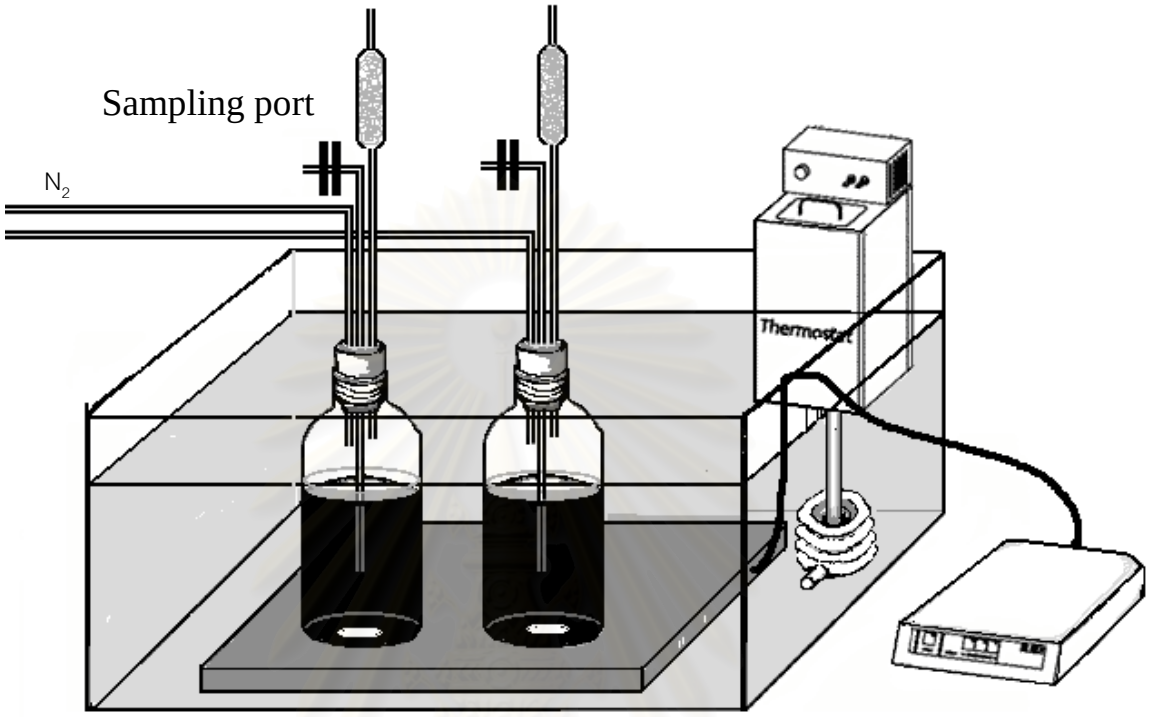


Figure. 3.2 Batch experiment use for anaerobic decolorization by bacteria

At the end of anaerobic phase (24 h), each incubated synthetic dye wastewater was aseptically transferred into 500 ml. Erlenmeyer flasks. Then the flasks were incubated at 30 ^{o}C using a shaker water bath at 200 rpm. The dye concentrations, COD values and decolorized by-products were monitored as a function of time during the batch decolorization runs.

3.7 Analytical methods

3.7.1 Dye concentration and biomass

Calibration graphs of absorbance versus dye concentration were constructed from solutions of each azo dye in synthetic wastewater for the calculation of the individual dye concentrations. Samples were withdrawn from the serum bottles with a syringe. Absorbance of the samples was measured at the maximum absorption wavelength (λ_{max}) in the visible region for each dye (λ_{max} = 495 nm for Remazol Brilliant Orange 3R, λ_{max} = 595 nm for Remazol Black B, λ_{max} = 557 nm for Remazol Brilliant Violet 5R and λ_{max} = 587 nm for Remazol Brilliant Blue R). With appropriate calibrations at specific wavelengths, concentrations of biomass and dyes were determined using a UV-visible Hitachi U 2000 spectrophotometer. The concentration of dye was primarily determined by measuring the optical density (OD) of the supernatant of the sample after centrifugation for 10 min at 8,000 rpm. A sterile cell-free synthetic wastewater was chosen as the control. As all samples contained biomass and dye, concentrations of biomass (i.e. (1) and (2)) and dye (i.e. (3)) were evaluated as follows:

1) OD_{600nm} of sample mixtures without centrifugation:
   OD_{600nm} (biomass+dye) = OD_{600nm} (dye) + OD_{600nm} (biomass)

2) OD_{600nm} of sample supernatant after centrifugation:
   OD_{600nm} (supernatant) = OD_{600nm} (dye), and

3) $OD\lambda_{max}$ of sample supernatant after centrifugation:
   $OD\lambda_{max}$ (supernatant) = $OD\lambda_{max}$ (dye)

Samples were diluted to an optical density of < 0.8 when absorbance was not within the linear range (0.2-0.8). For determination of bacterial biomass, the calibration curves between bacterial cell dry weight and optical density were made. Twenty-five ml. of bacteria suspensions at various optical density (OD_{600nm}) were concentrated and dehydrated in oven at 103 ^{0}C until getting the constant dry weight.

3.7.2 HPLC analysis of decolorization metabolites

The anaerobic and aerobic metabolites of four reactive dyes were extracted with equal volume of ethyl acetate after acidification to pH 2-3 with 6N HCl. The extracts were dried over anhydrous Na_2SO_4. HPLC analysis was carried out on a Shimadzu model LC-3A chromatograph equipped with Shimadzu model SPD-2A detector and Pegasil ODS, (4.6 mm x 150 mm [inside diameter] column, Senshu Scientific Co., Ltd., Tokyo, Japan). Mobile phase composed of 50% methanol, 0.3 % H_3PO_4, and 49.7% water with the flow rate of 0.5 ml min^{-1}. The effluents were monitored by UV absorption at 275 nm.

3.7.3 Chemical oxygen demand (COD)

COD was measured by closed reflux titrimetric method following Standard methods (5220 C). See appendix 4 for details.

3.8 Data treatment.

For calculation of specific decolorization rate and specific growth rate, the experimental data were smooth with Microsoft© Excel™. Stepwise cubic spline functions g(t) (Reinsch, 1967) were applied in order to fit the following conditions, for every couple of experiment points (t_i, x_i):

$$\int_{t_0}^{t_n} g''(t_i)^2\,dt \text{ minimum } \& \quad \sum_{i=0}^{i=n} \left(\frac{g(t_i) - x_i}{\sigma(x_i)} \right)^2 < S$$

where,

x_i = experimental biomass concentration or dye concentration at time t_i
t_i = sampling times
n = number of total experimental points
S = smoothing constant.
It determines how remote can the smoothed curve be from the experiment points. To choose this constant, the condition should be done as the following directives:

- g" (t) is ture when a peak corresponds to decolorization of dyes
- g (t) should not overpass the error bars of the experimental points.
- The minimum value of S matching the former two conditions was maintained.

The macro was built to capable of choosing S value manually, examining its impact on the curves in real time. Smoothed data led to determination of specific growth rate (μ_i) or specific decolorization rate (r_{dye}):

$$\mu_i = \frac{1}{x_i}\left(\frac{dx}{dt}\right)_i = \frac{g'(t_i)}{g(t_i)}$$

$$r_{dye} = \frac{1}{x_i}\left(\frac{d[dye]}{dt}\right)_i = \frac{g'(t_i)}{g(t_i)}$$

3.9 Decolorization under different operation and medium conditions

After aerobic cultivation on synthetic wastewater, early stationary cultures of decolorizing bacteria were centrifuged (8,000 g, 10 min) to harvest bacterial cells, which were subsequently transferred into synthetic wastewater containing reactive dye for removal of dye color. The decolorization experiments were performed under static-incubation condition (i.e., neither aeration nor agitation was employed). The decolorization was done at different initial cell concentration (75-600 mg/l), temperatures (22-60 ^{0}C), pH values (4.0-11.0), initial dye concentration (50-1,000 mg/l), nitrate concentration (0-1,000 mg/l) and copper concentration (0-200 mg/l). The experiments were operated at 30 ^{0}C, pH 7.0, and with an initial dye concentration of 100 mg/l.

3.10 Analysis of color removal in the medium containing mixture of dyes

All selected dyes including Remazol Brilliant Orange 3R, Remazol Black B, Remazol Brilliant Violet 5R and Remazol Brilliant Blue R, each at a concentration of 100 mg/1, were dissolved together in synthetic wastewater medium. The mixture of dyes did not have a well-defined peak at the visible absorption spectra. Therefore, the detection of color level was made using the alternated American Dye Manufacturers Institute (ADMI) Method (Eaton et al., 1995). Spectrophotometric measurements were made with a Hitachi spectrophotometer, model U-2000 (see appendix 2 for detail).

3.11. Determination of decolorization of individual dyes in the mixture of dyes

The spectrophotometric determination of four reavtive dyes can be a difficult problem in view of the complexity of their absorption spectra. The results from the univariate calibration show significant errors, due to the multiple interferences mentioned above. By using a multivariate calibration the accuracy is increased significantly, because by monitoring various wavelengths the contribution of the interference signals is greatly reduced.

The analytical methodology for determination of dye mixture1 (Remazol Brilliant Orange 3R, Remazol Black B and remazol Brilliant Violet 5R) and dye mixture 2 (Brilliant Orange 3R, Remazol Brilliant Violet 5R and Remazol Brilliant Blue R) using a spectrophotometric method and a multivariate calibration technique (partial least-squares regression, PLSR). The calibration was made by by PLSR, using the recorded adsorbance values between 400 and 700 nm as the dependent variables, with intervals of 10 nm.

Hitachi spectrophotometer, model U-2000 was used for multi-components of dye determination because it has some unique features: standard curves for multivariate calibration and multi-component value calculation equations are installed in this instrument (see appendix 3 for more detail).

Chapter 4

4.1 Enrichment and isolation of reactive dyes-decolorizing bacteria

A microorganism was newly isolated from wastewater and soil samples collected in textile industries in Thailand by a synthetic textile wastewater medium containing four reactive dyes (Remazol Brilliant Orange, Remazol Brilliant Violet, Remazol Black B and Remazol Brilliant Blue R). Soils or wastewaters were added in the medium. The cultivation was carried out in serum bottles containing 250 ml of synthetic watewater medium. From the moment that the decolorization was observed, 100 µl of the culture was transferred to newly prepared medium. After five transfers, it was plated on synthetic wastewater agar containing all four reactive dyes. The plate were incubated at 30 ^{0}C under aerobic and anaerobic incubation. After 2 days, some colonies made clear zones around themselves under anaerobic condition, indicating decolorization of all reactive dyes. Growth of cultures and color of inoculated plates during growth was compared with that of the uninoculated plates, i e. with the original color at 0 h. before the isolates growth.

Under aerobic condition, many bacterial isolates were found to capable of growing in synthetic wastewater medium containing all four dyes. However, no decolorization of dyes was apparent, either in solid or liquid cultures under aerobic cultivation after two weeks. Bacterial colonies under aerobic condition is shown in Figure 4.1. On the other hand, six bacterial isolates were isolated from the anaerobic system and all of them were found capable of decolorizing dyes as shown in Figure 4.2.

Bacteria colonies surrounded by an almost decolorized zone were isolated and then tested for color removal capability using submerge cultures, among these colonies, six of them with the highest decolorization ability in synthetic wastewater medium, designated as S1, A1, A2, A4, A5 and A6, were selected for a further study. Decolorization of various dyes by the growing cells of the six isolates were shown in Table 4.1.

Among the 9 reactive dyes, monoazo and diazo dyes were decolorized by all the strains only under anaerobic condition, while anthraquinone dye was reduced slightly under both anaerobic and aerobic incubation. The effectiveness of all the six isolates in decolorizing these 9 dyes may depend on the structure and complexity of the dyes, particularly on the nature and position of substituent in the aromatic rings and the resulting interactions with the azo bond (Zimmerman et al., 1982). The most monoazo dyes except Remazol Brilliant Violet 5R, a metal complex monoazo dye, had color removal higher than diazo dyes and anthraquinone dyes test under the anaerobic incubation. The different efficiency may be due to the number of azo groups.

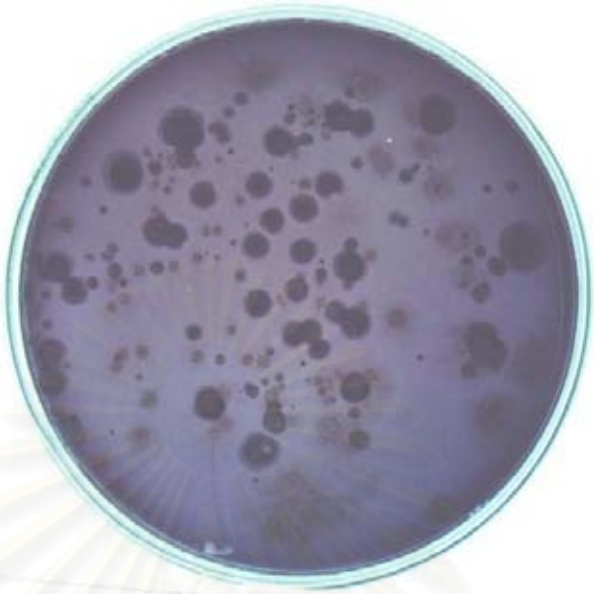


Figure 4.1 Characterization of bacterial isolates on synthetic textile wastewater agar under aerobic incubation for 48 h

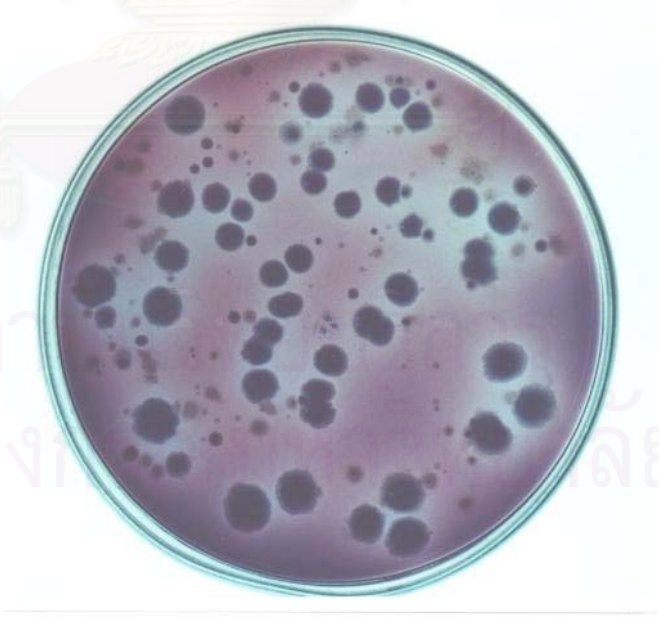


Figure 4.2 Characterization of bacterial isolates on synthetic textile wastewater agar under anaerobic incubation for 48 h

Table 4.1 Decolorization of textile dyes by various decolorizing-bacteria

Dyes	Chromophores	λ_{max} (nm)	Decolorization (%) at initial dye concentration (100 mg/l)											
			S1		A1		A2		A4		A5		A6	
			AN*	AE	AN	AE	AN	AE	AN	AE	AN	AE	AN	AE
Remazol Brilliant Orange 3R	monoazo	495	++	-	++	-	++	-	+	-	++	-	++	-
Remazol Brilliant Violet 5R	monoazo	557	+	-	+	-	-	-	-	-	++	-	+	-
Remazol Brilliant Orange 3G	monoazo	487	++	-	+	-	+	-	+	-	++	-	++	-
Remazol Golden Yellow 5	monoazo	420	++	-	+	-	+	-	+	-	++	-	++	-
Remazol Red 3B	monoazo	520	+	-	+	-	+	-	-	-	++	-	++	-
Remazol Black B	diazo	595	++	-	+	-	+	-	+	-	++	-	+	-
Remazol Red RB	diazo	525	+	-	-	-	-	-	-	-	+	-	+	-
Remazol Navy Blue GG	diazo	590	++	-	+	-	+	-	+	-	++	-	+	-
Remazol Brilliant Blue R	anthraquinone	587	+	+/-	+	+	+	+	+	+	+	+	+	+

Six decolorizing strains were incubated at 30 ^{0}C under static or shaking conditions in synthetic wastewater containing 100 mg/l of the various dyes. After 24 and 72 h of incubation, the decolorization of each dye was determined by measuring the decrease of absorbance in the supernatant fraction at the absorption maximum of the dye by using a UV/visible spectrophotometer.

AN = anaerobic incubation, AE = aerobic condition
++ = completely decolorization within 24 h.
+/- = significantly decolorization within 72 h.
- = no decolorization

4.2 Strain identification of decolorizing bacteria

Two of the colonies were isolated as the azo dyes-decolorizing strains. Two strains (S1 and A5) were selected for further study as the most active reactive dyes-decolorizing microorganisms. Both of them are Gram-variable, endosopre-forming rods, facultatively anaerobic and nitrate reduction positive. Catalase and oxidase were positive. No strain produced special pigments. According to taxonomic criteria in Bergey's manual of systematic bacteriology, they belong to *Bacillus* due to the morphological and physiological properties. For instance, strain S1 and A5 were designed as *Bacillus* sp. strain S1 and A5, respectively. The morphology, physiology and biochemistry characteristics of strain S1 and A5 were summarized in Table 4.2.

Table 4.2 Biochemical and morphological characteristics of reactive dyes-decolorizing bacteria

Characteristics	**Results**	
	Strain S1	**Strain A5**
Bacteriological test		
Gram reaction	Variable	Variable
Shape	rod	rod
Endospore former	+	+
Growth tests		
Glucose	+	+
Galactose	+	+
Maltose	+	-
Sorbitol	-	+
Manitol	+	+
Sucrose	+	+
Xylose	+	+
Simmon citrate	-	+
Biochemical tests		
Oxidase	+	+
Catalase	+	+
MacConkey	-	-
Nitrate reduction	+	+

Table 4.2 (continue)

Characteristics	Results	
	Strain S1	**Strain A5**
Lysine decarboxylation	A/A	A/A
TSI agar	K/A	K/K
Urease	-	-
Ornithine decarboxylation	-	-
Gelatinase	-	-
MR test	+	+
VP test	-	-
Indole formation	-	-
Hydrogen sulfide	-	-

+: positive; -: negative

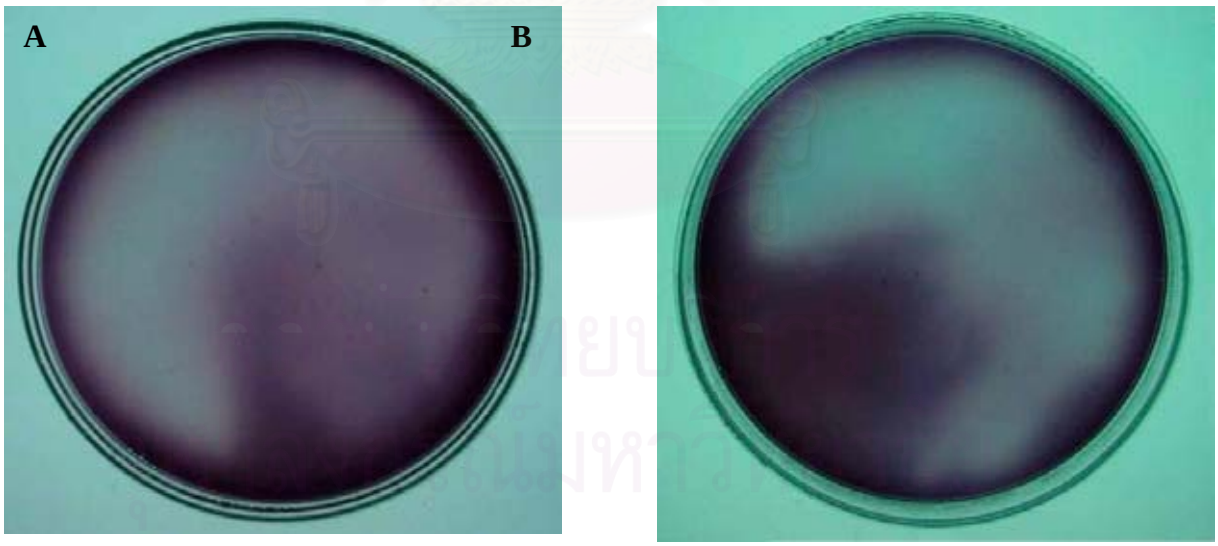


Figure 4.3. Appearance of clear zones around colonies of bacterial isolate (**A**) strain A5 and (**B**) strain S1 on synthetic textile wastewater agar containing mixture of azo dyes (Remazol Brilliant Orange 3R, Remazol Black B and Remazol Brilliant Violet 5R) under an anaerobic condition for 24 h

4.2.1 16S rRNA gene sequence analysis of strain A5 and S1

The members of the genus *Bacillus* are widely distributed in nature and have physiologically diverse characteristics (Claus and Berkeley, 1986). Traditionally, lack of incisive differentiating criteria caused all aerobic, endospore-forming bacteria to be classified as *Bacillus*. In addition, the absence of discriminating assays has limited recognition of new species, and promoted grouping of microorganisms to show clear-cut differences. Identification of *Bacillus* species remains difficult for most bacteriologists because of taxonomic and characterization problems. To alleviate the latter, the identification schemes of *Bacillus* sp. have been developed by using the molecular biological methods. By using 16S rRNA gene sequencing, the genus *Bacillus* cloud be separated into several distinct genera such as *Alicyclobacillus*, *Aneurinibacillus*, *Brevibacillus*, *Halobacillus*, *Paenibacillus* (Meehan, et al., 2001) and *Virgibacillus* (Yoon et al., 2002). There is now general agreement that the classification and identification of Gram-positive, endospore-forming rods should be performed by using a polyphasic taxonomic approach that integrates phylogenetic analysis based on 16S DNA sequence, genomic relatedness and extensive phenotypic characteristics (Yoon et al., 2002).

To correctly classify the genus of strain S1 and A5, 16S rRNA gene sequence analysis was carried out to identify the genus of both strains. 16S rRNA gene sequencing of strain S1 and A5 were carried out using protocols and universal primers as a detail for the Microseq 16S rDNA Bacterial Identification System (Applied Biosystems) and then compared with the 16S rRNA gene sequence obtained from the GenBank databases. The comparison result of 16S rRNA sequences of strain S1 and A5 with each of other sequences available in GenBank database are shown in Table 4.3.

Table 4.3 16S rRNA gene sequence similarities to 16S rRNA gene sequence of strain S1 and A5

Species name	Accession no.	Similarity (%)
Paenibacillus macerans	AB073196.1	97
Paenibacillus sp. WN9	AF164345.1	97
Paenibacillus turicensis clone	AF378700.1	97
B3Paenibacillus polymyxa	AY135394.1	97
Paenibacillus cf *polymyxa*	AF181573.1	97
Paenibacillus sp. LCSAOTU12	AF506061.2	97
Paenibacillus turicensis clone A	AF378697.1	97

In order to identify the strain S1 and A5, partial 16S rRNA gene sequences were determined, which had highest similarities with *Paenibacillus macerans* (97%) (Table 4.3). Somewhat lower matching sequence identities (97%) also occurred with other *Paenibacillus* isolates (Table 4.3).

In conclusion, the phenotypic data and 16S rRNA gene sequences clearly demonstrated that strain S1 and A5 are member of the genus *Paenibacillus*. Furthermore, the special phenotypic properties of both strains are:

Sporulation induced by the addition of manganese (Mn^{2+})

Menaquinone as the sole quinone components

Elongated-cell phenotypes found under anaerobic condition.

4.3 Special phenotypic properties of *Paenibacillus* sp. strain S1 and A5

4.3.1 Sporulation induced by the addition of manganese (Mn^{2+})

Typically, bacterial species of *Paenibacillus* are endospore-forming Gram-positive rod bacteria. Strain A5 and S1, however, sporulation of these strains have not been found during the classical cultivation on enriched culture medium (Luria Bertani medium) nor minimum culture medium (synthetic textile wastewater). Moreover, the Gram's reaction of both strains is usually variable depending on their growth phases and have been rather considered to be Gram-negative than positive.

To clarify this unusual property of both strains, manganese (Mn^{2+}) was added into the culture medium of both strains to induce the sporulation. The cells were taken from cultures in sporulation medium (synthetic textile wastewater containing $MnSO_4 \bullet H_2O$ 5 mg/l) 24 h after the onset of sporulation and determined by light microscopy.

The results in Figure 4.4A and 4.5A show that strain A5 and S1 could not produce the endospore during growth in the culture medium without Mn^{2+}. On the other hand, single ellipsoidal endospore in the subterminal/terminal region of the cells of both strains were observed in the culture medium containing manganese. Light micrographs showing spores produced by the strains A5 and S1 are shown in Figure 4.4B and 4.5B, respectively. This finding confirms that the strain A5 and S1 are similar to other *Paenibacillus* sp. which capable of production their endospores.

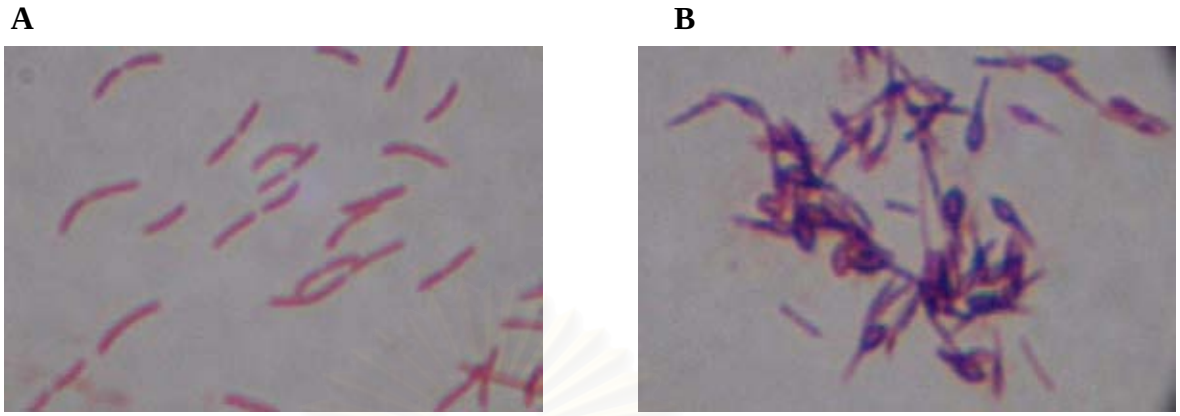
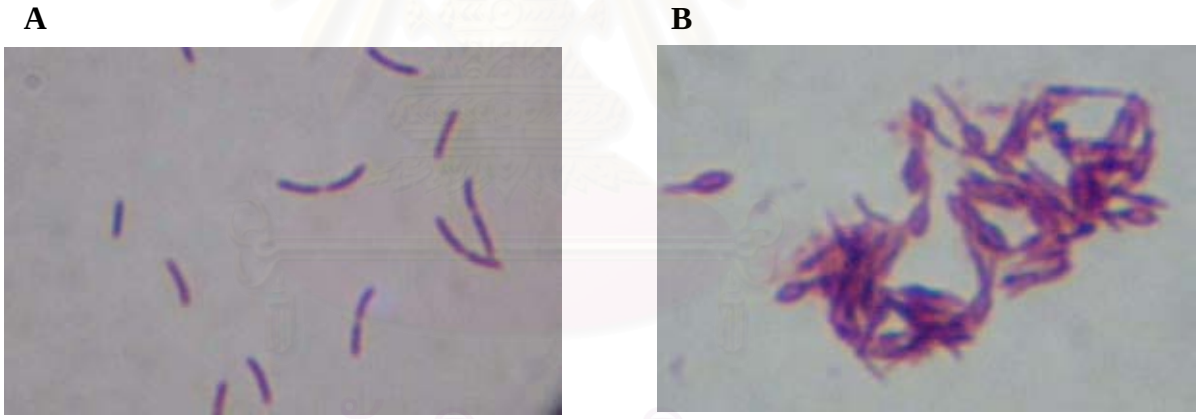


Figure 4.4 Characteristics of *Paenibacillus* sp. strain A5 after grown on the synthetic textile wastewater under aerobic condition for 24 h; (**A**) in the absence of Mn^{2+} and (**B**) in the presence of Mn^{2+}

Figure 4.5 Characteristics of *Paenibacillus* sp. strain S1 after grown on the synthetic textile wastewater under aerobic condition for 24 h; (**A**) in the absence of Mn^{2+} and (**B**) in the presence of Mn^{2+}

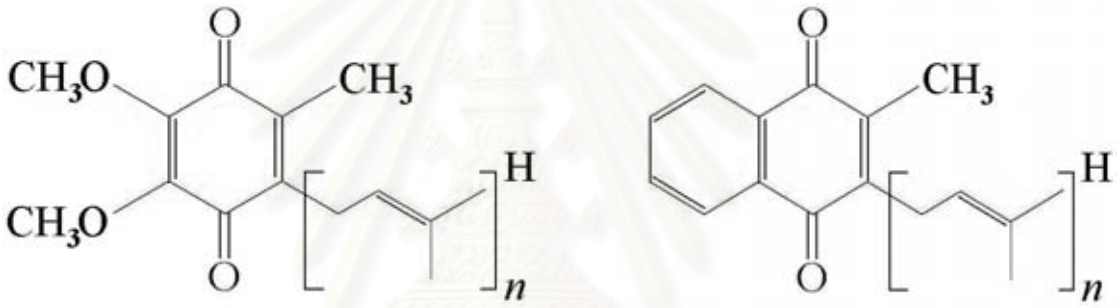


The induction of sporulation of *Paenibacillus* sp. by using of Mn^{2+} have been formery reported (Meehan et al., 2001). The ability of Mn^{2+} to stimulate the bacterial sporulation is involving to the preparation of spore coat proteins (Henriques et al., 1998).

Archibald and Fridovich (1982) suggested that cells of *Bacillus* sp. in the early sporulation phase and/or premature spores are sensitive to superoxide anion (O_2^-) and also that both superoxide dismutase (SOD) and Mn^{2+} protect sporulating cells from oxidative stress. Manganese is known to have a SOD-like activity in vitro, and several Mn^{2+} complexes and manganous porphyrins catalyze the elimination of O_2^- (Archibald and Fridovich, 1982). A Mn-dependent SOD encoded by the *sodA* locus of *B. subtilis* was found to be associated with spore coat proteins (Henriques et al.,1998).

4.3.2 Menaquinone as the sole quinone components

Quinone profile technique has been used to clarify the classification of some bacterial groups (Meganathan and Coffell, 1985). Quinone, a constituent of the bacterial respiratory chain, exists in almost all bacteria and plays an important role in electron transport. The major species of quinone in bacteria cell are ubiquinone (Q-n) and menaquinone (MK-n), respectively, where n is the length of the isoprene unit of side chain (Figure 4.6). Menaquinone is a lipophilic naphthoquinone with a prenyl side chain of variable length that participates in the transfer of electrons to low-potential electron acceptors and normally found within the lipid bilayer of bacterial cell membrane (Newman and Kolter, 2000).



Ubiquinone (Q-n) Menaquinone (MK-n)

Figure 4.6. Molecular structures of ubiquinone (Q-n) and menaquinone (MK-n)

Because each bacterium has one predominant quinone, thus the quinone determination is able to carry out in bacteria systematic studies (Meganathan and Coffell, 1985). Every *Bacillus* sp. so far examined contains menaquinone as the sole quinone component and general absence of ubiquinone (Meganathan and Coffell, 1985). Most of *Paenibacillus* sp. contains MK-7 as the predominant menaquinone (Yoon et al., 2002). To confirm the identification results, *Paenibacillus* sp. strain A5 and S1 were also determined their quinone species present in their plasma membrane.

Determination of quinone component(s) in plasma membrane

Paenibacillus sp. strain A5 cells were grown either aerobically or anaerobically in 100 ml. volumes. Cells were harvested by centrifugation for 10 min at 10,000 x *g* after the cultures reached an $OD_{600} \approx 2.0$. Pellets were resuspended in 6 ml chloroform:methanol (2:1, v/v) and the suspension was gently mixed overnight in complete darkness at room temperature. The suspension was filtered though Whatman no. 1 filter paper and filtrate was evaporated under reduced pressure. The residue was resuspended in ethyl acetate, and solution was spotted on Silica Gel 60 F254 aluminum-packed TLC plates (E. Merck, Germany). Menaquinone (MK-1) and ubiquinone (Q1) (Sigma Chemical Co., St. Louis, Mo.) were used as standards. Samples were eluted with a mixture of n-hexane and diethyl ether (85:15, v/v). Quinones were detected on TLC plates by brief irradiation with short-wave ultraviolet light (Meganathan and Coffell, 1985).

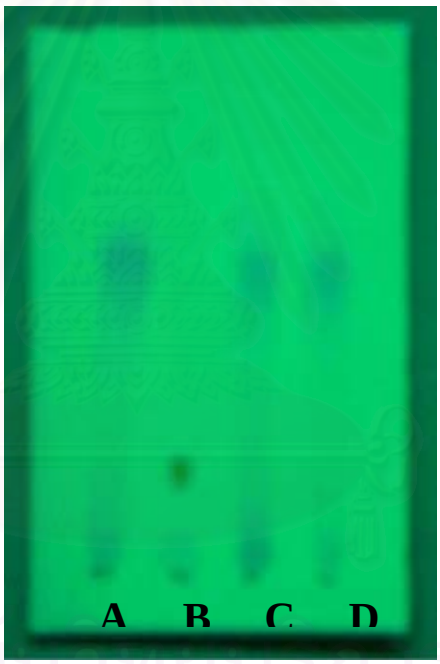


Figure 4.7. Thin-layer chromatogram of quinone species of *Paenibacillus* sp. strain A5 and S1. (**A**) standard menaquinone (**B**) standard ubiquinone (**C**) quinone from *Paenibacillus* sp. strain A5 (**D**) quinone from *Paenibacillus* sp. strain S1

Like all the other bacilli, lipid extraction of culture grown aerobically showed that only menaquinone was found in *Paenibacillus* sp. strain A5 and S1 (as assayed by thin layer chromatography) (Figure 4.7).

4.3.3 Elongated-cell phenotypes found under anaerobic condition

Incubation the log-phase cultures of *Paenibacillus* sp. strain A5 and S1 under anaerobic condition led to the formation of the long filamentous, aseptate cells (Figure 4.8 and 4.9). Septation and division resume immediately after exposure to oxygen. These findings suggest that anaerobic condition have an indirect effect on the general division machinery of bacterial cells, probably via cellular proteins involved in an early stage of cell division.

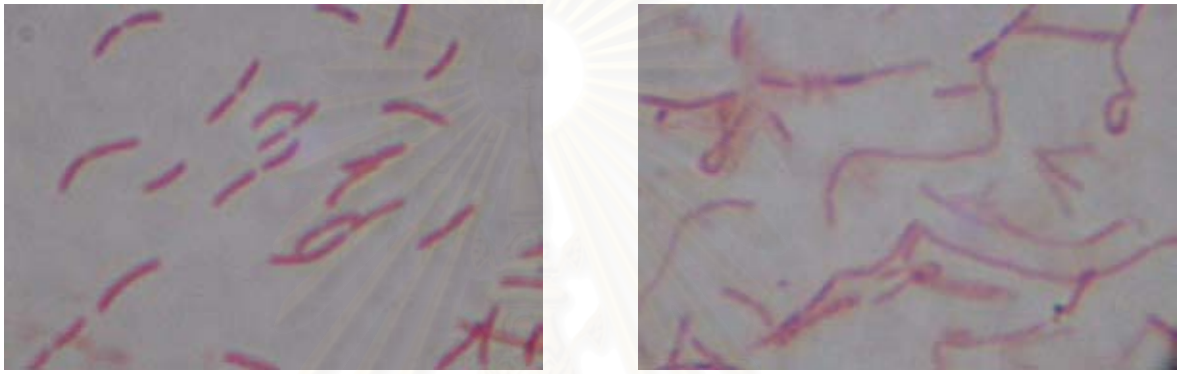


Figure 4.8 Morphology of *Paenibacillus* sp. strain A5 cells under an aerobic and anaerobic environment (**A**) Light-micrograph showing normal-size after aerobic incubation for 24 h. (**B**) Light-micrograph showing filamentous cells after anaerobic incubation for 24 h

Figure 4.9 Morphology of *Paenibacillus* sp. strain S1 cells under an aerobic and anaerobic environment (**A**) Light-micrograph showing normal-size after aerobic incubation for 24 h. (**B**) Light-micrograph showing filamentous cells after anaerobic incubation for 24 h

Many bacteria divide by binary fission, forming medially positioned septa and identical daughter cells. The process of cell division is governed by several gene products, the most conserved of which appears to be the tubulin-like protein FtsZ (Lowe and Amos, 1998). Prior to septation, FtsZ polymerizes as a ring structure at the nascent division site. Formation of the FtsZ ring is essential but not sufficient for invagination of the cell wall (Levin et al., 1997).

Ramirez and co-worker (2001) found that the expression of *Neisseria gonorrhoeae* cell division genes, ftsZ, ftsE and minD is influenced by environmental conditions. These three genes are upregulated under anaerobiosis.

Maier and co-workers (1999) proposed that polyphosphate (polyP) may have an effect on the ubiquitous bacterial cell division protein FtsZ, whose GTPase activity is known to be strictly dependent on divalent metal ions. It is tempting to speculate that polyP, because of its metal ion-chelating nature, indirectly blocks the dynamic formation (polymerization) of the Z ring, which would explain the aseptate phenotype.

As mentioned above, it can be hypothesized in this study that inhibition of Fts proteins may be responsible for anaerobic-induced filamentous phenotype of *Paenibacillus* sp. strain A5 and S1.

Chapter 5

Characteristics of bacterial decolorization of reactive dyes by an anaerobic-aerobic treatment system

5.1 Decolorization of Remazol Brilliant Orange 3R

The results obtained for the treatment of Remazol Brilliant Orange 3R by using single strain A5, S1 and mixed culture of strain S1 and A5 are shown in Figure. 5.1, 5.2 and 5.3, respectively. From these figures, it can be seen that the color was completely removed in the anaerobic phase. The mixed culture had higher decolorization ability and faster decolorization than the single strains. A rapid decrease in dye concentration during first 10 min of incubation occurred as a result of abiotic decolorization. This resulted in an actual initial dye concentration of approximately 80-85 mg/l for bacterial decolorization. The bacterial biomass increased slowly in anaerobic phase but increased more rapidly in a subsequent aerobic phase. The majority of COD was removed in the aerobic phase. The major COD in synthetic wastewater are due to soluble starch, acetic acid and yeast extract, all of which are known as anaerobically and aerobically biodegradable. In this study, bacteria aerobically oxidized most energy source available in synthetic wastewater for cellular growth and maintenance resulting in rapid COD removal in aerobic phase.

The HPLC analysis performed to supernatants taken during the different duration of the anaerobic and aerobic phase showed the correspondence of the evolution of decolorizing-metabolite to the color removal. The chromatographic peak areas corresponding to the dye-degradation metabolite (retention time = 5.3 min) increased as incubation time of anaerobic phase increased. At the end of aerobic phase, the HPLC analyzes seem to indicate that the decolorizing-metabolites produced during the anaerobic phase were decreased in the subsequent aerobic phase.

The specific decolorization rates of Remazol Brilliant Orange 3R by single and mixed culture of strain A5 and S1 are shown in Figure 5.4. In the case of mixed culture, it is clearly shown that not only the highest specific decolorization rate was achieved but also no lag phase of decolorization was presented. In contrast, the lag period of 3 and 6 h were observed in the decoloization of the orange dye by the single strain A5 and S1, respectively. These results show the synergism effects of strain A5 and S1 on the decolorization of Remazol Brilliant Orange 3R.

The characteristic of synthetic textile wastewater before and after anaerobic-aerobic incubation of the mixed culture were shown in Figure 5.5.

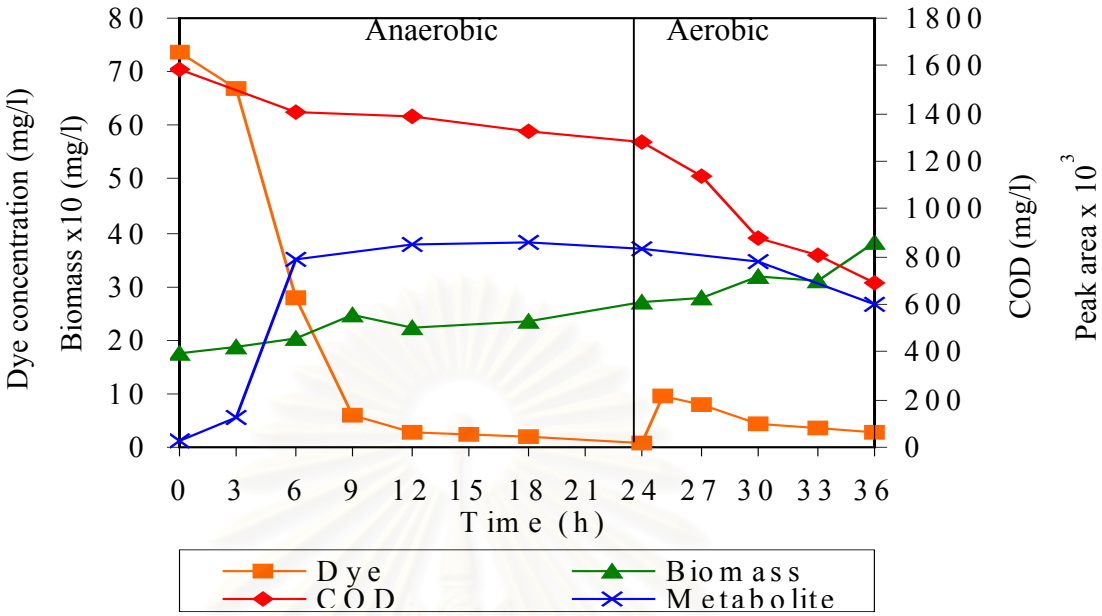


Figure. 5.1 Time-course profile of Remazol Brilliant Orange 3R, biomass, COD and decolorized metabolite (retention time = 5.3 min) during decolorizing cultivation of *Paenibacillus* sp. strain A5 in synthetic textile wastewater under anaerobic and aerobic condition

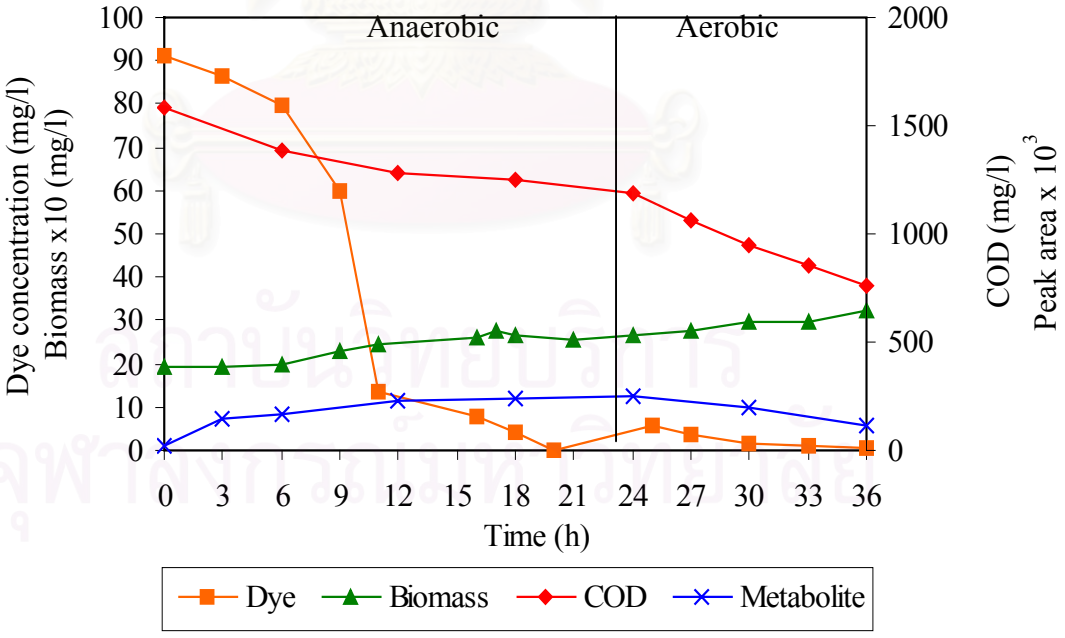


Figure. 5.2 Time-course profile of Remazol Brilliant Orange 3R, biomass, COD and decolorized metabolite (retention time = 5.3 min) during decolorizing cultivation of *Paenibacillus* sp. strain S1 in synthetic textile wastewater under anaerobic and aerobic condition

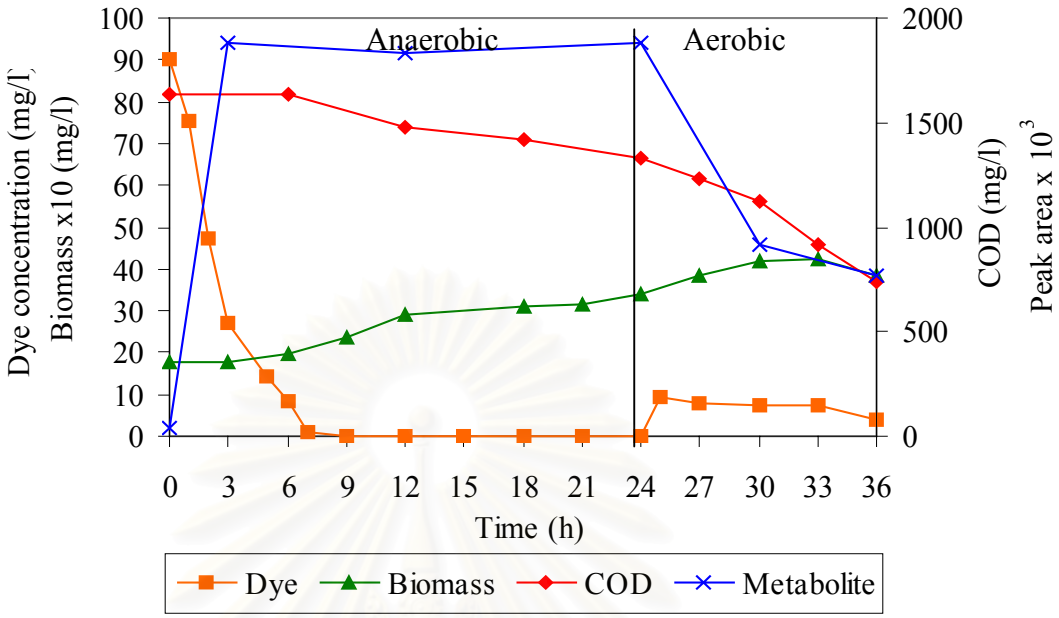


Figure. 5.3 Time-course profile of Remazol Brilliant Orange 3R, biomass, COD and decolorized metabolite (retention time = 5.3 min) during decolorizing cultivation of a mixed culture of *Paenibacillus* sp. strain S1 and A5 in synthetic textile wastewater under anaerobic and aerobic condition

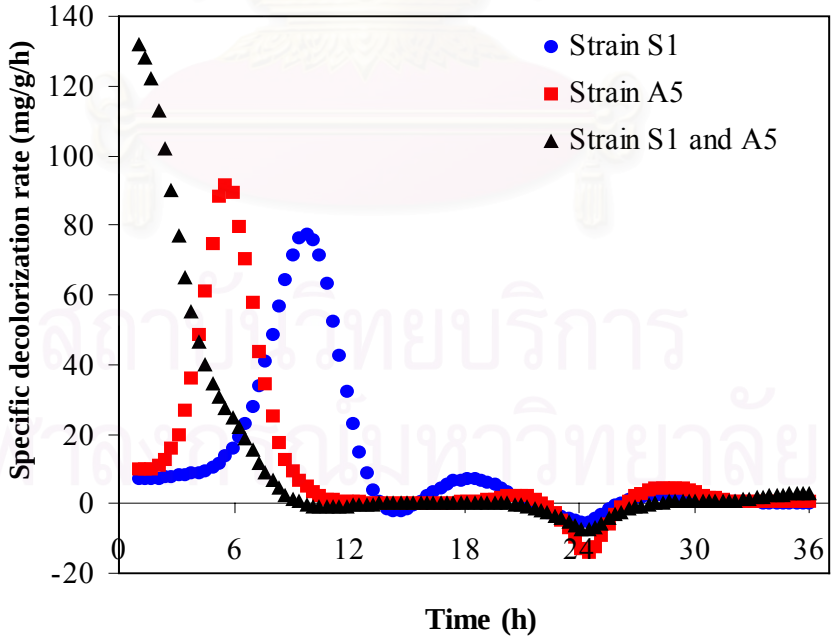


Figure 5.4 Time courses of specific decolorization rate of Remazol Brilliant Orange 3R by single and mixed culture of *Paenibacillus* sp.strain A5 and S1

Figure 5.5. Characteristics of synthetic wastewater containing of Remazol Brilliant Orange 3R before and after batch decolorization by the mixed culture of strain S1 and A5

UV-visible spectra (Figure. 5.6) obtained for the filtered samples of mixed culture (*Paenibacillus* sp. strain A5 and S1) showed marked alterations with reaction time. These changes could be explained by structural modifications of dye molecule due to azo bond reduction as under anaerobic conditions many types of bacteria perform this reaction (Wuhrmann et al., 1980), producing metabolites such as aromatic amines. In order to clarify this phenomenon, the ethyl acetate extracts taken during anaerobic and aerobic phases of decolorization operations of Remazol Brilliant Orange 3R were analyzed by HPLC.

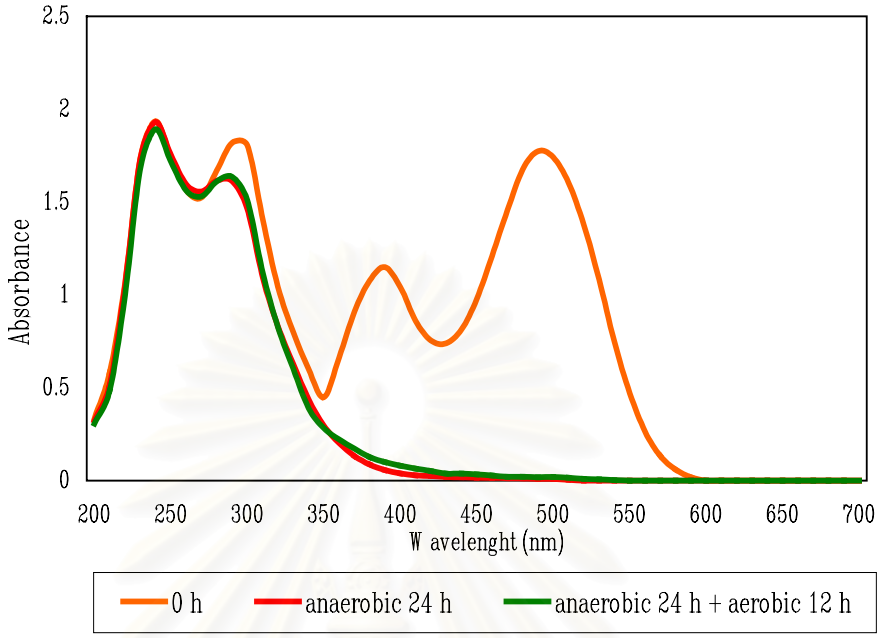


Figure. 5.6 Typical UV-visible spectra of supernatants from batch decolorization of Remazol Brilliant Orange 3R by the mixed culture of strain S1 and A5 at various reaction times.

Figure 5.7 shows the result of HPLC analysis on the decolorizing metabolites of Remazol Brilliant Orange 3R by mixed bacterial culture. At the beginning of anaerobic incubation, the parent compound was not detected under these chromatographic conditions. After anaerobic incubation for 24 h, the intensity of peaks A and B (retention time = ca. 5.3 min and 6.6 min, respectively) increased significantly. As the decolorization proceeded (Figure 5.1, 5.2 and 5.3), the area of peak A increased along with a decreased in concentration of Remazol Brilliant Orange 3R. At this point, It is thus reasonable to assume that the mixed culture caused cleavage of the azo bond of Remazol Brilliant Orange 3R, which decomposed to form two aromatic amines represented by peak A and B, respectively. It should be noted that due to the unavailability of authentic standards, the chromatographic peaks appearing in samples taken during the anaerobic phase could not be identified or quantified. However, the HPLC patterns of decolorizing metabolites in this study are similar to the HPLC patterns of metabolites formed from the decolorization of an azo dye, Reactive Red 22 (Chang et al., 2001).

At the end of aerobic phase, the HPLC analyzes seem to indicate that the decolorizing-metabolites produced during the anaerobic phase were mineralized in the subsequent aerobic phase. Figure 4.25B and 4.25C also shows that when such metabolites were degraded aerobically they formed less aromatic, more polar compounds, since the metabolite peak area decreased and shifted towards lower retention time (3.7 min). Degradation in the aerobic stage may result in the formation of oxidized and very polar derivatives (e.g., aldehydes, carboxylic acids) having a lower aromaticity, as suggested by Nortemann et al. (1986) in a study of 6-aminonaphthalene-2-sulfonic acid degradation. No new UV absorbance was found in uninoculated controls nor inoculated synthetic wastewater without dye controls.

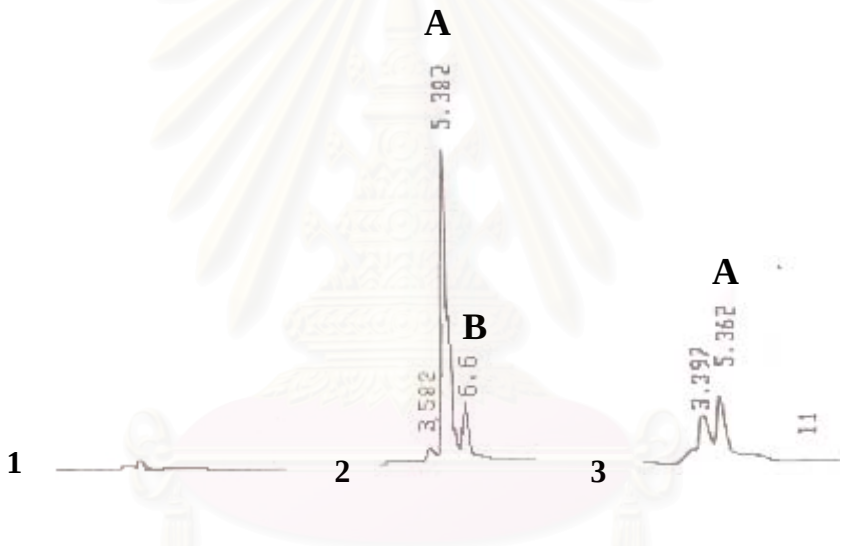


Figure. 5.7 The HPLC analysis on metabolites resulting from decolorization of Remazol Brilliant Orange 3R under anaerobic-aerobic condition **(1)** at the beginning of anaerobic incubation **(2)** after anaerobic incubation for 24 h, and **(3)** after aerobic incubation for 12 h

5.2 Decolorization of Remazol Black B

The typical profiles for Remazol Black B and COD removal and its decolorzing-metabolite revolution by using *Paenibacillus* sp. strain A5, S1 and mixed culture of strain S1 and A5 are represented in Figure 5.8, 5.9 and 5.10, respectively. Similar with what has been revealed for the decolorization of the Remazol Brilliant Orange 3R that the majority of color was removed in the anaerobic phase and the majority of COD was removed in the aerobic phase. However, the specific decolorization rate of Remazol Black B of individual single strains were slower than the specific decolorization rate of the mixed culture of both strains at the same incubation time (Figure 5.11). The ethyl acetate extracts taken during anaerobic and aerobic phases of decolorization operations of Remazol Black B were analyzed by HPLC. The result of HPLC analysis on the decolorization metabolites of Remazol Black B by the single and mixed bacterial culture showed that after anaerobic incubation for 24 h, the intensity of major metabolite peak (retention time = ca. 5.5 min) increased significantly. It is thus reasonable to suggest that this peak represent the decolorization metabolites, confirming the formation of additional aromatic metabolites. The characteristic of synthetic wastewater before and after anaerobic/aerobic incubation of mixed culture were shown in Figure 5.12.

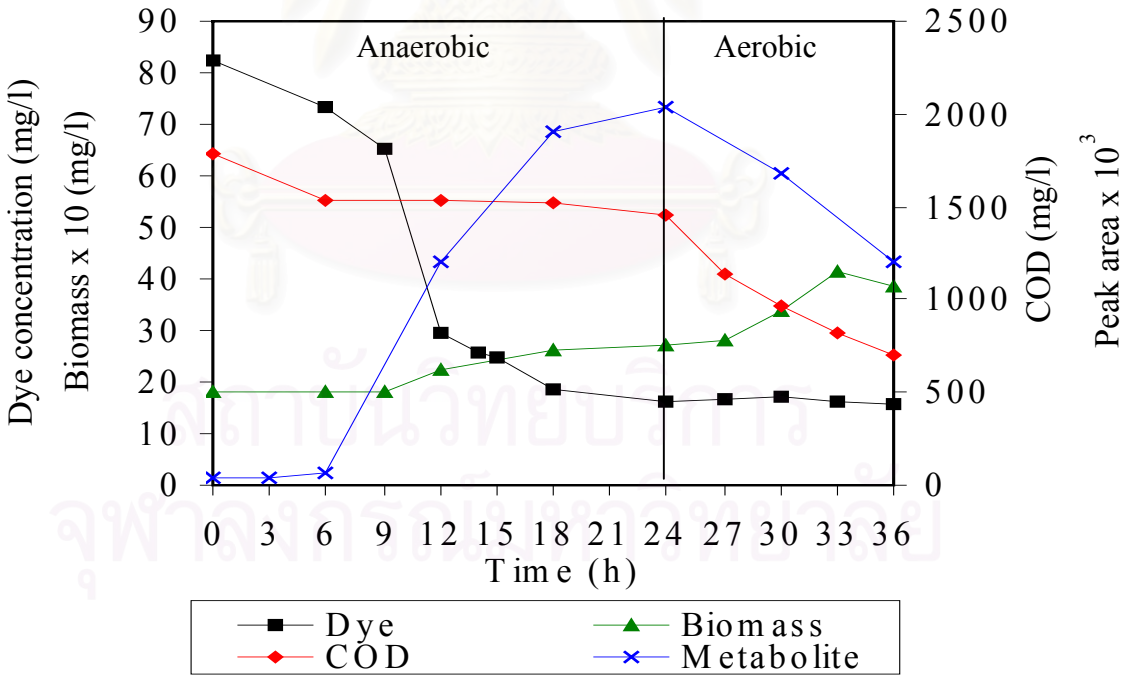


Figure. 5.8 Time-course profile of Remazol Black B, biomass, COD and decolorized metabolite (retention time = 5.3 min) during decolorizing cultivation of *Paenibacillus* sp. strain A5 in synthetic textile wastewater under anaerobic and aerobic condition

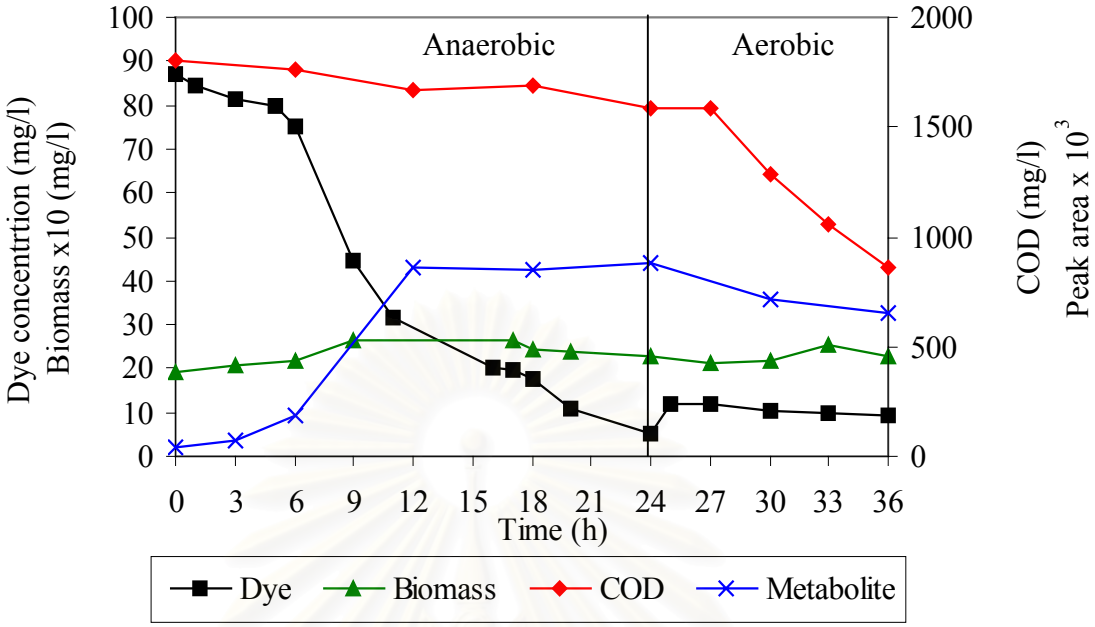


Figure. 5.9 Time-course profile of Remazol Black B, biomass, COD and decolorized metabolite (retention time = 5.3 min) during decolorizing cultivation of *Paenibacillus* sp. strain S1 in synthetic textile wastewater under anaerobic and aerobic condition

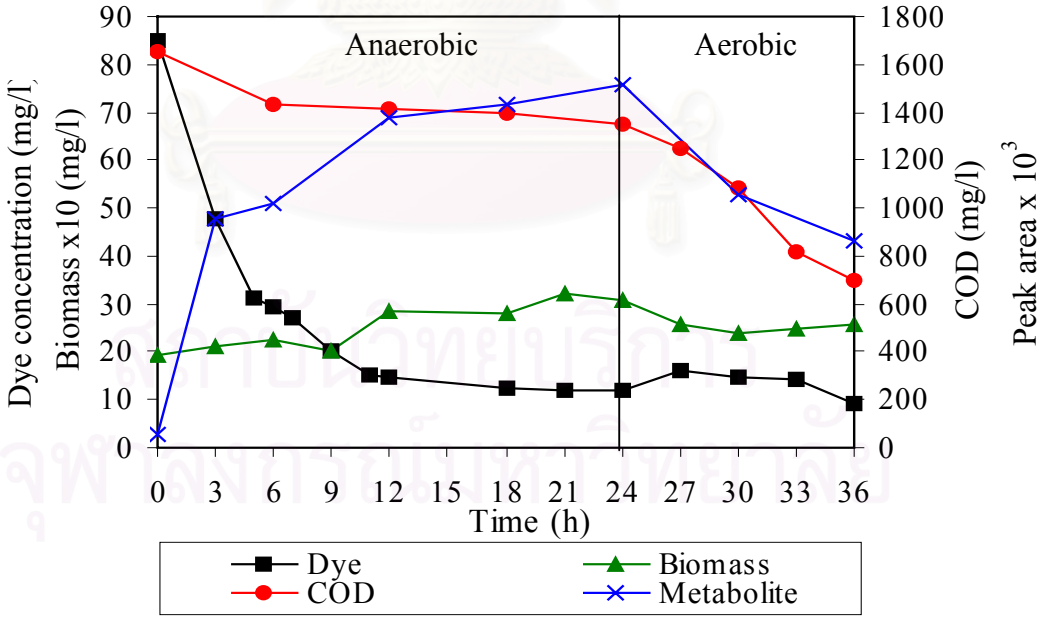


Figure. 5.10 Time-course profile of Remazol Black B, biomass, COD and decolorized metabolite (retention time = 5.3 min) during decolorizing cultivation of the mixed culture of *Paenibacillus* sp. strain S1 and A5 in synthetic textile wastewater under anaerobic and aerobic condition

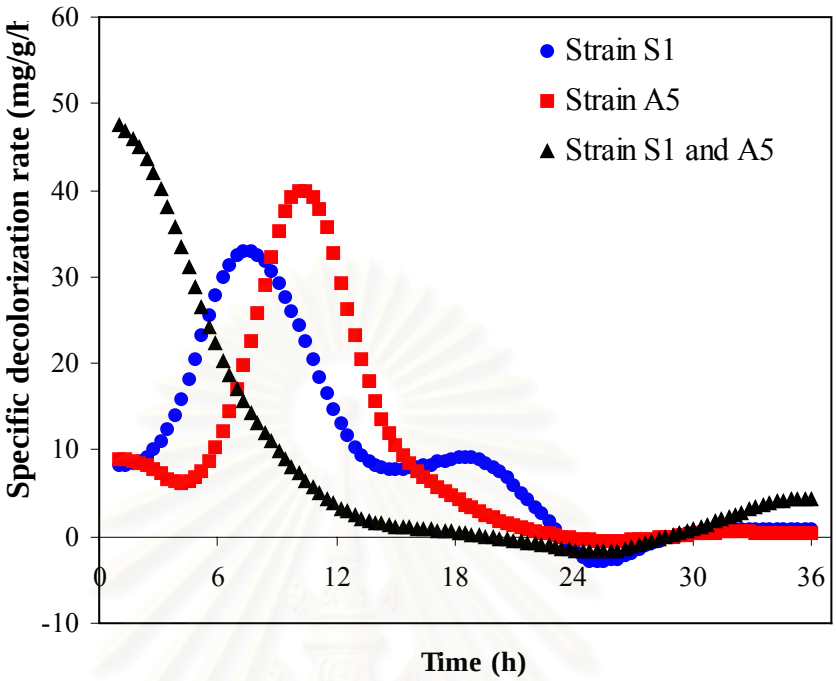


Figure 5.11 Time courses of specific decolorization rate of Remazol Black B by single and mixed culture of *Paenibacillus* sp.strain A5 and S1

Figure. 5.12 Characteristics of synthetic textile wastewater containing of Remazol Black B before and after batch decolorization by the mixed culture of strain S1 and A5

From Figure 5.8, 5.9 and 5.10, the rate of decolorization Remazol Black B of all experiments were high for the early period of operation, but it decreased with an increase in the operation time. The decolorization of Remazol Black B may be undertaken in two steps as follows: fast decolorization which decolorization of this dye decreased rapidly and slow decolorization which decolorization decreased to a smaller value for a longer period of time. This two steps of decolorization of Remazol Black B has also been found by several researchers. Panswad and Luangdilok (2000) reported that the decolorization rate of the black dye in an anaerobic/aerobic SBR reactor was 11.9 SU/h in the first 2 h of anaerobic stage and after that the decolorization rate dropped to 0.44 SU/h. In the study of Chen (2003), the first order kinetic of decolorization of Remazol Black B was 0.413 h^{-1} and 0.0129 h^{-1} within 2 h and after 2 h of anaerobic incubation time, respectively.

A mechanism for Remazol Black B reduction shown in Figure 5.13 was proposed by Chen (2003) involving a two stage reduction of the azo bond as given in reactions (1) and (2). One of decolorizing-products of reaction (1) is still contain azo bond in its molecule.

$$4e^- + 4H^+ + (R–N=N–R^1–N=N–R) \rightarrow (R–NH_2) + (R–N=N–R^1–NH_2) \qquad (1)$$

$$4e^- + 4H^+ + (R–N=N–R^1–NH_2) \rightarrow (R–NH_2) + (R^1–NH_2) \qquad (2)$$

where R and R^1 are variously substituted phenyl and naphthol residues.

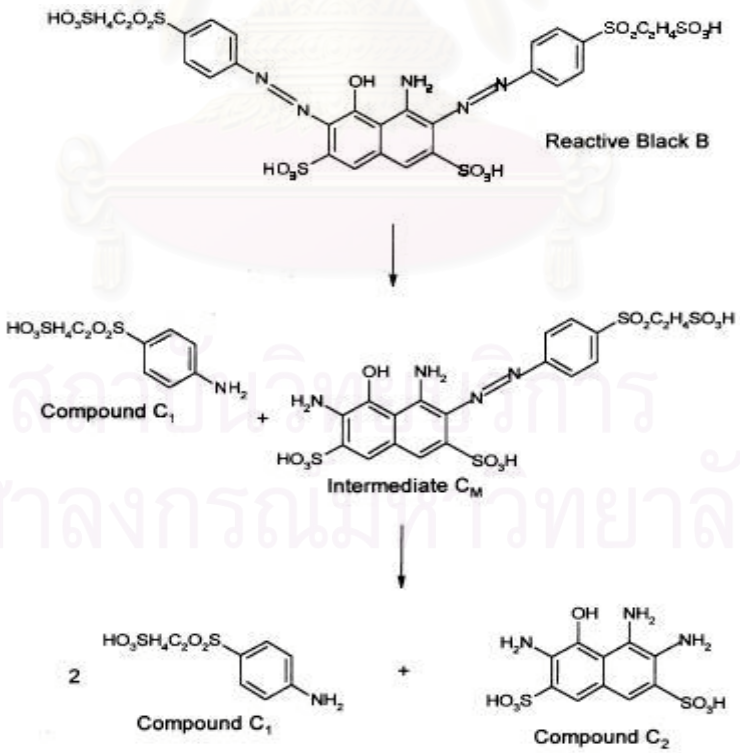


Figure 5.13 The proposed two-steps mechanism for decolorization of Remazol Black B (Chen, 2003)

This analysis also indicated that decolorizing-metabolites are likely to be more chemically stable than the parent compound, as metabolite ($R–N=N–R^1–NH_2$) is more persistent than Remazol Black B (Strauss, 1997). This two-step decolorization occurred due to different reactivities of two azo-bonds existing in Remazol Black B. Such difference in azo-bond cleavage may be attributed to the reactivity difference of hydroxyl (-OH) and amino ($-NH_2$) group, which are the activating groups of aromatic electrophilic substitution, present on the naphthalene structure in Remazol Black B at the position *ortho* (Chen, 2003).

UV-visible spectra (Figure 5.14) obtained for the supernatants from the mixed culture of *Paenibacillus* sp. strain A5 and S1 showed marked alterations with reaction time. These changes could be explained by structural modifications of dye molecule due to azo bond reduction as the same phenomenon in the case of decoloization of Remazol Brilliant Orange 3R. In order to clarify this phenomenon, the ethyl acetate extracts taken during anaerobic and aerobic phases of decolorization operations of Remazol Black B were analyzed by HPLC.

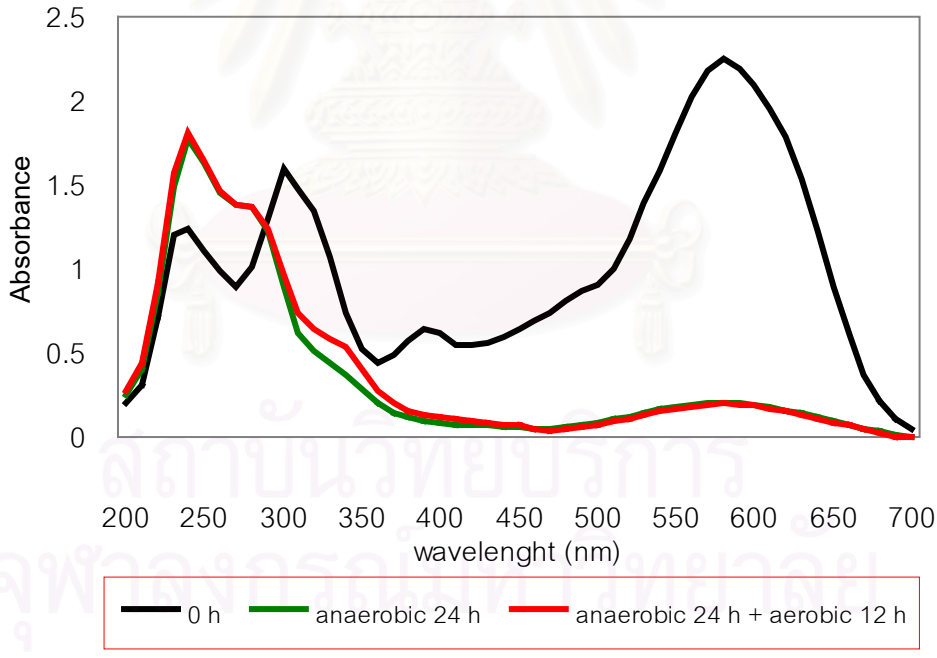


Figure. 5.14 Typical UV-visible spectra of supernatants from batch decolorization of Remazol Black B by mixed culture of strain S1 and A5 at various reaction times

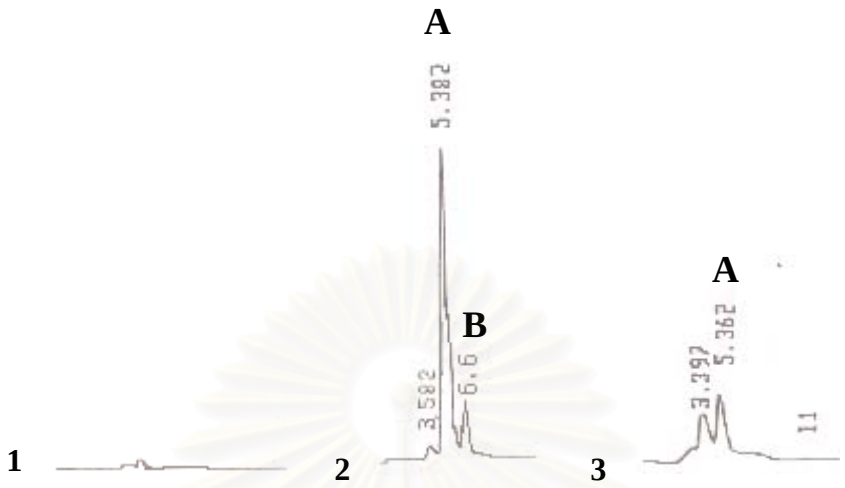


Figure. 5.15 The HPLC analysis on metabolites resulting from decolorization of Remazol Black B anaerobic-aerobic condition **(1)** at the beginning of anaerobic incubation **(2)** after anaerobic incubation for 24 h, and **(3)** after aerobic incubation for 12 h.

Figure. 5.15 shows the results of HPLC analysis on the decolorization metabolites of Remazol Black B by the mixed culture of strain A5 and S1. After anaerobic incubation for 24 h, the intensity of peaks A and B (retention time = ca. 5.3 min and 6.6 min, respectively) increased significantly. It is thus reasonable to suggest that peaks A and B represent the decolorization metabolites, confirming the formation of additional aromatic metabolites. Figure 5.15B and 5.15C also show that when such metabolites were degraded aerobically they formed less aromatic, more polar compounds, since the metabolite peak area decreased and shifted towards lower retention time (3.3 min).

5.3 Decolorization of Remazol Brilliant Violet 5R

The results obtained for the treatment of Remazol Brilliant Violet 5R by using *Paenibacillus* sp. strain A5, S1 and mixed culture of strain S1 and A5 are shown in Figure. 5.16, 5.17 and 5.18 respectively. The trend in terms of percentage removal of dye, COD, and its decolorizing-metabolite are similar to that of Remazol Brilliant Orange 3R and Remazol Black B. However, this violet dye were decreased just only in the presence of strain A5. Though Remazol Brilliant Violet 5R has a monoazo bond in complex with copper as its chromophore, this dye was decolorized completely within 24 h under anaerobic incubation of strain A5 and the mixed culture. The specific decolorization rate of the violet dye by the single strain A5 was slightly higher than the mixed culture. However, the shorter of lag phase before the beginning of decolorization was observed in the case of the mixed culture (Figure 5.19). The COD removal of the single and mixed culture was mainly occurred in aerobic phase.

The ethyl acetate extracts taken during anaerobic and aerobic phases of decolorization operations of Remazol Brilliant Violet 5R were analyzed by HPLC. The result of HPLC analysis on the decolorization metabolites of Remazol Brilliant Violet 5R by single and mixed bacterial culture are shown that after anaerobic incubation for 24 h, the area of appearing peak (retention time = ca. 4.3 min) increased significantly. It is thus reasonable to suggest that this peak represents the decolorization metabolite, confirming the formation of additional aromatic metabolite. The retention time of metabolite formed from decolorization of violet dye is different from the decolorizing metabolites of orange and black dye. In this case, it may be due to the formation of the different decolorizing metabolites resulting from decolorization of the different chemical structure of parent compounds. Moreover, the decolorizing metabolite was degraded aerobically, since the peak area of such metabolite decreased during the aerobic incubation as shown in this study. The characteristic of synthetic textile wastewater before and after anaerobic-aerobic incubation of the mixed culture are shown in Figure 5.20.

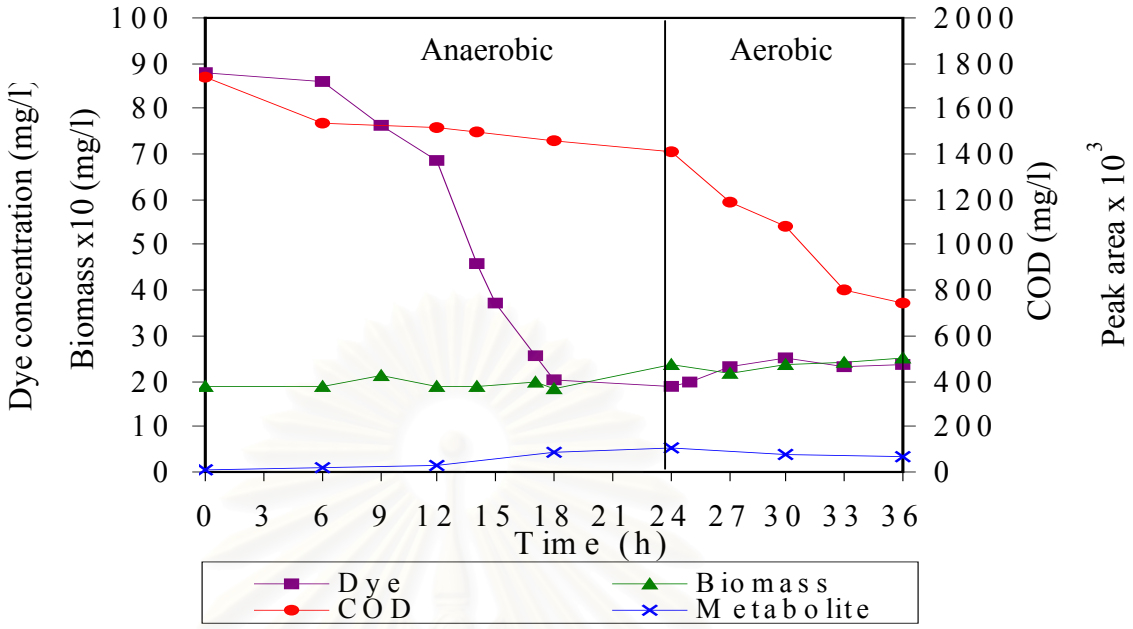


Figure. 5.16 Time-course profile of Remazol Brilliant Violet 5R, biomass, COD and decolorized metabolite (retention time = 4.0 min) during decolorizing cultivation of *Paenibacillus* sp. strain A5 in synthetic textile wastewater under anaerobic and aerobic condition

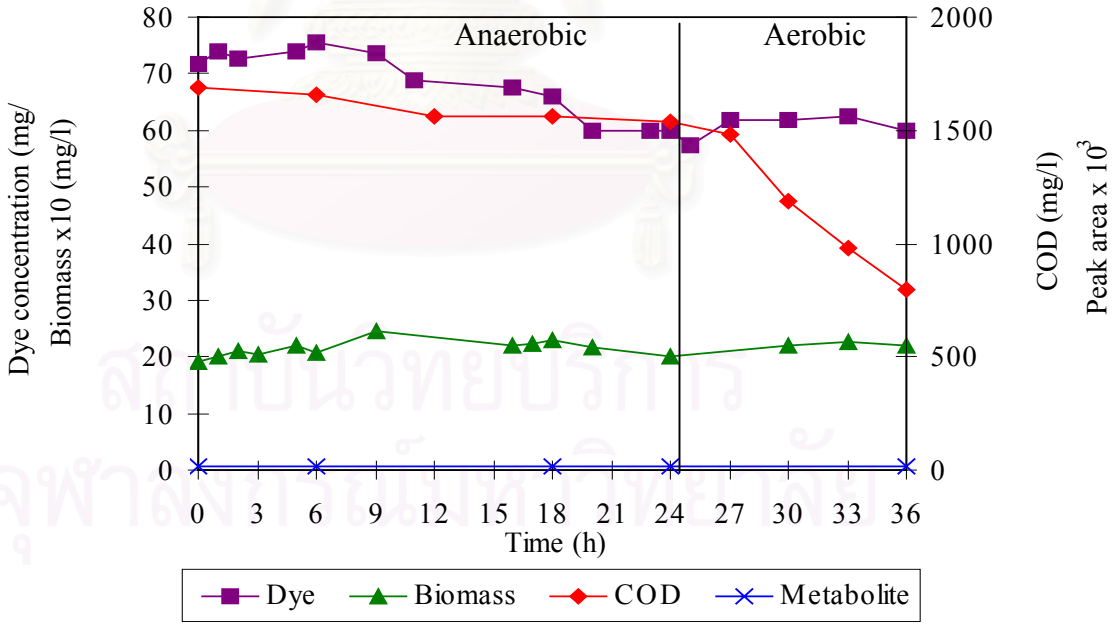


Figure. 5.17 Time-course profile of Remazol Brilliant Violet 5R, biomass, COD and decolorized metabolite (retention time = 4.0 min) during decolorizing cultivation of *Paenibacillus* sp. strain S1 in synthetic textile wastewater under anaerobic and aerobic condition

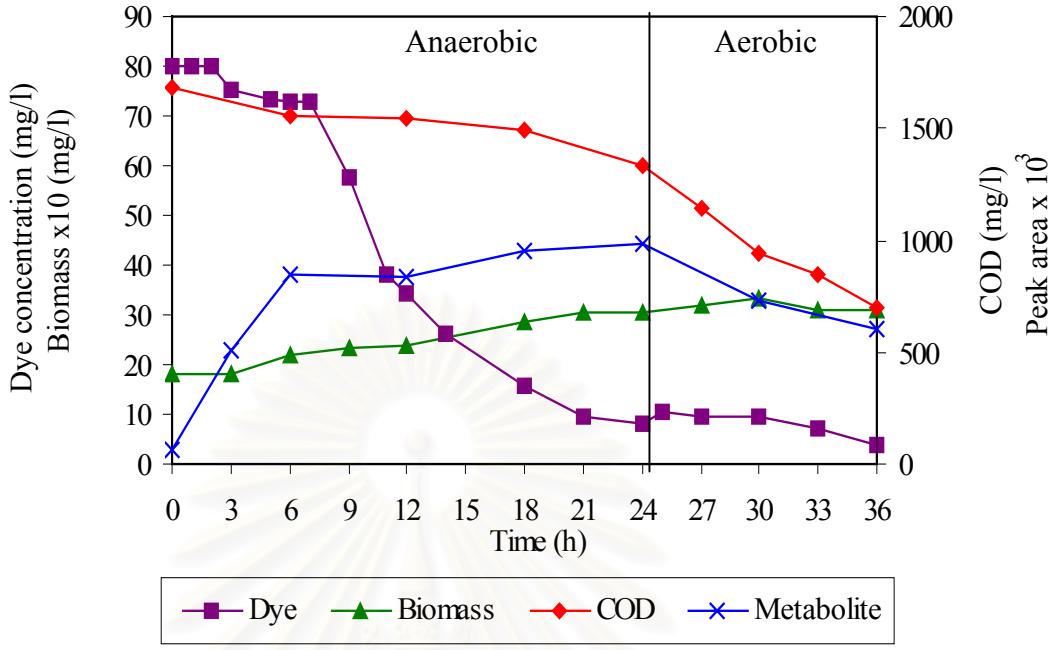


Figure. 5.18 Time-course profile of Remazol Brilliant Violet 5R, biomass, COD and decolorized metabolite (retention time = 4.0 min) during decolorizing cultivation of the mixed culture of *Paenibacillus* sp. strain S1 and A5 in synthetic textile wastewater under anaerobic and aerobic condition

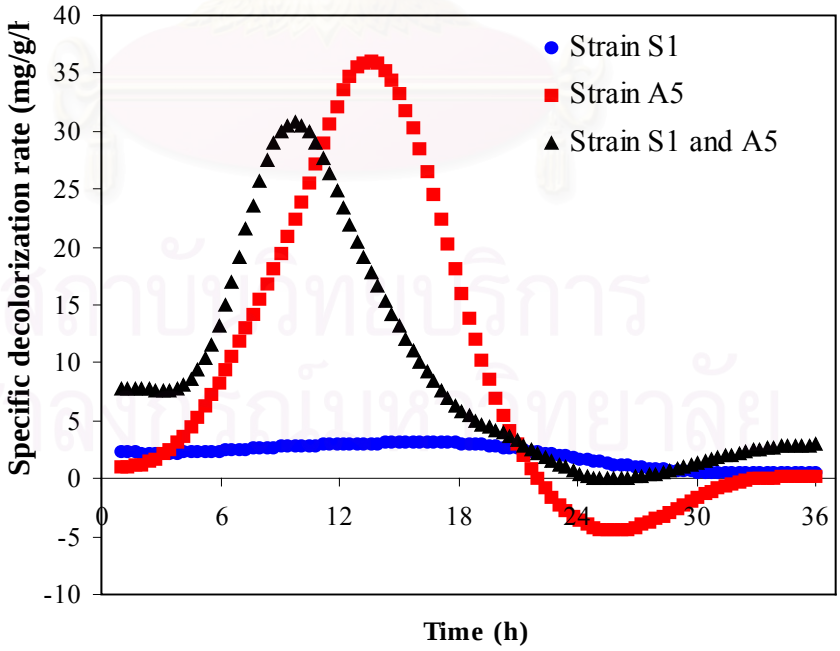


Figure 5.19 Time courses of specific decolorization rate of Remazol Brilliant Violet 5R by single and mixed culture of *Paenibacillus* sp.strain A5 and S1

Figure. 5.20 Characteristics of synthetic textile wastewater containing of Remazol Brilliant Violet 5R before and after batch decolorization by the bacerial mixed culture of strain S1 and A5

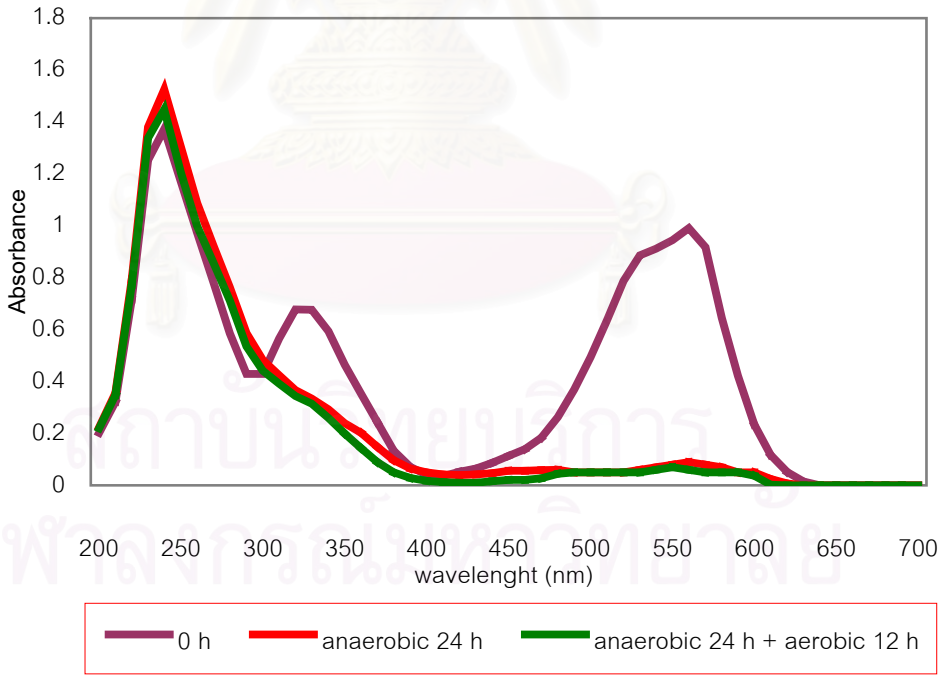


Figure. 5.21 Typical UV-visible spectra of the supernatants from batch decolorization of Remazol Brilliant Violet 5R by mixed bacterial culture strain S1 and A5 at various reaction times

UV-visible spectra (Figure 5.21) obtained for the filtered samples of mixed culture of *Paenibacillus* sp. strain A5 and S1 showed marked alterations with reaction time. These changes could be explained by structural modifications of dye molecule due to azo bond reduction. In order to clarify this phenomenon, the ethyl acetate extracts taken during anaerobic and aerobic phases of decolorization operations of Remazol Brilliant Violet 5R were analyzed by HPLC.

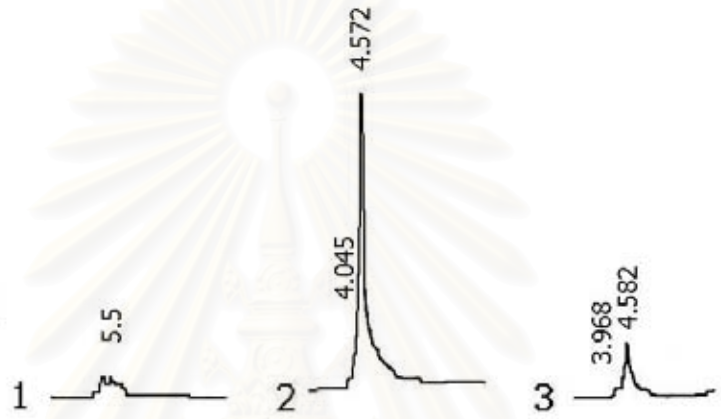


Figure. 5.22 The HPLC analysis on metabolites resulting from decolorization of Remazol Brilliant Violet 5R under anaerobic-aerobic condition **(1)** at the beginning of anaerobic incubation **(2)** after anaerobic incubation for 24 h, and **(3)** after aerobic incubation for 12 h

The result of HPLC analysis on the decolorization metabolites of Remazol Brilliant Violet 5R by mixed bacterial culture strain A5 and S1 is shown in Figure. 5.22. After anaerobic incubation for 24 h, the area of appearing peak (retention time = ca. 4.0 min) increased significantly. It is thus reasonable to suggest that this peak represents the decolorization metabolite, confirming the formation of additional aromatic metabolite. The retention time of metabolite formed from decolorization of violet dye is different from the decolorizing metabolites of orange and black dye. In this case, it may be due to the formation of the different decolorizing metabolites resulting from decolorization of the different chemical structure of parent compounds.

It has been reported that decolorization of Remazol Brilliant Violet 5R can occur easily under anaerobic condition, however, certain its decolorizing metabolites are known to be resistant to degradation by aerobic bacteria (Lourenco et al., 2001;Sosath et al., 1997). Contrary to several reports, which difficulties with the removal of violet dye metabolites, the decolorizing metabolites was removed aerobically, since the peak area of such metabolite decreased during the aerobic incubation as shown in this study.

5.4 Decolorization of Remazol Brilliant Blue R

The results obtained for the treatment of Remazol Brilliant Blue R, an anthraquinone dye, by using *Paenibacillus* sp. strain A5, and S1 and mixed culture of strain S1 and A5 are shown in Figure. 5.23, 5.24 and 5.25, respectively. From these figures, it can be seen that the blue dye was removed in both anaerobic and aerobic phase. The decolorization of this dye was different form the decolorization of the reactive azo dye and it depended on the concentration of the bacterial biomass. It seems to indicate that the decolorization mechanism of this blue dye is biosorbtion (see Chapter 7.2 for more details) to the bacterial cell materials. The bacterial biomass increased slowly in the early phase of anaerobic incubation but increased more rapidly in a subsequent aerobic phase. Dye adsorption can be judged clearly by inspecting the cell mats. Cell mats become deeply blue colored after anaerbic/aerobic incubation because of adsorbing Remazol Brilliant Blue R. The majority of COD was also removed in the aerobic phase. The characteristics of synthetic wastewater before and after anaerobic/aerobic incubation of mixed culture are shown in Figure (5.27).

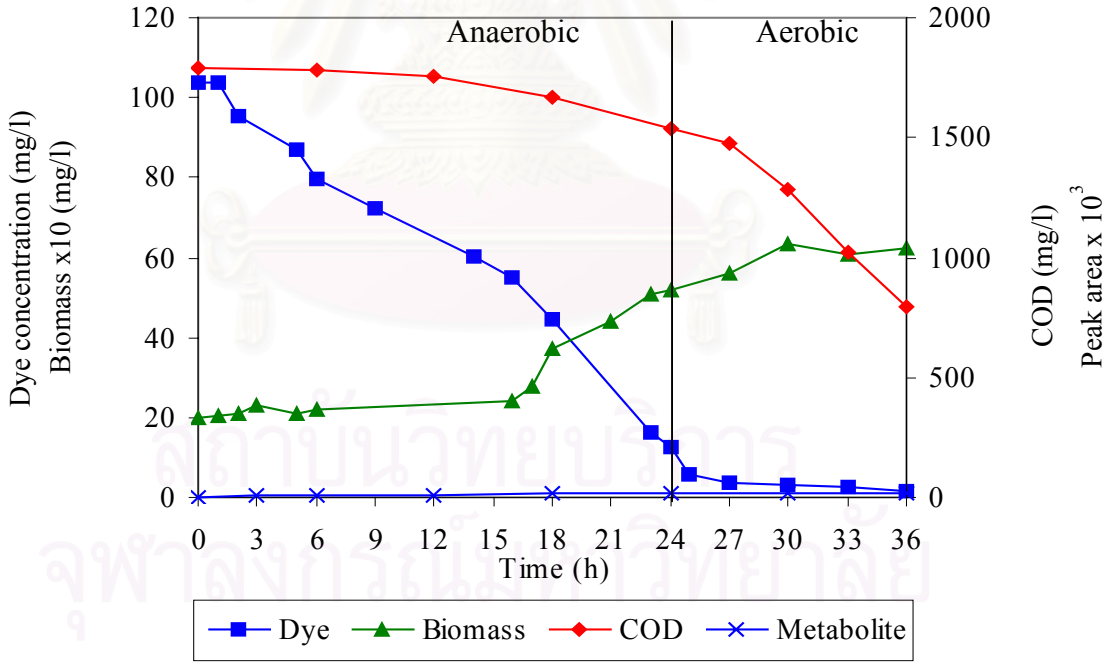


Figure. 5.23 Time-course profile of Remazol Brilliant Blue R, biomass and COD during decolorizing cultivation of *Paenibacillus* sp. strain A5 in synthetic textile wastewater under anaerobic and aerobic condition

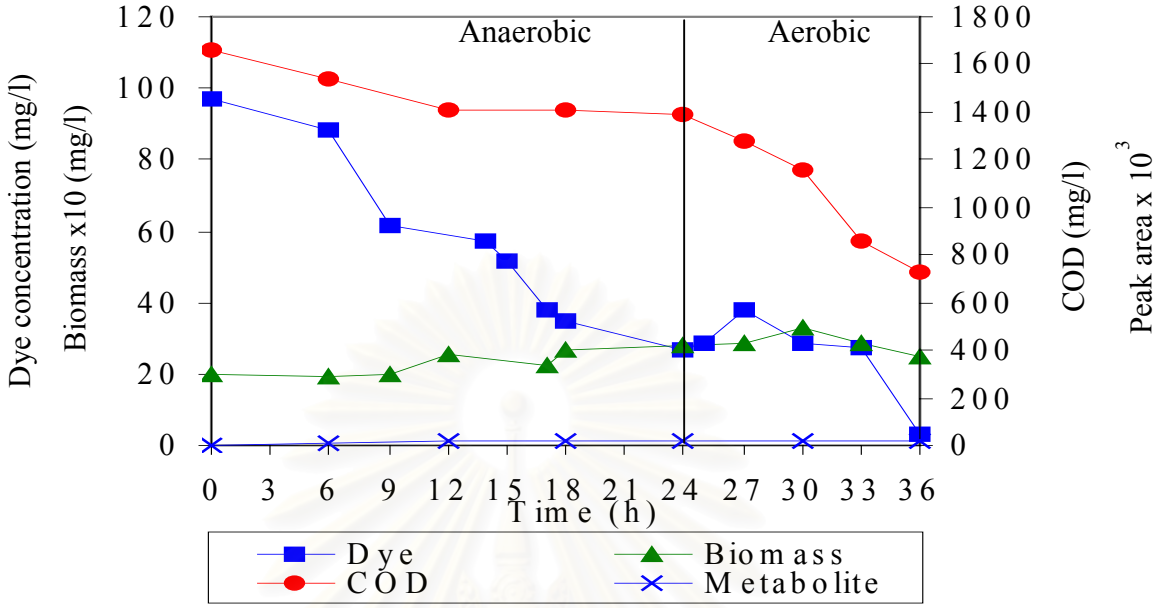


Figure. 5.24 Time-course profile of Remazol Brilliant Blue R, biomass and COD during decolorizing cultivation of *Paenibacillus* sp. strain S1 in synthetic textile wastewater under anaerobic and aerobic condition

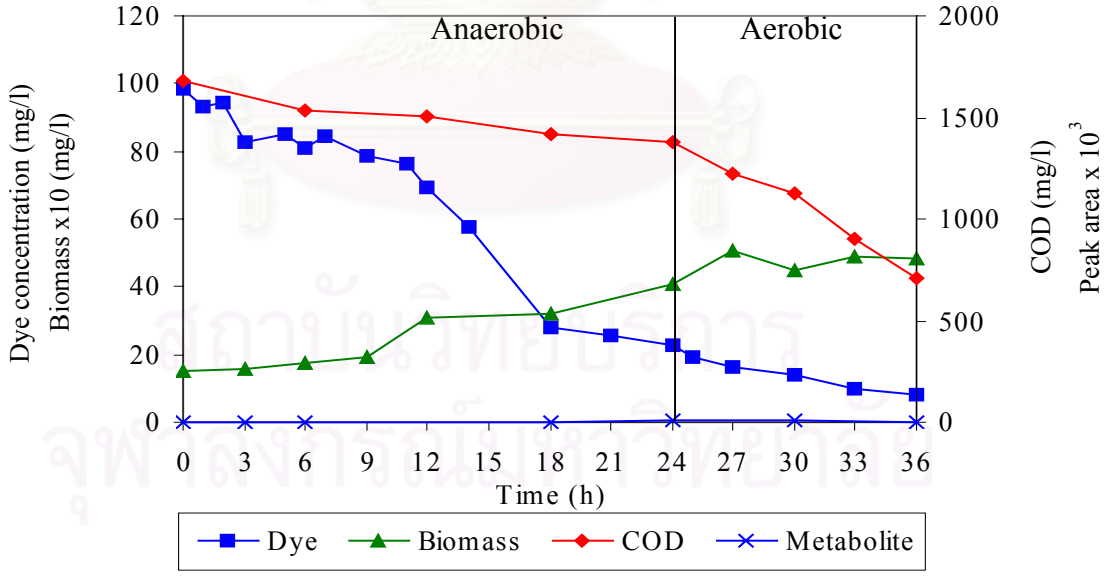


Figure. 5.25 Time-course profile of Remazol Brilliant Blue R, biomass and COD during decolorizing cultivation of the mixed culture of *Paenibacillus* sp. strain S1 and A5 in synthetic textile wastewater under anaerobic and aerobic condition

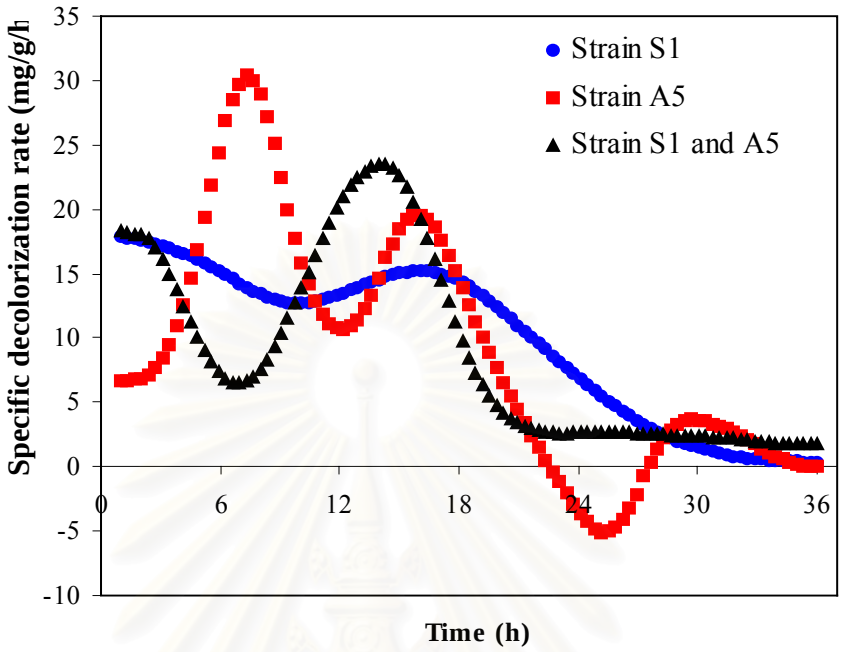


Figure 5.26 Time courses of specific decolorization rate of Remazol Brilliant Blue R by single and mixed culture of *Paenibacillus* sp.strain A5 and S1

Figure. 5.27 Characteristics of synthetic textile wastewater containing of Remazol Brilliant Blue R before and after batch decolorization by the mixed culture of strain S1 and A5

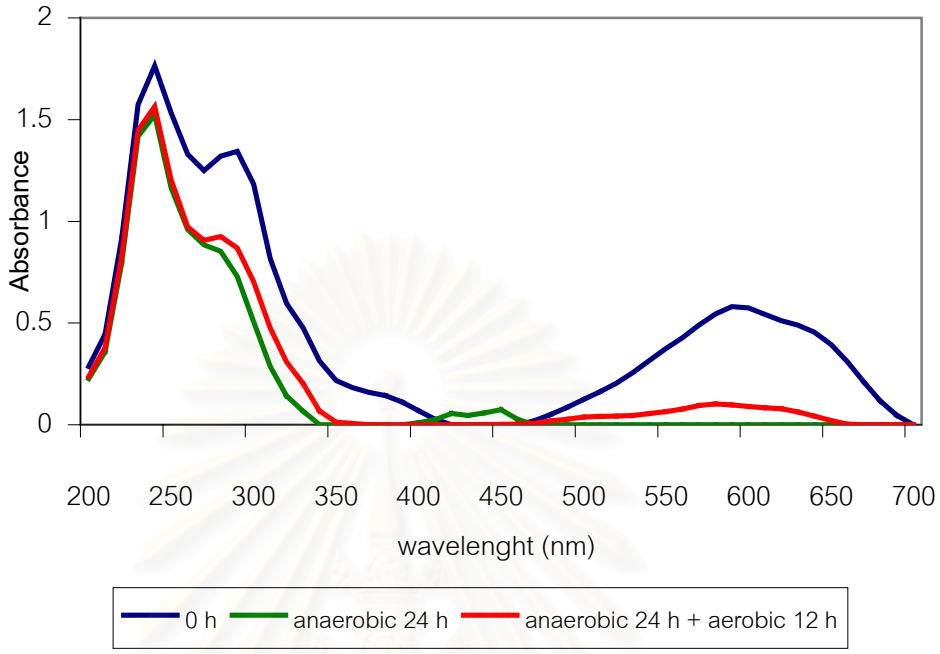


Figure. 5.28 Typical UV-visible spectra of the supernatants from batch decolorization of Remazol Brilliant Blue R by mixed bacterial culture strain S1 and A5 at various reaction times

UV-visible spectra (Figure 5.28) obtained for the filtered samples from mixed culture of *Paenibacillus* sp. strain A5 and S1 showed marked alterations with reaction time. These decreases could be explained by decolorization of dye molecule due to absorption to bacterial biomass.

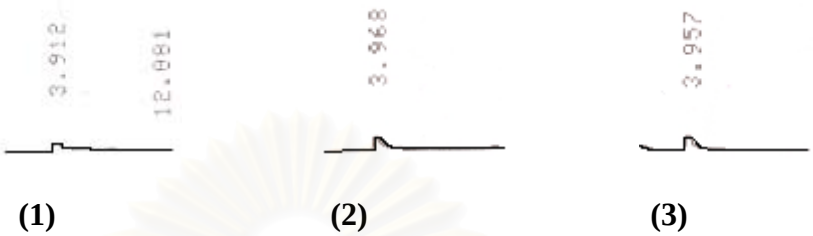


Figure. 5.29 The HPLC analysis on metabolites resulting from decolorization of Remazol Brilliant Blue R anaerobic-aerobic condition **(1)** at the beginning of anaerobic incubation **(2)** after anaerobic incubation for 24 h, and **(3)** after aerobic incubation for 12 h\\

Also, the HPLC analysis performed to supernatants taken during the different duration of the anaerobic and aerobic phase did not show the correspondence of the evolution of the suspected decolorizing-metabolite to the color removal (Figure 5.29).

5.5 Conclusions

Microbial decolorization of three reactive azo dyes in an anaerobic environment occurred as a result of reduction of azo bonds. This gave rise to the liberation of decolorization metabolites. No ready biodegradation of these metabolites was expected in the anaerobic system that gives rise to decolorization. However these metabolites could be further reduced by means of either biodegradation or autooxidation under subsequent aerobic treatment. As a characteristic of the textile-processing industry, a wide range of structurally diverse dyes is used within short periods in one and the same factory, and therefore effluents from textile industry are extremely variable in composition. Results obtained from this work show that the mixed bacterial culture possesses high decolorization efficiency. The mixed bacterial cultures decolorized three structurally dissimilar azo dyes, suggesting that anaerobic decolorization was not a specific process. Meanwhile, the non-degradable anthraquinone dye was decolorized by the biosorption onto the bacterial biomass.

It was clear that the majority of the color removal occurred in the anaerobic stage. On the other hand, the anaerobic phase of decolorization operations gave low COD removal and most of the COD was removed in the subsequent aerobic phase, including the decolorized metabolites formed from anaerobic phases. It is possible that the mixed culture metabolized small amounts of COD under anaerobic conditions to the source of reduction equivalents (for example, NADH) to reduce the azo bonds present in reactive dyes. The color removal yield with the more complex diazo dye Remazol Black B was, however, much lower than that obtained with the monoazo dye Remazol Brilliant Orange 3R and Remazol Brilliant Violet 5R. The rate of decolorization of azo dyes is affected by their molecular weights, substitution groups of the dye molecules and the intramolecular hydrogen bond between the azo and hydroxy groups. It is considered that the low decolorization of Remazol Black B is attributed to this factor. A longer retention time of anaerobic phase may be, therefore, required to enhance the rate of elimination of black dye.

Hence, decolorization of Remazol Brilliant Orange 3R, Remazol Black B and Remazol Brilliant Violet 5R by a mixed bacterial culture are most likely due to complete breakdown of the reactive azo dyes to form aromatic amines. Removal of azo dye metabolites after anaerobic reduction can be done in aerobic phases by the same mixed bacterial culture. However, further investigation should be clarified the removal mechanism of decolorization metabolites under aerobic phase.

The mixed culture of *Paenibacillus* sp. strain A5 and S1 showed the synergism effect on the decolorization of all azo dyes used. The fastest decolorization time and the highest specific decolorization rate of all azo dyes were achieved by the mixed culture of strain A5 and S1. The advantages of mixed cultures are apparent as some strains can collectively carry out complex biodegradation tasks that no individual strain can achieve independently. Similar other mixed bacterial cultures have also been reported recently (Knapp and Newby, 1995; Nigam et al., 1996). Therefore, the defined mixed culture of both strains would be selected to carry out the further experiments in this study.

Chapter 6

Influencing factors for bacterial decolorization of reactive dyes

In the decolorization process by bacteria, there are various influencing factors. They can be grouped into two kinds: one is related to bacterial growth conditions; the other is related to the characteristics of the dye solution or wastewater.

Bacterial growth conditions

As different component possess different abilities to decolorize dyes, it is necessary to create an optimal environment favorable to bacterial growth and thus make the bacteria possess the maximum ability to decolorize dyes in wastewater. Important bacterial growth conditions are discussed as follow:

Carbon or co-substrates

Glucose, starch, yeast extract and municipal wastewater, among others, have been reported, as examples of an essential co-substrate needed to obtain good color removal. Low color removal capacities reported for some treatment systems could be due to low organic load of the synthetic wastewater used. Several carbon sources have been compared and glucose, glycerol and lactose gave the best results in relation to color removal efficiency (82, 71 and 71 %, respectively), while starch and distillery waste resulted in poorer decolorization (52 and 39 %, respectively) (Nigam et al., 1996). Glucose has been added to enhance the decolorization performance of biological systems in some studies (Haug et al., 1991; Carliell et al., 1995). However, others reported that glucose inhibited the decolorizing activity (Chung et al., 1978; Knapp and Newby, 1995; Chen et al., 2003). The variability may be due to the different microbial characteristics.

Chen et al., (2003) studied the effects of glucose concentration on decolorization of dyes by *Aeromonas hydrophila* and observed that glucose concentration of higher than 0.15 g/l inhibited appreciably the azo reduction of azo dye. The author explained that glucose inhibited decolorization activity because the consumed glucose was converted to organic acids that might decrease the pH of the culture medium, thus inhibiting the cell growth and decolorization activity.

Decolorization of the original azo dye also proceeded with a volatile fatty acids mixture (VFA) as a carbon source, though less efficiently than glucose (Donlon et al., 1997). Pansuwan and Panswad (1997) and Pansuwan et al. (1999) concluded from a study on dye decolorization by an Anaerobic/Oxic (A/O) or Phoredox process that external carbon sources such as sucrose or acetic acid could be used for the co-metabolism, resulting in a better color reduction efficiency.

Nutrients (nitrogen and phosphorus)

Dyebath additives containing nitrogen and phosphorus (e.g. urea, ammonium acetate, ammonium sulfate and phosphate buffers) are the main sources of nutrients in the textile wastewater. The mean value reported for a silk and lycra printing plant was 129 mg NH_4^+-N /l (Rigoni-Stern et al., 1996). Nitrogen removal is commonly performed by nitrification/denitrification in anoxic/aerobic biological plants. Textile effluents were found to be major inhibitors of the nitrifying bacteria in aerobic treatment system, thus hampering nitrogen removal (Gruttner et al., 1994). It should be emphasised that anaerobic treatment can be effective in the removal of organic compounds, i.e. COD, but it is unable to remove inorganic compounds such as NH_4^+ and PO_4^-. COD/N ratio is considered as a paramount importance for anaerobic treatment of textile effluents. If the effluent has a too low COD/N ratio, nitrogen removal is insufficient. Thus, the addition of an external COD is essential for nitrogen removal in such case (Rigoni-Stern et al., 1996). Effort should be put into the reduction of the amounts of dye bath additves appiled and the selection of nitrogen- and phosphorus-free alternative auxiliaries in the production process.

pH effects

Acids and alkalies are used in the dyeing process depending on the dye class involved and large quantities of alkali are used in bleaching, desizing, scouring and mercerizing. Extreme of pH have to avoided in order to maintain good wastewater treatment performance by biological processes, thus making costly pH adjustment necessary. The used of acetic acids does not contribute to increased salinity but this component can account for 50-90% of dyehouse organic load (Bortone et al., 1995).

Heavy metals

The main source of heavy metals in the textile industry is the dyeing process. The maximum heavy metal contents of dyeing wastewater have been reported to be 12,1 mg Cu^{2+} /l for direct dye on cotton, 2.7 mg Cr^{6+} /l for direct dyes on viscose, 7.5 mg Cd /l for basic dyes on wool, and 3.4 mg Zn^{2+} /l for acid dyes on wool (Horning, 1978). Metal ions would be a factor influencing biosorption. They might compete with dye molecules for binding sites or stimulate the biosorption of dye onto biomass.

Dye types

Different dyes have different molecular structures. So a bacteria capable of decolorizing one dye may have different capacities for other dyes. This occurs more than often for living cells than dead ones. The are two divergent views about the effect of dye structures, substitution patterns and substitutents. Azo dye was enzyme (azoreductase) substrate while anthraquinone dye was not the substrates of azoreductase. The aromatic rings with substitutents such as hydroxy, amino, acetamido, or nitro functions were mineralized to a reater extent than unsubstituted rings in dye decolorization by *Phanerochatete chrysosporium* (Spado et al., 1992).

6.1 Effect of aeration on the decolorization of reactive dyes by mixed culture *Paenibacillus* sp. strain S1 and A5

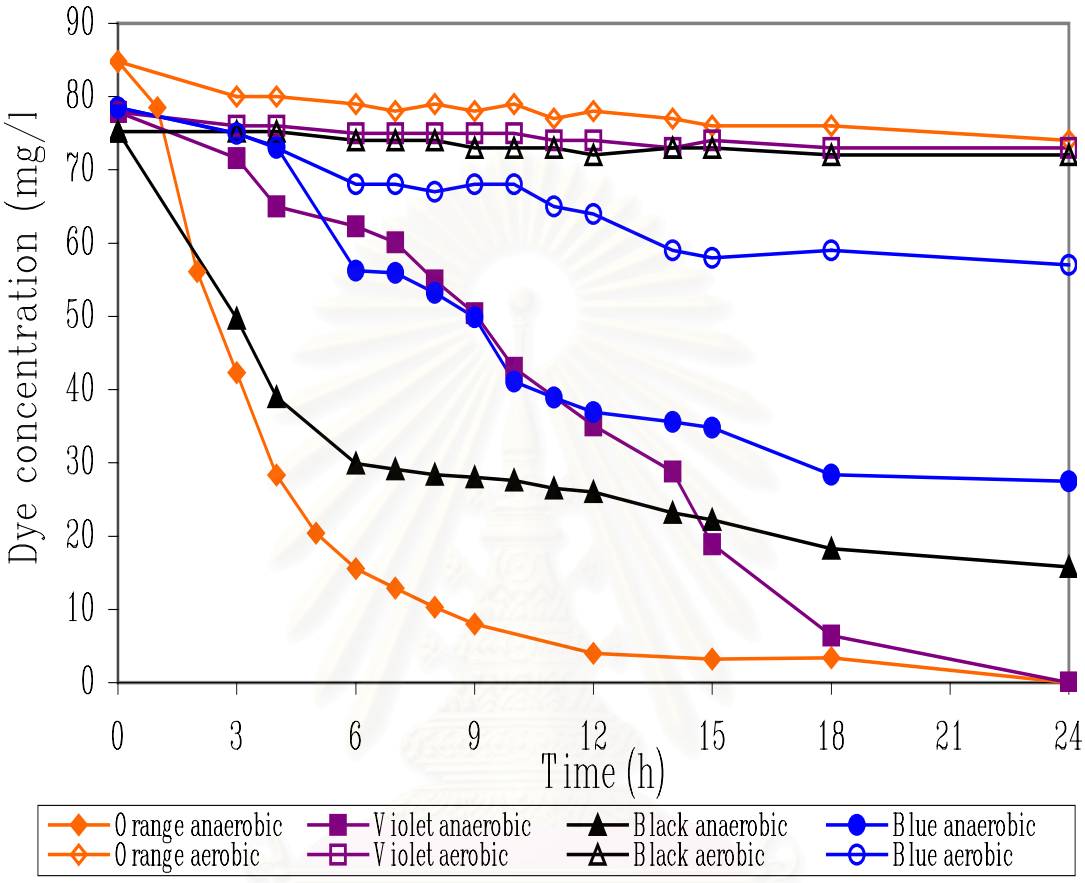


Figure 6.1 Effect of aeration on decolorization of four reactive dyes by mixed culture *Paenibacillus* sp. strain S1 and A5

The results in Figure 6.1 clearly showed that aeration inhibited the decolorization of reactive azo dyes used in this study. Meanwhile the azo dyes were decolorized rapidly under anaerobic condition. It can be assumed that the decolorization mechanism of *Paenibacillus* sp. strain S1 and A5 was the reduction of azo bonds present in chemical structure of azo dyes and this mechanism was inhibited by the presence of oxygen in medium. The azo reductase have been repoeted by many bacteria and the enzymatic reaction linked to anaerobiosis, because it is inhibited by oxygen that is in competition with the azo group as the electron receptor in the oxidation of the reduced electron carrier, i.e. NADH (Wuhrmann et al., 1980; Zimmerman et al., 1982; Banat et al., 1996). Seldom are bacteria able to decolorize azo compounds in the presence of oxygen (Wuhrmann et al., 1980). Therefore, the results indicated that the decolorization by mixed bacterial cultures was very sensitive to dissolve oxygen presented in culture medium. To achieve an effective azo dye removal, agitation and aeration should be avoided.

6.2 Effect of initial cell concentrations on decolorization of reactive dyes by mixed culture of *Paenibacillus* sp. strain S1 and A5

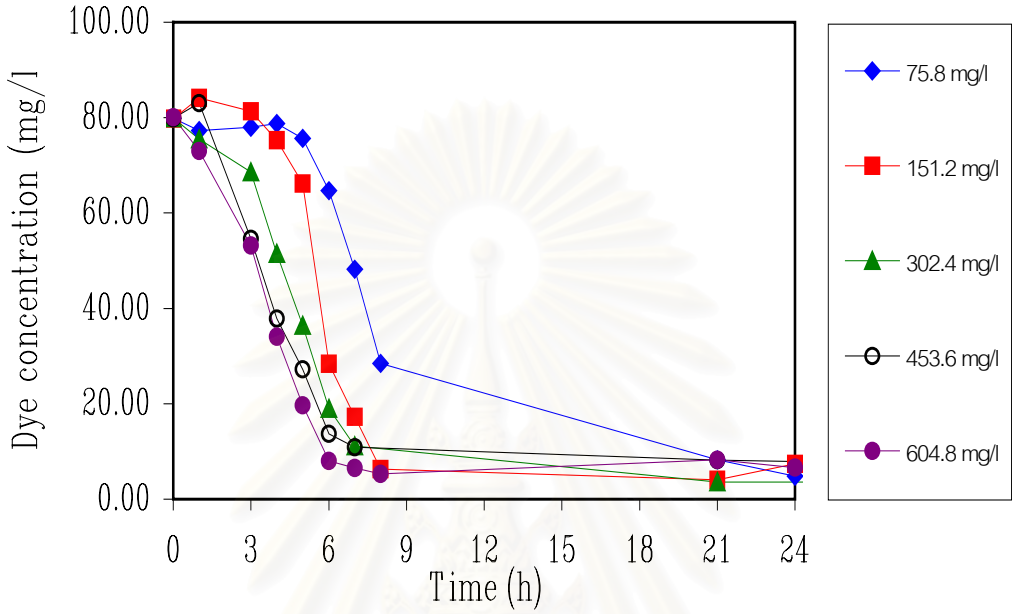


Figure. 6.2 Phase curve profiles of decolorization of Remazol Brilliant Orange 3R by mixed culture *Paenibacillus* sp. strain S1 and A5 at various initial cell concentrations

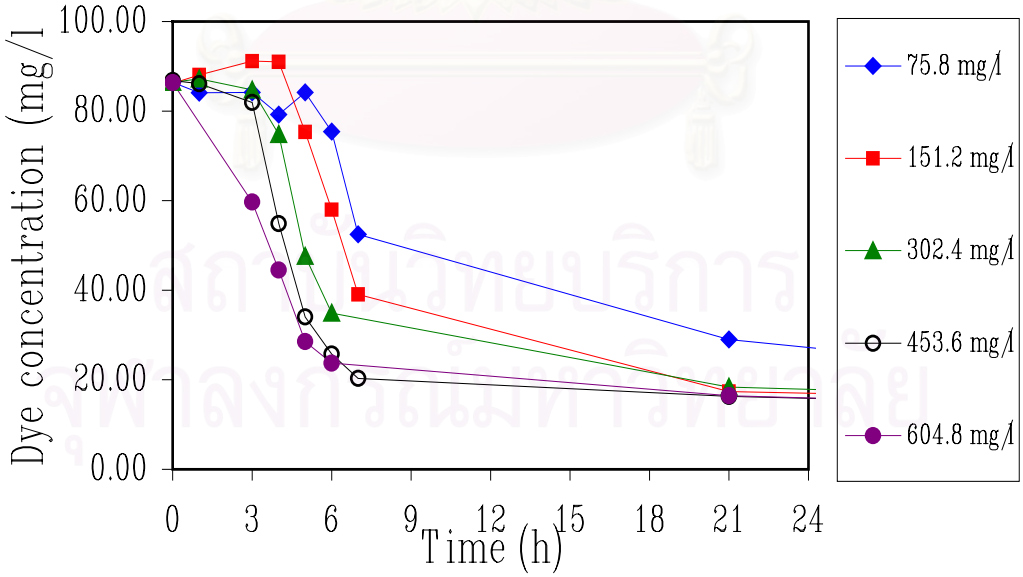


Figure. 6.3. Phase curve profiles of decolorization of Remazol Black B by mixed culture *Paenibacillus* sp. strain S1 and A5 at various initial cell concentrations

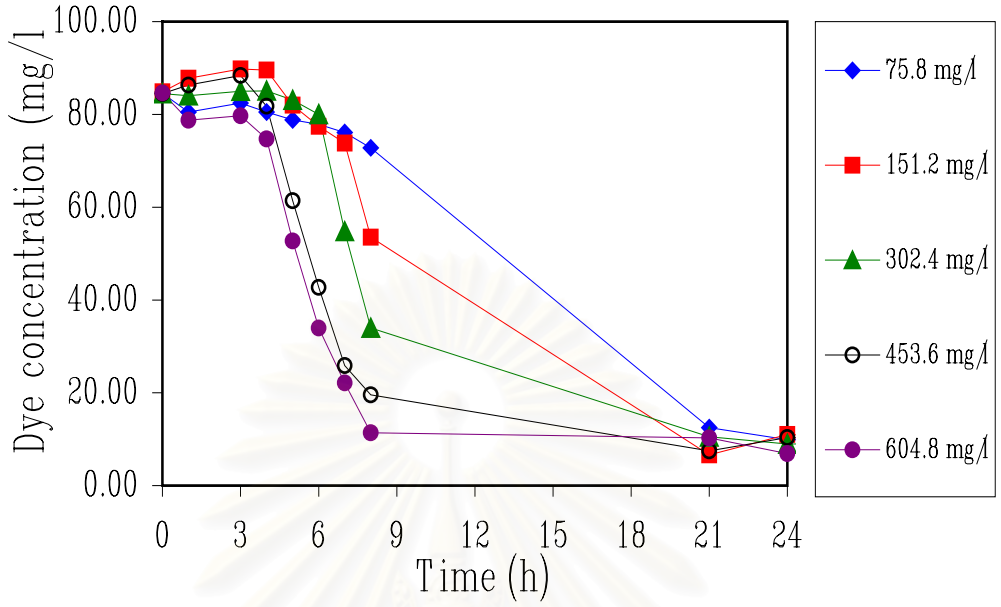


Figure. 6.4. Phase curve profiles of decolorization of Remazol Brilliant Violet 5R by mixed culture *Paenibacillus* sp. strain S1 and A5 at various initial cell concentrations

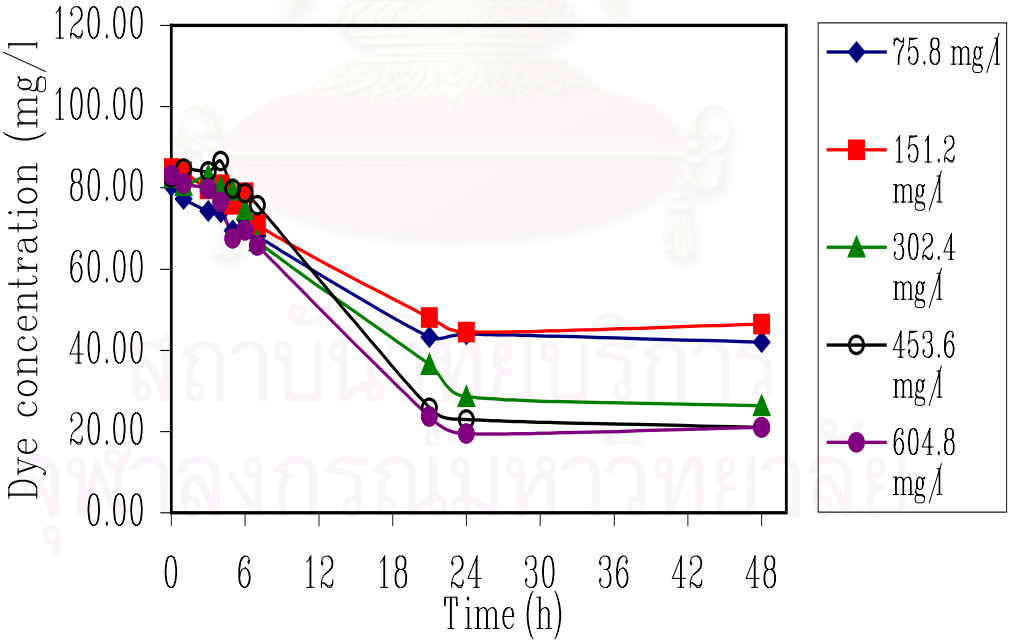


Figure. 6.5 Phase curve profiles of decolorization of Remazol Brilliant Blue R at by mixed culture *Paenibacillus* sp. strain S1 and A5 at various initial cell concentrations

6.3 Effect of temperatures on the decolorization of reactive dyes by mixed culture *Paenibacillus* sp. strain S1 and A5 under anaerobic condition

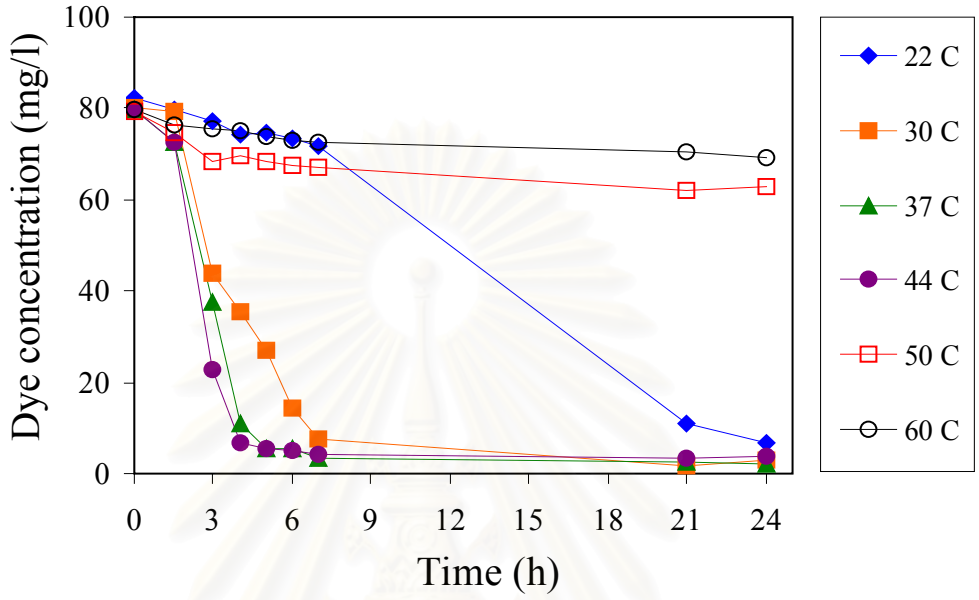


Figure. 6.6 Phase curve profiles of decolorization of Remazol Brilliant Orange 3R at by mixed culture *Paenibacillus* sp. strain S1 and A5 at various incubation temperatures

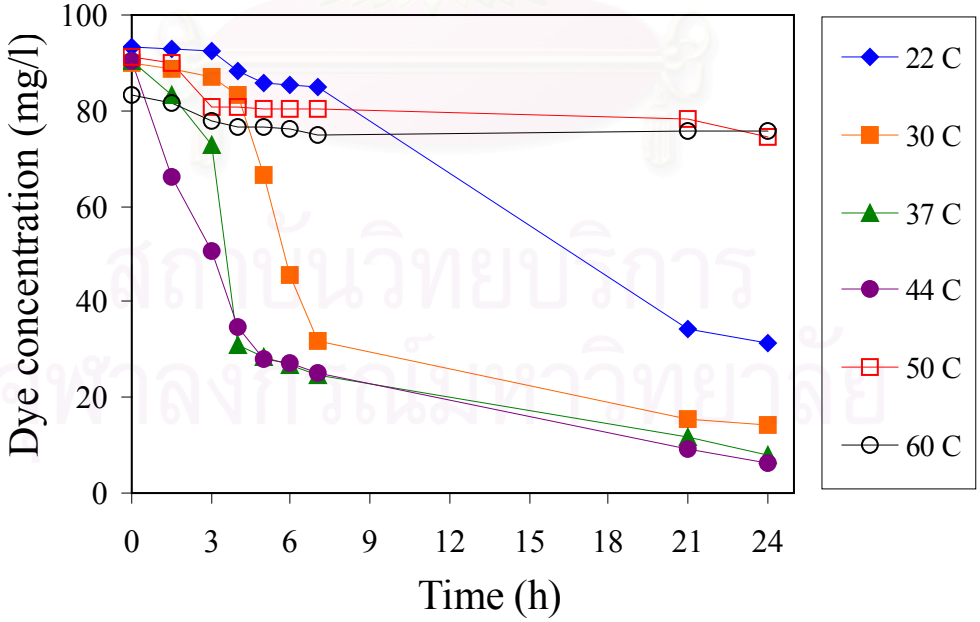


Figure. 6.7 Phase curve profiles of decolorization of Remazol Black B at by mixed culture *Paenibacillus* sp. strain S1 and A5 at various incubation temperatures

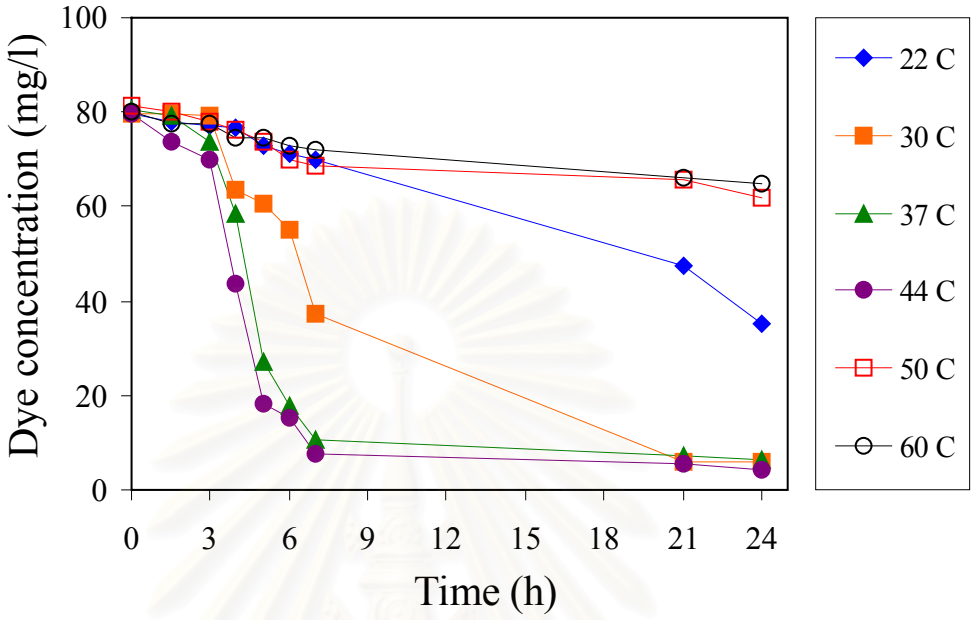


Figure. 6.8 Phase curve profiles of decolorization of Remazol Brilliant Violet 5R at by mixed culture *Paenibacillus* sp. strain S1 and A5 at various incubation temperatures

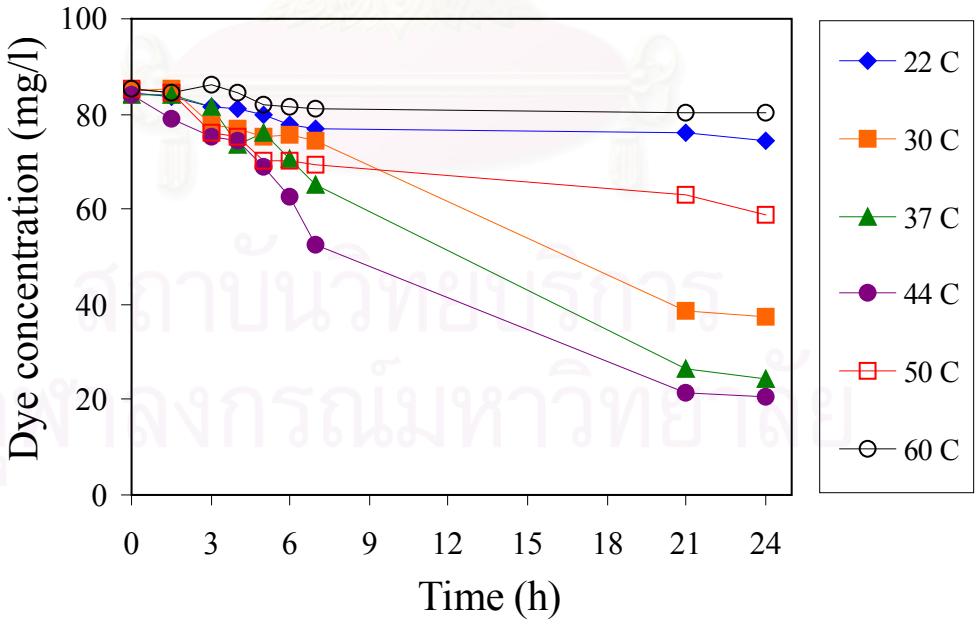


Figure. 6.9 Phase curve profiles of decolorization of Remazol Brilliant Blue R at by mixed culture *Paenibacillus* sp. strain S1 and A5 at various incubation temperatures

In order to study the effects of temperature on decolorization of reactive dyes by mixed culture of *Paenibacillus* sp. strain A5 and S1, experiments were performed at 22, 30, 37, 44, 50 and 60 ^{0}C. Over a range of 22 – 44.^{0}C, the decolorization of reactive dyes by the mixed culture of *Paenibacillus* sp strain A5 and S1 increased as the temperature rose (Figure 6.6, 6.7, 6.8 and 6.9) and then declined above 44 ^{0}C. The latter could be attributed to cell death or enzyme denaturation at high temperatures because the decolorization rate depends on cell mass concentration as shown in Figure 6.2, 6.3, 6.4 and 6.5.

The rate of decolorization can be described as being proportional to temperatures, if first-order kinetics is assumed:

$$\ln[(C_t/C_0)] = -K_d t$$

where C_t is concentration of dyes at any particular time (mg/l)
K_d is first order kinetic of decolorization
C_0 is initial concentration of dye (mg/l)
t is time (h)

K_d is calculated from a plot of $\ln[(C_t/C_0)]$ vs t.

The activation energy of the decolorization reaction can be estimated by the use of Arrhenius equation:

$$K_d = A\exp(-E_a/RT)$$

Thus,
$$\ln(K_d) = (-E_a/R)\,(1/T) + \ln(A)$$

where A is the frequency factor and has the same unit as K_d,
E_a is the activation energy (cal/mol),
R is the ideal gas law constant (1.987 cal/mol/ K), and
T is the temperature (K).

The values of E_a and A were calculated from the slope and intercept of plot ln (K_d) vs. $1/T$.

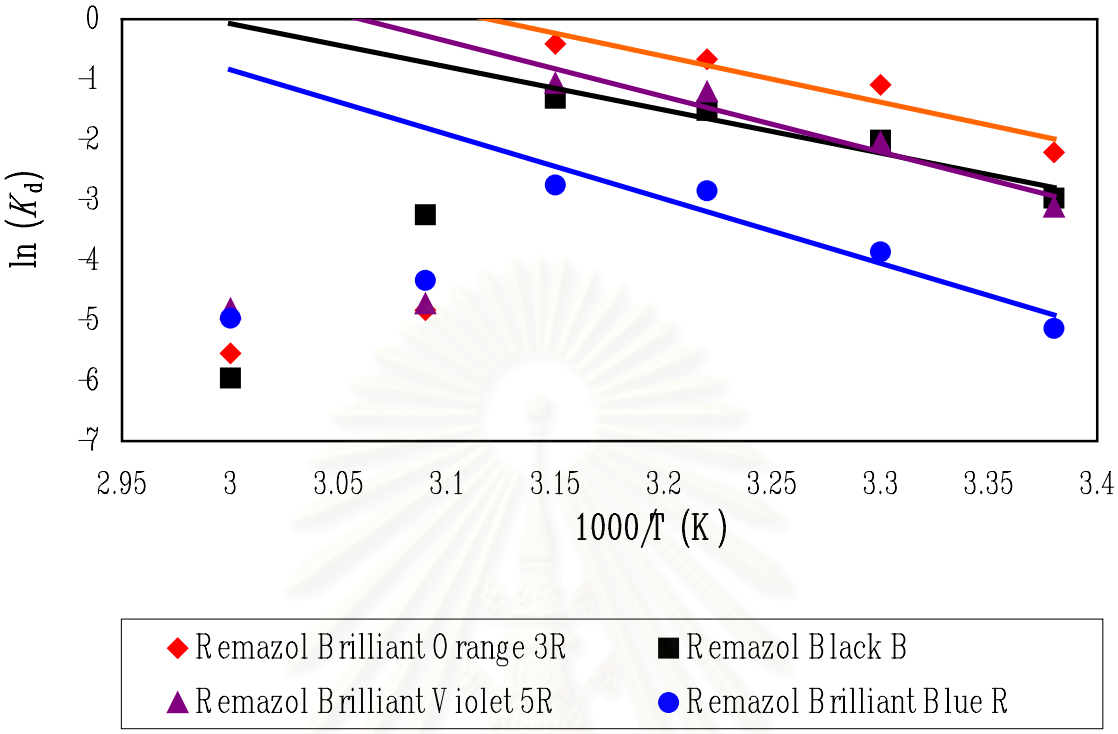


Figure 6.10 Arrhenius plot: dependence of the first order kinetic of decolorization (K_d) on temperature

In the temperature range of 22 – 44 ^{0}C, the first order kinetic of decolorization increase with temperature depends on the activation energy of the reaction as given by Arrhenius equation. According to the slopes and intercepts of the Arrhenius plot, the activation energy (E_a) for decolorization of four reactive dyes were calculated from the slope in Figure 6.10. The estimated E_a values for decolorization of Remazol Brilliant Orange 3R, Remazol Black B, Remazol Brilliant Violet 5R and Remazol Brilliant Blue R by a mixed culture of *Paenibacillus* sp. strain A5 and S1 were 15.12, 14.22, 18.22 and 21.24 kcal/mol, respectively. The lower values of activation energy shows that the bacterial decolorization of Remazol Black B and Remazol Brilliant Orange 3R are easier than the decolorization of Remazol Brilliant Violet 5R.

The value of E_a supports the previous findings that the rate of bacterial decolorization was temperature dependent. Similar observations have been recorded for decolorization of C.I. Reactive Red 22 by *E. coli* NO3 which its E_a value was 6.57 kcal/mol (Chang and Kuo, 2000). In addition, E_a value for decolorization of C.I. Acid Violet 7 (monoazo dye) by *Pseudomonas* sp. strain GM3 was 16.87 kcal/mol (Yu et al., 2001). Thus, this decolorization activation energy by a mixed culture of *Paenibacillus* sp strain A5 and S1 is at the high value side of the general activation energy range of enzyme-catalyzed reactions, which are usually within 4 – 20 kcal/mol range, mostly about 11 kcal/mol (Shuler and Kargi, 1992).

6.4 Effect of initial pH on the decolorization of reactive dyes by mixed culture *Paenibacillus* sp. strain S1 and A5 under anaerobic condition

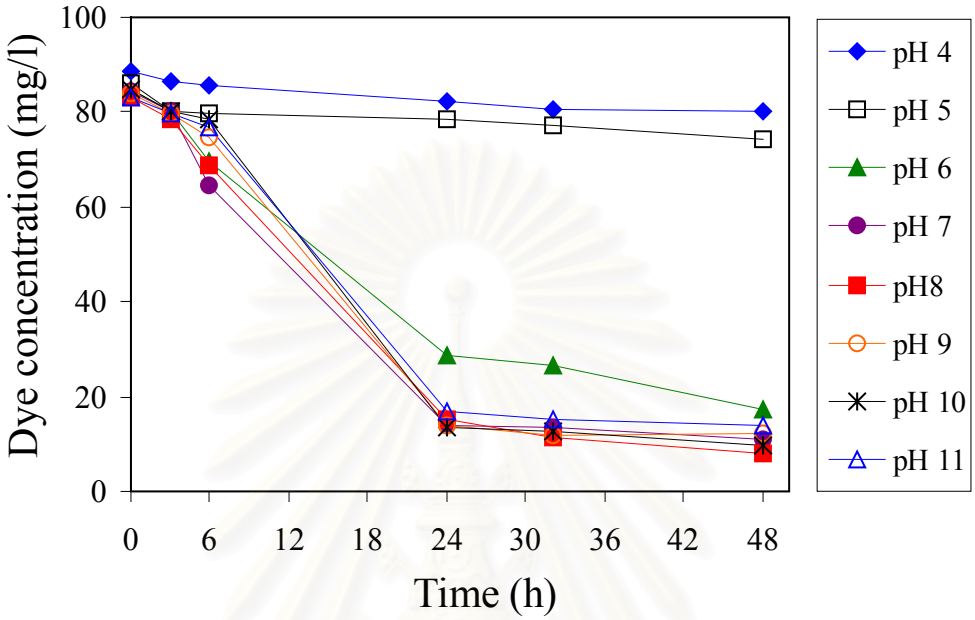


Figure. 6.11 Phase curve profiles of decolorization of Remazol Brilliant Orange 3R at by mixed culture *Paenibacillus* sp. strain S1 and A5 at various initial pH

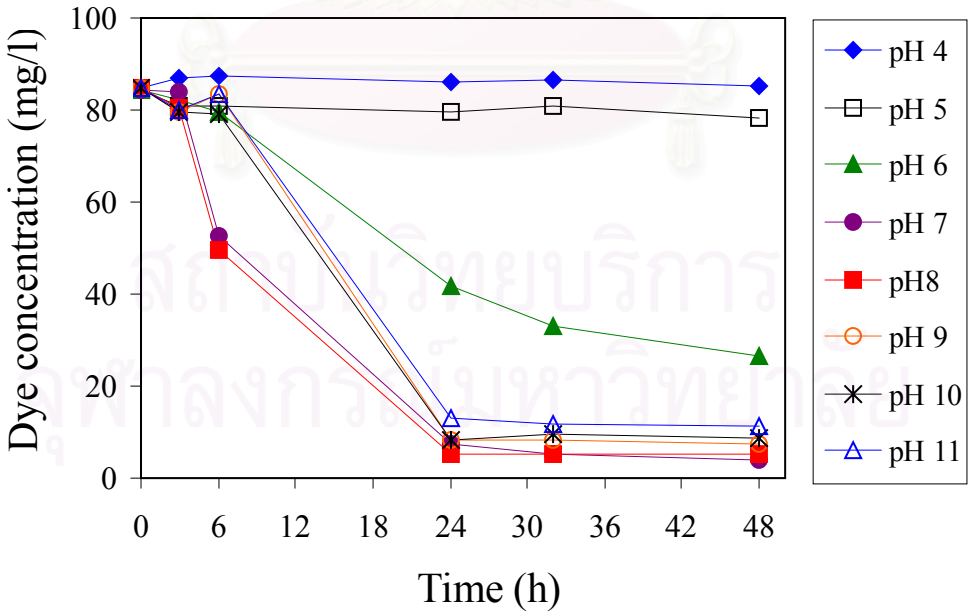


Figure. 6.12 Phase curve profiles of decolorization of Remazol Black B at by mixed culture *Paenibacillus* sp. strain S1 and A5 at various initial pH

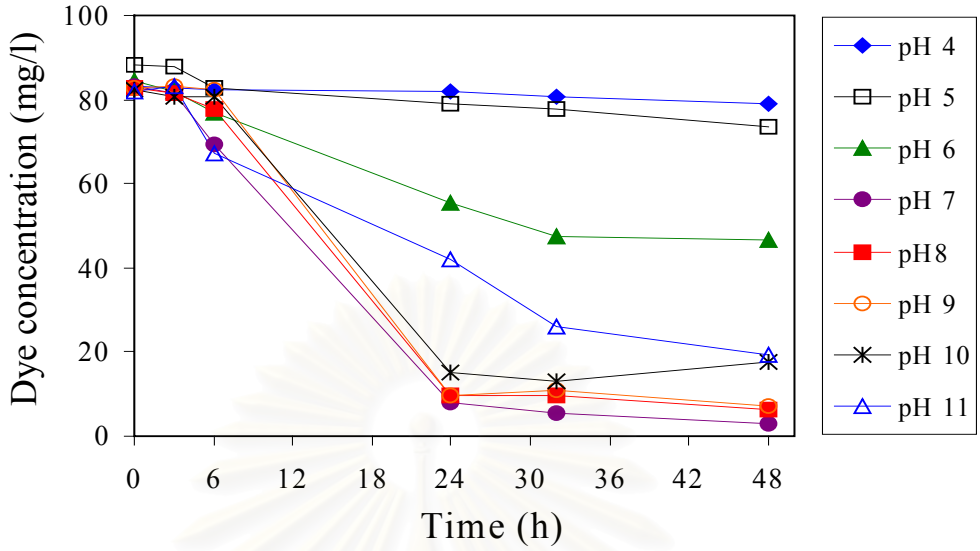


Figure. 6.13 Phase curve profiles of decolorization of Remazol Brilliant Violet 5R at by mixed culture *Paenibacillus* sp. strain S1 and A5 at various initial pH

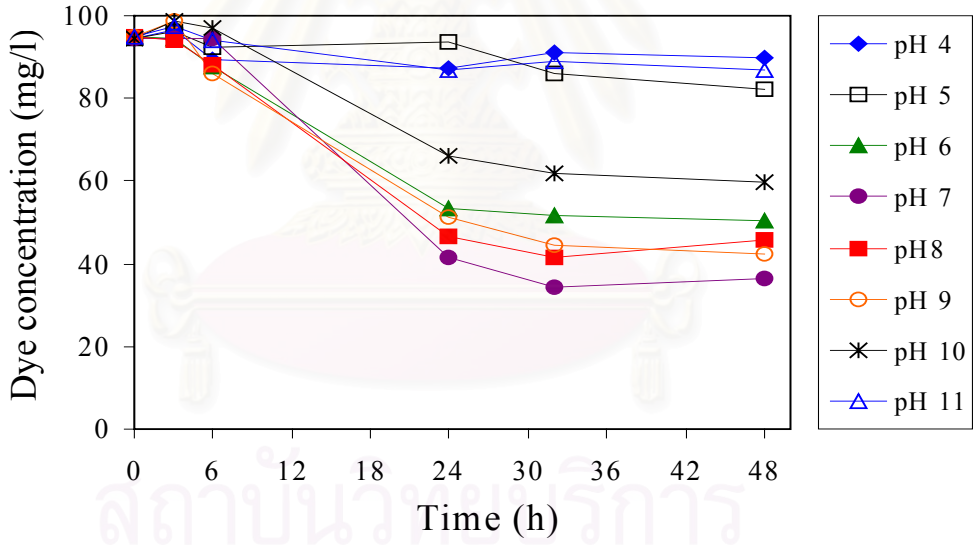


Figure. 6.14 Phase curve profiles of decolorization of Remazol Brilliant Blue R at by mixed culture *Paenibacillus* sp. strain S1 and A5 at various initial pH

Comparison of decolorization of four reactive dyes at various initial pH are presented in Figure 6.11, 6.12, 6.13 and 6.14. It shows that an increase in pH from 5.0 to 7.0 led to a significantly increase in the decolorization rate, which remained essentially the same for pH of 7 to 10. This seems to indicate that neutral and basic pH values would be more favorable for decolorization of the reactive dyes, especially azo dyes. The trend of pH dependence on decolorization is similar to that observed in *Pseudomanas luteola* (Chang et al., 2000) and *Escherichia coli* NO3 (Chang and Kuo, 2000).

6.5 Effect of nitrate concentration on the decolorization of reactive dyes by mixed culture of *Paenibacillus* sp. strain S1 and A5 under anaerobic condition

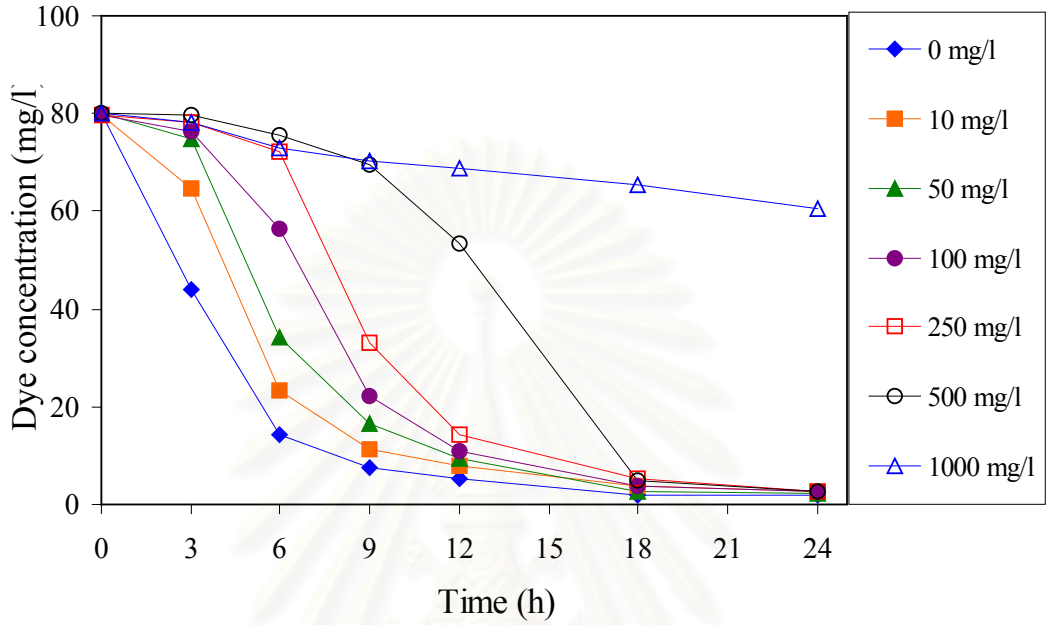


Figure. 6.15 Effect of nitrate concentrations on the decolorization of Remazol Brilliant Orange 3R by mixed culture *Paenibacillus* sp. strain S1 and A5

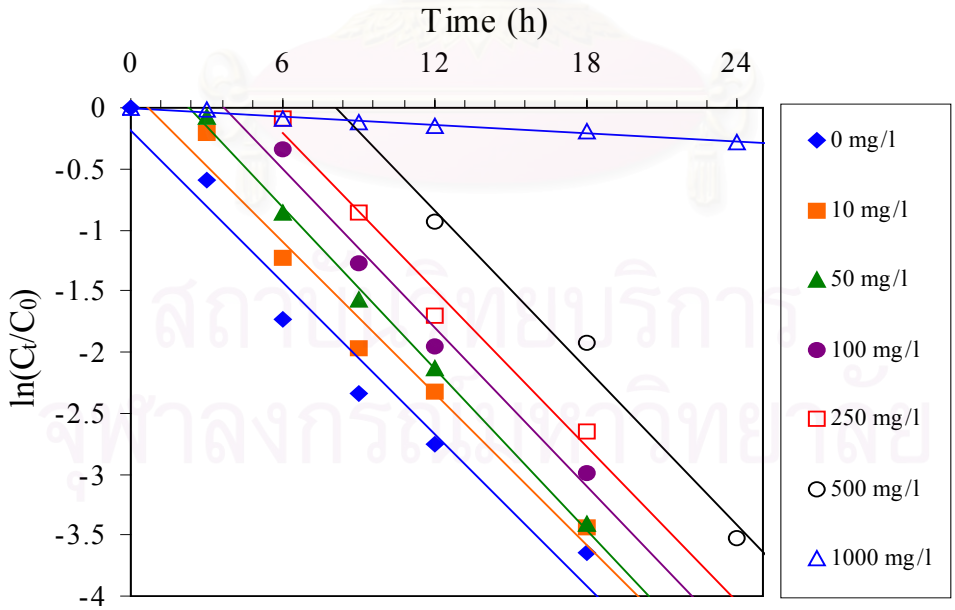


Figure. 6.16 Rates of decolorization of Remazol Brilliant Orange 3R after delay induced by addition of nitrate

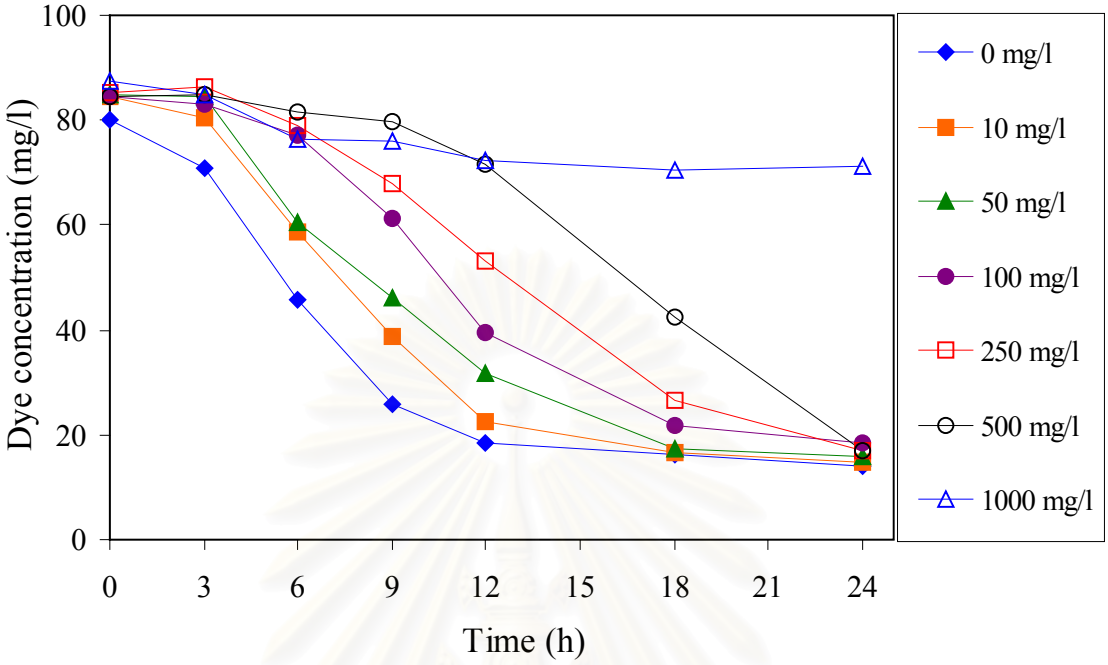


Figure. 6.17 Effect of nitrate concentrations on the decolorization of Remazol Black B by mixed culture *Paenibacillus* sp. strain S1 and A5

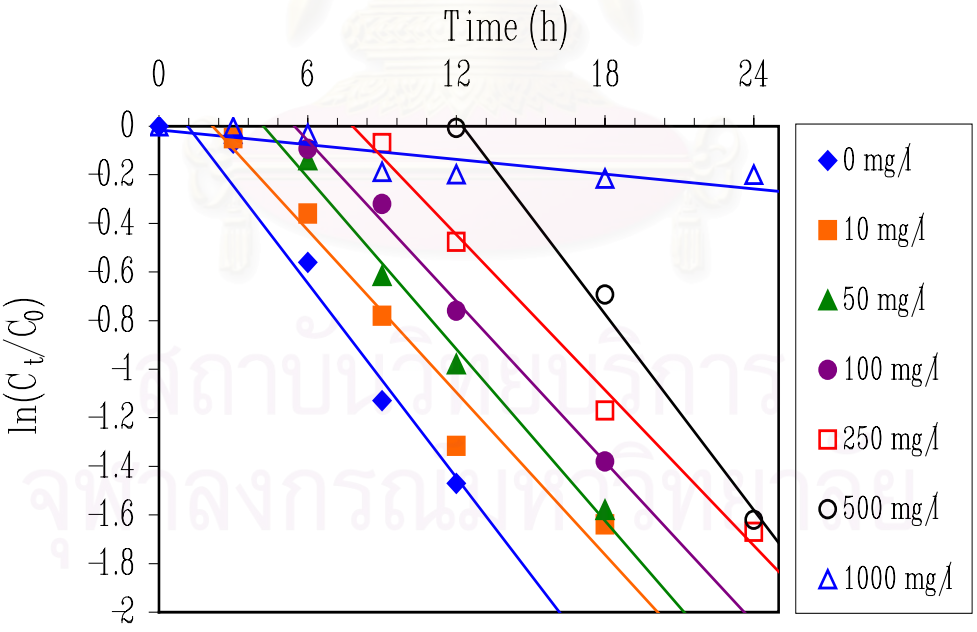


Figure 6.18 Rates of decolorization of Remazol Black B after delay induced by addition of nitrate

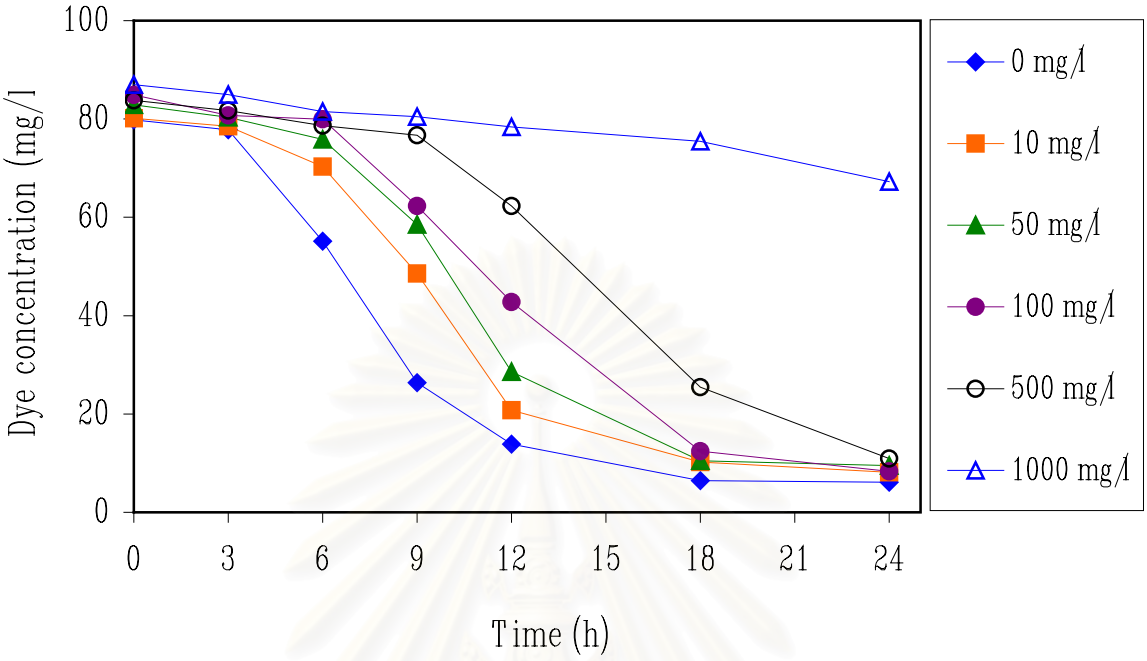


Figure. 6.19 Effect of nitrate concentrations on the decolorization of Remazol Brilliant Violet 5R by mixed culture *Paenibacillus* sp. strain S1 and A5

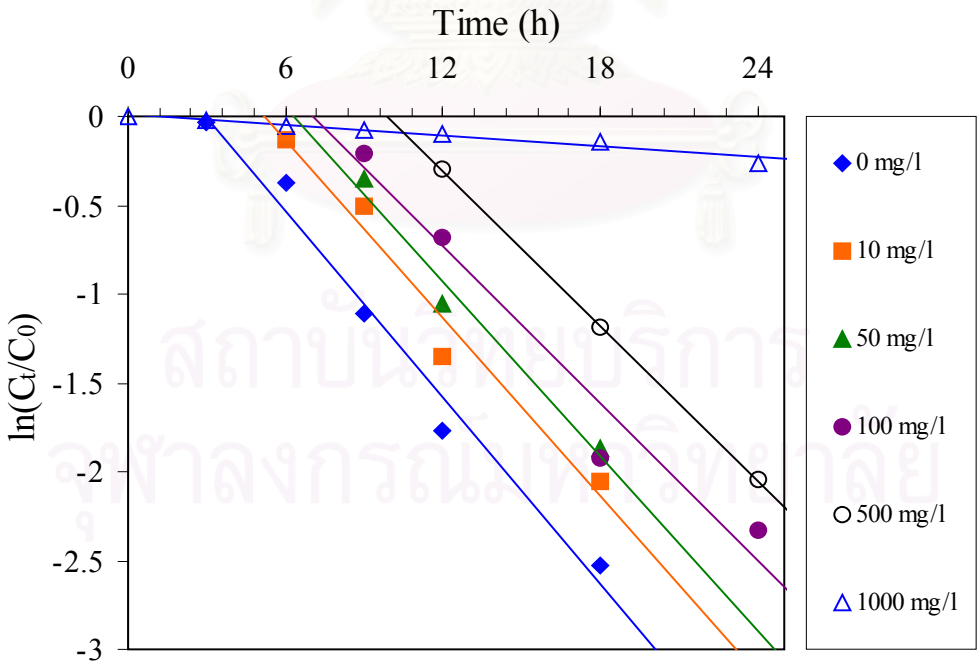


Figure 6.20 Rates of decolorization of Remazol Brilliant Violet 5R after delay induced by addition of nitrate

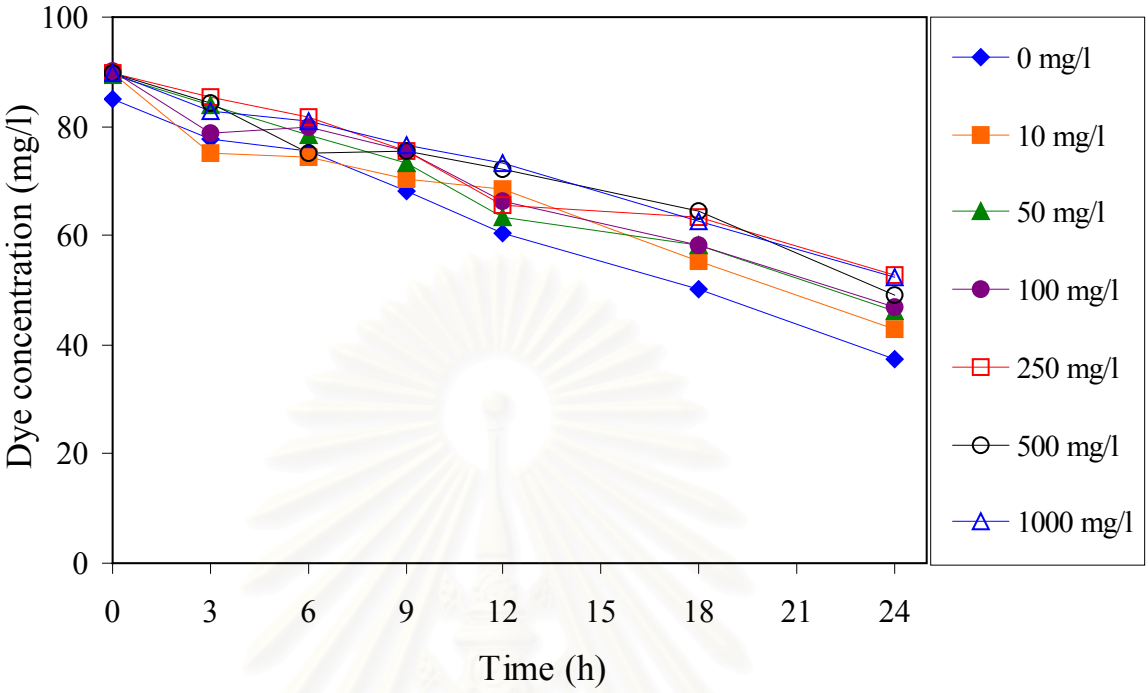


Figure. 6.21 Effect of nitrate concentrations on the decolorization of Remazol Brilliant Blue R by mixed culture *Paenibacillus* sp. strain S1 and A5

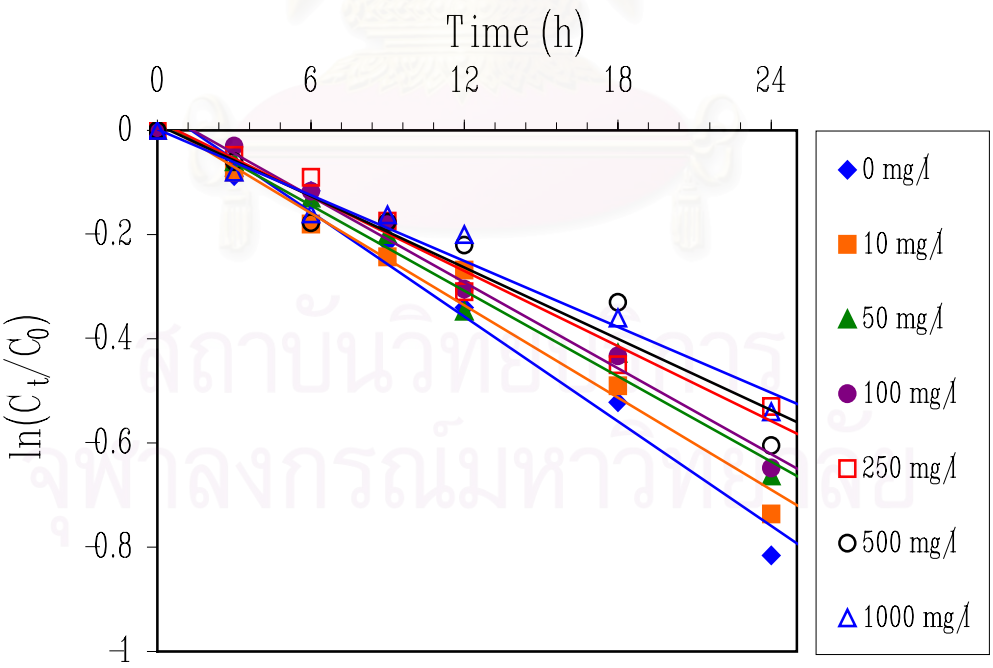


Figure 6.22 Rates of decolorization of Remazol Brilliant Blue R after delay induced by addition of nitrate

Figure 6.15, 6.17 and 6.19 show that the presence of nitrate in the anaerobic system delays the onset of decolorization of azo dyes, Remazol Brilliant Orange 3R, Remazol Black B and Remazol Brilliant Violet 5R, respectively. The duration of these delay phases is related to the initial concentration of nitrate in the system. This is well illustrated by comparing the duration of the delays phase in the system containing 10, 50, 100, 500 mg/l (approximately 3, 6, 9 and 12 h, respectively). The close relationship between nitrate concentrations and the duration of delay suggests that nitrate reduction occur preferentially to reduction of all azo dyes used. Once decolorizations of azo dyes were initiated they conformed to the original first order relationship regardless of the starting concentration of nitrate, as shown in Figure 6.16, 6.18 and 6.20. Similar decolorization rates of all azo dyes were recorded in the control (which no nitrate) and in culture medium containing increasing concentrations of nitrate (Table 6.1).

Table 6.1. Rate of decolorization of reactive dyes following nitrate-induced delay

Nitrate concentrations	First order kinetics of decolorization (k_d/h)			
	Orange	Black	Violet	Blue
0 mg/l	0.207	0.133	0.175	0.033
10 mg/l	0.206	0.111	0.166	0.029
50 mg/l	0.219	0.118	0.164	0.027
100 mg/l	0.216	0.110	0.147	0.027
500 mg/l	0.215	0.134	0.145	0.022
1000 mg/l	0.011	0.010	0.010	0.021

The addition of nitrate had little or no marked effect on decolorization of the Remazol Brilliant Blue R, an anthraquinone dye (Figure 6.21 and 6.22). It is suggested that the mechanism of bacterial decolorization of Remazol Brilliant Blue R differ from the azo dyes.

From the practical point of view, for instance in the disposal of wastewater from the dying industry and with city sewage in activated sludge plants, the inhibition of the azo dyes decolorization by nitrate is most pertinent. The present trend in biological waste treatment is due to use fully nitrifying plants, i. e. complete oxidation of all nitrogen containing compounds in the waste flow. One of the incentives for this practice is the possibility of completely eliminating nitrogen from wastes by denitrification. In this study indicate that all azo dyes are not decolorized until all nitrate has been denitrified. Consequently, it is not possible in practice to combine denitrification and azo dye reduction in a simultaneous process unless the detention time in the anaerobic step corresponds to the sum of the detention times required by the two reactions involved.

6.6 Effect of copper concentration on the decolorization of reactive dyes by mixed culture of *Paenibacillus* sp. strain S1 and A5 under anaerobic condition

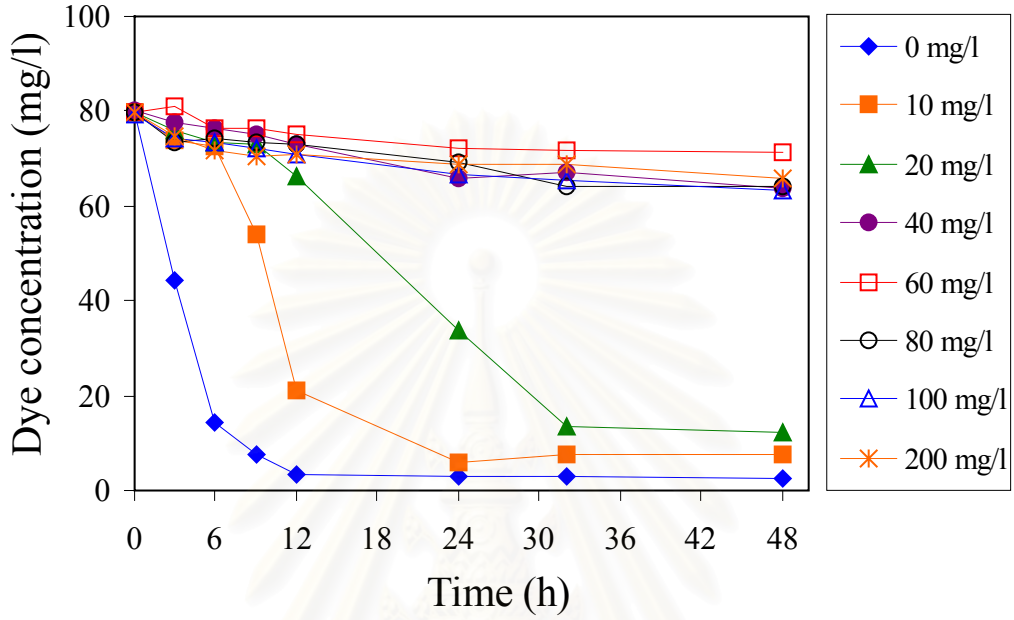


Figure. 6.23 Effect of copper concentrations on the decolorization of Remazol Brilliant Orange 3R by mixed culture *Paenibacillus* sp. strain S1 and A5

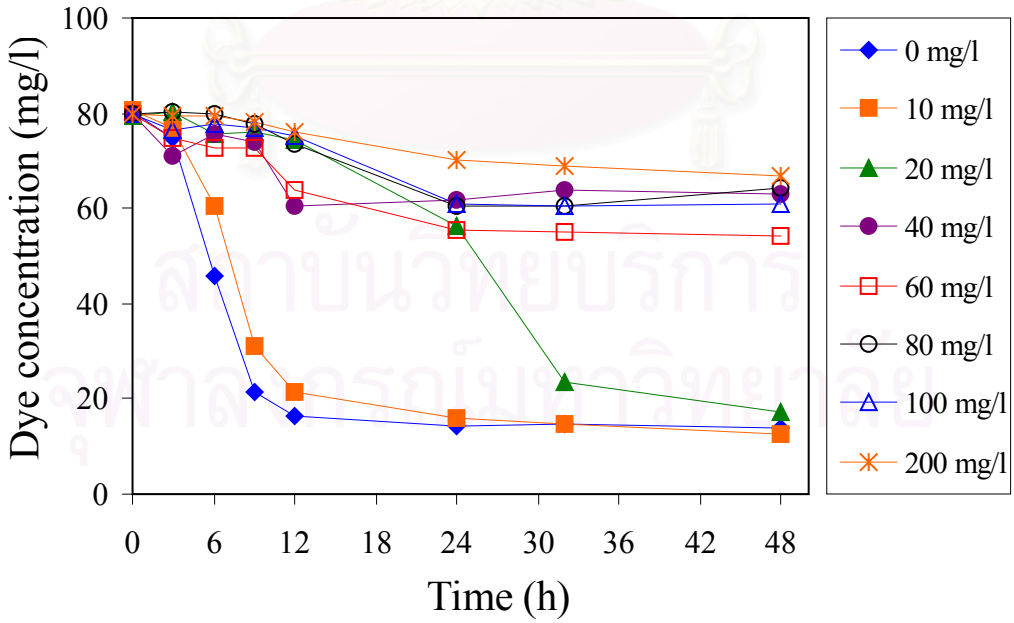


Figure. 6.24 Effect of copper concentrations on the decolorization of Remazol Black B by mixed culture *Paenibacillus* sp. strain S1 and A5

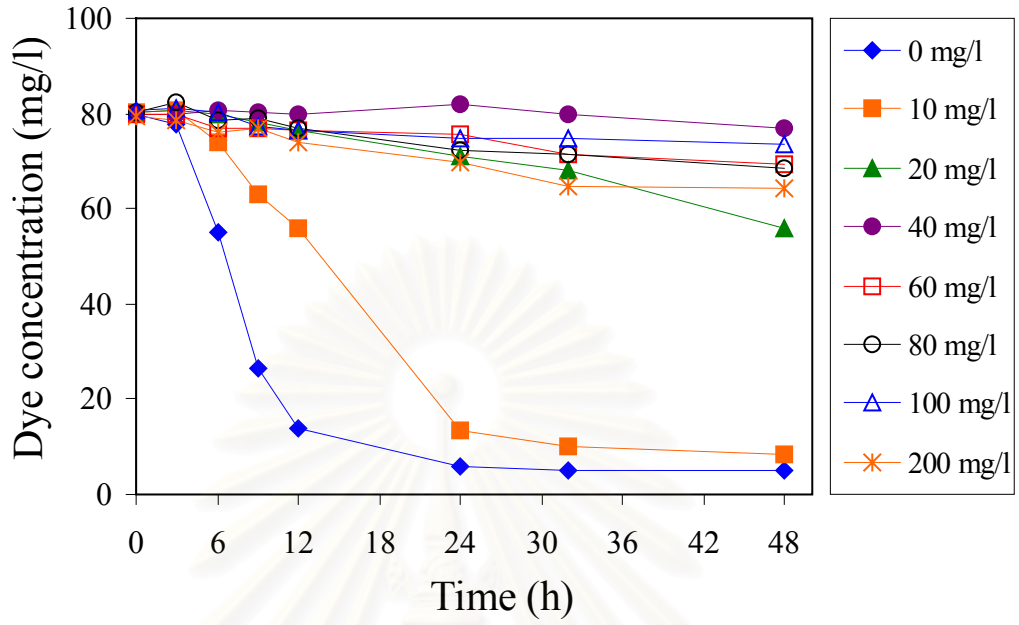


Figure. 6.25 Effect of copper concentrations on the decolorization of Remazol Brilliant Violet 5R by mixed culture *Paenibacillus* sp. strain S1 and A5

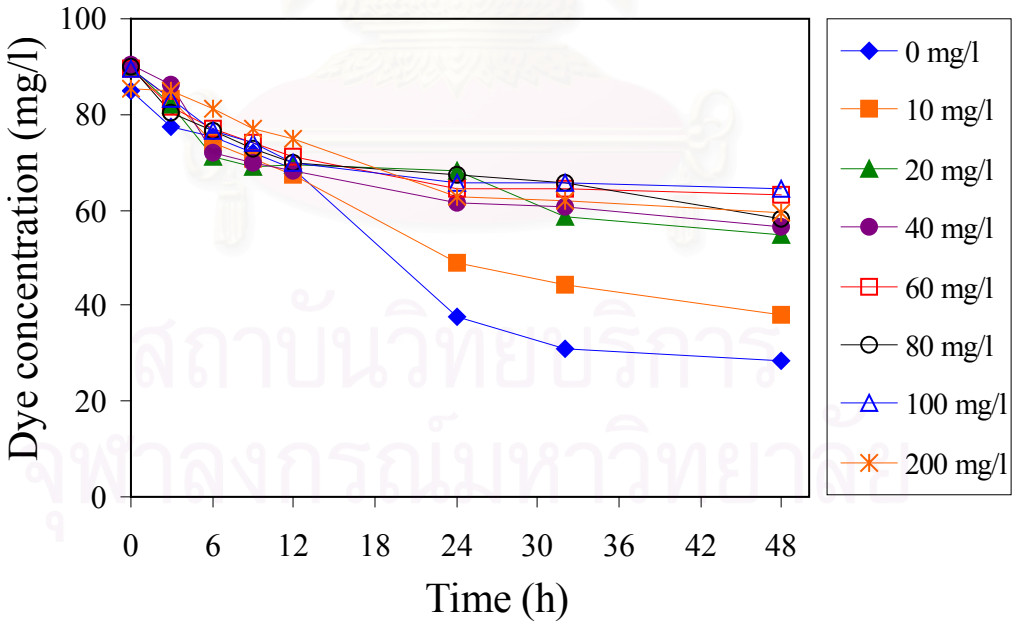


Figure. 6.26 Effect of copper concentrations on the decolorization of Remazol Brilliant Blue R by mixed culture *Paenibacillus* sp. strain S1 and A5

In addition to complex mixture of dyes, the textile mill effluents often contain variable concentrations of heavy metals. Copper (Cu^{2+}) is often toxic to microbial growth and can originate from the use of metallized azo dyes that may contain copper ions both as part of their colorant molecules, and as ionic copper impurities. However, results of earlier studies have shown most of the copper-complexed dyes to contain little free Cu^{2+} relative to their dye-complexed form (Kim and Baughman, 1999). Copper is believed to result mainly from the use of metallized dyes.

Microorganisms require Cu^{2+} at low concentration as essential micronutrient for vital cofactors for metalloproteins and certain enzymes (Nies, 1999). However, at higher concentrations, it has been reported that Cu^{2+} interfere with important microbial processes including aerobic and anaerobic degradation of organic matter (Kuo and Genther, 1996). Toxic effects include ion displacement and/or substitution of essential ions from cellular sites and blocking of functional groups of important molecules, e.g., enzymes, polynucleotides, and essential nutrient transport systems (Nies, 1999). This results in denaturation and inactivation of enzymes and disruption of cell organelle membrane integrity (Ochiai, 1987). The present investigation examines the effect of copper (a well-known toxic metal) on the decolorization of reactive dyes by mixed culture of *Paenibacillus* sp. strain A5 and S1.

Figure 6.23, 6.24, 6.25 and 6.26 show that the presence of copper in the anaerobic system inhibits the decolorization of all reactive dyes used, especially in the case of azo dyes. In the presence of even low copper concentrations (10 mg/l), the mixed culture required more time than in the copper-free control to complete the decocolorization. It can be seen from Figure 6.23, 6.24, 6.25 and 6.26 that under the same conditions, Cu^{2+} at 40 mg/l completely inhibited dolorization of all dyes used, since no colors removal were observed for up to 48 h.

Comparison the decolorization of Remazol Brilliant Orange 3R (Figure 6.23) and Remazol Black B (Figure 6.24) with Remazol Brilliant Violet 5R (6.25) indicates that the copper concentration (20 mg/l) showed more inhibitory effects on the decolorization of the violet dye than the orange and black dye. Since the Remazol Brilliant Violet 5R is known as a copper-complexed azo dye, it is possible that the copper moiety present in the violet dyes molecules could increase the inhibitory effects of free Cu^{2+} present in culture medium on bacterial decolorization.

Sani and co-worker have reported that the presence of Cu^{2+} significantly inhibited the rate at which *Desulfovibrio desulfuricans* lowered the redox potential (E_h) of the medium; thus, lag times were increased compared to copper-free control. The same authors have also suggested that Cu^{2+} likely inhibits periplasmic hydrogenase, which would in turn impair the organism's ability to scavenge trace oxygen, thus lengthening the time required to lower the medium E_h.

In the case of Remazol Brilliant Blue R (Figure 6.26), the inhibition effect of Cu^{2+} on its decolorization might be due to the inhibition of cell growth by Cu^{2+} or competitive adsorption between Cu^{2+} and the dye molecules on binding-sites available on bacterial biomass (Baughman, 2000).

As an approach to solving copper inhibition problems, textile mills should consider the following: (1) Changing the order in which process chemicals (e.g., Cu^{2+}) are released. (2) Decreasing the use of copper-complexing chemicals. (3) Reducing the amount of strong sequestrant (e.g., EDTA), which can increase the concentration of dissolved copper in textile effluents.

6.7 Effect of initial dyes concentration on color removal performance of the mixed culture *Paenibacillus* sp. strain S1 and A5

To determine the maximum reactive azo dyes concentration tolerated by a mixed culture of *Paenibacillus* sp. strain A5 and S1, experiments with different initial dye concentrations (50-1000 mg/l) were performed. The decolorization efficiencies of all azo dyes were above 90% for initial dye concentration less than 1000 mg/l after 2 days cultivation (Figure 6.27, 6.28, 6.29 and 6.30). This means that an acceptable high color removal can be achieved by the defined mixed culture of *Paenibacillus* sp. strain A5 and S1 in an extensive range of azo dye concentrations.

The dye content in an industrial azo waste stream typically varies from 10 to 50 mg/l (Coughlin et al., 2002). However, changes in dyeing condition do occur and it is important to know how well an azo dye-decolorizing by bacterial cultures can handle higher concentrations. The data in Figure 6.27, 6.28, 6.29 and 6.30 clearly demonstrate the capacity of mixed-culture *Paenibacillus* sp. strain A5 and S1 to remove sudden increase in dye concentration.

Azo dyes generally contain one or more sulfonic-acid groups on aromatic rings, which might act as detergents to inhibit the growth of microorganisms (Wuhrmann et al., 1980). In addition, it was also reported that dyes were the inhibitors for nucleic acid synthesis (Ogawa et al., 1988) or for cell growth (Ogawa et al., 1989). However, substrate inhibitions were not observed at the concentration of dyes used in this study (50 – 1000 mg/l).

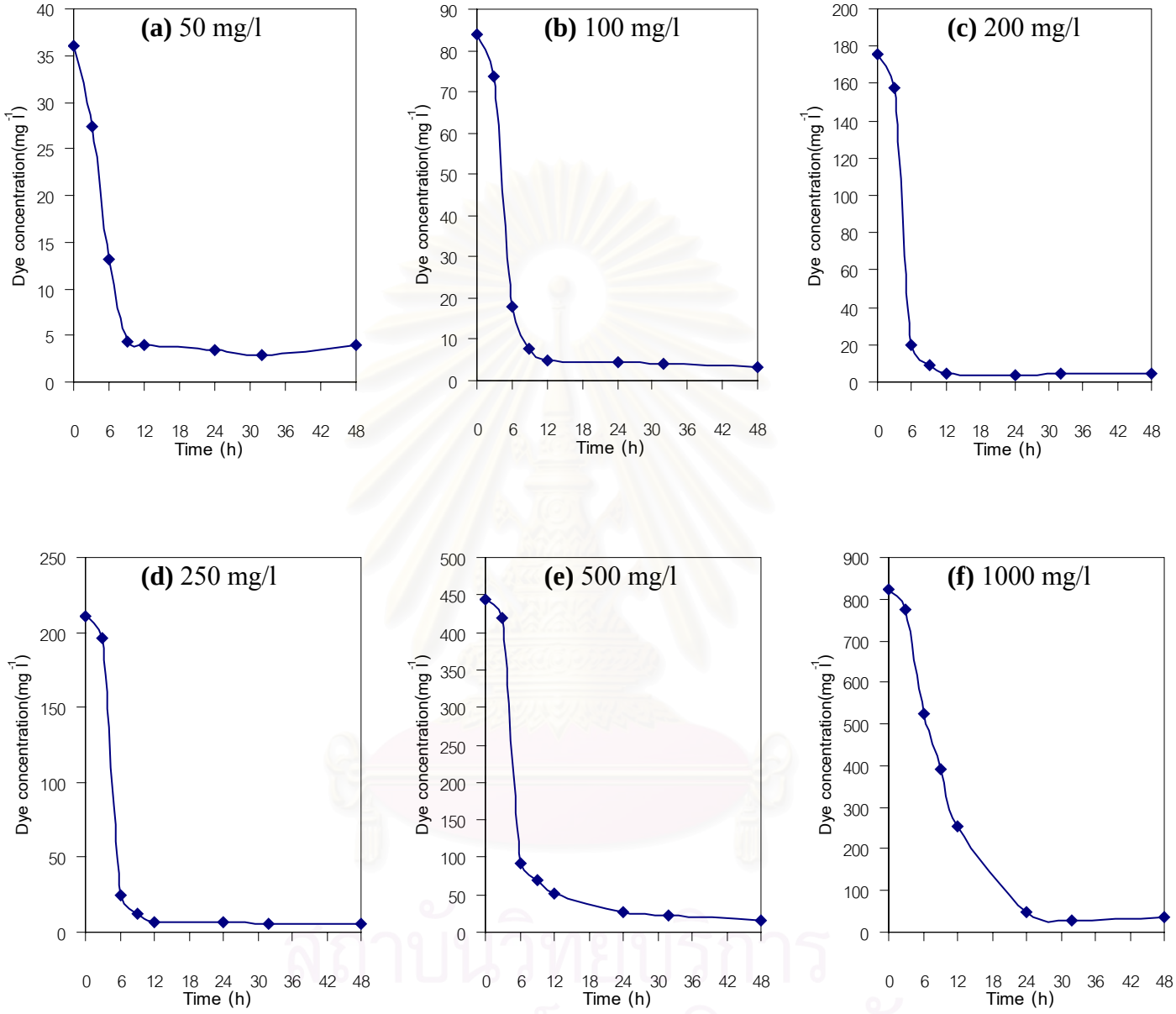


Figure 6.27 . Time-course decolorization profiles at the different initial concentrations of Remazol Brilliant Orange 3R by mixed culture *Paenibacillus* sp. strain A5 and S1 under anaerobic condition

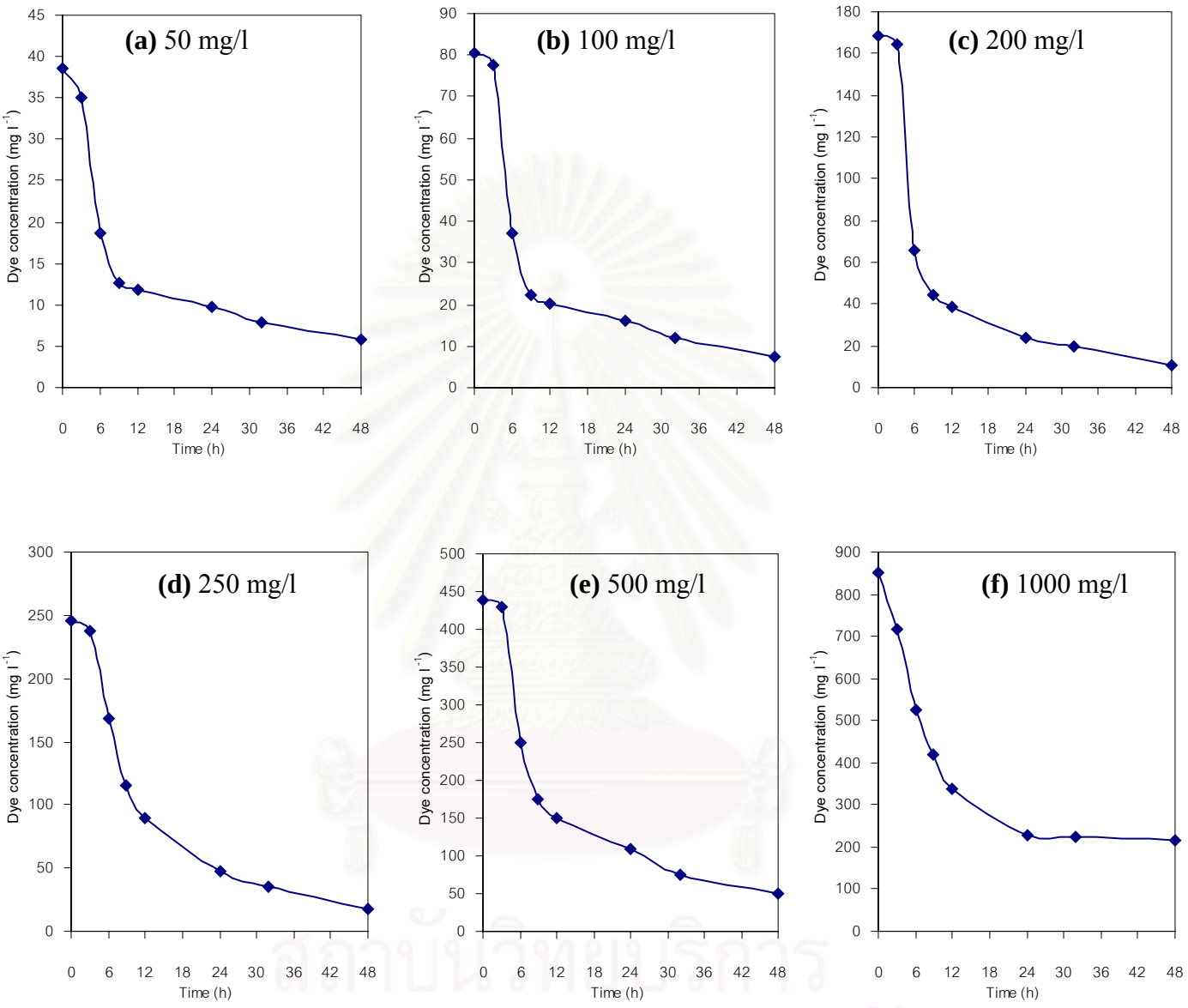


Figure. 6.28 Time-course decolorization profiles at the different initial concentration of Remazol Black B by mixed culture *Paenibacillus* sp. strain A5 and S1 under anaerobic condition

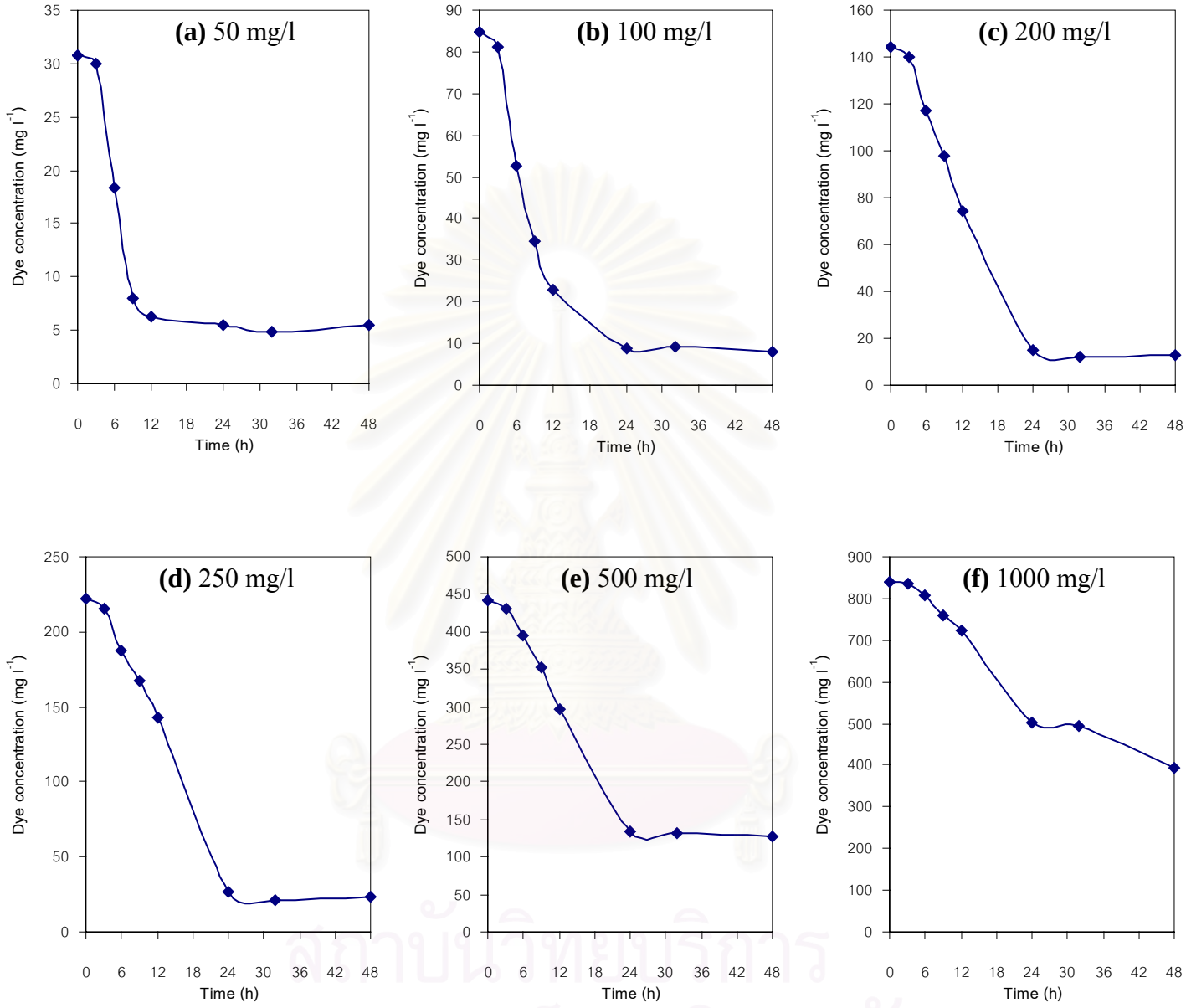


Figure. 6.29 Time-course decolorization profiles at the different initial concentrations of Remazol Brilliant Violet 5R by mixed culture *Paenibacillus* sp. strain A5 and S1 under anaerobic condition

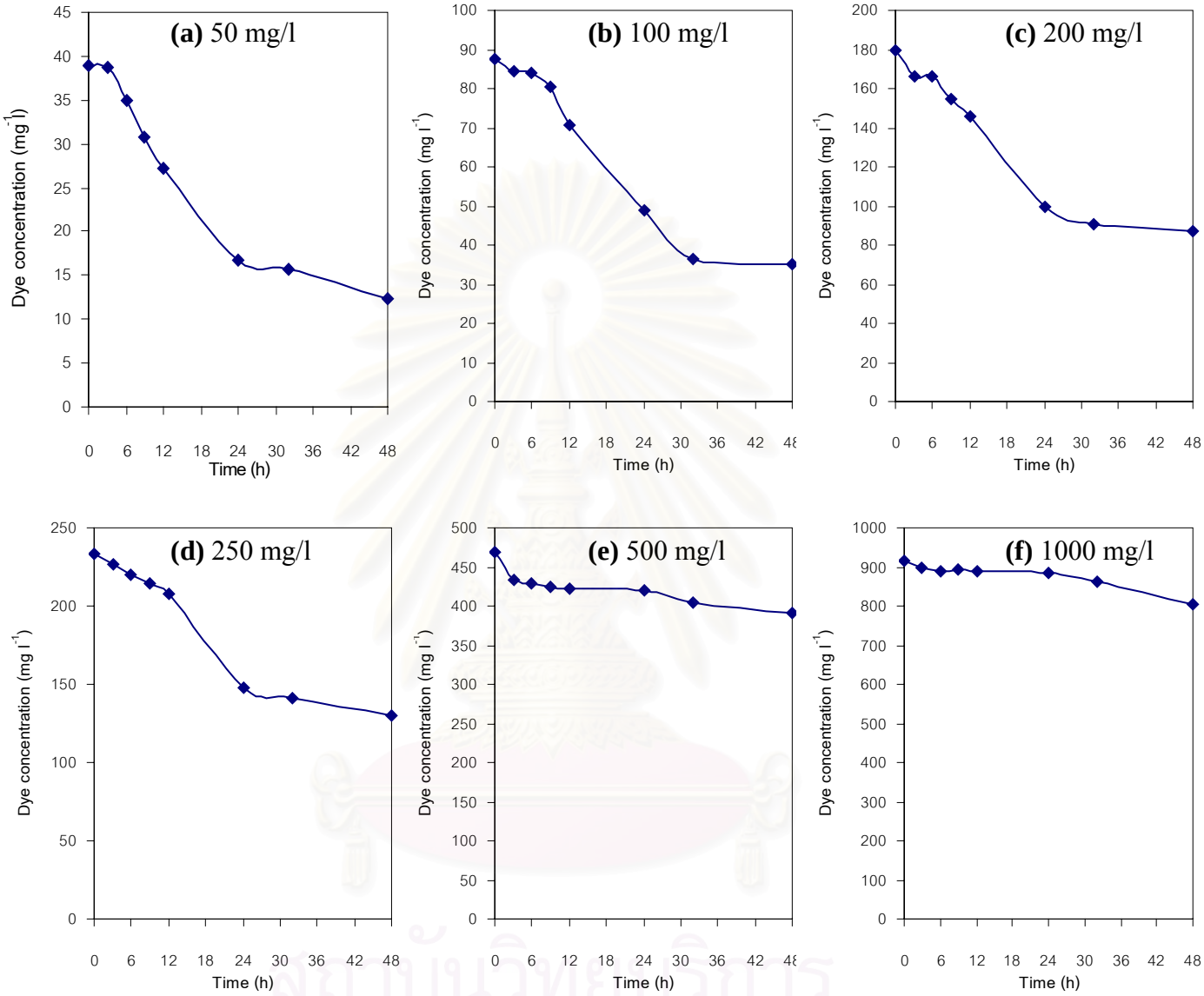


Figure. 6.30 Time-course decolorization profiles at the different initial concentrations of Remazol Brilliant Blue R by mixed culture *Paenibacillus* sp. strain A5 and S1 under anaerobic condition

The dependence of specific decolorization rate (r_{dye}) on the dye concentration is shown in Figure 6.31. The r_{dye} value tended to increase as the concentration of Remazol Brilliant Orange 3R, Remazol Black B, and Remazol Brilliant Violet 5R was increased from 50 to 1000 mg/l.

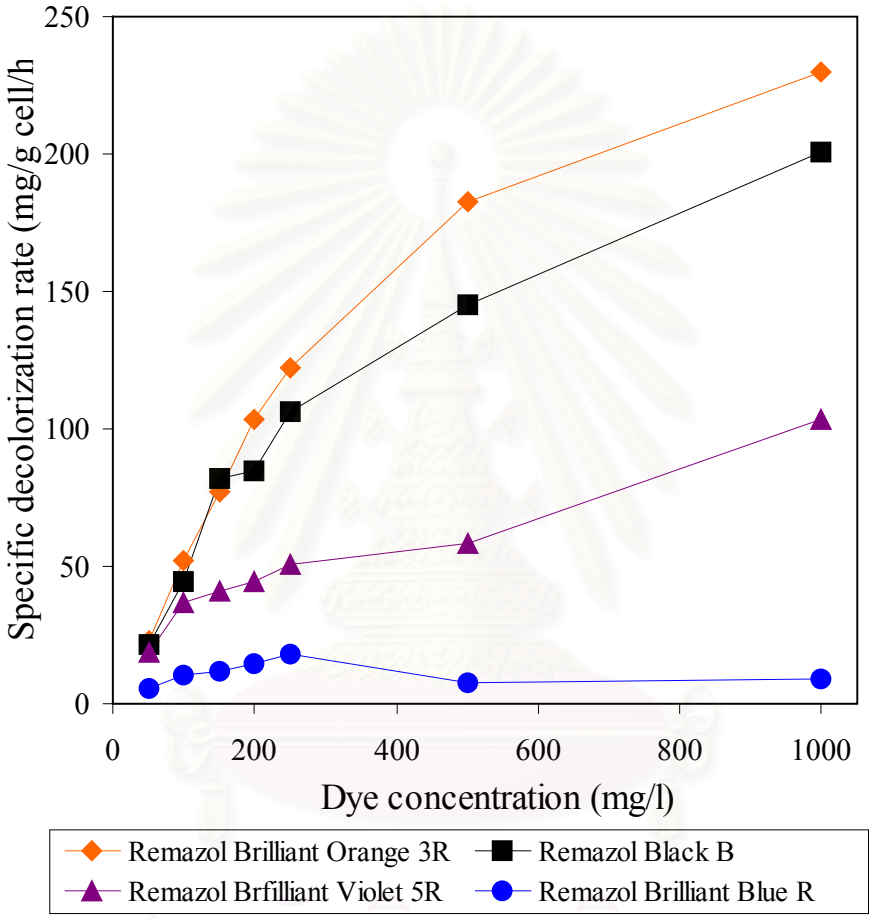


Figure 6.31 Dependence of specific decolorization rate on the initial concentration of reactive dyes

The correlation between specific decolorization rate and dye concentration can be described by Michaelis-Menten kinetics (Roberts, 1977),

$$r_{dye} = r_{dye,max}[Dye]/(K_m + [Dye])$$

where; $r_{dye,max}$ is the maximum specific decolorization rate (mg/h/g cell)
K_m is Michaelis constant (mg/l)
[Dye] is the concentration of reactive azo dye (mg/l).

The kinetic constants $r_{dye,max}$ and K_m were calculated for each of the reactive dye-bacteria systems by use of the Lineweaver-Burk equation.

$$1/r_{dye} = (K_m/r_{dye,max})\,(1/[Dye]) + 1/r_{dye,max}$$

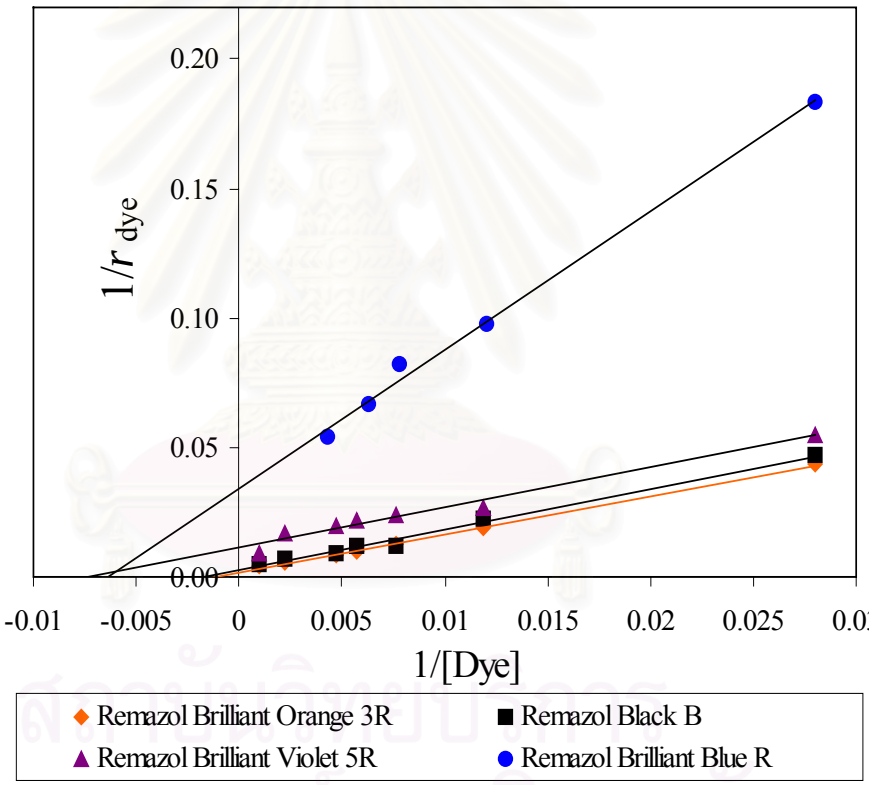


Figure 6.32. Lineweaver-Burk plot of reactive dye decolorization by the mixed culture of *Paenibacillus* sp. strain A5 and S1

The Michaelis-Menten kinetic constants (K_m) estimated from the experimental data (Figure 6.32) are 578.8p, 595.6, 186.5 and 712.0 mg/l for the orange, black, violet and blue dye, respectively. The maximum specific decolorization rate ($r_{dye,max}$) for decolorization of Remazol Brilliant Orange 3R, Remazol Black B, Remazol Brilliant Violet 5R and Remazol Brilliant Blue R are 392.2, 378.8, 120.5 and 133.3 mg/gcell/h, respectively.

6.8 Decolorization of mixture of dyes by the mixed culture *Paenibacillus* sp. strain S1 and A5 under aerobic and anaerobic condition.

Dyes of different structures are often used in the textile processing industry, and, therefore, the effluents from the industry are markedly variable in composition. A nonspecific biological process may be vital for treatment of the textile effluents containing a mixture of dyes.

6.8.1 Analysis of color removal in synthetic textile wastewater containing mixture of four reactive dyes by mixed culture of *Paenibacillus* sp. strain A5 and S1

All reactive dyes including Remazol Brilliant Orange 3R, Remazol Black B, Remazol Brilliant Violet 5R and Remazol Brilliant Blue R, each at a concentration of 100 mg/1, were dissolved together in synthetic wastewater medium. The mixture of dyes did not have a well-defined peak at the visible absorption spectra. Therefore, the detection of color level was made using the alternated American Dye Manufacturers Institute (ADMI) Method (Eaton et al., 1995). Spectrophotometric measurements were made with a Hitachi spectrophotometer, model U-2000 (see appendix 2 for detail).

From the results in Figure 6.33, it is shown that the synthetic textile wastewater containing the mixture of four reactive dyes was rapidly decolorized under the anaerobic incubation and the color removal was almost 60.5% within 24 h of anaerobic cultivation, followed by an insignificant change in decolorization for the next 10 days (data not shown). Figure 6.34 displayed a well-growth of *Paenibacillus* strains under aerobic condition, however, no color removal are observed in an aerobic condition.

According to the reports (Knapp and Newby, 1995; Sani and Banerjee, 1999) decolorization of dyes by bacteria can be due to adsorption to microbial cells or to biodegradation. In adsorption, examination of the absorption spectrum will reveal that all peaks decrease approximately in proportion to each other. If the dye removal is attributed to biodegradation, either the major visible light absorbance peak will completely disappear or a new peak will appear. The typical visible spectra obtained during the decolorization of mixed dyes are shown in Figure 6.35.

Dye adsorption can also be judged clearly by inspecting the cell mats. Cell mats become deeply colored because of adsorbing dyes, whereas those retaining their original colors are accompanied by the occurrence of biodegradation (Knapp and Newby, 1995). Also, the bacterial cells in this study were stained with the blue color under both anaerobic and aerobic incubated conditions therefore, the decolorization of Remazol Brilliant Blue R are suspected to causing by biosorption mechanism.

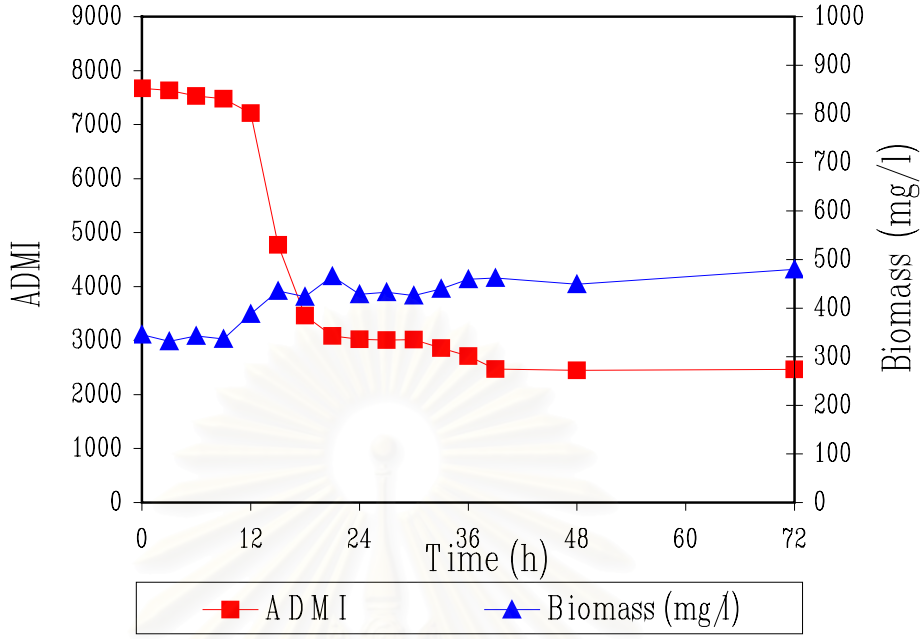


Figure. 6.33 Time courses of growth and decolorization of mixture dyes including Remazol Brilliant Orange 3R, Remazol Black B, Remazol Brilliant Violet 5R and Remazol Brilliant Blue R by mixed culture *Paenibacillus* sp. strain A5 and S1 under anaerobic condition

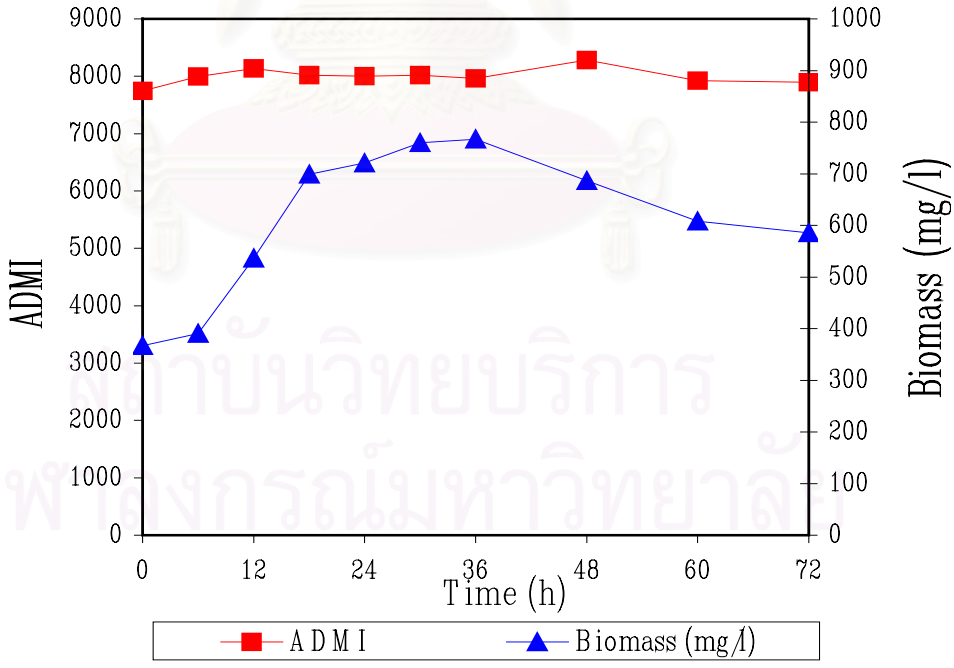


Figure. 6.34 Time courses of growth and decolorization of mixture dyes including Remazol Brilliant Orange 3R, Remazol Black B, Remazol Brilliant Violet 5R and Remazol Brilliant Blue R by mixed culture *Paenibacillus* sp. strain S1 and A5 under aerobic condition

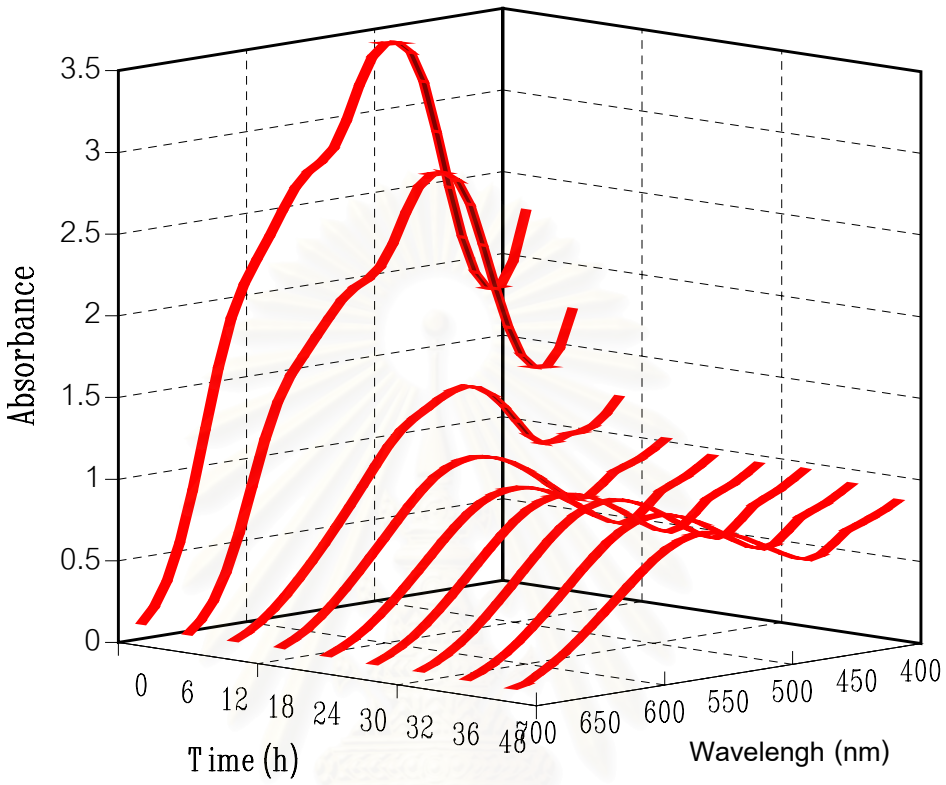


Figure. 6.35 Typical visible spectra of centrifuged samples from anaerobic decolorization of dyes mixture including Remazol Brilliant Orange 3R, Remazol Black B, Remazol Brilliant Violet 5R and Remazol Brilliant Blue R by mixed culture *Paenibacillus* sp. strain S1 and A5 at various incubation time

The absorbance peak at approximate 500 nm, which is the maximum adsorption wavelength of Remazol Brilliant Orange 3R, dramatically decreased after 12 h of anaerobic cultivation (Figure 6.35).

6.8.2 Determination of decolorization of individual dyes in the synthetic textile wastewater containing mixture of reactive dyes by Paenibacillus sp. strain A5 and S1

The spectrophotometric determination of four reactive dyes can be a difficult problem in view of the complexity of their absorption spectra. The results from the univariate calibration show significant errors, due to the multiple interferences mentioned above. By using a multivariate calibration the accuracy is increased significantly, because by monitoring various wavelengths the contribution of the interference signals is greatly reduced (Figure 6.36).

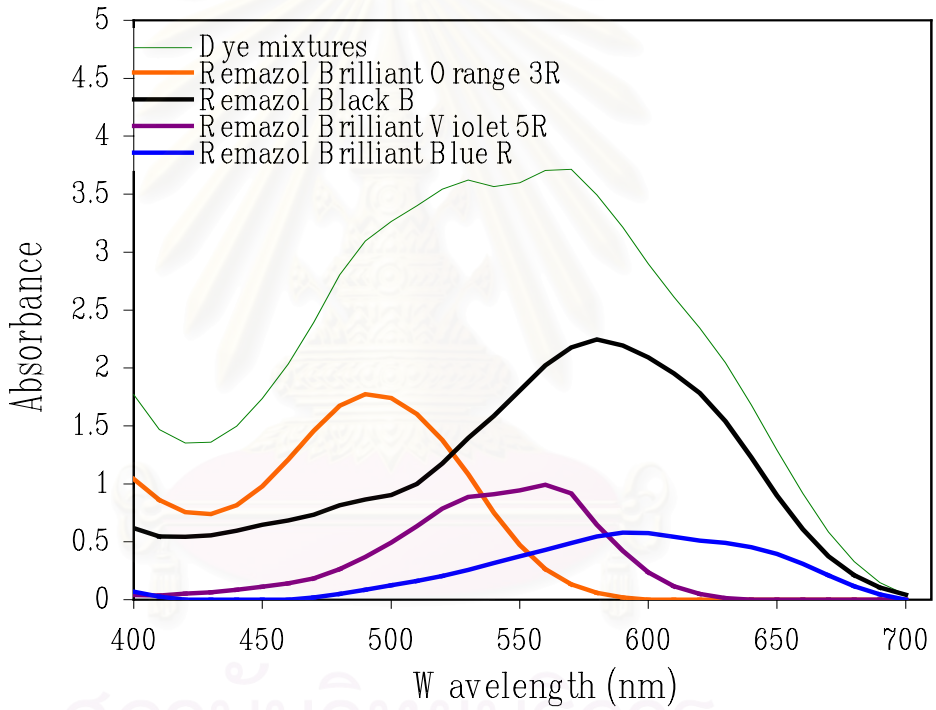


Figure 6.36. Typical visible spectrum of Remazol Brilliant Orange 3R, Remazol Black B, Remazol Brilliant Violet 5R, Remazol Brilliant Blue R and four dye mixtures

The analytical methodology for determination of the remaining concentration of the individual dye during decolorization of the dye mixtures in the first experiment (Remazol Brilliant Orange 3R, Remazol Black B and Remazol Brilliant Violet 5R) and dye mixtures in the second experiment (Remazol Brilliant Orange 3R, Remazol Brilliant Violet 5R and Remazol Brilliant Blue R) had been carried out by using the spectrophotometric method with a multivariate calibration technique (partial least-squares regression, PLSR).

The calibration was made by by PLSR, known amounts of the standard dye solutions were placed in a 10 ml volumetric flask and completed to the final volume with deionized water (final pH: 7.5). The final concentration of these solution varied between 10 and 100 mg/1 of each dye. The calibration was made by the PLSR, using the recorded adsorbance values between 400 and 700 nm as the dependent variables, with intervals of 10 nm. Hitachi spectrophotometer, model U-2000 was used for multi-components of dye determination because it has some unique features: standard curves for multivariate calibration and multi-component value calculation equations are installed in this instrument (see appendix 3 for more detail).

Figure 6.37 and 6.38 show the decolorization profile for synthetic wastewater containing mixture of all azo dye: Remazol Brilliant Orange 3R, Remazol Black B and Remazol Brilliant Violet 5R, each concentration of 100 mg/l. It can be noted from these figures that the extent of decolorization of azo dye occurred rapidly under anaerobic condition. Meanwhile the well-growth are observed under the aerobic condition. Similar results are observed with the dye mixtures in the second experiment (Figure 6.40 and 6.41) that the majority of azo dye was decolorized under anaerobic condition.

In the dye mixtures containing Remazol Brilliant Blue R (the second experiment), it is shown that the azo dyes, Remazol Brilliant Orange 3R and Remazol Brilliant Violet 5R, were rapidly decolorized under the anaerobic condition with the same decolorization rate obtained form the single dye decolorization experiments (Figure 6.40). In the other hand, only the small amounts of Remazol Brilliant Blue R were removed under aerobic condition (Figure 6.41). Figure 6.41 also displayed a well-growth of *Paenibacillus* strains A5 and S1 under aerobic condition, however, no color removal of azo dyes are observed in aerobic condition.

From Figure 6.37, as the Remazol Brilliant Orange 3R, Remazol Black B, and Remazol Brilliant Violet 5R were removed, the *Paenibacillus* strains remained colorless. Different results were observed in a mixture of dyes which containing of Remazol Brilliant Blue R, which the biomass were strained to deep blue color after both aerobic and anaerobic incubation.

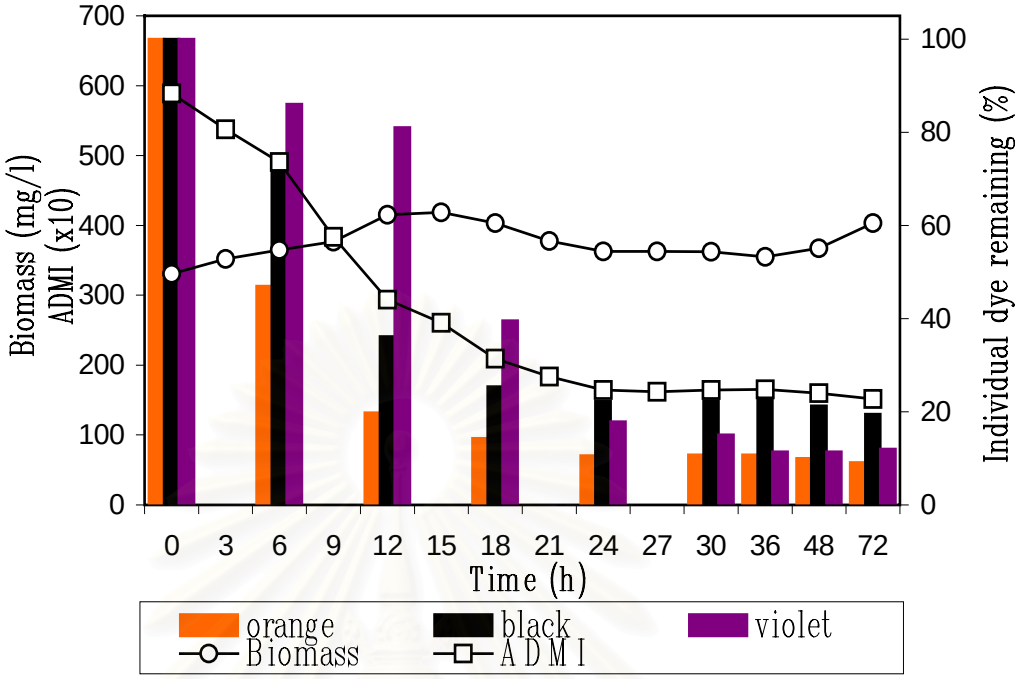


Figure. 6.37 Time courses of growth and decolorization of mixture dyes including Remazol Brilliant Orange 3R, Remazol Black B and Remazol Brilliant Violet 5R by mixed culture *Paenibacillus* sp. strain S1 and A5 under anaerobic condition

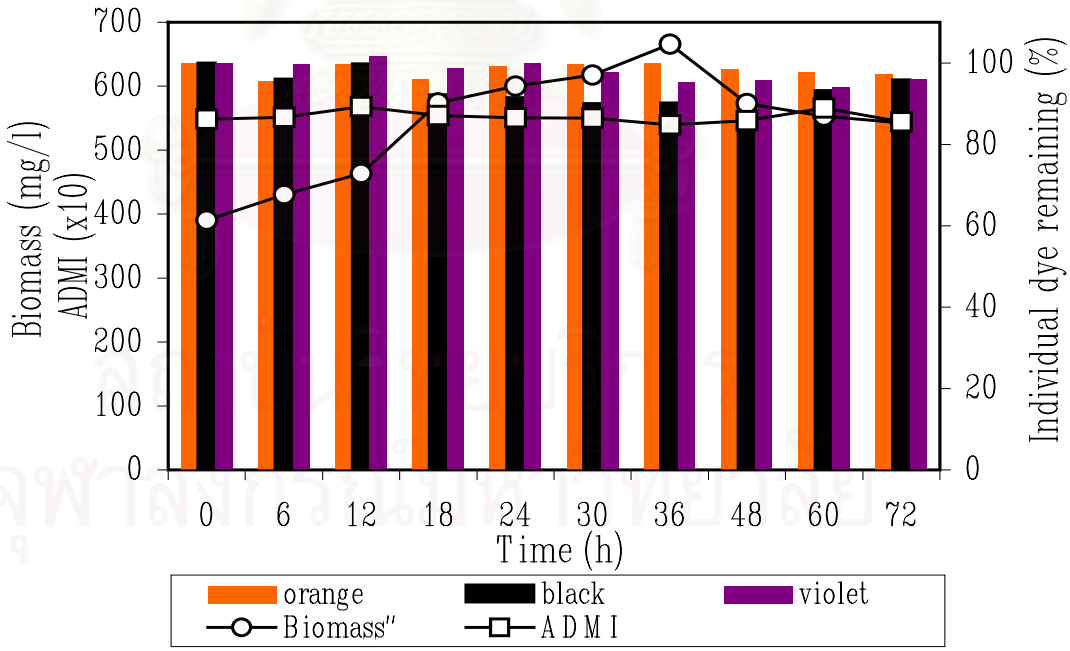


Figure. 6.38 Time courses of growth and decolorization of mixture dyes including Remazol Brilliant Orange 3R, Remazol Black B and Remazol Brilliant Violet 5R by mixed culture *Paenibacillus* sp. strain S1 and A5 under aerobic condition

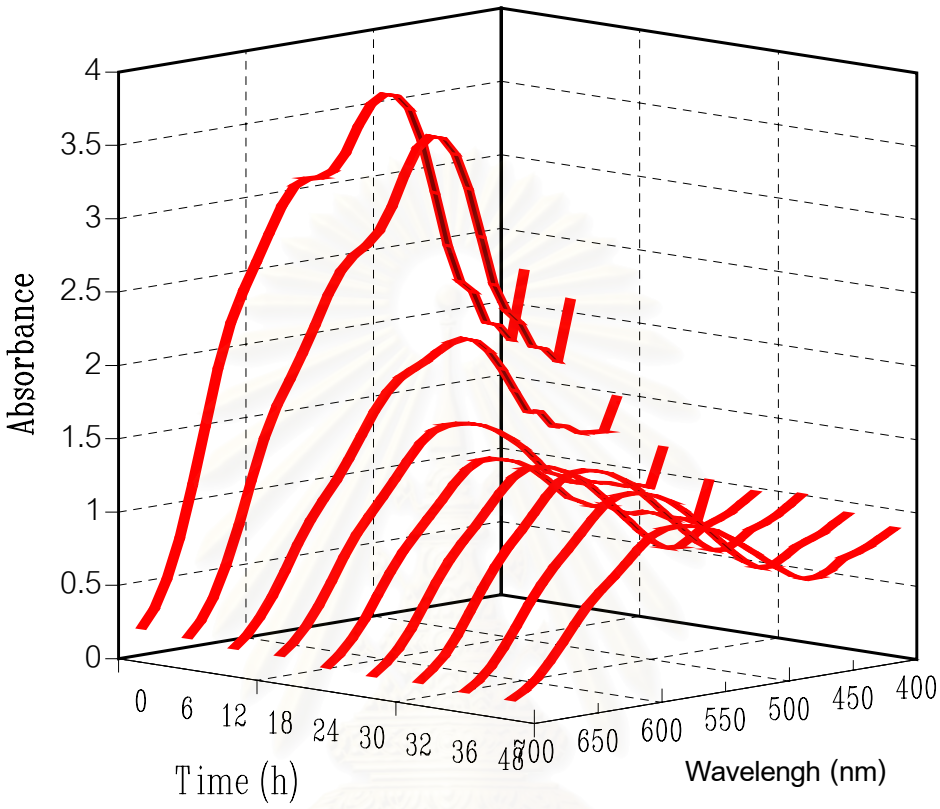


Figure. 6.39 Typical visible spectra of centrifuged samples from anaerobic decolorization of dyes mixture including Remazol Brilliant Orange 3R, Remazol Black B and Remazol Brilliant Violet 5R by mixed culture *Paenibacilluss* sp. strain S1 and A5 at various incubation times

The progressive decolorization of the dye mixture by mixed culture of strain A5 and S1 can be observed in the spectral sequence presented in Figure 6.39 and 6.42. The absorbance peak at approximate 500 nm, which is the maximum adsorption wavelength of Remazol Brilliant Orange 3R, dramatically decreased after 12 h of anaerobic cultivation (Figure 6.39). The same phenomenon has been appeared in Figure.6.42, there was a significant decrease in color intensity or in the peak absorbance at 500 nm. before decreased in other wavelength.

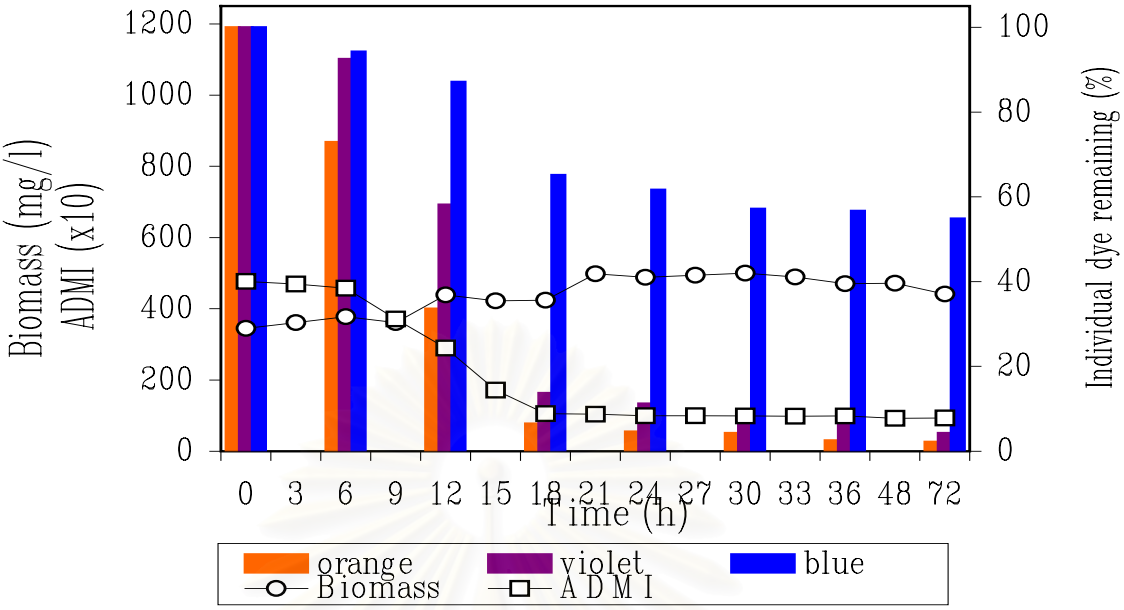


Figure. 6.40 Time courses of growth and decolorization of mixture dyes including Remazol Brilliant Orange 3R, Remazol Brilliant Violet 5R, and Remazol Brilliant Blue R by mixed culture *Paenibacillus* sp. strain A5 and S1 under anaerobic condition

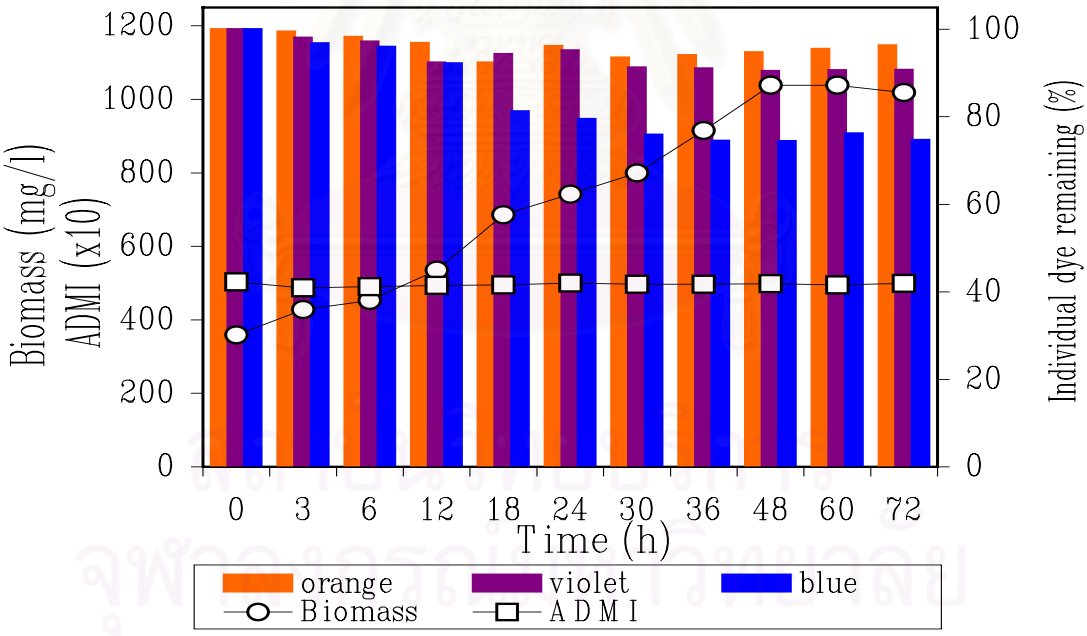


Figure. 6.41 Time courses of growth and decolorization of mixture dyes including Remazol Brilliant Orange 3R, Remazol Brilliant Violet 5R, and Remazol Brilliant Blue R by mixed culture *Paenibacillus* sp. strain A5 and S1 under aerobic condition

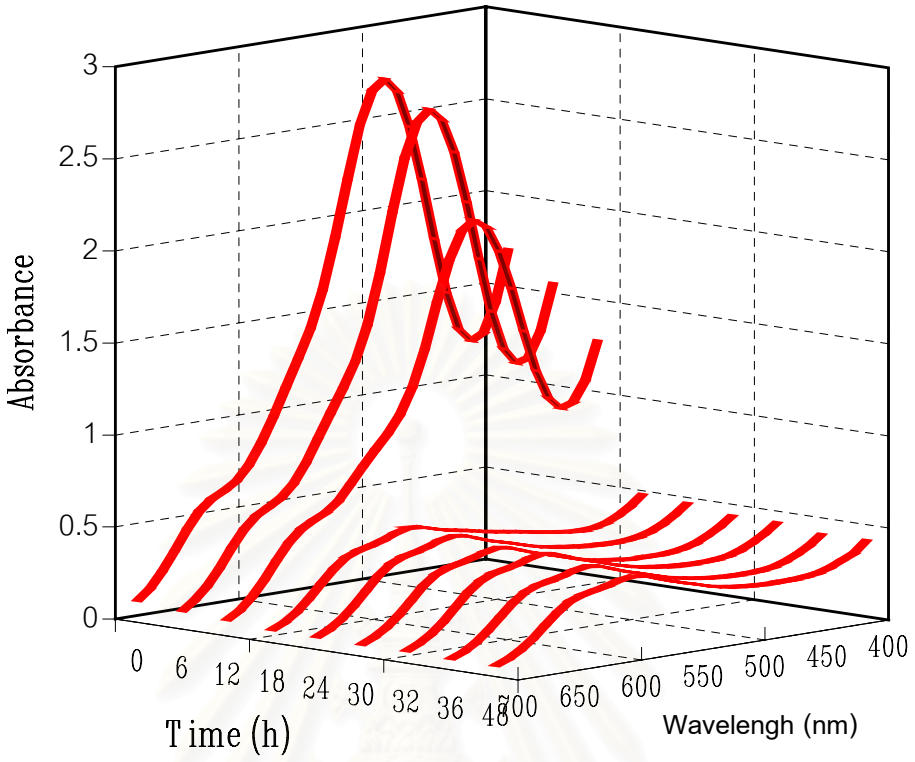


Figure. 6.42 Typical visible spectra of centrifuged samples from anaerobic decolorization of dye mixtures including Remazol Brilliant Orange 3R, Remazol Brilliant Violet 5R, and Remazol Brilliant Blue R by mixed culture *Paenibacilluss* sp. strain S1 and A5 at various incubation times

Consequently, according to the above results, the azo dyes removal by mixed culture of *Paenibacillus* strains might be largely attributed to anaerobic reduction (Figure 6.39 and), and the biosorption onto the bacterial biomass was significant to the decolorization of Remazol brilliant Blue R (Figure 6.40 and 6.41).

Further all the dyes are taken up simultaneously without preference. The decolorization of mixture of dyes by a defined mixed culture of *Paenibacillus* sp. strain A5 and S1 is a crucial piece of information with regard to the adaptability of bacterial process to field conditions. Such a nonspecific biological process may vital for treatment of textile mill discharge effluents containing mixture of dyes rather than a specific dye. The ability of a mixed culture of *Paenibacillus* sp. strain A5 and S1 to decolorize mixture reactive dyes would be appear to be comparable with that seen for PDW mixed bacterial culture (Nigam et al., 1996), which could decolorize 80% of color present in effluent sample containing mixture of azo- and diazo-reactive dyes after 4 days of anaerobic incubation.

Chapter 7

Mechanisms for decolorization of reactive dyes by *Paenibacillus* sp. strain A5

7.1 Decolorization mechanism of azo dyes

7.1.1 Activity of azoreductase in crude cell-free extract of *Paenibacillus* sp. strain A5 on various reactive azo dyes

Various bacterial strains reduce azo dyes under anaerobic conditions. The most generally accepted hypothesis for this phenomenon is that many bacterial strains possess rather unspecific cytoplasmic enzymes, which act as "azoreductases" and under anaerobic conditions transfer electrons via soluble flavins to the azo dyes (Chung et al., 1978; Rafii et al., 1990). Although the exact mechanism of the bacterial azo reduction is still a matter of discussion, it seems to involve biological water-soluble electron carriers such as FAD (flavin adenine dinucleotide), FMN (flavin mononucleotide), riboflavin, NAD (nicotinamide adenine dinucleotide) or NADP (nicotinamide adenine dinucleotide phosphate). According to some authors, the biological cofactors, acting as electron mediators, are firstly reduced by azoreductase, while the azo compounds act as electron receptors at the end of the electron transport process (Gingell and Walker, 1971). This mechanism suggests that azo reduction is in part mediated by low molecular weight electron carriers rather than by specific azoreductase enzymes.

There are numerous reports which describe the reduction of azo compounds by bacteria under anaerobic conditions. The main interest in this field has focused on bacteria from the intestine which are involved in the metabolism of azo dyes ingested as food additives, and there are some studies which refer to bacteria isolated from soil or sewage treatment systems (Gingell and Walker, 1971; Chung et al., 1978; Rafii et al., 1990). Table 7.1 shows some example reports of bacterial decolorization of various azo dyes by use of azoreductases.

Therefore, to determine the decolorization mechanism which *Paenibacillus* sp. using for the decolorization of various azo dyes, the investigation of azoreductase activity of *Paenibacillus* sp. strain A5 was performed in the preliminary experiment.

Determination of azoreductase activity for crude cell extract

Azo dye-decolorizing bacterial cells prepared from designated culture conditions were harvested by centrifugation (10,000 g, 10 min), and resuspend in 50 mM Tris-HCl buffer (pH 7.5). The cell solution (approximately 3-5 mg cell/ml) was sonicated (number 4; 5min) with an ultrasonic processor (Misonix Inc.) The crude cell extract was collected after removal of cell debris from the disrupted cell solution by centrifugation at 10,000 g and 4 ^{0}C for 10 min. Azoreductase activity was determined as following procedures. In general, cell-free extract was added to 50 mM Tris-HCl buffer (pH 7.5).to give final concentration of 1 mg protein/ml. The enzyme reaction mixture was consisting of 24 μM of reactive azo dye and 0.35 mM of NADH (Sigma); the total volume of reaction mixture was 1 ml. The reactions were started by addition of cell-free extract. The residual dyes concentration in the reaction mixtures were measured as a function of time, and the initial rate of dye disappearance was determined from the concentration profiles to determine the enzyme activity. The experiments were designed to reveal the effect of oxygen on the enzyme activity by evacuated the dissolved oxygen by flushing with oxygen-free nitrogen before the addition of cell-free extract.

Results and discussions

The results of Figure 7.1, 7.2 and 7.3 are clearly shown that the decolorization of azo dyes occurred only in the presence of cell-free extracts of *Paenibacillus* sp. strain A5.

Like the whole cell experiments (chapter 6.1), the presence of oxygen in the reaction mixtures totally inhibited the reduction of all azo dyes by cell-free extract of strain A5. Chung et al. (1992) and Chang et al. (2001) also determined that the inhibition by oxygen of enzymatic reduction of azo dyes for *Pseudomonas luteola* affected the azoreductase. This was attributed to competition for NADH utilization by aerobic respiration, which triggers electron transfer from NADH to oxygen to form ATP. Since NADH acts as an electron donor for the reduction of azo bonds that leads to bacterial decolorization of azo dyes, the consumption of NADH by oxidative phosphorylation would result in a negative effect on the azoreductase-driven decolorization. However, Chang et al. (2001) determined that the presence of oxygen did not directly inhibit the activity of azoreductase, thus oxygen inhibition is more likely to be a metabolism-dependent event.

Table 7.1 Reports on bacterial cultures capable of azo dye decolorizations

Culture	Azo dye and concentration	Percent removal/time	Mechanism	Reference
Aeromonas hydrophila var 24B	Various azo dyes (0.2 mM)	40-100% (24 h)	Azoreductase	Yatome et al. (1987)
Aeromonas hydrophila var 24B	Various azo dyes (10-100 mg/l)	50-90% (24 h)	Azoreductase	Idaka and Ogawa (1978)
Bacillus subtilis	2-carboxy 4' dimethyleamino benzene (0.045 mM)	100% (20 min)	Azoreductase	Yatome et al. (1991)
Bacillus subtilis IFO 3002	*p*-aminozaobenzene (30 mg/l)	80-90% (30 h)	Azoreductase	Horitsu et al. (1977)
Klebsiella pneumoniae RS-13	Methyl Red (100 mg/l)	100% (24 h)	Azoreductase	Wong and Yuen (1996)
Pseudomonas cepacia 13 NA	C. I. Acid Orange 12 (10 mg/l)	65% (8 h)	Azoreductase	Ogawa et al. (1986)
	C. I. Acid Orange 20 (10 mg/l)	87% (8 h)	Azoreductase	
Pseudomonas luteola	Red G (100 mg/l)	34.2% (2 days)	Azorecuctase	Hu (1994)
	Remazol Black B	93.2% (2 days)	Azoreductase	
Pseudomonas stutzeri	Various azo dyes (0.1 mM)	90% (20 min)	Azoreductase	Yatome et al. (1990)
Mixed bacterial culture	Mordant Yellow (unknown)	50% (5 days)	Azoreductase	Haug et al. (1991)
Mixed bacterial culture PDW	Remazol Black B	67% (24 h)	Azoreductase	Nigam et al., 1995)
Proteus mirabilis	RED RBN	95% (20 h)	Azoreductase	Chen et al. (1999)

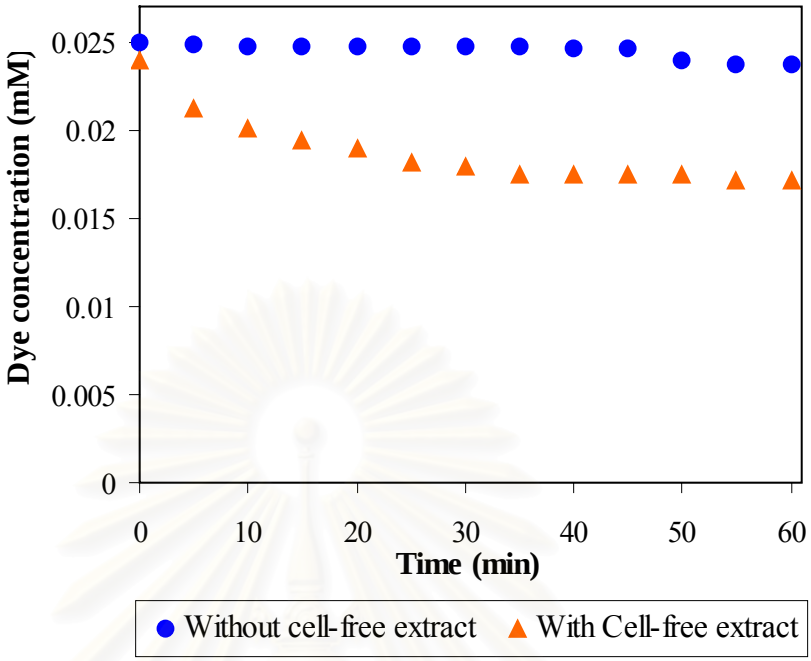


Figure. 7.1 Azo reductase activity of crude cell-free extract of *Paenibacillus* sp. strain A5 on decolorization of Remazol Brilliant Orange 3R

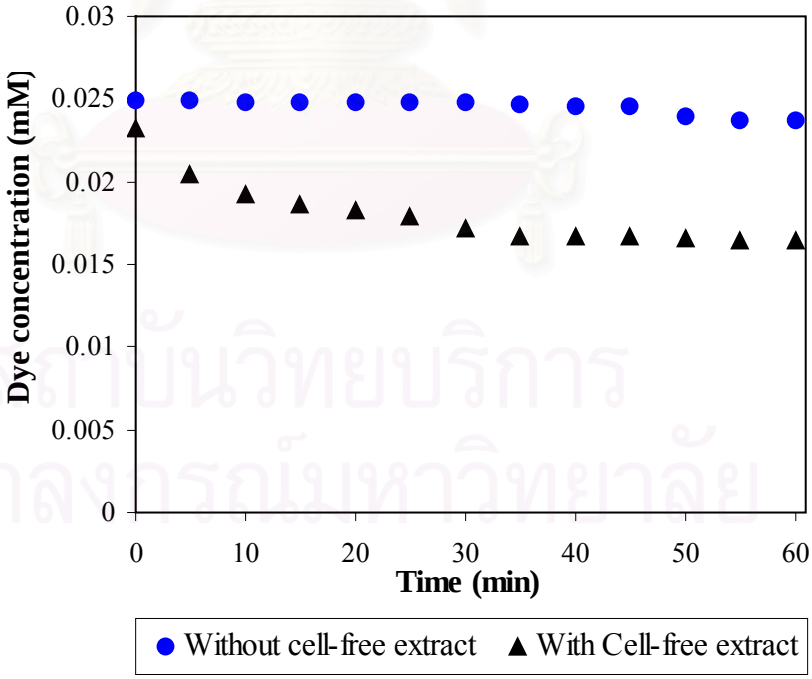


Figure. 7.2 Azo reductase activity of crude cell-free extract of *Paenibacillus* sp. strain A5 on decolorization of Remazol Black B

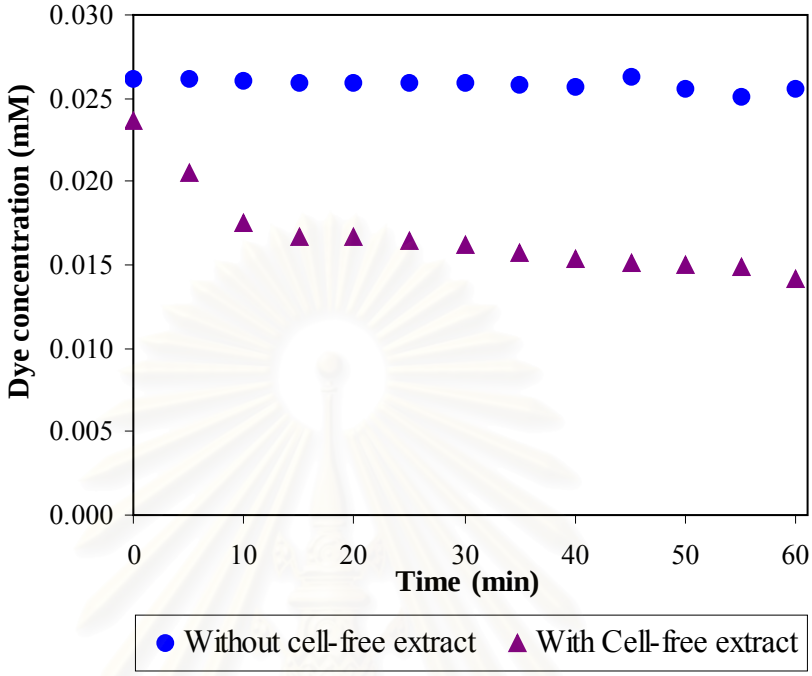


Figure. 7.3. Azoreductase activity of crude cell-free extract of *Paenibacillus* sp. strain A5 on decolorization of Remazol Brilliant Violet 5R

7.1.2 Effect of temperature on azoreductase activity in crude cell-free extract of *Paenibacillus* sp. strain A5

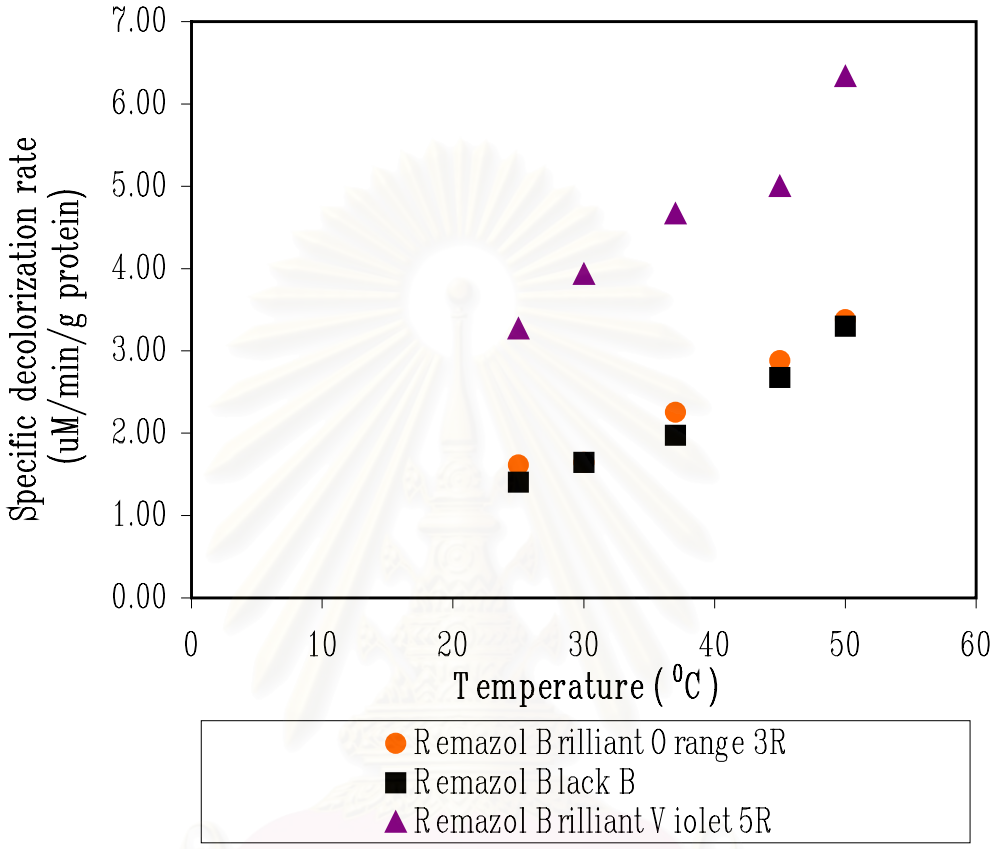


Figure 7.4 Dependence of specific decolorization rate of cell-free extract of strain A5 on reaction temperatures

In order to study the effects of temperature on decolorization of reactive dyes by cell-free extract of *Paenibacillus* sp. strain A5, experiments were performed at 25, 30, 37, 45 and 50 ^{0}C. Over a range of 25 – 50.^{0}C, the decolorization of reactive dyes by the cell-free extract of strain A5 increased as the temperature rose (Figure 7.4).

The dependence of specific decolorization rate (r_{dye}) on the temperatures of reaction is shown in Figure 7.4. The r_{dye} value tended to increase as the temperatures was increased from 25 to 50 ^{0}C.

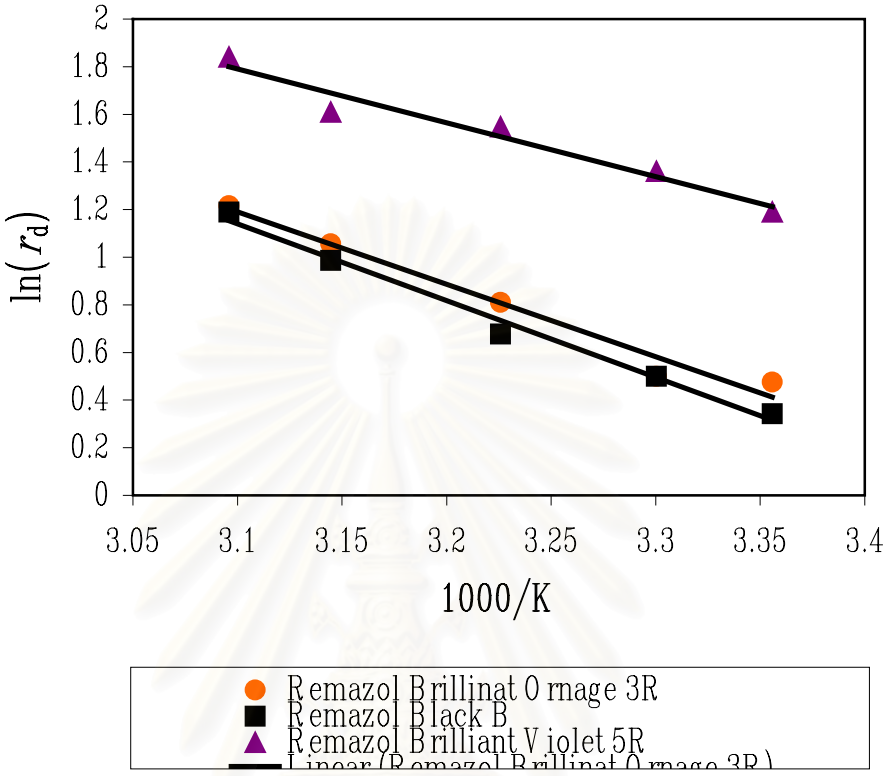


Figure 7.5 Arrhenius plot: dependence of the specific decolorization rate (r_{dye}) on temperatures

In the temperature range of 25 – 50 ^{0}C, the specific decolorization rate increase with temperature depends on the activation energy of the reaction as given by Arrhenius equation (Figure 7.5). According to the slopes and intercepts of the Arrhenius plot, the activation energy (E_a) for decolorization of four reactive dyes were calculated from the slope in Figure 7.5. The estimated E_a values for decolorization of Remazol Brilliant Orange 3R, Remazol Black B and Remazol Brilliant Violet 5R by cell free extract of strain A5 were 6.04, 6.41 and 4.49 kcal/mol, respectively. The lower value of activation energy shows that the decolorization of Remazol Brilliant Violet 5R by cell-free extract of strain A5 is easier than the decolorization of Remazol Brilliant Orange 3R and Remazol Black B.

The value of E_a supports the previous findings that the specific decolorization rate was temperature dependent. Similar observations have been recorded Thus, this decolorization activation energy by cell-free extract of *Paenibacillus* sp strain A5 is at the high value side of the general activation energy range of enzyme-catalyzed reactions, which are usually within 4 – 20 kcal/mol range (Shuler and Kargi, 1992).

7.1.3 Determination of decolorized products formed from azo dyes decolorization.

To confirm the assumption obtained from preliminary experiment that the decolorization of azo dyes by *Paenibacillus* sp. strain A5 is due to the reduction of azo bonds and then formation of the colorless aromatic amines, the further identification of decolorizing products was carried out.

7.1.3.1 Identification of reactive dyes decolorization products

The anaerobic metabolites of Remazol Brilliant Orange 3R, Remazol Black B and Remazol Brilliant Violet 5R were separated and identified liquid chromatography. The samples were taken from the synthetic textile wastewater cultures initially containing approximately 0.5 g cell/l of *Paenibacillus* sp. strain A5 cells and 200 mg/l of individual reactive azo dye. HPLC analysis was carried out on a Shimadzu model LC-10AVP chromatograph equipped with Shimadzu model SPD-10AVP UV-visible detector and Pegasil ODS, (4.6 mm x 150 mm [inside diameter] column, Senshu Scientific Co., Ltd., Tokyo, Japan). Mobile phase composed of 50% methanol, 0.3 % H_3PO_4, and 49.7% water with the flow rate of 0.5 ml/min. The effluents were monitored by UV absorption at 275 nm.

7.1.3.2 Chemically reduction of reactive azo dyes by sodium dithionite (Garrigos et al., 2002)

Several analytical methods for the determination of aromatic amines in dyes have been described in the literature. These methods were applied to the determination of amines in specific matrices, such as industrial effluents and inks, but most of them were applied to textiles and leather. The general process consisted of the reductive cleavage of dye samples with sodium dithionite ($Na_2S_2O_4$) in an aqueous media, followed by solvent extraction and further determination of the amines by liquid or gas chromatography.

$$R_1N{=}NR_2 \xrightarrow{Na_2S_2O_4} R_1NH_2 + R_2NH_2$$

Completely reduction of azo bond by sodium dithionite

Sodium dithionite ($Na_2S_2O_4$) was also obtained from Sigma (St. Louis, MO, USA). Stock solutions in methanol (~ 10 μg/g) of each reactive azo dye, Remazol Brilliant Orange 3R, Remazol Black B and Remazol Brilliant Violet 5R, were prepared. First ~ 5 g of dye solution in methanol (equivalent to 50 μg of dye) was weighed into round bottomed dark flask and placed into a waterbath shaker at 65 ^{0}C for 10 min. Then, 100 μl of a freshly prepared $Na_2S_2O_4$ solution (1 g/l in water), were added to the flask and lelf for 5 min or until complete decolorization was reached. The flask was then placed in an ice bath until the solution cooled to room temperature and then the extract was immediately analyzed by HPLC. Quantification of amines was performed by comparing chromatographic peak areas for sample extracts with those of chemical reduction in the same concentration range.

Results and Discussions

The HPLC analysis for the sample taken at the beginning of static incubation shows that no major peak was found within retention time 10 min for all azo dyes used (Figure 7.6A, 7.7A and 7.8A), thus decolorization did not occur at the early stage of static incubation.

As the decolorization proceeded, the intensity of new peaks (retention time = 3.6, 4.0 and 5.3 min for decolorization of Remazol Brilliant Orange 3R and Remazol Black B (Figure 7.6B and 7.7B) and retention time = 3.6, 3.8 and 4.7 for Remazol Brilliant Violet 5R (Figure 7.8B) increased along the anaerobic incubation time. At this point, it seems reasonable to assume that the *Paenibacillus* sp. strain A5 caused cleavage of the azo bond of all azo dyes, which decomposed to form aromatic amines. The newborn peaks due to decolorization of Remazol Brilliant Orange 3R are similar to the new peaks formed from decolorizattion of Remazol Black B. However, the HPLC pattern obtained from decolorization of the orange and black dye are different from the HPLC pattern found in the decolorization of Remazol Brilliant Violet 5R. It may be assumed that the decolorizing-products formed from decolorization of Remazol Brilliant Orange 3R and Remazol Black B are similar to each other but different form the decolorizing-products of Remazol Brilliant Violet 5R.

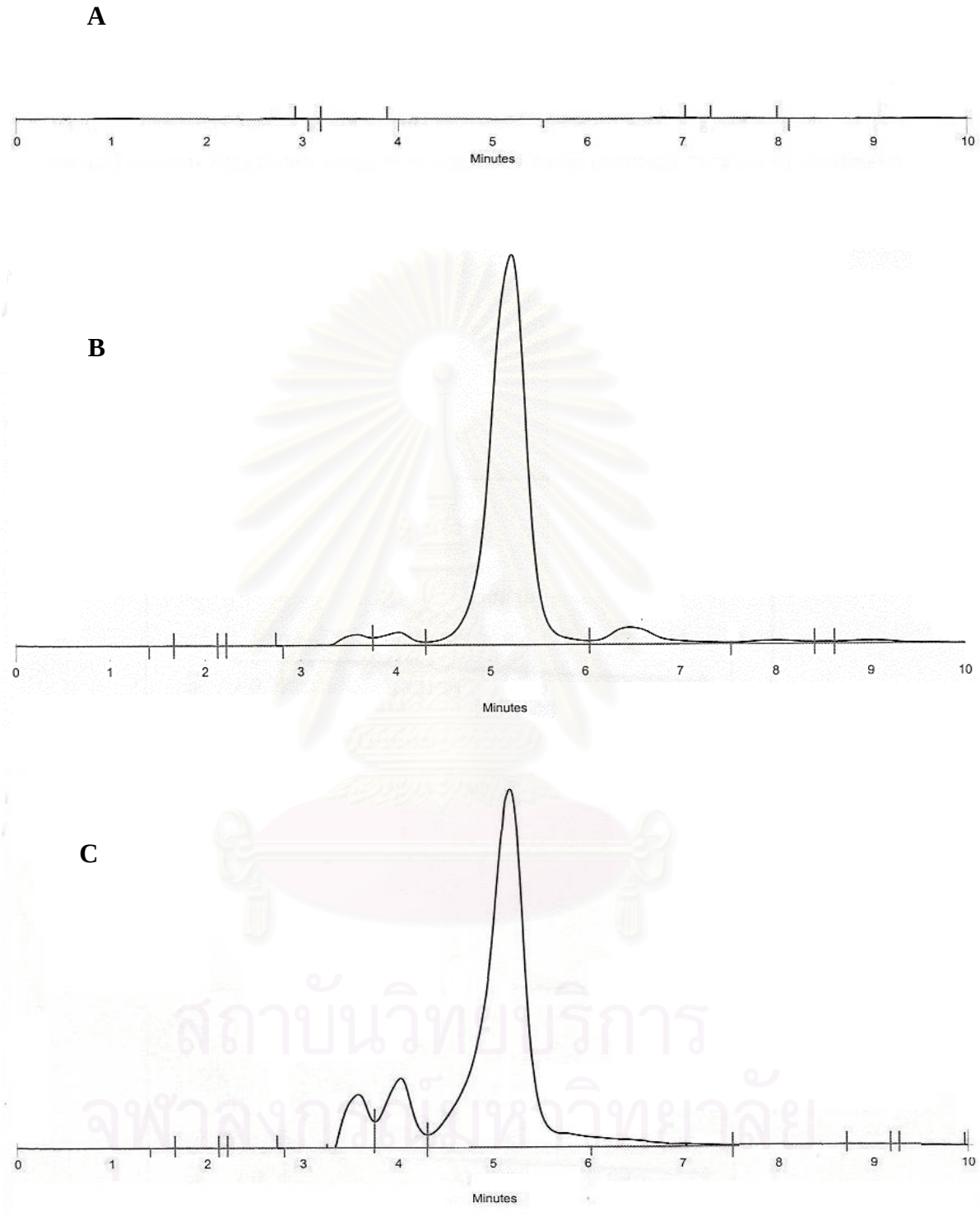


Figure. 7.6 The HPLC analysis on metabolites resulting from decolorization of Remazol Brilliant Orange 3R by *Paenibacillus* sp. strain A5 (**A**) at the beginning of anaerobic incubation (**B**) after anaerobic incubation for 24 h, and (**C**) products resulting from decolorization of Remazol Brilliant Orange 3R by chemical reduction with $Na_2S_2O_4$

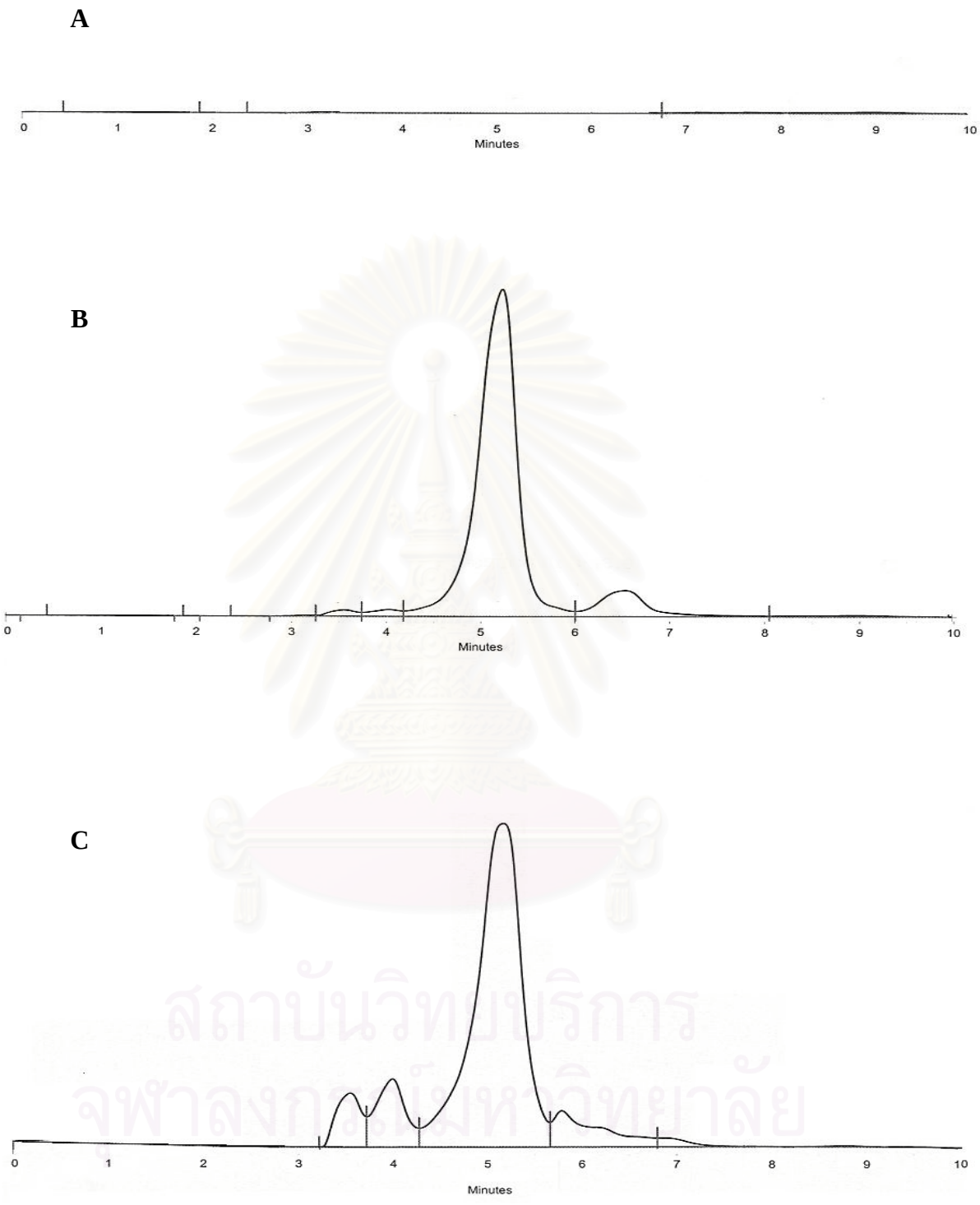


Figure. 7.7. The HPLC analysis on metabolites resulting from decolorization of Remazol Black B by *Paenibacillus* sp. strain A5 (**A**) at the beginning of anaerobic incubation (**B**) after anaerobic incubation for 24 h, and (**C**) products resulting from decolorization of Remazol Brilliant Orange 3R by chemical reduction with $Na_2S_2O_4$

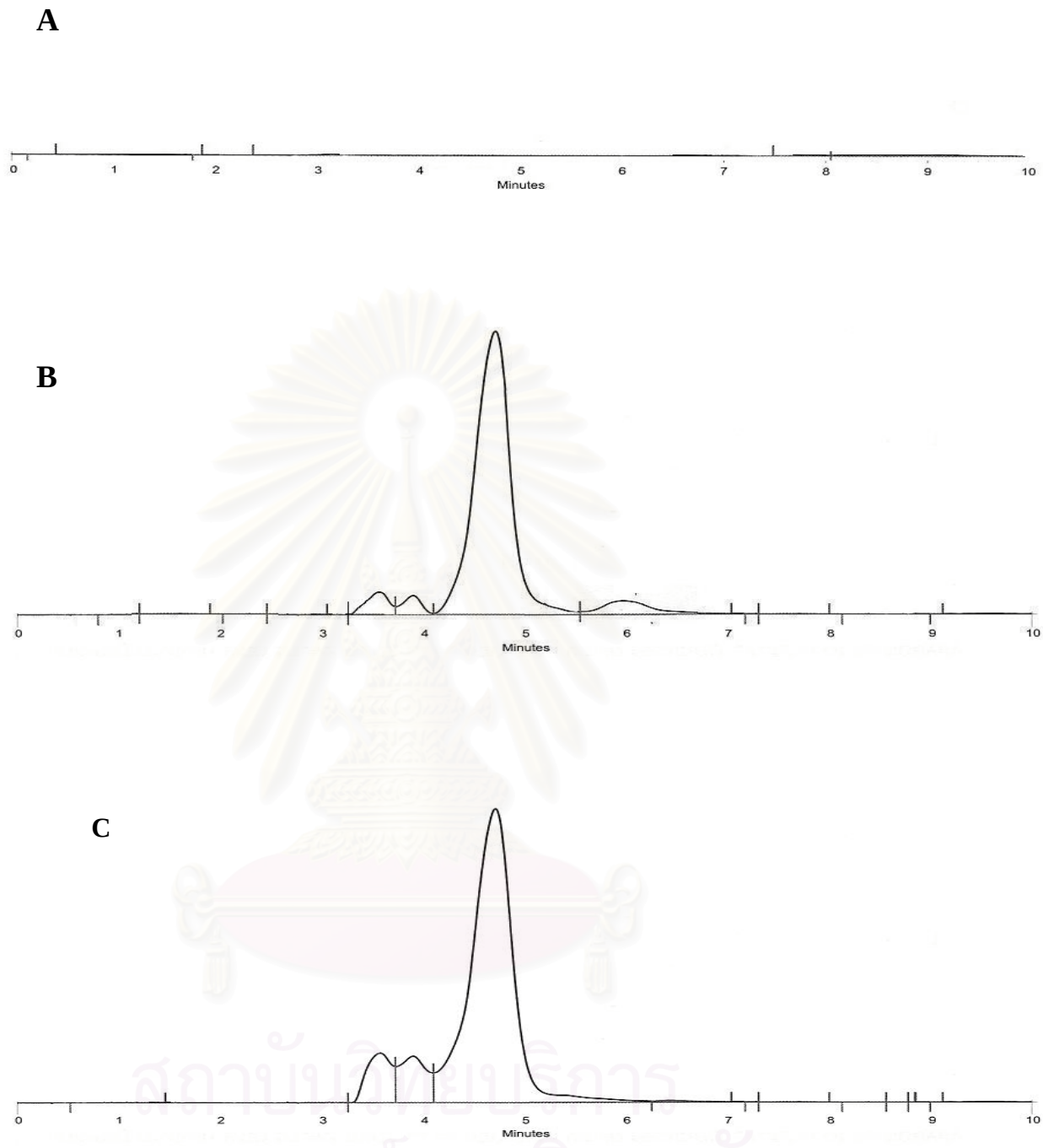


Figure. 7.8. The HPLC analysis on metabolites resulting from decolorization of Remazol Brilliant Violet 5R by *Paenibacillus* sp. strain A5 (**A**) at the beginning of anaerobic incubation (**B**) after anaerobic incubation for 24 h, and (**C**) products resulting from decolorization of Remazol Brilliant Orange 3R by chemical reduction with $Na_2S_2O_4$

Though, the lack of standards for the identification of HPLC peaks, which assumed as aromatic amines resulting from anaerobic reduction of each azo dyes, this work have attempted to identify the unknown metabolites by comparison them with the similar structure amines available commercially for example, 4-aminosulfonic acid (sulfanilic acid) and 1-amino-8-hydroxynaphthalene-3, 6-disulfonic acid (H-acid). The results showed that the retention time of sulfanilic acid and H-acid were 4.0 min and 5.4 min, respectively (data not shown). Thus, it is possible that peak at retention time 4.1 and 5.3 min (Figure 7.6B and 7.7B) which formed from orange dye and black dye decolorization, corresponding to the benzene-based and naphthalene-based amines, respectively. Also, the further study was taken to confirm the formation of aromatic amines after anaerobic decolorization of azo dyes by comparison HPLC patterns obtained form bacterial decolorization to the HPLC patterns obtain from chemical reduction of each azo dye. The results shown the similar pattern obtained from both bacterial and chemical reduction in all dye used.

Conclusions

The use of $Na_2S_2O_4$ for the clevage of the –N=N- linkage followed by HPLC analysis of the reduction products allows monitoring and determination of aromatic amines formed from the reduction of azo dyes. At this point, it seems reasonable to assume that the *Paenibacillus* sp. strain A5 caused cleavage of the azo bond of the Remazol Brilliant Orange 3R, Remazol Black B and Remazol Brilliant Violet dye, which decomposed to form colorless aromatic amines.

7.1.4 Humic substance enhanced anaerobic reduction of sulfonated azo dyes by *Paenibacillus* sp. strain A5

7.1.4.1 Introduction

Azo dyes are characterized by the presence of one or more azo groups (-N=N-). They are the largest and most versatile class of dyes, and more than half of the annually produced dyes are azo dyes (Zollinger, 1987). Presumably more than 2,000 different azo dyes are currently used for the dyeing of various materials such as textiles, leather, plastics, cosmetics, and food (Chudgar, 1985). The textile industry consumes the largest amount of azo dyes, and its estimated the approximately 10-15% of dyes used for coloring textiles might be lost in waste streams (Anliker, 1979). Sulfonated azo dyes are known to be resistant to degradation by aerobic bacteria due to the strong electron-withdrawing property of the azo group thought to protect against attack by oxygenases (Knackmuss, 1996) so that there generally pass biodegradative processes in conventional sewage treatment systems untreated (Pagga and Brown, 1986; Shaul et al., 1991). The azo dyes-containing effluents from these industries have caused serious environment pollution, because the presence of dyes in water is highly visible and affects their transparency and aesthetics even if the concentration of the dyes is low (Hao et al., 2000).

Microbial decolorization of sulfonated azo dyes readily occurs under anaerobic conditions by a wide variety of bacteria utilizing several intracellular reductases to reductive cleavage of the azo dyes to produce corresponding colorless aromatic amines (Carliell et al., 1995; Chung et al., 1978; Dubin and Wright, 1975; Gingell and Walker, 1971; Razo-Flores et al., 1997; Roxon et al., 1967). These reactions usually occur with rather low specific activities but are extremely unspecific with regard to the microorganisms involved and the dyes converted. In the textile processing industry, a wide range of structurally diverse dyes is used within short time periods in one and the same factory and, therefore, the effluents from the industry are markedly variable in composition (Keck et al., 2002). Moreover, in the case of sulfonated azo dyes, sulfonic acid substitutions seem to be an effective inhibitor of permeation of the dyes through the cell membrane (Wuhrmann et al., 1980) and therefore, intracellular reductases do not function (Russ et al., 2000).

Thus, an extracellular nonspecific biological process may be vital for treatment of the textile effluents. From the currently known biological systems, the required unspecifity may be obtained by using the suitable redox mediator system. The unspecific anaerobic reduction of azo compounds very often low-molecular-weight redox mediators (e.g., flavins or quinones) are involved (Chung et al., 1992; Keck et al., 1997; Kudlich et al., 1997; Rau and Stolz, 2003; Rau et al., 2000; Stolz, 2001). These mediators are reduced by bacterial enzymes to corresponding their reduced forms (e.g., reduced flavins or hydroquinones) which enable the transfer of redox equivalents to extracellular azo dyes in a purely chemical reaction (Chung et al., 19921; Rau et al., 2000; Stolz, 2001).

These alternative redox mediators either occur naturally in groundwater and sediments or are possible additives for stimulating in situ biodecolorization processes. Also, organic matter in the natural environment may contain humic substances, which are known to accelerate reductive processes by redox mediation (Bradley et al., 1998; Cervantes et al., 2000; Loveley et al., 1996; Scott et al., 1998).

In the present study, humic substance is evaluated as a potential redox mediator for the reduction of sulfonated azo dyes. Humic substance is the stable organic matter accumulating in soils and sediments (Stevenson, 1994). Although humic substance is generally consider to inert for microbial catabolism, it has recently been reported to play an active role in anaerobic oxidation of a wide variety of ecologically relevant organic substrate (Cervantes et al., 2000; Coates et al., 1998; Lovley et al., 1996). These studies have demonstrated that the reduction of humic substances may be important mechanisms for organic substrate oxidation in many anaerobic environments. Quinone moieties of humus have been implicated as redox active groups (Scott et al., 1998) accepting the electrons. Such quinone moieties presented in humus is shown in Figure.7.9.

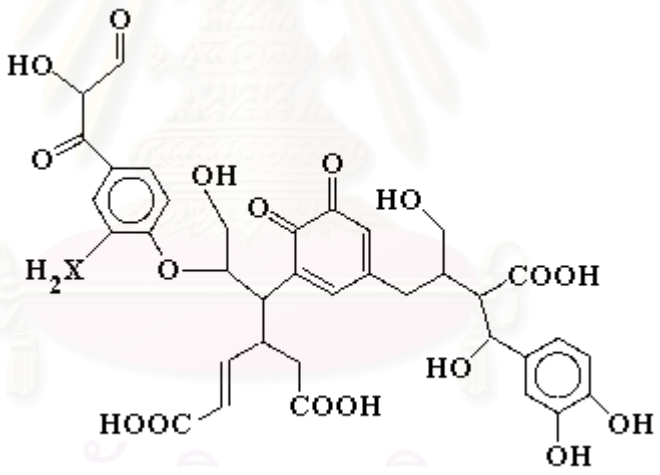


Figure. 7.9 A proposed humic acid building block (Mao, et al., 2000)

Anthraquinone-2,6-disulfonate (AQDS) have been used as a defined model for such moieties (Cervantes et al.,2000; Lovley et al., 1996). The molecular structure of AQDS is shown in Figure 7.10. Most AQDS-respiring microorganisms are capable of transferring electrons to AQDS, reducing it to anthraquinone-2,6-disulfonate (AH_2QDS).

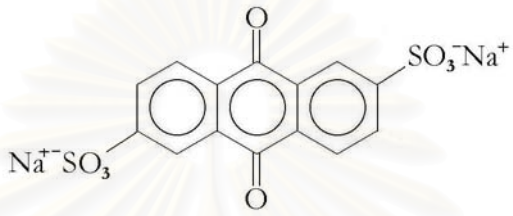


Figure 7.10 A molecular structure of anthraquinone-2,6-disulfonate (AQDS)

The role of humic analog AQDS as an electron shuttle has been demonstrated previously and thought to provide a strategy for Fe (III) reducers to access insoluble Fe (III) compounds (Lovley et al., 1996). The anaerobic microbial oxidation of phenol and *p*-cresol in granular sludge was recently found to be coupled to the reduction of AQDS (Cervantes et al., 2000). The addition of humic acids or AQDS was also shown to stimulate the mineralization of the priority pollutants vinyl chloride and dichloroethene by a humus-respiring consortium under anaerobic conditions (Bradley et al., 1998). The rates of azo dye decolorization are also enhanced in the presence of different quinoid redox mediators, especially anthraquinone-2-sulfonate. (Kudlich et al., 1997; Rau et al., 2000; Zee, et al., 2000).

Because the utilization of humic substance as a redox mediator should allow very unspecific reduction processes with various azo dyes, in the present study it was therefore to examined the mechanism which, humic substance stimulate anaerobic reduction of sulfonated azo dyes by *Paenibacillus* sp. strain A5. The location of the enzyme system(s) which are responsible for the reduction of sulfonated azo dyes by whole cells of strain A5 in the presence of humic substance was also determined.

7.1.4.2 Materials and Methods

7.1.4.2.1 Culture medium

The basic composition of minimal medium (MMG) was (in g/l): glucose 0.9, $(NH_4)_2SO_4$ 0.28, NH_4Cl 0.23, KH_2PO_4 0.067, $MgSO_4 \bullet 7H_2O$ 0.04, $CaCl_2 \bullet 2H_2O$ 0.022, $FeCl_3 \bullet 6H_2O$ 0.005, NaCl 0.15, $NaHCO_3$ 1.0 and 1 ml/l of a trace element solution containing (in g/l) $ZnSO_4 \bullet 7H_2O$ 0.01, $MnCl_2 \bullet 4H_2O$ 0.1, $CuSO_4 \bullet 5H_2O$ 0.392, $CoCl_2 \bullet 6H_2O$ 0.248, $NaB_4O_7 \bullet 10H_2O$ 0.177 and $NiCl_2 \bullet 6H_2O$ 0.02.

7.1.4.2.2 Anaerobic reduction of sulfonated azo dyes with whole cells

Cells of *Paenibacillus* sp. strain A5 were grown aerobically in MMG to an optical density at 600 nm (OD_{600}) of approximately 1. Then cells were harvested by centrifugation, washed twice, and resuspended in 50 mM Na-K phosphate buffer (pH 7.5). The cell suspension (protein concentration of approximately 0.1 g/l) was transferred into screw cap-glass tubes (10 ml). The reaction mixture contained in a final volume of 10 ml, 5 mM of glucose, 50 mM of Na-K phosphate buffer (pH 7.5), and different concentration of redox mediators. Oxygen was removed from reaction mixture by evacuation and flushing with oxygen-free nitrogen gas for 5 min. The reaction was started by the addition of sulfonated azo dyes (final concentration = 100 μM) from anaerobic stock solution into reaction mixture. To prevent possible contamination with oxygen during sampling, tubes were opened only once, and only as many glass tubes were incubated as measurements were planned. The cells were removed by centrifugation (10,000 x *g*, 10 min), and the concentration of sulfonated azo dyes in supernatant was determined spectrophotometrically at λ_{max} of each azo dye.

7.1.4.2.3 Standard assay for determination of azo reducatase activities with cell extracts and soluble cell membrane in the presence of different redox mediators

The azo reductase activity was determined anaerobically in 1.5 ml-rubber-stoppered cuvettes which were flushed before the assay with oxygen-free nitrogen gas. Azo reductase activity was routinely measured by a modification of the spectrophotometric assay described previously by Kudlich et al. (1997) and Russ et al. (2000). For the standard assay, the anaerobically prepared reaction mixtures contained in 800 μl of 50 mM of Tris-HCl buffer (pH 7.5), 25 μM of the respective sufonated azo dyes, and 50 μM of redox mediators (FAD or AQDS). The cell extracts or solubilized cell membrane from *Paenibacillus* sp. strain A5 were added (200 μl, about 0.05 to 0.1 mg of protein), and the reaction mixtures were flushed again with nitrogen gas. Finally, the reaction was started by the addition of 300 μM of NADH, and the initial rate was measured at 30 ^{0}C with a model UV WINLAB, Perkin Elmer molecular

spectrophotometer for 30 min (by using 1-min measuring intervals). Reaction rates were calculated by using molar extinction coefficient of 15.4, 29.1, 6.8 /mM/cm for Remazol Brilliant Orange 3R, Remazol Black B and Remazol Brilliant Violet 5R, respectively.

7.1.4.2.4 Preparation of cell membranes

Strain A5 was grown aerobically under the conditions described above until they reached the late exponential growth phase. Cells were harvested by centrifugation at 10,000 x *g* for 10 min, washed twice, and resuspended in Na-K phosphate buffer (50 mM, pH 7.5) to an OD_{600} of about 5. Cell-free extracts and cell membranes were prepared by disruption of a suspension of whole cells (protein content, about 1 g/l) by ultrasonication using an ultrasonic processor (Sonicator® W-385, Heat systems-Ultrasonics, Inc.). After removing the cell debris by centrifugation at 10,000 x *g* for 15 min at 4 ^{0}C, the supernatant was ultracentrifuged at 100,000 x *g* for 30 min at 4 ^{0}C (Kuldlich et al., 1997; Rau and Slotz, 2003). The supernatant of ultracentrifugation was used in certain experiment as the "cytoplasmic fraction". In the same time, the transparent pellet formed after ultracentrifugation was resuspended in a volume of about 10 ml in 50 mM Tris-HCl buffer (pH 7.5) and used in enzyme assay as the "membrane fraction" (Kuldlich et al., 1997; Rau and Slotz, 2003). For the isolation of the membrane-bound azo reductase, 1 ml of this preparation was incubated on ice for 5 min with 950 µl of 50 mM Tris-HCl buffer (pH 7.5) and 50 µl of a solution of Triton X-100 (20%, v/v). Finally, 200 µl of this mixture was used for the azo dye reduction as described above. The remaining membrane-bound protein was stored at –20 ^{0}C until used.

7.1.4.2.5 Enzyme assays

(i) NADH:quinone oxidoreductase

The NADH:quinone oxidoreductase was measured spectrophotometrically by a modification of the method given by Matsushita et al. (2001). In this anaerobic assay, this enzyme activity is determined by the reduction of menaquinone (MK-2) or NADH. In standard assay, cell extract and soluble cell membrane were added to a solution (final volume, 1ml) containing 50 mM of Tris-HCl (pH 7.5), 50 µM of menaquinone dissolved in dimethyl sulfoxide, and 2.5 mM of NaCN. The reaction was started by the addition of 200 µM of NADH. Due to the behavior of menaquinone, which has an intense peak at 336 nm in the oxidized form and 326 nm in reduced form (Cook et al., 2000), thus, the reduction rate of NADH was measured at 348 nm (molar extinction coefficient = 5.9 /mM/cm), where reduced and oxidized forms of menaquinone have identical absorbtivity.

(ii) NAD(P)H:flavin oxidoreductase

The NAD(P)H:flavin oxidoreductase was determined by a modification of the method given by Izumi and Ohshiro (2001) and Russ et al (2000). The reaction mixture (final volume, 1 ml) for the measurement of the enzyme activity contained 50 mM of Tris-HCl (pH 7.5), 0.5-1 mg protein of cell extract or soluble cell membrane, and 3 μM of riboflavin. The reaction was started by the addition of 250 μM of NADH. The decrease in the concentration of NADH was determined spectrophotometrically at 340 nm, and reaction rates were calculated by using a molar extinction coefficient of 6.2 /mM/cm.

7.1.4.2.6 Determination of protein content

The protein content of cell extracts and soluble cell membrane were determined by the method of Bradford (1976) with bovine serum albumin (Sigma, Fraction V) as the standard. Proteins in whole cells were assayed after adding NaOH to the cells to 1 N concentration, then boiling the cultures for 5 min. The supernatant fractions obtained by centrifuging the suspensions were assayed by the Bradford method, using 1 N NaOH as diluent for the standards.

7.1.4.2.7 Reduction of AQDS by whole cells of strain A5 and subsequent chemical reduction of sulfonated azo dyes by reduced AQDS

Strain A5 was aerobically grown in MMG until reach the late-exponential growth phase. The cells were harvested by centrifugation, washed and resuspended in Na-K phosphate buffer (50 mM, pH 7.5). One millilitre of the cell suspension (protein content = 0.1 g/l) was transferred to screw cap-glass tube (10 ml.) containing 9 ml of 50 mM Na-K phosphate buffer (pH 7.5) plus glucose (5 mM). The anaerobic incubation was started by the addition of AQDS (final concentration = 0.25 mM) form anaerobic stock solution into reaction mixture. The concentrations of reduced AQDS (AH_2QDS) were determined spectrophotometrically at 450 nm. After 10 h of incubation, the cells of strain A5 were removed form suspension by filtration through a 0.2-μm-pore diameter filter under anaerobic atmosphere. The resulting supernatant was then transferred into 1 cm disposable plastic cuvettes. The chemical reduction of azo dyes by reduced AQDS was started by the addition of anaerobic azo dyes solutions (final concentration = 0.1 mM) into cell-free suspension. The reduction of azo dyes was determined spectrophotometrically at λ_{max} of each azo dye for 30 min (using 30 sec measuring intervals).

7.1.4.2.8 Analytical techniques

The concentration of sulfonated azo dyes was determined spectrophotometrically (UV WINLAB, Perkin Elmer). Concentration of AH_2QDS were determined spectrophotometrically by monitoring the absorbance at 450 nm by using a molar extinction coefficient of 2.7 /mM/cm obtained from a calibration curve of AQDS chemically reduced by dithionite as previously described (Cervantes et al., 2000).

7.1.4.2.9 Chemicals

NADH, FAD, ubiquinone (Q1), and menaquinone (MK) were purchased form Sigma Chemical Co., St. Louis, Mo. AQDS, riboflavin, and humic acid were purchased from Aldrich Chemical (Milwaukee, Wis.). All other chemicals used for minimum medium and buffer solutions were obtained from E. Merck AG. (Darmstadt, Germany) and Aldrich Chemical (Milwaukee, Wis).

7.1.4.3 Results and Discussions

7.1.4.3.1 AQDS stimulation of sulfonated azo dyes reduction by whole cells of strain A5

To determine whether AQDS stimulated bacterial azo dyes reduction, cell suspensions of azo dyes-reducing bacterium *Paenibacillus* sp. strain A5 were added to phosphate buffer containing glucose as the electron donor and various sulfonated azo dyes and were incubated under anaerobic condition. Cell suspensions of strain A5 only slowly reduced azo dyes in the absence of additional redox mediator, but when the AQDS was also added to the buffer, azo dyes reduction was greatly stimulated (Figure 7.11, 7.13 and 7.15). The observed variations in the relative increase of the azo dye reduction rates for different dyes were presumably due to differences in the redox potentials of the individual azo compounds. The lesser but significant stimulation was observed in this study with the commercially available Aldrich humic acids that was previously found to stimulate the dissimilatory reduction of ferric iron (Lovley et al., 1996). Abiotic reduction of sulfonated azo dyes did not occur in controls without cells or added electron donor (Figure 7.11, 7.13 and 7.15).

Several different mechanisms have been proposed for reduction or degradation of azo dyes and similar compounds. A description of a nonspecific azo reductase system involved in azo dye reduction has been provided for selected bacterial species, and it has been shown that the relevant gene is relatively conserved in various anaerobic and facultative bacteria (Kudlich et al., 1997; Rafii and Coleman, 1999). In this research it was also hypothesized that coenzyme reducing equivalents (e.g., NADH) involved in normal electron transport through oxidation of organic substrates may act as electron donors for reduction of azo dyes. This would likely explain the observation that azo dye reduction occurs more readily as a co-metabolic event when additional readily degradable substrates (glucose) are provided (Figure 7.11, 7.13 and 7.15). There were at least two possible ways for glucose to enhance reduction of sulfonated azo dyes. It could act as a donor of reducing equivalents [e.g., via NADH or $FADH_2$], or its addition could result in more actively respiring cells, thus rapidly removing the oxygen in culture medium and enabling corresponding enzymes to transfer reducing equivalent to azo dyes (Russ et al., 2000).

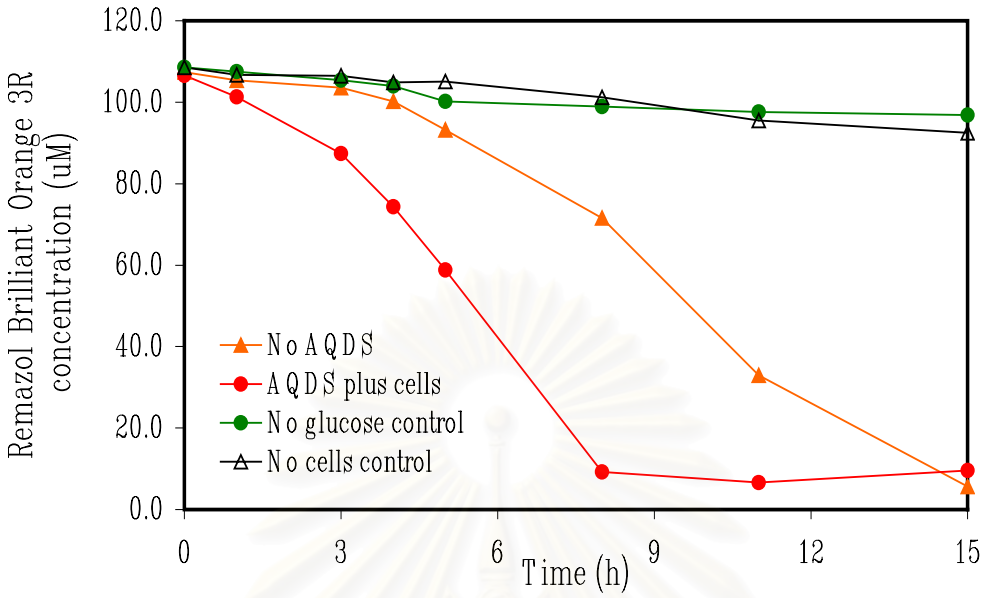


Figure 7.11 Reduction of Remazol Brilliant Orange 3R by *Paenibacillus* sp. strain A5 in the presence and absence of AQDS

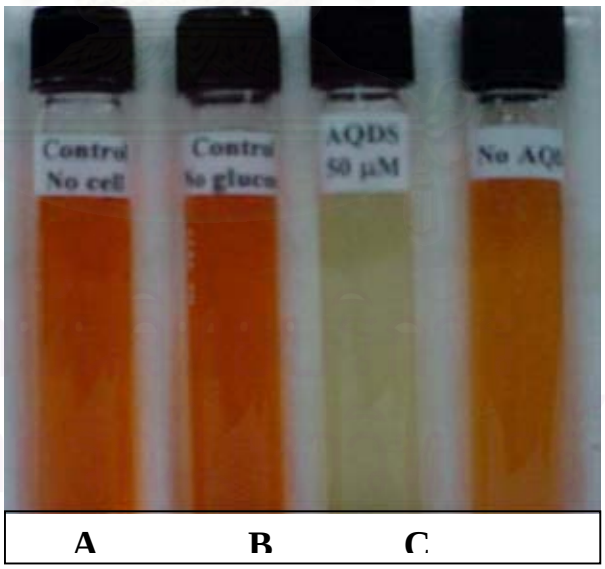


Figure 7.12 Reduction of Remazol Brilliant Orange 3R by *Paenibacillus* sp. strain A5 on mineral medium containing glucose in the presence and absence of AQDS. (**A**) Control without cells (**B**) control without glucose (**C**) cells plus AQDS and glucose (**D**) cells plus glucose without AQDS

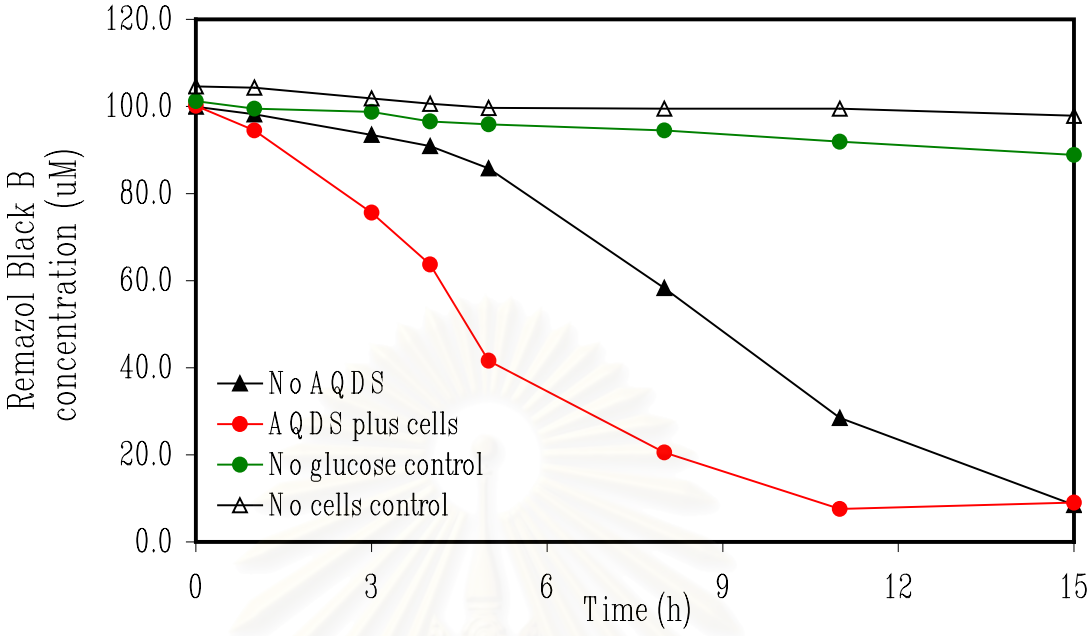


Figure 7.13 . Reduction of Remazol Black B by *Paenibacillus* sp. strain A5 in the presence and absence of AQDS

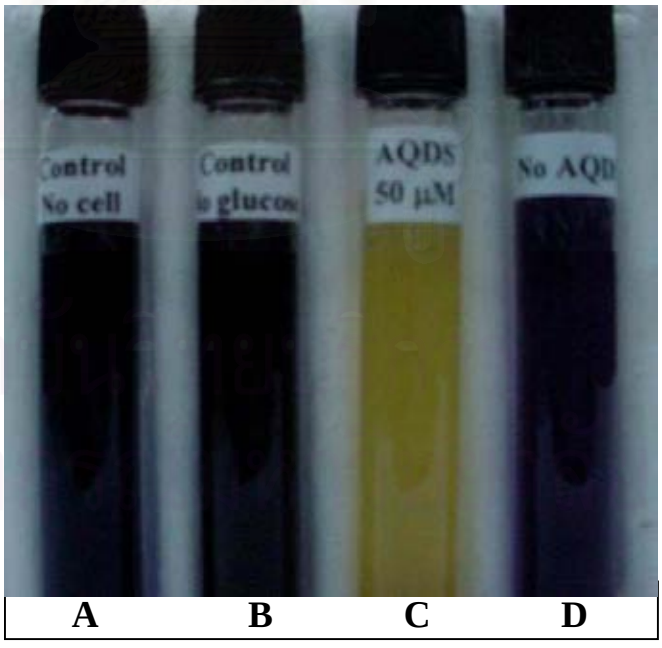


Figure 7.14 Reduction of Remazol Black B by *Paenibacillus* sp. strain A5 on mineral medium containing glucose in the presence and absence of AQDS. (**A**) Control without cells (**B**) control without glucose (**C**) cells plus AQDS and glucose (**D**) cells plus glucose without AQDS

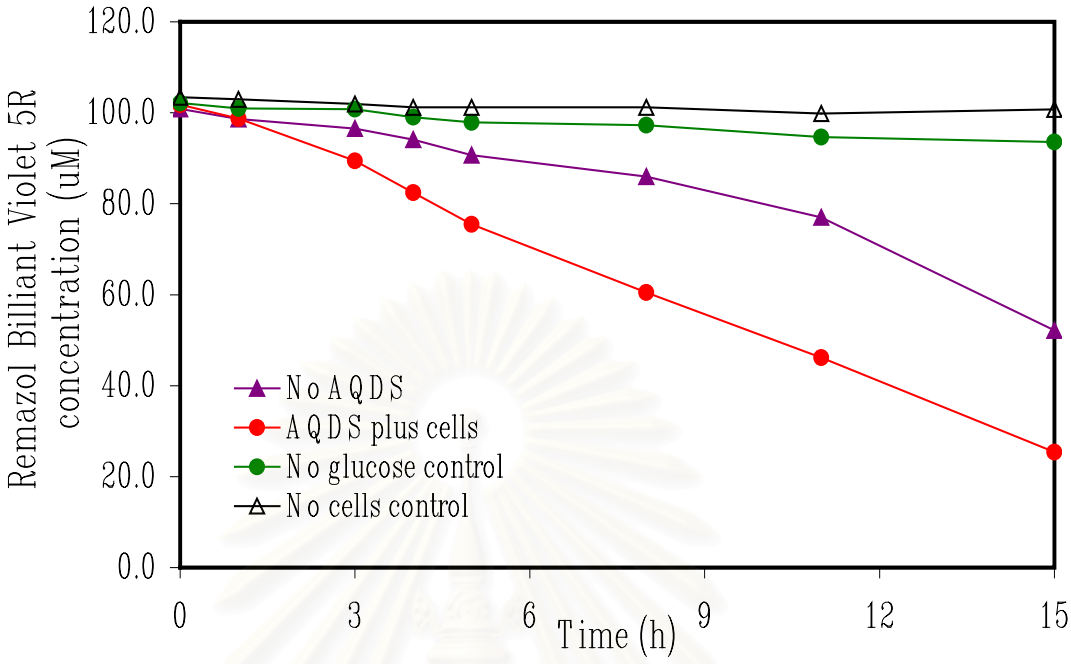


Figure 7.15 Reduction of Remazol Brilliant Violet 5R by *Paenibacillus* sp. strain A5 in the presence and absence of AQDS

Figure 7.16 Reduction of Remazol Brilliant Violet 5R by *Paenibacillus* sp. strain A5 on mineral medium containing glucose in the presence and absence of AQDS. (**A**) Control without cells (**B**) control without glucose (**C**) cells plus AQDS and glucose (**D**) cells plus glucose without AQDS

7.1.4.3.2 Reduction of different sulfonated azo dyes by whole cells of strain A5 in the presence of different concentrations of AQDS

The addition of quinoid redox mediators resulted in an increased reduction rate for azo compounds by various bacteria (Keck et al., 1997; Kudlich et al., 1997Laszlo, 2000; Rau et al., 2000; Rau and Stolz, 2003,). To demonstrate the general applicability of this system for the treatment of textile wastewaters, various sulfonated azo dyes were incubated under anaerobic condition with whole cells of *Paenibacillus* sp. strain A5 in the presence of different concentrations of AQDS. Thus, it was found that the addition of AQDS significantly increased the rate of decolorization of various sulfonated azo dyes (Figure 7.17). Concentration of AQDS as low as 50 µM significantly stimulated all sulfonated azo dyes reduction by cell suspensions of *Paenibacillus* sp. strain A5 (Figure. 7.17). The rate of azo dyes reduction was also dependent on the concentration of AQDS added.

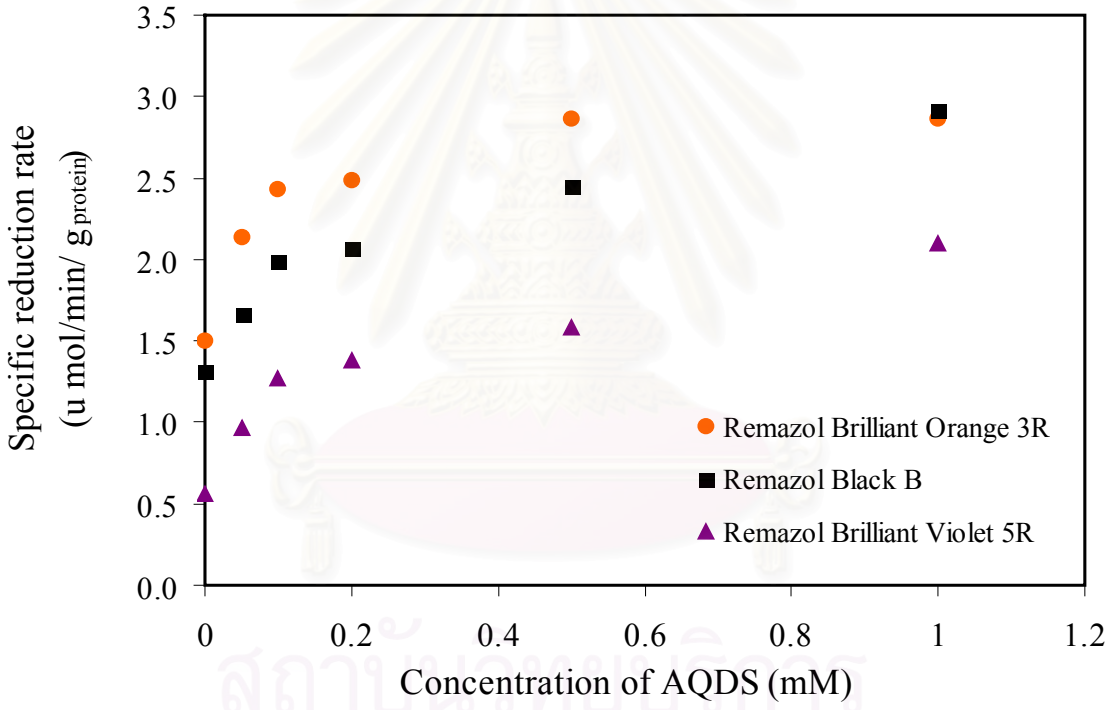


Figure. 7.17. Anaerobic reduction of sulfonated azo dyes in the presence of different concentrations of AQDS by whole cells of *Paenibacillus* strain A5. The cells of strain A5 were grown aerobically in a minimal medium with glucose (5 mM). When cell reached the lated-exponential growth phase, cells were harvested by centrifugation and resuspended anaerobically in Na-K phosphate buffer (pH 7.5; 50 mM). This cell suspension (protein content, 0.09 g/l) was completely filled into cultured tubes (10 ml). The oxygen-free reaction mixtures contained sulfonated azo dyes (0.1 mM), glucose (5 mM), and different concentration of AQDS. The decolorization of the sulfonated azo dyes was measured spectrophotometrically at λ_{max} of each azo dye.

7.1.4.3.3 Comparison of the azo reductase activities in different cellular components of *Paenibacillus* sp. strain A5 with different industrially sulfonated azo dyes

In the present study it was attempted to determine the location of the enzyme system(s) responsible for the reduction of sulfonated azo dyes by *Paenibacillus* sp. strain A5 in the presence of AQDS. Therefore, azo reductase activities of cellular membrane and cytoplasm of strain A5 were compared in the presence of AQDS (0.05 mM) in reaction mixtures. Thus, it was found that the majority of AQDS-dependent azo reductases activities present in membrane fraction of strain A5 (Table 7.2).

Table 7.2 Effect of the different redox mediators on anaerobic reduction of sulfonated azo dyes by *Paenibacillus* sp. strain A5

Azo dye	Redox mediator[a]	Specific reduction rate[b] (μmol/min/g of protein) with:		
		Whole cells	Membrane	Cell extract
Remazol Brilliant Orange 3R	None	1.5	5.1	1.9
	AQDS	2.2	9.4	2.2
	FAD	1.6	6.5	4.8
Remazol Black B	None	1.2	1.9	1.7
	AQDS	1.7	2.9	2.7
	FAD	1.3	1.9	3.4
Remazol Brilliant Violet 5R	None	0.6	5.9	3.3
	AQDS	1.0	20.2	5.0
	FAD	0.7	9.4	7.0

[a]The redox mediators were added in concentration of 50 μM each. The E_0' values of AQDS and FAD are –184 mV and –210 mV, respectively.

[b]Specific reduction rate of sulfonated azo dyes with whole cells, cellular membrane or cell extract was determined as described in Materials and Methods.

Up to the present, the most generally accepted hypothesis for bacterial reduction of azo dyes is that many bacterial cells possess a rather unspecific cytoplasmic azo reductases which transfers electrons via soluble or bound-flavins to the azo dyes (Roxon et al., 1967; Wuhrmann et al., 1980). The involvement of different low molecular weight redox mediators (e.g., flavins and quinones) in the bacterial reduction of azo dyes have been repeatedly suggested (Chung et al., 1978, Keck et al., 1997; Kudlich et al., 1997; Rau et al., 2000; Rau ans Stolz, 2003). In this manuscript, it was also attempted for the first time to perform the comparison of the effects of both flavin-type (FAD) and quinoid-type mediator (AQDS) on azo reductase activities present in different cellular components.

Without the addition of any redox mediators (FAD nor AQDS), the specific decolorization rates of orange, violet and black dye of whole cells were significantly lower than the specific decolorization rates of the same dyes determined with cell extracts (Table 7.2). This suggested that either the cell membrane limited the uptake of highly polar sulfonated azo dyes or the lack of some cofactors (e.g., free flavins) limited the reduction of azo dyes by whole cells. The addition of FAD (0.05 mM) to whole cell suspensions and the cell membrane fraction of *Paenibacillus* sp. strain A5 did not significantly increase the azo reductase activities of whole cells and slightly increased the azo reducase activities present in membrane fraction. In contrast, it was observed that the addition of the same concentration of FAD in the cytoplasmic fraction of strain A5 resulting in dramatically increased in the specific reduction rates of all azo dyes used.

On the other hand, the addition of the same concentration of AQDS, a quinoid redox mediator, was significantly enhanced azo reductase activities of whole cells and greatly stimulated the azo reductase activities present in membrane fraction of strain A5. In cyto plasmic fraction, however, this externally added quinoid mediator had rather lower stimulating effect on azo dyes reduction than the addition of FAD (Table 7.2). Thus the membrane-bound and cytoplasmic azo reductases of strain A5 are probably different enzyme systems which the latter may have insignificant importance in the reduction of sulfonated azo dyes in vivo.

7.1.4.3.4 Quinone reductase and flavin reductase activity in membrane and cytoplasmic fraction of strain A5

In cytoplasmic membranes of almost all prokaryotes, it has been shown that the reduction of soluble quinones is catalyzed by the membrane-bound respiratory NADH:quinone reductase (Bertsova et al., 1998; Bjorklof et al., 2000;Matsushita et al., 2001; Yano, 1991). Furthermore, it was previously suggested that in *Sphingomonas xenophaga* BN6 the (membrane-bound) NADH:quinone oxidoreductase of respiratory chain is responsible for the reduction of anthraquinone-2-sulfonate (and thus the azo reductase activity) (Kudlich et al., 1997). In the other hand, in earlier studies with facultatively anaerobic bacteria, it was repeatedly suggested that the reduced flavins generated by cytosolic flavin-dependent reductases (flavin reductases) were responsible for unspecific reduction of azo dyes (Gingell and Walher, 1971; Roxon et al., 1967; Russ et al., 2000). Because quinone reductases and flavin reductase are hypothesized involving the unspecific reduction of azo dyes by bacteria, therefore, in this study we attempted to determine the activity of these enzymes present in membrane and cytoplasmic fractions of strain A5. For strain A5, it has shown that the high level of NADH: quinone oxidoreductase activity was found in membrane fraction meanwhile the high level of NAD(P)H:flavin oxidoreductase activity was found in cytosolic fraction of the cells (Table 7.3).

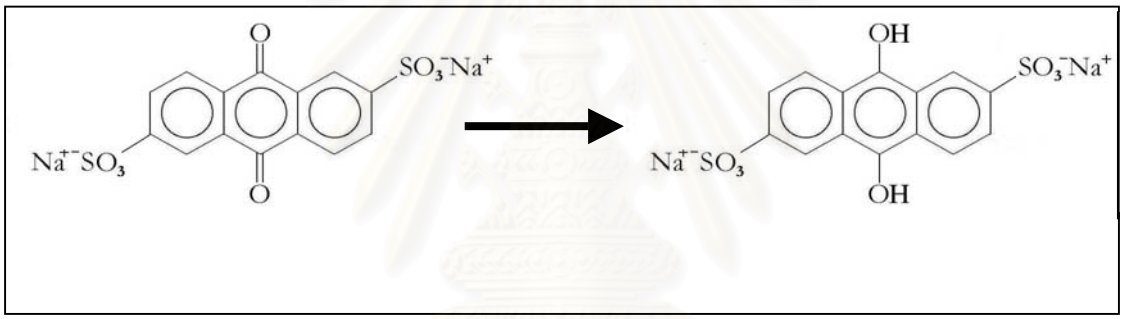
Table 7.3 Specific activities of quinone reductase and flavin reductase in membrane-bound and cell extract of *Paenibacillus* sp. strain A5

Enzyme	Specific activity (μmol/min/g of protein)[a]	
	Membrane	Cell extract
Quinone reductase	81.8	8.8
Flavin reductase	6.8	72.1

[a]The enzyme activities were determined spectrophotometrically as described in Materials and Methods.

7.1.4.3.5 Mechanism which AQDS enhance sulfonated azo dyes reduction

The proposed mechanism by which AQDS enhance reduction of azo compounds encloses two independent reactions: first, the quinones are enzymatically reduced to the corresponding hydroquinones, and second, the hydroquinones cleave the azo dyes in a purely chemical reaction (Lovley et al., 1996; Rau et al., 2000; Stolz, A., 2001) Therefore, both reactions were analyzed separately (Figure 7.19). For the first reaction, the experimental results shown that the anaerobic *Paenibacillus* sp. strain A5 suspensions could drive a large fraction of AQDS to the reduced state (anthrahydroquinone-2,6-disulfonate, AH_2QDS) as evidenced by the orange color formation and increased absorbance at 450 nm (Figure 7.18) of the culture medium within 10-h incubation period (Figure 7.19). At the end of anaerobic incubation, the buffer turned orange owing to the accumulation of AH_2QDS. The fact that the introduction of oxygen at the end of the anaerobic incubation resulted in immediate loss of orange color and decreased absorbance confirmed that AQDS was enzymatically reduced during the anaerobic incubation with strain A5.



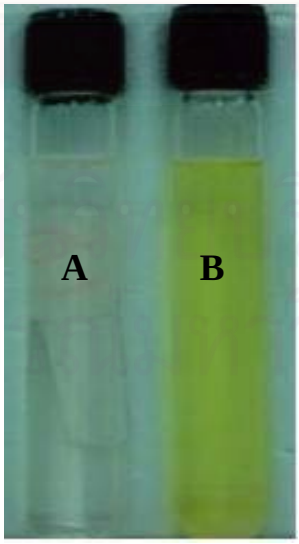


Figure 7.18 Enzymatic reduction of AQDS to AH_2QDS by *Paenibacillus* sp. strain A5 after anaerobic incubation for (**A**) 0 h and (**B**) 10 h

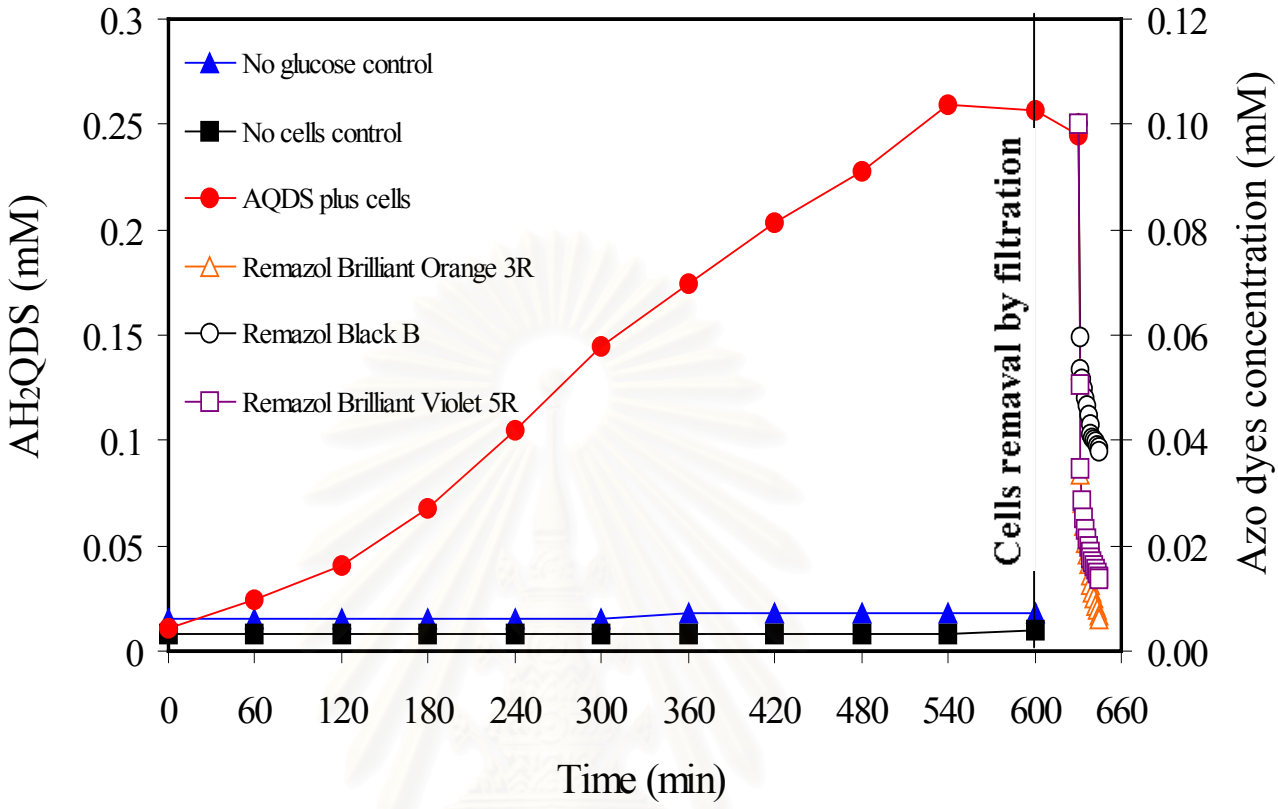


Figure 7.19 Enzymatic reduction of AQDS to AH_2QDS by *Paenibacillus* strain A5 and subsequent chemical reduction of sulfonated azo dyes by AH_2QDS. Washed cells of *Paenibacillus* sp. strain A5 (0.09 g of protein/l) were suspended in 10 ml. Na-K phosphate buffer (50 mM; pH 7.5) containing glucose (5 mM) as the electron donor and AQDS (100 µM) as electron acceptor. The concentration of reduced AQDS (AH_2QDS) was determined spectrophotometrically at 450 nm. After 10 h of anaerobic incubation, cells were removed by filtration (0.2-µM-pore-diameter filter). The cell-free culture supernatant was then filled into gas-tight cuvettes (final volume = 1 ml.) under anaerobic condition and anaerobic stock of each sulfonated azo dyes was added to 100 µM final concentration. The cuvettes were transferred to a spectrophotometer and the decolorization of sulfonated azo dyes was determined at λ_{max} of each azo dye.

To evaluate the second reaction in proposed mechanism, sulfonated azo dyes were added into cell-free culture filtrates containing of AH_2QDS, it was found that the orange color of AH_2QDS disappeared immediately. Furthermore, it is evident that the amounts of three sulfonated azo dyes reduced rapidly and Remazol Brilliant Orange 3R and Remazol Brilliant Violet 5R were completely removed within a few minutes after addition into filtrates of 10-h anaerobic incubated cell suspensions containing 0.25 mM AQDS. In the other hand, AQDS not incubated with *Paenibacillus* sp. strain A5 did not reduced all sulfonated azo dyes and filtrates of cell suspensions that did not contain AQDS did not reduced all azo dyes.

The addition of fixed concentration of all sulfonated azo dyes (0.1 mM) into the cultured-filtrates containing known concentration of AH_2QDS (≈ 0.25 mM) demonstrated that the chemical reduction of orange and violet dye required two moles of AH_2QDS to produce the corresponding aromatic amines and by four moles of AH_2QDS to produce the corresponding aromatic amines of black dye. The stoichiometry suggested a complete reduction of sulfonated azo dyes to the corresponding aromatic amines.

Kudlich et al. (1997) localized a quinoide redox mediator-dependent azo reductase activity in the membrane of a gram-negative bacterium, *Sphingomonas* sp strain BN6. Therefore, in the present study, the AH_2QDS is suggested to shuttle reduction equivalents in the cells to extracellular sulfonated azo dye and reduce the azo compound in a purely chemical reaction (Figure 7.20).

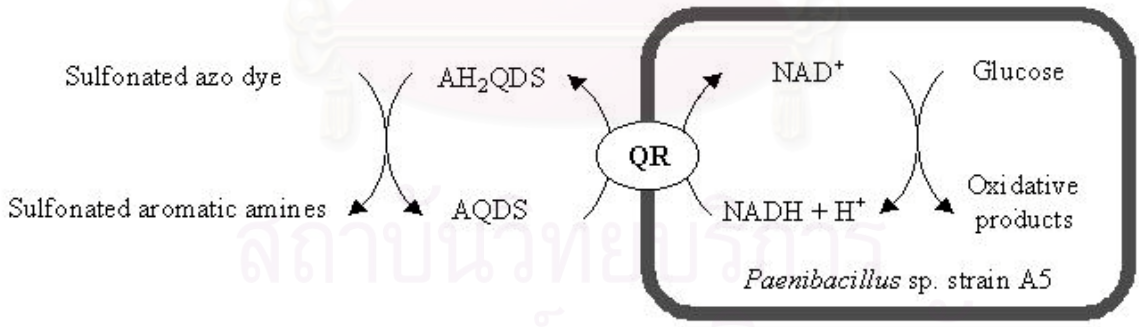


Figure 7.20.Model for mechanism by which AQDS, a humic analogue stimulate sulfonated azo dyes reduction by *Paenibacillus* sp. strain A5. QR is quinone reductase

The anaerobic reduction of AQDS occurred only in the presence of glucose, which seems to be the source of reduction equivalents to reduce the AQDS outside the membrane of strain A5. The proposed mechanism demonstrated in Figure 7.20 could be useful in the treatment of sulfonated azo dyes containing wastewaters by strain A5 or other bacteria which able to reduce AQDS. Under anaerobic conditions, the chemical reactions of the reduced AQDS (AH_2QDS) with the sulfonated azo dyes allows for extremely unspecific reduction processes which are mainly governed by the redox potentials of the AH_2QDS and azo compounds therefore, a wide range of azo dyes can be reduced. The main restriction to this mechanism is that the amines that are formed are usually not further metabolized under anaerobic conditions. Since aromatic amines and also sulfonated aromatics are aerobically degraded by bacteria (Brown and Laboureur, 1983; Feigel and Knackmuss, 1993; Ohe and Watanabe, 1986; Nortemann et al., 1986; Nortemann, 1994), it has been repeatedly suggested to combined the anaerobic reduction of the azo dyes with an aerobic treatment system for the amines formed (Fitzgerald and Bishop, 1995; Haug et al., 1991; O'Neill et al., 2000; O'Neill et al., 2000; Tan et al., 1999).

In many intestinal bacterial isolates, a flavin compound (riboflavin, flavin adenine dinucleotide, of flavin mononucleotide (FMN)) is required for azoreductases activity (Gingell and Walker, 1971; Roxon et al., 1967; Russ et al., 2000). The most generally accepted hypothesis for this phenomenon is that many bacterial cells possess a rather unspecific cytoplasmic favin-dependent reducatses (flavin reductases) which transfers electron under anaerobic conditions via (soluble) flavins to the azo dyes (Russ et al., 2000; Stolz, 2000). In the present study, a rather rapid decolorization of all azo dyes was observed when incubated them with cytoplasmic fraction of strain A5 in oxygen-free buffer with NADH as a source of reduction equivalents (Table 7.2). However, the reaction rate increased dramatically in the presence of flavin adenine dinucleotide (FAD). A possible explanation for this phenomenon is that FAD is reduced enzymatically by NADH and reduced FAD ($FADH_2$) can then spontaneously reduce the three sulfonated azo dyes to the corresponding amines (Dubin and Wright, 1975 Gingell and Walker, 1971, Roxon et al., 1967; Russ et al., 2000). In contrast, it was shown that the addition of FAD did not lead to enhancement of the reduction rates of sulfonated azo dyes by whole cells of strain A5 (Table 7.2). Thus, this has generally been explained by the low permeability of the cell membranes for the highly polar sulfonated azo compounds (Wuhrmann et al., 1980). Moreover, the bacterial membrane are also hardly permeable for flavin-containing cofactors and restrict the transfer of reducing equivalents by flavins from cytoplasm of intact cells to extracellular sulfonated azo dyes (Wuhrmann et al., 1980).

It was clearly demonstrated in this study that the almost activity of flavin reductase, which hypothesized to function under adequate conditions as flavin-dependent azo reductase, was present in the cytoplasmic fraction (Table 7.3). Therefore, it appears reasonable that, with intact cells, intracellular enzymes like flavin reductases are of little importance for reduction of extracellular sulfonated azo compounds by strain A5. These results supported the hypothesis of Russ and coworkers (2000) that the reduction of sulfonated azo dyes by reduced flavins formed by cytosolic flavin-dependent azo reductases is mainly observed in vitro and in vivo is of insignificant importance (Kudlich et al., 1997; Stolz, 2000).

Thus, in the intact cells, other enzyme systems, which does not require transport of the azo dyes though the cell membrane, are presumably responsible for the unspecific reduction of various sulfonated azo dyes by *Paenibacillus* sp. strain A5. Several quinoide redox mediator compounds, for example; anthaquinone-2-sulfonate (AQS), 2-hydroxy-1,4-naphthoquinone (lawsone), 4-amino-1,2-naphthoquinone and AQDS, have been shown to enhance degradation of sulfonated azo dyes by acting as electron shuttles that facilitate reduction of the azo dye (Keck et al., 1997; Keck et al., 2002; Kudlich et al., 1997; Rau et al., 2000; Rau and Stolz, 2000). In this study, it shown that the external added AQDS not only stimulate quinone-dependent azo reductase activity in detergent-soluble membrane fractions but also enhance anaerobic reduction of intact strain A5 cells. In addition, it could be demonstrated in the cell-fractioning experiments that the NADH: quinone oxidoreductase activity was almost restrictively present in the membrane fraction (Table 7.3). These results suggest the existence of an NADH-dependent quinone reductase in *Paenibacillus* sp. strain A5 membranes that catalyze the reduction of endogenous quinones (e.g., menaquinone), may responsible for the reduction of exogenous quinones (e.g., AQDS).which then transfer reduction equivalents to sulfonated azo dyes outside the cells.

The anaerobic decolorization occurred only in the presence of cells, indicting that the cells reduced the AQDS to the corresponding hydroquinone (AH_2QDS). This reaction may be catalyzed anaerobically by NADH-quinone oxidoreductase (NDH) of the respiratory chain, which appeared to have a low substrate specificity of the quinone-binding site in several bacterial genera (Davis and Doroshow, 1986; Kudlich et al., 1997; Rau et al., 2000; Rau and Stolz, 2003; Stolz, 2000; Yagi, 1991). NDH play their most important role as a primary dehydrogenase, linked with the central metabolism, in the respiratory chain of all organisms having an aerobic or anaerobic electron-transport system. Several types of NDH occur in bacteria, the most common of which are referred to as NDH-1 and NDH-2 (Cook and Shiemke,2002). NDH-1 is an energy-transducing enzyme meanwhile NDH-2 appears to have no role in energy transduction (Cook and Shiemke,2002). Some gram-positive bacteria such as *Bacillus subtilis* have non-energy generating NDH II but not NDH I (Bergsma et al., 1981).

In addition, it has recently suggested that oxygen-insensitive nitroreductase (NfsA and NfsB) are capable of effectively reducing not only nitro compounds but also quinones, which may not be natural substrates (Zenno et al., 1996). The nitroreductase, and other enzymes in different families, which had sequence homologies to a certain group of NfsA and NfsB such as flavin reductase (FRP) (Zenno et al., 1996), may be also able to function under anaerobic conditions in the appropriate conditions as quinone-dependent azo reductases in many bacterial genera (Rau and Stolz, 2003; Zenno et al., 1996). Indeed, the further study is necessary to identification the real enzyme system which is responsible for the ability of *Paenibacillus* sp. strain A5 to reduce AQDS and thus to reduce sulfonate azo dyes under anaerobic condition in the presence of AQDS or other quinoide mediators.

In biological systems, quinones were also shown to accelerate azo dye reduction by anaerobically incubated aerobic biomass as well as granular sludge (Kudlich et al., 1997; Rau et al., 2000; Rau et al., 2003). Theoretically, feasible quinoide redox mediators should have redox between those of the two eventual half reactions, the reduction of azo dyes and the oxidation of a primary electron donor. Although standard redox potential (E_0') for the reduction of the sulfonated azo dyes to their constituent aromatic amines are not available, an indication can be derived from paragraphic data. Redox potentials of the azo compounds are approximately vary between –180 mV and –430 mV (Dubin and Wright, 1975; Rau et al., 2000). For bacterial azo dye reduction, i.e., coupled to the oxidation of organic primary electron donors by anaerobically incubated bacteria, the E_0' value of NAD(P)H, the cellular redox cofactors with the lowest electron potential (-320 mV), can be taken into account (Zee et al., 2003). It was recently suggested that quinoide redox mediators with standard redox potentials (E_0') between approximately –320 mV and –50 mV could in general function as effective redox mediators in the bacterial reduction of sulfonated azo dyes (Rau et al., 2000). Thus quinones with a rather negative redox potential such as AQDS are suitable as redox mediators for the anaerobic treatment of azo dyes.

The ability of quinone compounds (i.e., AQDS) to convoy electrons between the bacterial membrane and the dye in solution at the same distance from the cell suggests that *Paenibacillus* sp. strain A5-AQDS couple can be used to reduce dye in a separate compartment without direct contact between the sulfonated azo dyes and bacterial cell membrane. Since hydroquinones are readily oxidized by sulfonated azo dyes, this hydroquinones only needs to be present at substoichiometric concentrations to be an effective electron carrier as long as these azo dyes are abundant in the wastewaters. Although the effective AQDS dosage levels were low, continuous dosing implies continuous expenses related to procurement of the chemical as well as continuous discharge of this recalcitrant sulfonated azo compounds. Therefore, it is desirable to immobilize the redox mediator in the bioreactor for treatment azo dye-containing wastewater continuously. For this propose, various reactor configurations were employed to demonstrate that, though the use of redox mediator such as AQDS, direct contact between azo dye and microbial cells is not required, which allows microbial activity to decoupled in space and time from azo dyes reduction process. For example, a system of two separated columns which one used for redox mediator reduction and the another one

use for azo dye reduction has been set up for stimulation of azo dye reduction by *Burkholderia cepacia* (Laszlo, 2000). Alternatively, the laboratory-scale upflow anaerobic sludge bed (UASB) containing activated carbon as an immobilized quinoid redox mediator in the sludge bed has been tested for its accelerating effect on anaerobic reduction of a recalcitrant azo dye (Zee et al., 2003).

The fact that exogenous extracellular molecules (such as humic substances) can participate in electron transfer to extracellular environmental contaminants indicates that they may make a significant contribution to biotransformation of such xenobiotics in many environments (Bradley et al., 1998; Cervantes et al., 2000; Cervantes et al., 2000). Whether microbially produced extracellular molecules have a similar role remains an important question. Another possibility for the reduction of extracellular quinoide redox mediator such AQDS, which does not require the transport of both redox mediators and azo dyes though the cell membrane, has been suggested for humate-respiring bacterium, *Shewanella putrefaciens* (Newman and Kolter, 2000). Non-proteinaceous small compound that has characteristics similar to a quinone and can be excreted into the medium is involved in electron transfer to AQDS and humic acid by this strain (Newman and Kolter, 2000). Moreover, a derivative of 1,4-dihydroxy-2-naphthoate (DHNA), precursor of menaquinone, is responsible for the carbon tetrachloride transformation activity observed in *Shewanella oneidensis* MR-1 after aerobic growth (Ward et al., 2003). Menaquinone (MK) is the only common link for the different electron transfer routes in *B. subtilis*, and it is tempting to propose that MK itself can be the component whose reduction-oxidation is controlled by energization (Newman and Kolter, 2000).

Because the rather high reduction rate of sulfonated azo dyes in the absence of any exogenous redox mediators found in whole cells experiment (Figure 7.11, 7.13 and 7.15 and Table 7.2), it could not eliminate the possibility that since some unknown enzymatic activities are involved in the anaerobic reduction of sulfonated azo dyes by whole cells of strain A5.

7.1.4.4 Conclusions

The humic quinone moiety model compound anthraquinone-2,6-disulfonate (AQDS) could function as redox mediator in the unspecific anaerobic reduction of different industrially relevant sulfonated azo dyes by *Paenibacillus* sp. strain A5. This compound was enzymatically reduced by the cells of strain A5 to corresponding hydroquinone, which subsequently reduced the azo dyes outside the cells in a purely chemical redox reaction, and ultimately causing decolorization. Cell fractioning experiments demonstrated that the AQDS-dependent azo reductase activity was located in the cell membranes of strain A5. For strain A5, the presence of both membrane-bound and cytoplasmic azo reductase activities was shown, and they were probably different enzyme system, and the former system has significant importance in the reduction of sulfonated azo compounds in vivo, when quinoid redox mediators may be present.

7.2 Declorization of Ramazol Brilliant Blue R by biosorption on bacterial biomass

7.2.1 Biosorption characteristics of *Paenibacillus* sp. strain A5 for Remazol Brilliant Blue R

Adsorption techniques are seen as an economic alternative. Activated carbon has proved effective for dye removal but it is expensive. Less costly alternative sources of adsorption materials have been investigated, including peat, steel plant slag, bentonite clay and fly ash (Ramakrishna and Viraraghavan, 1997). Many bacteria and fungi have shown value as potential adsorbents (Nigam et al., 1996). The uptake or accumulation of chemicals by microbial mass is termed biosorption and may involve a combination of active, metabolism-dependent and passive transport mechanisms. Binding to sites at or near the cell surface which exhibit chemical affinity for the adsorbate is generally non-metabolism dependent and so occurs for both viable and denatured microbial cells (Nigam et al., 1996).

Results from the preliminary study in Chapter 5 and Chapter 6 previously demonstrated that the decolorization mechanism of Remazol Brilliant Blue R by *Paenibacillus* sp. strain A5 and S1 are the different mechanism which the bacteria using for decolorization of azo dyes. The biosorptions are suspected to be the main decolorization mechanism of Remazol Brilliant Blue R by *Paenibacillus* sp. strain A5 and S1. However the potential and behavior of bacterial biosorption processes to remove Remazol Brilliant Blue R ,an anthraquinone dye, are not determine in greatly details. Therefore, the objectives of this study are focused on the uptake characteristics of *Paenibacillus* sp. strain A5 biomass for Remazol Brilliant Blue R.

7.2.1.1 Equilibrium modelling in a batch system

The Langmuir and Freundlich equations are in common use for describing adsorption isotherms at a constant temperature for water and wastewater treatment applications.

Langmuir model

The Langmuir model is based on the assumption that maximum adsorption corresponds to saturated monolayer of dye molecules on the adsorbent surface with a finite number of identical sites, that the energy of adsorption is constant and that there is no transmigration of adsorbate in the plane of the surface. Langmuir isotherm is obtained by agitating the adsorbate of fixed dose and the dye solution of different concentrations for a constant time greater than the equilibrium time (Aksu and Tezer, 2000).The well known expression of the Langmuir model is given by the following equation

$$q_{eq} = \frac{Q_o b C_{eq}}{1 + bC_{eq}}$$

where

q_{eq} is the amount of adsorbed dye per unit weight of biomass (mg/g),
C_{eq} is the equilibrium concentration (mg/l),
Q_o is the Langmuir constants (mg/g), and
b is a constant related to the affinity of the binding sites (l/mg)

The Langmuir constants Q_o and b are calculated from the following equation

$$C_{eq}/q_{eq} = 1/Q_o b + C_{eq}/Q_o$$

Thus, a plot of C_{eq}/q_{eq} vs C_{eq} should be linear if Langmuir adsorption are operative, permitting calculations of Q_o and b.

Freundlich model

The Freundlich model is basically empirical, but is often useful as a means for data description. The isotherm assumes that adsorbent surface sites have a spectrum of different binding energies. The Freundlich isotherm indicates, first of all, whether the adsorption proceeds with ease or with difficulty. The expression of the Freundlich model is given by the following equation

$$x/m = k_f C_{eq}^{1/n}$$

where

x is the amount adsorbed dye (mg),
m is the biomass dose used (g),
k_f is the Freundlich constants (related to adsorption capacity),
n is a constant related to intensity of adsorption, and
C_{eq} is the equilibrium concentration (mg/l)

The Freundlich constants k_f and n are calculated from the following equation (Aksu and Tezer, 2000):

$$\log_{10}(x/m) = \log_{10} k_f + 1/n \log_{10} C_{eq}$$

Thus, a plot of $\log_{10}(x/m)$ vs $\log_{10} C_{eq}$ should be linear if Freundlich adsorption are operative, permitting calculations of k_f and n from the slope and intercept, respectively.

7.2.1.1.1 Methodology

Preparation of the *Paenibacillus* sp. strain A5 cells and Remazol Brilliant Blue R solutions for biosorption

After the growth period, strain A5 cells were harvested by centrifugation at 8000 x g for 10 min. The pellet was washed twice with deionized, distilled water before drying at 60 ^{0}C for 24 h. For the biosorption studies, 1 g of dried bacterial cells was suspended in 100 ml distilled water and then stored in the refrigerator. Ten millilitre dried biomass suspension was contacted with 90 ml of test solution containing a known concentration of dye in an Erlenmayer flask at 30 ^{0}C and at the pH 7. All the final solutions contained 1 g/l of biosorbent. The test solutions containing different concentrations of Remazol Brilliant Blue R reactive dye were prepared by diluting 1 g/l of stock solution of the dye in 1 l of double-distilled water. The range of concentrations of prepared dye solutions was 50 – 500 mg/l.

The flasks were agitated on a shaker at 150 rpm for 96 h to ensure that equilibrium was reached. Samples (5 ml) were taken before mixing the biosorbent solution and dye bearing solution and at predetermined time intervals for the residual dye concentration in the solution. Before analysis the samples were centrifuged at 8,000 rpm for 10 min and the supernatant liquid was analyzed for the remaining dye. All the experiments were carried out in duplicate.

7.2.1.1.2 Results and discussions

Initial concentration provides an important driving force to overcome all mass transfer resistance of the dye between the aqueous and solid phases. Hence a higher initial concentration of dye will enhance the adsorption process. The effect of initial dye concentration on the dye sorption capacity and yield of bacterial biomass was investigated between 50 and 500 mg/l at the initial pH value of 7.0, the results are presented in Table 7.4.

Table 7.4 Comparison of equilibrium adsorbed quantities and adsorption efficiencies of Remazol Brilliant Blue R dye obtained at different initial dye concentrations (temperature: 30 ^{0}C, pH: 7, agitation rate: 150 rpm, biomass: 1 g/l)

C_0 (mg/l)	q_{eq} (mg/g)	Adsorption efficiencies (%)
50	27.6	55.1
100	51.2	51.2
200	100.9	50.5
250	144.8	48.3
500	178.8	44.7

Paenibacillus sp.strain A5 was capable of removing more than 40% of the coloring material at 500 mg/l of initial dye concentration and did not appear to reach saturation over the concentration range involved (Table 7.4). This suggests that the adsorbent capacities of strain A5 effectively utilized in solutions of this initial dye concentration. The dye uptake increased with a further increasing in initial dye concentration in solution. When the initial dye concentration increased from 50 to 500 mg/l, the dye loading capacity of dried biomass increased from 27.6 to 178.8 mg/g for this strain (Table.7.4). However, higher adsorption efficiencies were observed at lower concentrations of dye.

Strain A5 was found to be effective for concentrating Remazol Brilliant Blue R dye at different capacities according to the dye concentration. Cell walls of *Paenibacillus* sp strain A5, a gram positive bacterium, contain peptidoglycan as basic building blocks which have ion exchange properties, and also proteins and lipids and therefore a host of functional groups capable of binding dye molecules. These functional groups such as hydroxy, amino, carboxylic, sulfydryl, phosphate and thiol groups, differ in their affinity and specificity for dye (Fu and Viraraghavan, 2001).

The adsorption isotherm of Remazol Brilliant Blue R to the biomass of the *Paenibacillus* sp. strain A5 is shown in Figure 7.21.

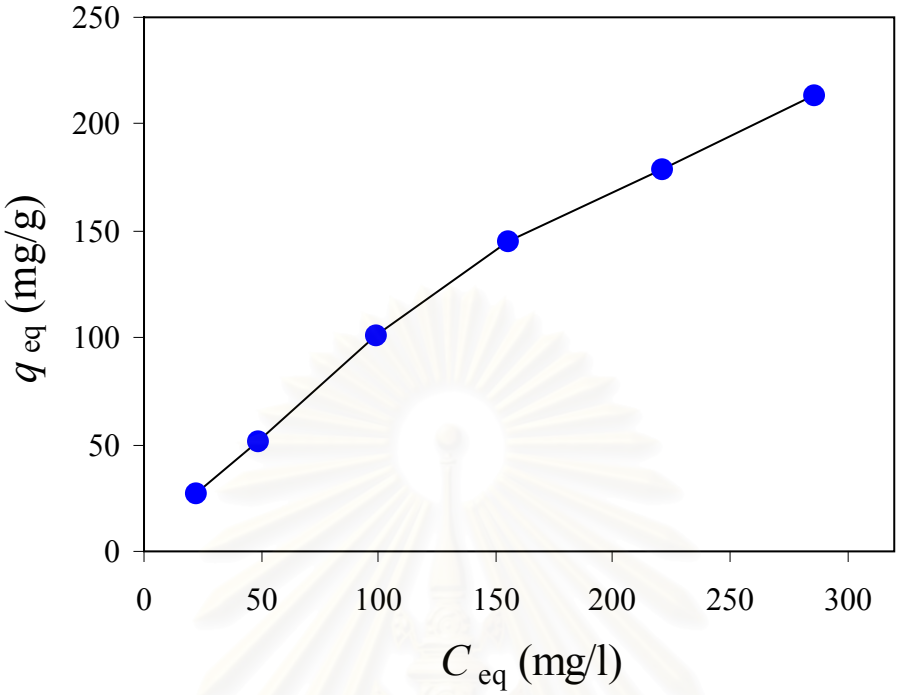


Figure 7.21. Remazol Brilliant Blue R adsorption isotherms to *Paenibacillus* sp. strain A5 biomass

Equilibrium modelling

Out of several isotherm equations developed to describe adsorption isotherm relationships, two were applied for the equilibrium data of *Paenibacillus* sp.strain A5: the Freundlich and Langmuir isotherms. The linearized Freundlich and Langmuir adsorption isotherms of Remazol Brilliant Blue R dye obtained at 30 ^{0}C are shown in Figure 7.22 and 7.23.

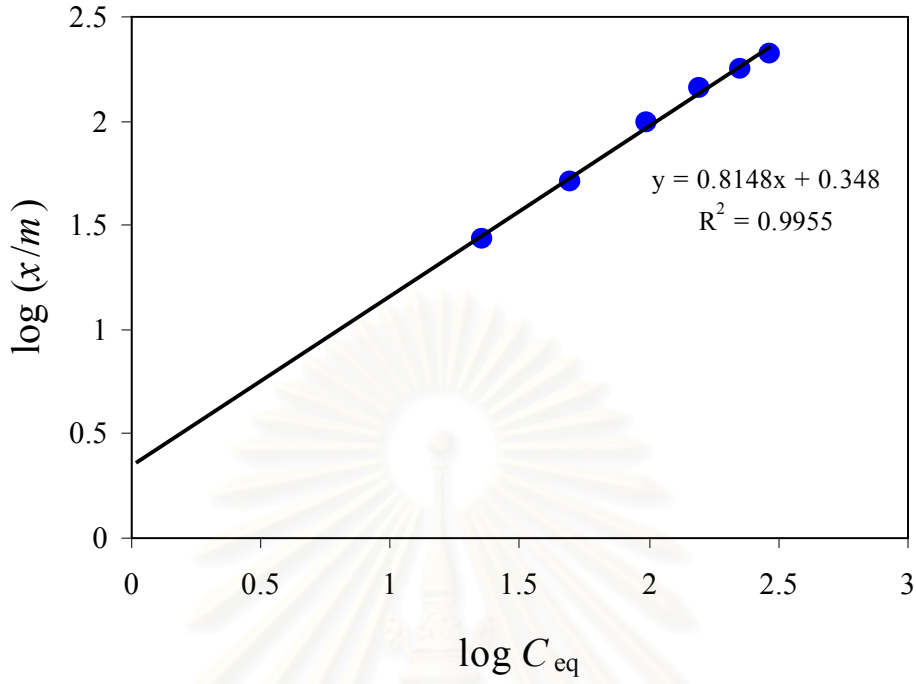


Figure 7.22. Linearized Freundlich adsorption isotherms of Remazol Brilliant Blue R for *Paenibacillus* sp. strain A5

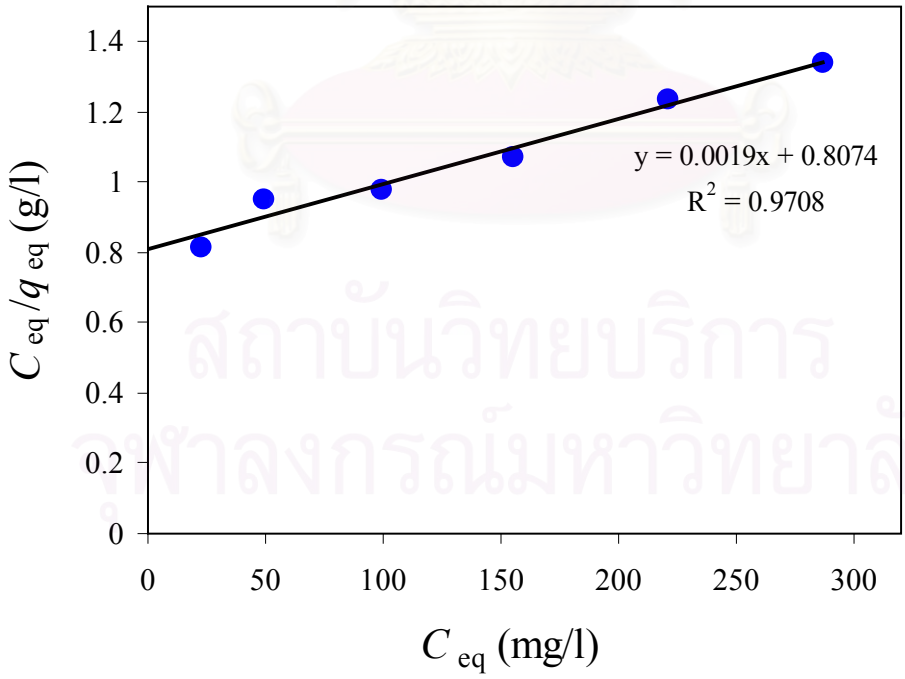


Figure 7.23. Linearized Langmuir adsorption isotherms of Remazol Brilliant Blue R for *Paenibacillus* sp. strain A5

Adsorption constants evaluated from isotherms with the correlation coefficients are presented in Table 7.5. Both the isotherms agreed well with the experimental data of dye considering that obtained linear regression coefficients are quite high. Adsorption isotherms are characterized by certain constants, the values of which express the surface properties and affinity of the sorbent and can also be used to determine the biosorptive capacity of strain A5 for the Remazol Brilliant Blue R.

The high values of k_f showed ready uptake of the dye from wastewater with high adsorptive capacities of the biomass of strain A5 (Table 7.5). In addition, n was greater than unity, indicating that the dye was favorably adsorbed by the dried strain A5 cells (Table 7.5). It is generally stated that the values of n between 1 and 10 indicates beneficial adsorption (Asfour et al., 1985).

Table 7.5 Comparison of the Freundlich and Langmuir adsorption constants

Freundlich constants			Langmuir constants		
k_f [(mg/g)(mg/l)^n]	n	R^2	Q_o (mg/g)	**b (l/mg)**	R^2
2.2	1.23	0.9955	526.3	2.35×10^{-3}	0.9708

Values of Q_o and b for strain A5 cells was calculated from the Langmuir plot (Figure 7.23) and the results are shown in Table 7.5. The value of Q_o (i.e. maximum uptake) appeared to be significantly high for the dye-*Paenibacillus* sp. strain A5 system indicating that biomass of *Paenibacillus* sp. strain A5 is a good sorbent for Remazol Brilliant Blue R.

The Langmuir model makes several assumptions, such as monolayer coverage and constant adsorption energy while the Freundlich equation deals with physicochemical adsorption on heterogeneous surfaces. It was seen that the biosorption equilibrium data of dried cells of *Paenibacillus* sp. strain A5 fitted very well to both Freundlich and Langmuir adsorption models in the studied concentration. It may be concluded that both monolayer adsorption and heterogeneous surface conditions exist under the experimental conditions used. The sorption of Remazol Brilliant Blue R on the cells of strain A5 is thus likely to be complex, involving more than one mechanism.

7.2.2 Desorption of Remazol Brilliant Blue R from biomass

Both incineration and land disposal represent possible options for final disposition of spent adsorbent material. However, both methods directly or indirectly pollute the environment. If regeneration of dyes from the spent adsorbent is possible then it would not only protect the environment but also help recycle the adsorbate and adsorbent and hence contribute to the economy of wastewater treatment.

Desorption studies also aim to evaluate the mechanisms of adsorption. If the adsorbed blue dye on the solid surface can be desorbed by water, then the attachment of the blue dye on the bacterial biomass is by weak bonds. If strong acid (i.e., HCl) or NaOH (0.1 N) can desorb the dye, then the adsorption is by ion exchange. If organic acids, like acetic acid, can desorb the dye, then the dye is held by the bacterial biomass through chemisorption (Namasivayam and Yamuna, 1992).

In this study, deionized water, acetic acid and NaOH (0.1 N) have shown 9.9, 6.0, and 63% desorption of Remazol Brilliant Blue R, respectively (Figure 7.24 and 7.25). The Remazol Brilliant Blue R is anionic in nature and its increased adsorption in an acidic medium and its increased desorption in alkaline medium show that this dye is held by bacterial biomass, most propably by ion exchange. That portion of the dye which could not be desorbed would likely be due to the binding of negatively charged dye molecules with the functional groups of the cellular component of *Paenibacillus* sp. strain A5.

It was noticed that the Remazol Brilliant Blue R was biosorbed into the exterior region of the rapidly setting biomass pellets of *Paenibacillus* sp. strain A5. The blue color from biomass could be slightly eluted with water, Tris-HCl buffer, acetic acid, and HCl (Figure 7.25) However, 63% of the adsorbed dye could be eluted from the loaded biomass by using 1 N NaOH. This result suggests simple mechanism of color removal via adsorption to cellular entities of the bacterium rather than biotransformation of dyes.

This would suggest that there could be various mechanisms involved in sorption process of Remazol Brilliant Blue R on different components present in biomass of *Paenibacillus* sp. strain A5.

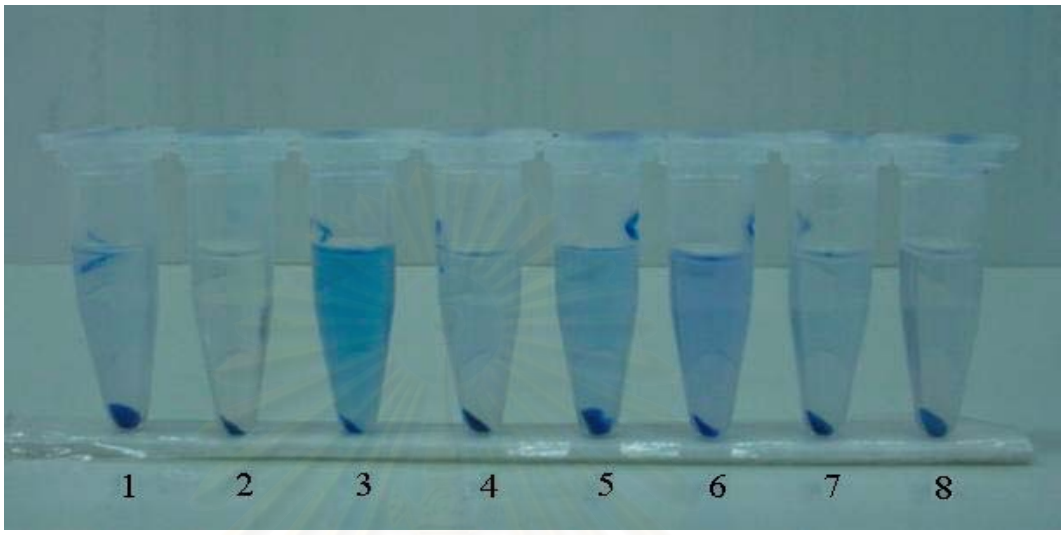


Figure 7.24. Desorption of Remazol Brilliant Blue R from dye-coated cells of *Paenibacillus* sp. strain A5 by various chemicals. (**1**). Deionized water (**2**) 1N HCl (**3**) 1N NaOH (**4**) 10 mM NADH (**5**) 0.1 mg/ml Lysozyme (**6**) 80% Methanol (**7**) 50 mM Tris-HCl buffer pH 7.5 (**8**) 1M Acetic acid

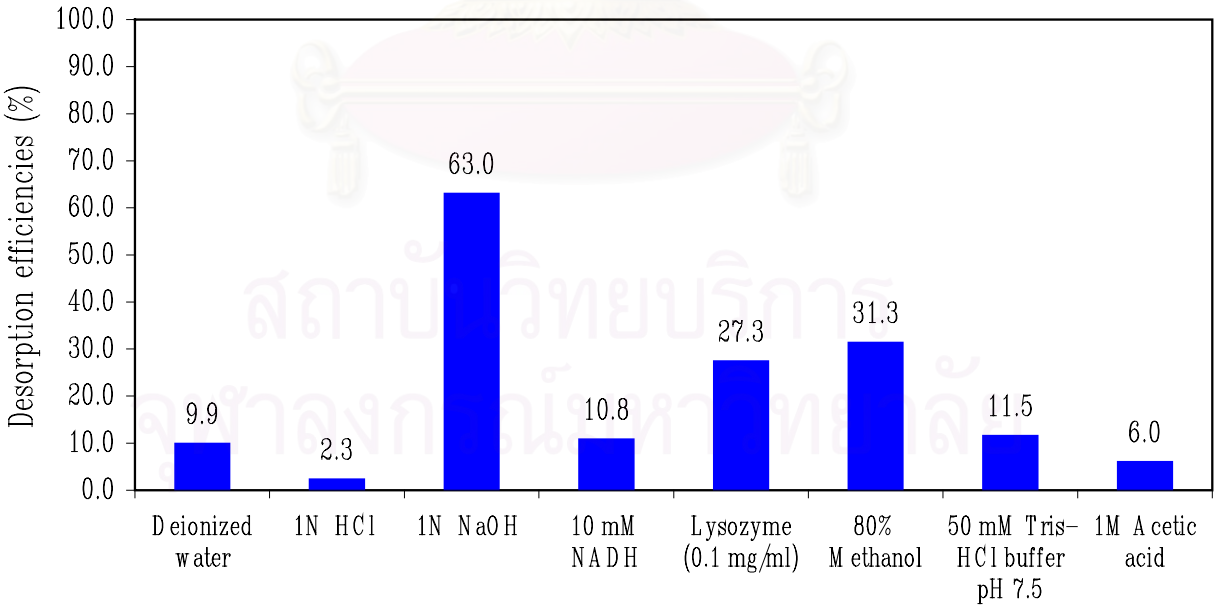


Figure 7.25. Desorption of Remazol Brilliant Blue R from dye-coated cells of *Paenibacillus* sp. strain A5 by various chemicals

The enhancement of uptake of reactive dyes at acidic pH may be explained in terms of electrostatic interactions between the biomass and the dye particles. Upon dissolution, ionic dyes release colored dye ions into solution (Ramakrishna and Viraraghavan, 1997). The adsorption of these charged dye groups onto the adsorbent surface is primarily influenced by the surface charge that in turn is influenced by the solution pH. With diminishing pH increasing numbers of weak base groups in the biomass become protonated and acquire a net positive charge (Tobin et al., 1984).

These charged sites become available for binding anionic groups such as the Remazol Brilliant Blue R used in this study. Remazol Brilliant Blue R contains anthraquinoid chromophore combined with vinylsulfone as a reactive groups which can interact with the active groups on the cell surface of bacteria such as peptidoglycans, acidic polysaccharides, lipids, amino acids and other cellular components of the microorganism (Fu and Viraraghavan, 2001).

Anthraquinone dye-ligands are able to bind most types of proteins, they are classified as affinity ligands because they interact with the active sites of many proteins mimicking the structure of the substrates, cofactors, or binding agents for those proteins. The interaction between the anthraquinone dye ligand and proteins can be by complex combination of electrostatic, hydrophobic, hydrogen bonding.

Interactions of the parent dyes especially Cibacron Blue F3G-A and their analogs with several oxidoreductases, phosphokinases, and ATPases have been investigated (Biellman et al., 1979). These studies have shown that both the anthraquinone and the adjacent benzene sulfonate rings on these dyes are important in binding to the enzymes. They do bind to the enzyme molecules at a similar position and in a way similar to the AMP moiety of the coenzyme. Nucleotide cofactors such as NADPH, NADH, ATP, and AMP have been used to elute dehydrogenases and other nucleotide-dependent enzymes from immobilized Cibacron Blue F3GA (an anthraquinone structure analogue of Remazol Brilliant Blue R) and other reactive dyes (Boyer and Hsu, 1993).

In this study, the addition of NADH, a nucleotide cofactor of many bacterial enzymes, could not effectively elute the cell-bound Remazol Brilliant Blue R from biomass of strain A5 (Figure 7.24 and 7.25). This result shows that the binding of anthraquinone dye and bacterial proteins is not the main sorption mechanism in the present study.

In the other hand, the rather high amount of Remazol Brilliant Blue R were eluted from the bacterial biomass into the solution in the presence of lysozyme (0.1 mg/ml) (Figure 7.24 and 7.25). Remazol Brilliant Blue R has been shown to bind the hydroxyl groups of sugars (Hardt et al., 2003) present in peptodoglycan, the major constituent of bacterial cell walls. Hydrolysis of the Remazol Brilliant Blue R dye-labled bacteria cells leads to release of soluble blue products in the solution (Hardt et al., 2003).

Cell walls of *Paenibacillus* sp strain A5, a gram positive bacterium, contain peptidoglycan as basic building blocks, which not only have ion exchange properties but also able to interact with the Remazol Brilliant Blue R effectively. Therefore, the peptidoglycan in cell wall of *Paenibacillus* sp. strain A5 and other bacteria may play an important role in the biosorption of Remazol Brilliant Blue R.

7.2.3 Conclusion

The ability of *Paenibacillus* sp. strain A5 for removing Remazol Brilliant Blue R from solution was examined. It is important to study the dye accumulation characteristics of the strain to identify its biosorption potentials and sorption behavior.

It was seen that biosorption can be accomplished with a high yield by increasing dye concentration up to 500 mg/l with the strain A5.

Biosorption equilibrium data of dried *Paenibacillus* sp. strain A5 fitted very well to both Freundlich and Langmuir adsorption models in the studied concentration range. Assuming the batch biosorption as a single staged equilibrium operation, the separation process can be mathematically defined using these isotherm constants to estimate the residual concentration of dye or amount of biosorbent for the desired levels of decolorization.

The suitability of the cells of *Paenibacillus* sp. strain A5 to remove Remazol Brilliant Blue R from textile mill are not only on its exchange capacity, but also its physical stability (resistance to solubilization by different desorpting-chemicals under experimental conditions).

Chapter 8

8.1 Decolorization of reactive dyes with calcium alginate-coimmobilized *Paenibacillus* sp. strain S1 and A5

For economical purposes, to decolorize reactive dyes it is very essential to immobilize the bacterium inside the carrier. Immobilization of microorganisms within gel beads is the most widely used technique for viable microbial cells (Kurosawa and Tanaka, 1990; Beunink and Rehm, 1990). Physical encapsulation inside polymeric gel matrixes is inexpensive, simple to prepare, and offers mild physicological conditions (Kurosawa, and Tanaka, 1990). Cells are mixed with a fluid precursor of a gel, which is then solidified by polymerization or precipitation. Hydrogels are generally used because of high water retention and porosity range, which allows better diffusion of smaller solutes, such as nutrients and waste products (Kühtreiber et al., 1999). As a consequence, define coimmobilization of microorganisms entrapped in a variety of gels, including polyacrylamide, cellulose, alginate, chitosan, agar, and K-carrageenan, has been either to utilize the complementary biocatalytic activities of two or more microorganisms.

Among the wide spectrum of materials used for preparation of immobilized gel beads, the use of alginate as an immobilizing agent in most applications rests in its ability to form heat-stable strong gels which can develop and set at room temperatures. (Fraser and Bickerstaff 1997). Alginates are linear unbranched polymers containing β-(1,4)-linked D-mannuronic acid (M) and α-(1,4)-linked L-guluronic acid (G) residues and is found in many algal species, specially inside the brown algae. This carboxylic polyelectrolyte is soluble in water and precipitates in the form of a co-acervate in the presence of multivalent metal ions like Ca^{2+}, Co^{2+} Zn^{2+}, Ba^{2+}, Fe^{2+}, Fe^{3+}, and Al^{3+} (Fraser and Bickerstaff 1997).

The alginate gel as an immobilization matrix is sensitive to chelating compounds such as phosphate, lactate and citrate, presence of anti-gelling cations such as Na^{+} or Mg^{2+}. There has been found a correlation between mechanical gel strength and affinity for cations. It has been found that gel strength may decrease in the following orders: Pb^{2+} > Cu^{2+} = Ba^{2+} > Sr^{2+} > Cd^{2+} > Ca^{2+} > Zn^{2+} > Co^{2+} > Ni^{2+} However, in applications involving immobilization of living cells toxicity is a limiting factor in the use of most ions, and only Sr^{2+}, Ba^{2+} and Ca^{2+} are considered as nontoxic for these purposes (Fraser and Bickerstaff 1997).

As encapsulation is by far the more widely used method for both single and defined coimmobilization systems. Table 8.1 presents a summary of various alginate encapsulation systems used.

Table 8.1 Systems for defined coimmobilization of mixed cultures by calcium alginate-encapsulation

Microorganisms	Target goals	References
Aspergillus awamori and *Zymomonas mobilis*	Ethanol production	Kurosawa and Tanaka, 1990
Alcaligenes sp. and *Enterobacter cloacae*	4-chloro-2-nitrophenol transformation	Beunink and Rehm, 1990
Kluyveromyces fragilis and *Zymomonas mobilis*	Ethanol production	Gunasekaran and Kamini,1991
Nitrosomonas europaea and *Paracoccus denitrificans*	Nitrification process	Kokufuta et al., 1988
Pseudomonas putida and *Cryptococcus elinovii*	Phenol degradation	Zache and Rehm, 1989
Lactobacillus casei and *Lactobacillus lactis*	Lactic acid production	Roukas and Kotzekidou, 1991
Streptococcus cremoris and *Streptococcus lactis*	Cheese production	Prevost, and Divies, 1987

It was also shown that an immobilized mixed culture was able to degrade phenol faster and at higher levels than was possible with pure cultures (Zache and Rehm, 1989).

Restricted diffusion rates of substrates such as oxygen and fermentation products, which can be limiting factors in alginate gel encapsulation products, which can be limiting factors in gel encapsulation, have, however, been used by some workers to advantage in defined coimmobilization. The most common approach is to utilize the oxygen-deficient inner part of alginate beads to encourage growth of anaerobic microorganisms, with aerobic microorganisms located nearer the surface, thereby increasing still further the oxygen gradient (Beunink and Rehm, 1990; Kurosawa et al., 1989). This enables two microorganisms with complementary biocatalytic activity but different oxygen requirements to be used in the same reactor in a way not possible in homogeneous systems. This creation of differental zones of growth within beads has been termed “habitat segregation” (Kurosawa et al., 1989).

The oxygen concentration profile in gel beads has been calculated to predict the gel volume that could be used by different aerobes and anaerobes (Kurosawa and Tanaka, 1990).

It was found that oxygen concentration in the center of beads (3-mm diameter) was too low to sustain growth off aerobic microorganisms. For example, growth of the aerobe *Aspergillus awamori* was dense only to a depth of 150 μm, and limited to 300 μm from the surface (Kurosawa and Tanaka, 1990). Reduction of bead size can remove this effect (Beunink, J. and Rehm, 1990), but increase problems of separation and attrition (Kurosawa and Tanaka, 1990).

Although oxygen-limiting environments in immobilized gel beads appear to be unfavorable for the aerobic growth of aerobic bacteria, entrapped cells within gel matrixes is particularly suitable for bacterial decolorization of azo dyes since it creates a local anaerobic environment favorable to oxygen-sensitive decolorization. In addition, despite the fact that the suspended –cell system allows better contact with the substrates, it may be less feasible in practical applications due to the requirement of downstream solid-liquid separation and the difficulty of achieving a high cell density.

Thus far only few researchers have been reported utilization of immobilized-cell systems for decolorization of wastewater and most cases have focused on immobilization of fungal biomass (Zhang et al., 1999) rather than bacterial cells which also hold potential for decolorization. In this chapter, cells of a defined mixed culture *Paenibacillus* sp. strain S1 and A5 were coimmobilized in calcium alginate gel matrices to investigate their decolorization ability.

In addition, the decolorization ability of immobilized cells in alginate bead can be affected by various factors, i. e. strength and rigidity of the bead, degree of cross-linking of alginate molecules, initial cell concentration and bead size. Among these factors, alginate bead size and the initial cell concentration are the most critical parameters to determine the decolorization ability of immobilized bacterial cells in alginate beads (Kudlich et al., 1996). Therefore, the preliminary experiments were set up to determine the effects of both factors on decolorization of reactive dyes by immobilized cells.

8.1.1 Materials and Methods

8.1.1.1 Immobilization of *Paenibacillus* sp. cells in calcium alginate

Sodium alginate (Fluka, Germany) was dissolved under constant stirring in hot water (80 ^{0}C). The concentrated homogeneous solution was diluted with water to a final concentration of 3% (w/v) and autoclaved for 10 min at 110 ^{0}C. The sodium alginate solution was cooled to room temperature. Then, the equal concentration of *Paenibacillus* sp. strain S1 and A5 cells, which pre-grown in the synthetic textile wastewater under aerobic condition for 18 h and suspended in 0.85% NaCl, were added in the cooled solution. The initial cell concentrations inside the bead varied between 10^5 and 10^8 CFU/ml of alginate. The mixture was completely homogenized by mixing with a sterile spatula and finally transferred to a 250-ml Erlenmeyer flask. A flexible tube with a canula (1.2 mm outer diameter) at the other end was soaked in the immobilized mixtures in the flask. The mixture of sodium alginate and bacterial cells was forced through the needle at a constant pump rate with a peristaltic pump into 500 ml of a $CaCl_2$ solution (2% w/v), which was incubated on ice and slightly stirred. The pump rate was fixed to obtain the desired bead sizes between 2.0 and 4.0 mm. The alginate beads were stirred for about 6 h in the $CaCl_2$ solution to enhance their mechanical stability and then washed twice for 20 min in 0.85% (w/v) NaCl. The average diameter of the beads were determined by using of verneir-caliper.

Figure 8.1. Morphology of calcium alginate beads

8.1.1.2 Batch decolorization operations with immobilized cells

Ten grams of immobilized *Paenibacillus* sp. strain S1 and A5 cells which different initial concentrations and diameter of beads were placed in a 50-ml synthetic textile wastewater. Remazol Brilliant Orange 3R, which was selected as a representative of all reactive dyes used, was added into the synthetic textile wastewater to achieve the final concentration of 100 mg/l for determination of color removal. Since the enzymatic decolorization activity is strongly inhibited by the presence of oxygen, the decolorization experiments were performed under the 'static-incubation' conditions (i.e., neither aeration nor agitation was employed). The decolorization efficiencies were obtained by the determination of the remaining color in cell-free synthetic textile wastewater after anaerobic incubation for 12 h. The COD removal efficiencies in subsequent aerobic incubation of the completely decolorized synthetic wastewater were also determined in the supernatant after 12 h of aerobic incubation. Decolorization with an identical amount of cell-free immobilization matrices was also performed as the blank control.

8.1.2 Results and Discussions

8.1.2.1 Abiotic color removal with cell-free matrixes

Cell-free beads were examined for their non-enzymatic color removal ability. This information served as the blank control for the results obtained from the coimmobilized cell experiments. The adsorption ability of the calcium alginate matrix for Remazol Brilliant Orange 3R, Remazol Black B, Remazol Brilliant Violet 5R and Remazol Brilliant Blue R was determined. With a fixed initial dye concentration (100 mg/l) and fixed volume of cell-free beads (20% w/v), the alginate beads caused only 8.1, 6.3, 7.3 and 9.4% color removal in 3 h for Remazol Brilliant Orange 3R, Remazol Black B, Remazol Brilliant Violet 5R and Remazol Brilliant Blue R, respectively. For all four reactive dyes, a complete concentration adjustment between the liquid phase and the alginate beads was found within 3 h. Generally, diffusion of small molecules seems to be scarcely affected by alginate matrixes (Kudlich et al., 1996). It has been previously reported that sulfonated azo dyes precipitate from aqueous solutions that contain high concentrations of Ca^{2+}-ions (Pagga and Taeger 1994). The rather low in adsorption capacity of alginate beads for all four sulfonated azo dyes may be related to the electrostatic interaction between the alginate matrixes and the dye molecules, since the alginate matrixes tend to possess negative charges and replusive force between negatively-charged sulfonated azo dyes may form to impede the contact of the dye molecules with the matrixes (Philips and Poon, 1988).

8.1.2.2 Effect of initial cell concentration

Different amounts of biomass were immobilized in alginate beads and the decolorization of Remazol Brilliant Orange 3R by these preparations was analyzed under anaerobic condition. If the concentration of immobilized cells were less than 10^7 CFU/ml immobilized phase, a strict correlation between the number of immobilized cells and the decolorization efficiency was found (Table 8.2). The lowest dye removal efficiency was found in the initial dye concentration of 10^5 CFU/ml alginate. The results show that decolorization efficiency increased as the initial immobilized cell rose, suggesting that the high cell density may provide a low-oxygen environment that is favorable for azo-dye decolorization. At higher initial cell loadings the diffusion barrier in alginate beads was built up by the oxygen consumption of the bacteria. Therefore, the oxygen concentration under the gel showed a rapid decrease with increasing cell content (Gosmann and Rehm, 1988).

However, no significant different of the color removal efficiency was found between the initial cell concentration of 10^7 and 10^8 CFU/ml alginate in all bead size experiments. High initial cell amount not only have a higher color removal efficiency, but also have a higher COD removal efficiency than the low initial immobilized cell concentration (Table 8.3).

8.1.2.3 Effect of bead size

Table 8.2 shows the effects of bead size on the removal of Remazol Brilliant Orange 3R. As the diameter of bead increases from 2 to 4 mm, the dye removal efficiency increases due to faster development of oxygen-free environments especially at the center of alginate beads of the larger bead sizes. In contrast, the efficiency of COD removal from the completely decolorized synthetic textile wastewater containing Remazol Brilliant Orange 3R decreased as the diameter of gel beads increased for each similar initial cell concentration experiment (Table 8.3). These results may be due to the lower resistance in diffusion of substrates and dissolved oxygen of the smaller bead sizes.

From the preliminary investigation as mentioned above, all further experiments would be carried out by using the initial immobilized cell concentration at 10^7 CFU/ml of alginate and fixed the bead diameter at 3 mm due to the high efficiency for both dye and COD removal.

Table. 8.2. Effect of initial cell concentration and bead size on decolorization efficiency of Remazol Brilliant Orange 3R using coimmobilized *Paenibacillus* sp. strain S1 and A5 in alginate at 12 h after anaerobic incubation

Bead sizes (mm)	Dye removal efficiencies (%)			
	Initial cell concentration (CFU/ml of alginate bead)			
	1×10^5	1×10^6	1×10^7	1×10^8
2.0	52.6	63.2	87.8	88.4
3.0	58.5	74.7	96.4	95.6
3.5	66.6	75.4	95.3	96.3
4.0	72.7	82.8	93.5	94.6

Table 8.3 Effect of initial cell concentration and bead size on COD removal efficiency of the complete anaerobic decolorized synthetic textile wastewater containing Remazol Brilliant Orange 3R by using coimmobilized *Paenibacillus* sp. strain S1 and A5 in alginate after aerobic incubation for 12 h

Bead sizes (mm)	COD removal efficiencies (%)			
	Initial cell concentration (CFU/ml of alginate bead)			
	1×10^5	1×10^6	1×10^7	1×10^8
2.0	62.5	64.5	85.2	84.3
3.0	54.8	57.9	82.6	84.1
3.5	44.1	59.3	77.8	78.0
4.0	35.5	56.7	72.3	76.7

8.2 Decolorization of reactive dyes by co-immobilized *Paenibacillus* sp. strain S1 and A5 in fluidized bed reactor

In the preceding first part of this chapter, the decolorizing performance of co-immobilized mixed cultures were preliminary investigated, which were operated in batch mode in synthetic textile wastewater containing reactive dye in shake flasks. To develop of the sequential anaerobic/aerobic co-immobilized cell process for treatment of textile wastewater, the scale-up experiments are indeed required. Therefore, in this part deals with the decolorization of this co-immobilized mixed population in the sequential anaerobic/aerobic bioreactor.

Dyestuff is a kind of refractory organic matter that has a complex molecular structure. The color of the dye present in wastewater is difficult to be removed by conventional wastewater treatment processes. The combined anaerobic and aerobic treatment has widely been used to treat dye-contaminated wastewater. In recent years, employing various high-rate biological reactors has been reported

O'Neill et al. (1999) demonstrated 68% and 86% color and COD removal efficiencies, respectively, in a upflow anaerobic sludge blanket (UASB)/activated sludge sequential reactor system treating reactive azo dyestuffs.

Rajaguru et al. (2000) studied the intermediate products during degradation period of Orange-G which contains sulfonated groups. Tan et al. (1999) observed 69% COD removal efficiency at an organic loading of 210 mg/l/day with ethanol–COD concentration of 2000 mg/l in an anaerobic/aerobic sequential reactor system removing Mordant Green dyestuff.

In a study performed by An et al. (1996), 83% color and 69% COD removal efficiencies were obtained in anaerobic (hydraulic retention times, HRT = 6–10 h) and aerobic (HRT = 6.5 h) sequential reactor degrading Acid Yellow and Basic Red dyes at a organic loading rate of 5.3 kgCOD/m^3/day and a COD concentration of 1200 mg/l.

Kalyuzhnyi and Sklyar (2000) obtained 56% decolorization efficiency in an anaerobic/aerobic hybrid reactor at a organic loading rate of 0.3 gCOD/l/day using ethanol as carbon source. It was observed that 400 mg/l of Procion Red (H-E7B) azo dye was degraded with 72% and 11% COD removal efficiencies in anaerobic and aerobic reactors, respectively at an organic loading rate of 3.34 kgCOD/m^3/day.

Zaoyan et al. (1992) observed 65% color and 74% COD removal efficiencies in an anaerobic/aerobic rotating biodisc system treating azo dyestuffs containing textile wastewaters at a HRT about 8 h and organic loading rate of 45 gCOD/m^3/day.

8.2.1 Sequencing Batch Reactor (SBR)

Sequencing batch reactors are known to have several advantages over conventional continuous flow systems. Bacterial decolorization of reactive dyes is possible in a single tank SBR if operating conditions are selected to introduce anaerobic and aerobic reactions during a cycle without any addition of separate reactors, recycling lines or clarifiers. The sequencing batch reactor technology has been recently implemented for nitrogen and phosphorus removal (Banas et al., 1999; Helmreich et al., 2000), particularly for piggery wastewater treatment (Bernet et al., 2000; Edgerton et al., 2000).

In a study performed by Luangdilok and Panswad (2000) the color and chemical oxygen demand (COD) removal efficiencies were investigated using anaerobic/aerobic SBR reactor for treatment of 20 mg/l of anthraquinone and diazo dyes. A total of 90% COD and 45% color removal efficiencies were obtained while acetic acid and glucose were used as carbon source.

The combination of SBR technology and anaerobic-aerobic treatment was used in this study due to its good operational flexibility, simple running and compact layout.

8.2.2 Fluidized bed reactors

During the last few years, different reactor designs have been proposed in order to obtain an effective continuous anaerobic/aerobic treatment of azo dyes: an anaerobic and an aerobic rotating biological contactor (Zaoyan et al., 1992), an anaerobic fixed-film fluidized bed reactor followed by an aerobic suspended-bed activated sludge reactor (Fitzgerald and Bishop, 1995; Seshadri et al., 1994), a combination of anaerobic and aerobic rotating-drum reactors (Harmer and Bishop, 1992; Sosath and Libra, 1997), and an anaerobic up-flow fixed bed column together with an aerobic agitated tank (An et al., 1996; O'Neill et al., 2000; Rajaguru et al., 2000).

Among fixed–film biological reactors, fluidized bed reactors have received considerable attention in biotechnology. In such reactors, biomass adheres and grows attached to an inert support which can be on static filling or on fluidizable particles e.g., sand. Fluidized bed technology can offer many advantages related to the use of small particles of support material for the accumulation of bacteria, and the recirculation (for anaerobic processes) or aeration (for aerobic processes) required to obtain sufficient upflow velocity which improves system mixing conditions and mass transfer.

8.2.2.1 Design considerations of a fluidized bed rector
(Carrondo et al., 1987; Iza, 1996; Marin, 1997)

a) **Bed expansion degree-hydrodynamic of fluidization.** The characterization variable of solid liquid fluidized system is the surface velocity needed to achieve 20% and 40% expansion. Consideration has to be given to the fact that the solid particles are covered by film of biomass, which at high surface velocities may lose this film by attrition, but very low surface velocities could produce crossed adherence between particles and change the fluidization condition of the bed.

b) **Support material.** One of the crucial aspects of fluidized bed reactor design is the support material. Sand and other high-density materials have relatively low adsorption capacity, while clay, volcanic stone and activated carbon show very high adsorptive power. The selection of the adequate support material has to consider several aspects, besides those related to fluidization, the experimental steps to choose the best are almost unavoidable. Other considerations have to do with the cost of the material and physical and chemical properties. The physical characteristics which have tobe considered are size, shape, particle density, hardness and surface area.

c) **Recycling**. In fluidized bed reactors the effluent is usually recycled, so as to achieve the fluidization velocity and the ratio used reaches moderately high values.

d) Yeild of biological solids and residual sludge. One of the main problems encountered in this technology is the growth of biomass on the particles and its control. Due to the large drag force generated between liquid and particle, it is sometimes possible that large amounts of adhered biomass may be carries away and leaves the reactor. This diminishes the yield of the process and even sometimes may destabilize its operation or cause washout. So some include an external settler, which at the same time serves as a support particle trap.

e) Temperature. All the known systems operate at mesophilic temperatures. The presence of solid support and small particle size provides somehow thermal stabilization to minor temperature change in the influent, provided it has a good conductivity.

f) Concentration of waste in the influent. The fluidized bed reactor have been used to treat all type of wastewaters, from sewage (low load) to wastewaters from beer and sugar plants (high load). The effect due to the excellent mass transfer conditions of these reactors allow this wide coverge and concentrations from 100 mg COD/l to 2000 mg COD/l have been processed without problem. The income or precipitation of a certain amount of solid within the reactor do not affect the global yield, since the bed expands further to hold the extra solids.

g) **Design strategy**. The design strategy may be described as follow: first consider the particles in their initial state and design a stationary biphasic or triphasic fluidized bed reactor, next incorporate the effect of the microbial growth on the operational variables, which considers nonstationary conditions, to finally incorperate the effect of the biogas produced. The recommended steps are:

Select the support material and its size
Select expansion of bed and aeration level
Calculate size of the bed (diameter and height) and peripherals
Evaluate the effects of the microbial growth
Incorporate the production of biogas

This has to be complemented with the determination of the residence time need for the removal of the organics of influent to an acceptable according to design conditions.

The use of fluidized bed technology for wastewater treatment has been studied at the laboratory and pilot plant level, but very few industrial plants have been built. Since many advantages, like high biomass concentration and surface area, good mixing of biomass and substrate, low pressure drop across the bed, high setting rate of carriers, inert sediment washes through reactor, no requirements for secondary clarifiers and small area requirement, have been claimed for fluidized bed reactor. Besides, the good setting characteristics of fluidizable particles allow a short standstill time for settling, allowing the suitable of fluidized bed reactor to operate as the sequencing batch reactor. This chapter has been carried out with the propose of verifying the applicability of a fluidized bed reactor working as a sequencing batch reactor to investigate the bacterial decolorization of reactive dyes. To achieve faster startup period of this reactor type, which is very complex process, the alginate co-immobilized *Paenibacillus* sp. strain S1 and A5 was used as the fluidizable bioparticles. The proposed of this study are also to investigate the effects of hydraulic retention time of anaerobic decolorization phase on the overall color and COD removal efficiencies.

8.2.3 Materials and Methods

8.2.3.1 Fluidized bed reactor set-up

The fluidized bed reactor was constructed form transparent acrylic column with a diameter of 6 cm and 60 cm height. A schematic of the reactor is shown in Figure 8.2. The working volume of the reactor was 1,300 ml. Air distributor was placed at the bottom of reactor to promote uniform fluidization of media and bioparticles. In an aerobic operational phase, the bed was expanded by aeration. Sterile air was supplied at the bottom of reactor by an air pump passed through the hydrophobic air filter. Air flow was regulated to achieve the desired aeration rate by rotameter. In an anaerobic operational phase, the effluent from the fluidized bed reactor was recycled through a variable speed peristaltic pump in order to provide upflow velocities for media and to maintain 10% biparticle expansion. Such upflow velocities also ensured that completely mixed conditions were maintained in the liquid phase and the active volume of the digester remained constant throughout the study. Recycle flow was drawn 3 cm below the free liquid surface in the enlarged section to avoid entrapment of gas accumulated in the headspace above, and pumped into the bottom assembly. Reactor temperature was maintained at 30 ^{0}C with external heating water jackets. Laboratory scale fluidized bed reactor was operated according to the sequencing batch reactor procedure. The system was periodically filled, operated as anaerobic-aerobic condition and drained in a proper time sequence, as described below. Operation of the peristaltic pump and air pump were manually controlled. A synthetic textile wastewater containing different reactive dyes was used to give reproducibility.

8.2.3.2 Start-up and steady-state operation

Approximately 20% (w/v) of alginate immobilized *Paenibacillus* sp. strain S1 and A5 were put into the surface sterilized-fluidized bed reactor. Initially, as startup fluidized bed reactor was continuously fed with synthetic textile wastewater without any reactive dyes with a HRT of 10 h. Air flow rate was controlled by a rotameter to achieve of 0.5 vvm. Every 6-h, samples were taken from reactor to determine the concentrations of COD and biomass. The reactor was operated with this operation mode until the steady state was achieved. The steady state was determined by either constant in COD reduction or stationary growth of alginate-immobilized biomass. From then onwards the reactor was fed with the synthetic textile wastewater containing individual reactive dyes and operated as a sequencing batch mode with a different experimental phases. The experimental operational conditions used in the decolorization studies are summarized in Table 8.4.

Table 8.4 Summary of the three SBR operational phases used for color removal studies with four reactive dyes

Cycle times	Phase I	Phase II	Phase III
Filled (min)	10	10	10
Anaerobic react (h)	24	18	12
Aerobic react (h)	10	10	10
Settle (min)	10	10	10
Draw (min)	10	10	10
Idle (min)	30	30	30
Other parameters			
Total one cycle range (h)	35	29	23
Day covered	3-10	11-17	18-23

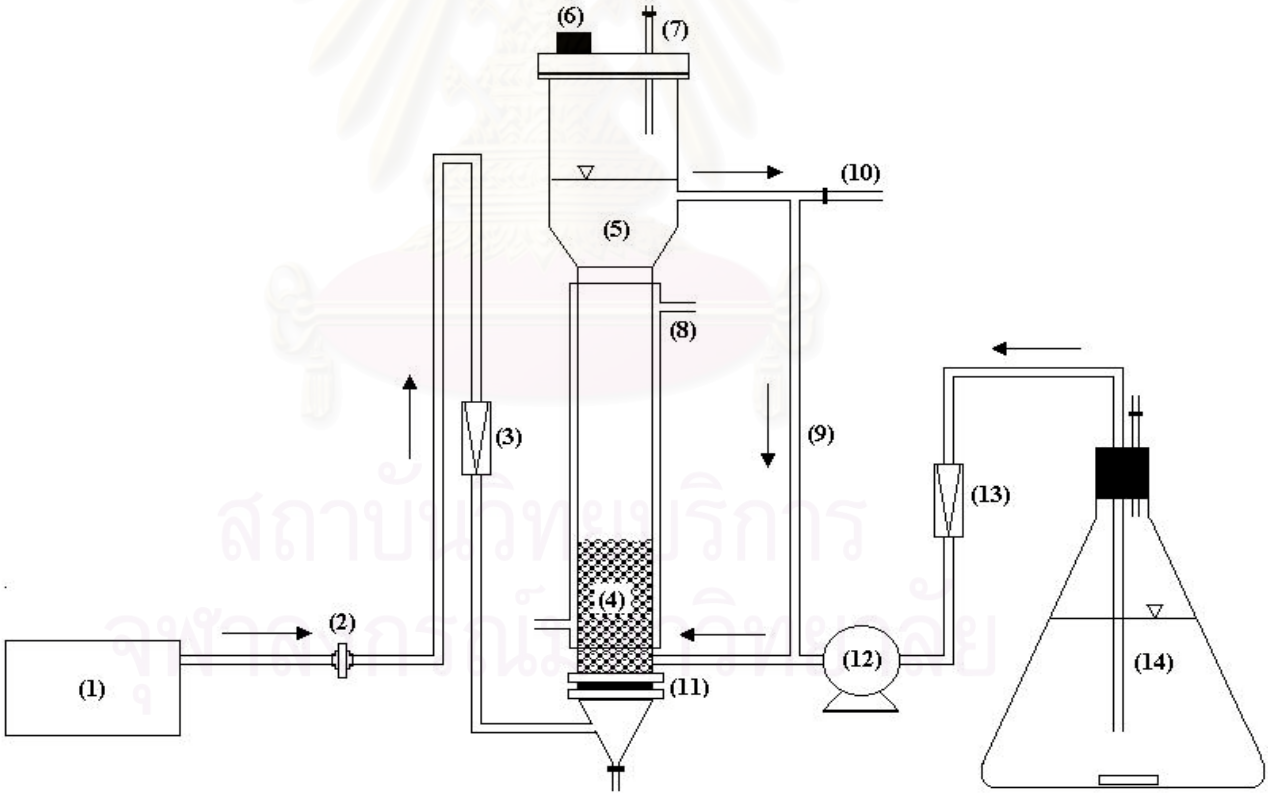


Figure 8.2. Schematic representation of the fluidized bed reactor (FBR). (1) Air pump, (2) air filter, (3) rotameter, (4) immobilized alginate gel beads, (5) fluidized bed reactor, (6) sampling port, (7) gas outlet, (8) water jacket, (9) recycle line, (10) effluent outlet, (11) air distributor, (12) peristaltic pump, (13) liquid flow meter, and (14) Feed flask

8.2.3.3 Determination of cell concentration

Viable cell concentration was determined by dilution plating. Several alginate beads were placed in tarred sterile tubes, and the actual gel weights were measured. The immobilized *Paenibacillus* sp. strain A5 and S1 were set free in a phosphate solution with 1.452% K_2HPO_4 and 0.226% KH_2PO_4. Successive tenfold dilutions of the resulting solutions were made with 50 mM Na/K phosphate buffer, pH 7.5, as diluent, and 100 µl aliquots of appropriate dilution were spread on LB agar plates. The viable cell concentration was calculated from the number of colonies appearing on the plates.

8.2.3.4 Morphological observations

Gel beads with entrapped *Paenibacillus* sp. strain A5 and S1 were put together with sodium alginate solution in a little glass test tube (ø 1 cm). The alginate was hardened for several hours in 2% $CaCl_2$-solution and the gel block with the embedded beads could be sliced with a very thin blade. The sectioned beads were determined under a light microscope.

8.2.3.5 Analytical techniques

Color, chemical oxygen demand (COD) and dye degradation and metabolite formation were measured according the analytical techniques in the Chapter 3. Dissolved oxygen and pH were determined according to standard procedures (American Public Health Association, 1995). Oxidation-Reduction Potential (ORP) was measured with an ORP electrode (platinum with silver/silver chloride as reference electrode, 3M KCl) connected to a digital pH meter (Radiometer, ION check 10), (pH 7.0 and 30 ^{0}C).

8.2.4 Results and Discussions

8.2.4.1 Distribution of biomass in alginate beads

Observations of sliced gel beads revealed following distributions of *Paenibacillus* sp. cells in alginate (Figure 8.3A and 8.3B):

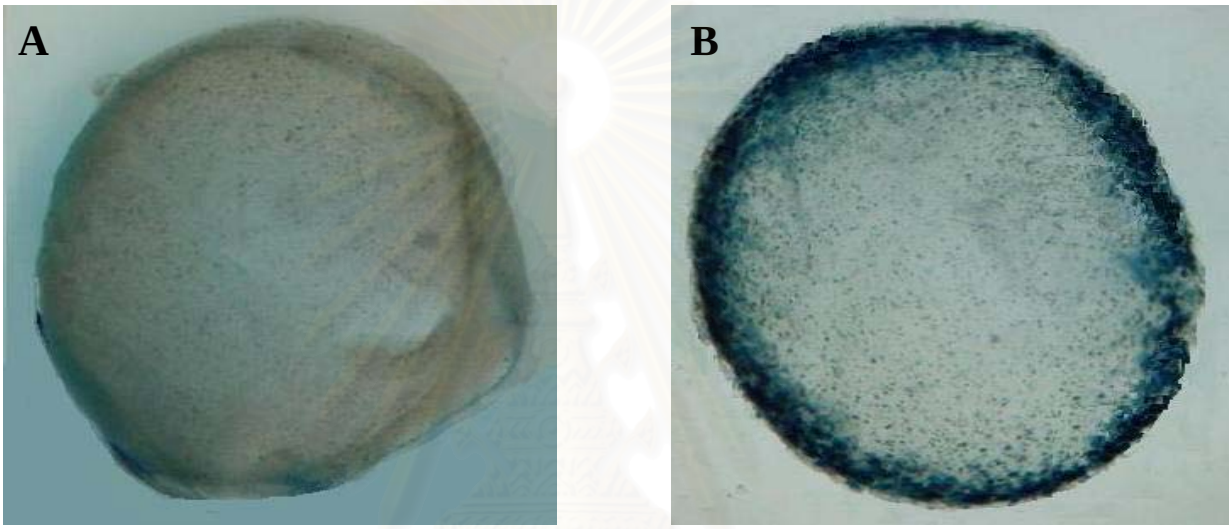


Figure 8.3. Morphological development of a defined mixed culture of *Paenibacillus* sp. strain A5 and S1 (initial ratio 1:1) in a calcium alginate bead with a diameter of 3 mm on synthetic wastewater effluent (A) after 0 h of incubation time (B) after 72 h of incubation time. Magnification x 40

Immediately after immobilization the cells were statistically distributed in the gel. After an incubation period growth in synthetic textile wastewater, gel beads showed a dense layer of cells in the surface area. This suggests the assumption that at high bacteria concentrations in alginate beads only the outer cells got sufficient oxygen for propagation, while the diffusion to the center of the beads was almost prevented. This growth behavior is already described in literature (Gosmann and Rehm, 1988; Beunink and Rehm, 1990; Kurosawa et al., 1989).

8.2.4.2 Fluidized bed reactor start-up

At start-up all the fluidized bed reactors for individual reactive dyes were continuously fed with synthetic textile wastewater without reactive dyes by setting HRT at 10 h under aerobic condition. Every alternate 6-h, the supernatant was taken to determine the concentration of COD and the alginate bead were withdrawn to measure the concentration of bacterial biomass. The reactors were operated with this operation mode until reduction in COD of up to 90% was achieved on 3 consecutive sampling time (6 h) in all reactors or the stationary growth phase of bacteria was reached. This was done to ensure that uniform biological activity was occurring in all reactors. The results of start-up period of all reactors for treatment of Remazol Brilliant Orange 3R, Remazol Black B, Remazol Brilliant Violet 5R and Remazol Brilliant Blue R are shown in Figure 8.4, 8.8, 8.12 and 8.16, respectively. The results show that the steady-state of all reactors is achieved after 72 h of start-up phase. From then onwards each reactor was changed to the sequencing batch operational conditions as shown in Table 8.4.

8.2.4.3 Performance of overall anaerobic-aerobic sequential system

8.2.4.3.1 Remazol Brilliant Orange 3R

The first biodecolorization studies with the orange dye were performed in a sequencing batchwise fluidized bed reactor with three imposed values of anaerobic retention time (operational phase 1, 2 and 3 of Table 8.4) and an aerated reaction phase of 10 h. Average results obtained after 20-day operation period are presented in Table 8.5.

During all operational phases (Table 8.5) it was observed that almost color removal performed under the anaerobic phase meanwhile, COD was mainly removed under the subsequent aerobic phase. In the present case, decolorization efficiency is apparently related to the capacity to lower the dissolved oxygen concentration in medium in the anaerobic phase, through generation of reducing equivalents and their transfer to adequate electron carriers which then reduce the azo bond present in Remazol Brilliant Orange 3R molecules. HPLC analyses seem to indicate that the intermediates produced during the anaerobic phase of reaction were decreased in the subsequent aerobic phase (Table 8.5).

The effluent residual color and COD values obtained during these operational phases are presented in Figure 8.4. It had not any interfering-effects of the longer retention times of the anaerobic phase form 12 to 18 and 24 h on the COD removal ability of immobilized bacteria in subsequent aerobic phase. The bacterial biomass (CFU/g of alginate bead) concentration in the steady state of sequencing batch reactor were approximately 2.4×10^9 CFU/g alginate bead.

Table 8.5 Average sequencing batch reactor performance parameter values for Remazol Brilliant Orange 3R decolorization with different anaerobic retention times of 12, 18 and 24 h, corresponding to operational phase I, II and III (Table 8.4), respectively.

Parameters	Sampling	Phase I	Phase II	Phase III
Color (mg/l)	Influent	101.5	101.2	100.6
	Anaerobic	7.7	7.8	7.6
	Aerobic	6.0	6.7	6.4
	Total removal (%)	96.9	95.6	94.8
COD (mg/l)	Influent	1778	1760	1789
	Anaerobic	1468	1540	1583
	Aerobic	137	204	234
	Total removal (%)	92.3	88.4	86.9
HPLC (area)	Influent	0	0	0
	Anaerobic	4968	4832	4775
	Aerobic	2157	2253	2242
DO (mg/l)	Anaerobic	0.42	0.5	0.48
	Aerobic	5.13	5.22	5.17

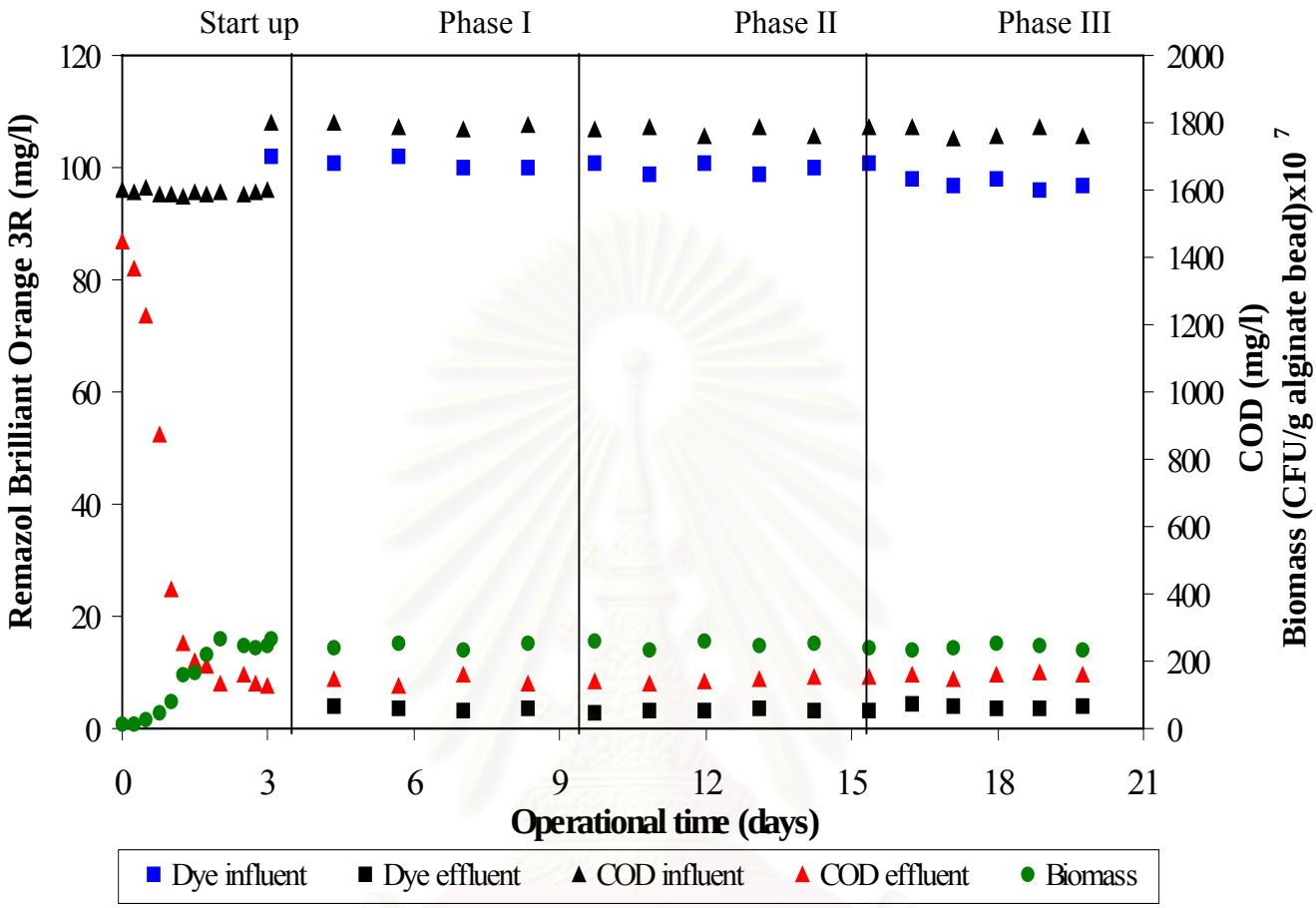


Figure 8.4 Performance of the reactor treating Remazol Brilliant Orange 3R

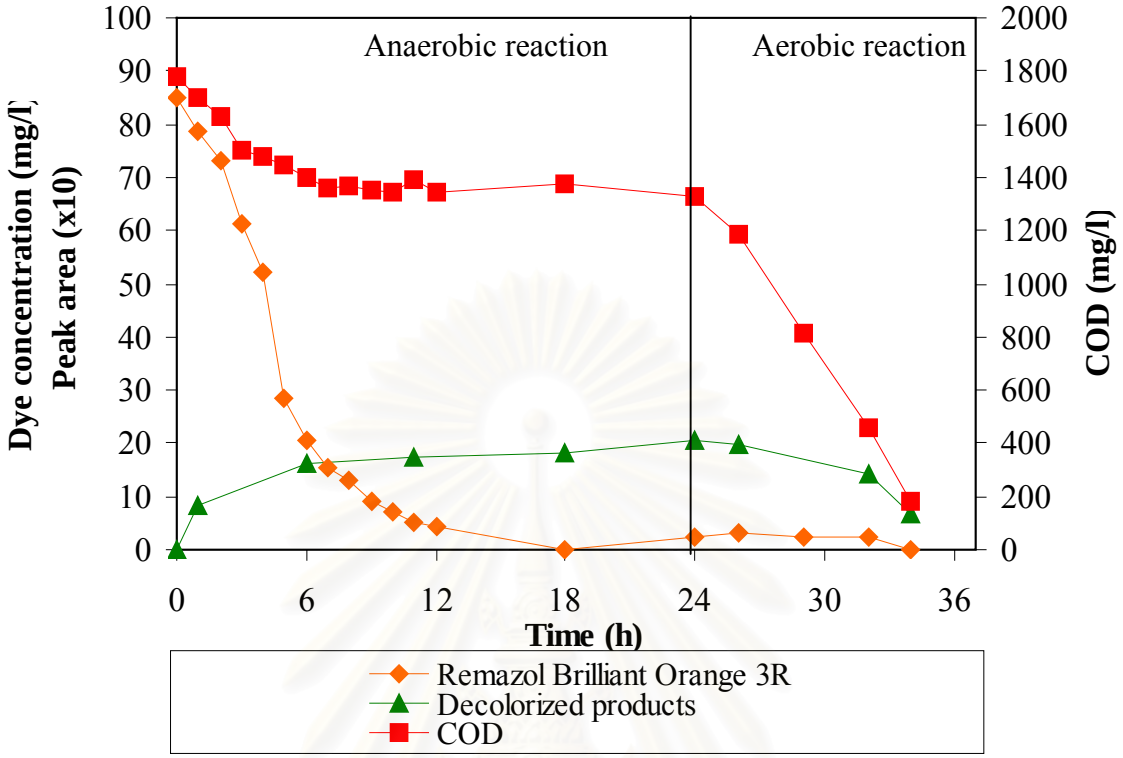


Figure 8.5. Profile of Remazol Brilliant Orange 3R, COD and decolorized products in a cycle at day 7

As shown in Figure 8.5, during the anaerobic phase, Remazol Brilliant Orange 3R concentration decreased from 84 to 2.3 mg/l within 12 h. During the following aerobic phase the concentration of COD sharply decreased form 1350 to 180 mg/l within 12 h. Moreover, the major decolorized products (R_t = 5.5 min) seemed to decrease in the subsequent aerobic phase. The characterization of the fluidized bed reactor before and after treatment of synthetic textile wastewater containing Remazol Brilliant Orange 3R by anaerobic-aerobic treatment system are shown in Figure 8.6A and 8.6B. The characteristics of synthetic textile wastewater obtained at different operational stages of the sequencing batchwise fluidized bed reactor are also shown in Figure 8.7.

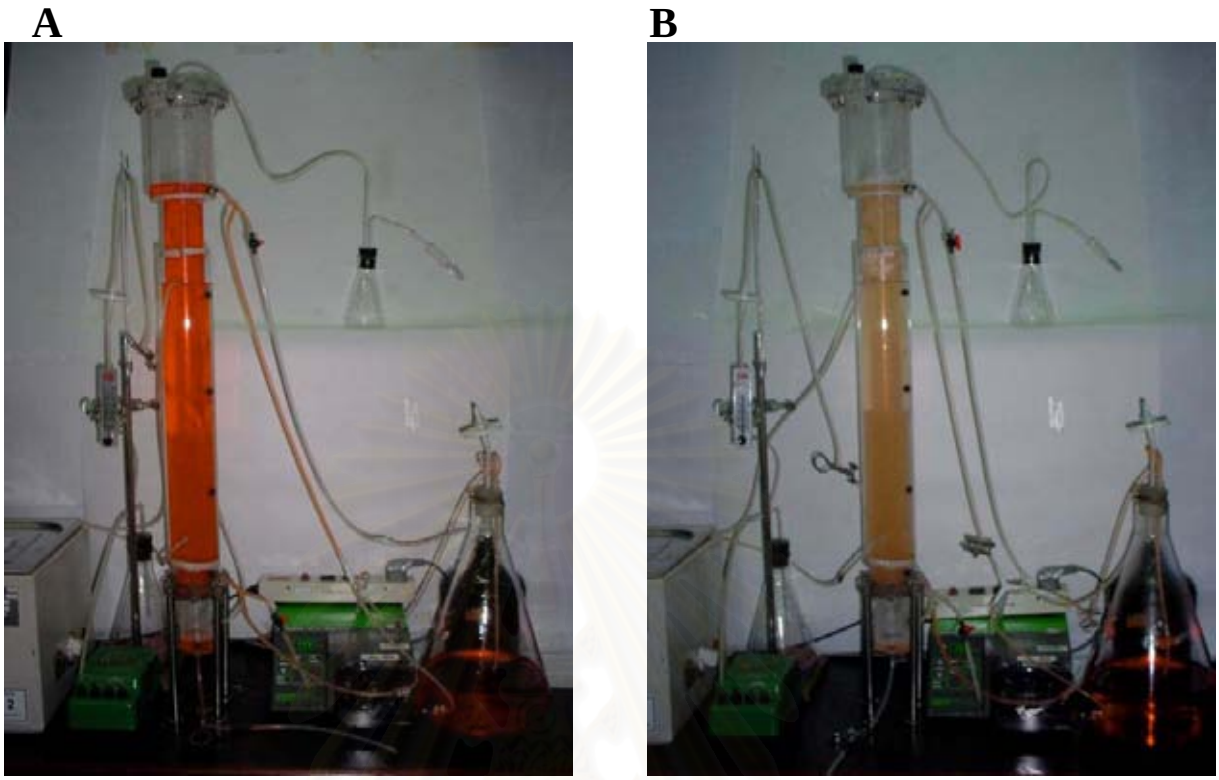


Figure 8.6. Fluidized bed reactor containing synthetic textile wastewater and Remazol Brilliant Orange 3R (A) before treatment with anaerobic-aerobic system, and (B) after treatment with anaerobic-aerobic system

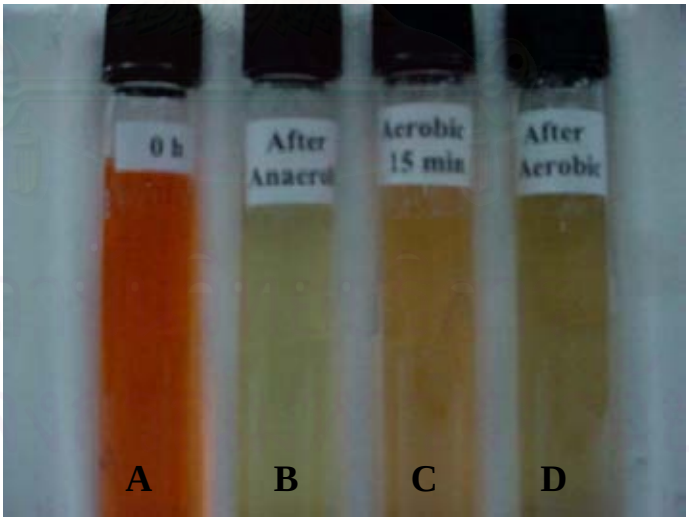


Figure 8.7 Characteristics of synthetic textile wastewater containing Remazol Brilliant Orange 3R at (A) the beginning of anaerobic phase, (B) after anaerobic phase (C) 15 min after aerobic phase and (D) after aerobic phase

8.2.4.3.2 Remazol Black B

The results of this work show that the Remazol Black B can ultimate mineralize and decolorize with very high COD and color removal efficiencies in a sequencing batchwise fluidized bed reactor system. The COD, color removal efficiencies, and other operation parameters in every operational phase are summarized in Table 8.6.

Table 8.6 Average sequencing batch reactor performance parameter values for Remazol Black B decolorization with different anaerobic retention times of 12, 18 and 24 h, corresponding to operational phase I, II and III (Table 8.4), respectively

Parameters	Sampling	Phase I	Phase II	Phase III
Color (mg/l)	Influent	99.7	100.8	98.9
	Anaerobic	11.1	18.6	17.6
	Aerobic	9.6	15.4	14.9
	Total removal (%)	90.4	86.0	83.1
COD (mg/l)	Influent	1752	1780	1720
	Anaerobic	1517	1557	1522
	Aerobic	180	206	223
	Total removal (%)	89.7	88.4	86.9
HPLC (area)	Influent	0	0	0
	Anaerobic	3685	3498	3545
	Aerobic	1651	1750	1716
DO (mg/l)	Anaerobic	0.38	0.58	0.67
	Aerobic	5.34	5.41	5.52

The findings of this study show that the decolorization of the black was significantly affected by anaerobic retention time. Decolorization efficiency increased with increasing anaerobic retention time. A total of 83.1–90.4% color removal efficiencies were obtained for Remazol Black B at retention time varying between 12 and 24 h in the anaerobic phases (Table 8.6). The Remazol Black B removal efficiencies obtained in this study are comparable higher than those obtained by Luangdilok and Panswad (2000), Nigam et al. (1996), Ganesh et al. (1994) and Beydilli et al. (1998). Nigam et al. (1996), found 67% color removal for 50 mg/l of Reactive Black B dye within 24 h in a batch reactor containing yeast extract as the carbon source.

A 68% color removal efficiency was obtained after 48 h of incubation period when glucose is used as carbon source (Beydilli et al., 1998). A 79% color removal efficiency was observed after 10 h of incubation at a Reactive Black B concentration of 200 mg/l (Beydilli et al., 1998). A 63% color removal efficiency was achieved in a sequential anaerobic/aerobic SBR reactor within 2 h (Luangdilok and Panswad (2000). Ganesh et al. (1994) also reported 67% decolorization efficiency in an anaerobic UASB reactor containing 50 mg/l Reactive Black B dye at a organic loading rate of 14 $kg/m^3/day$.

The performance of the reactor treating the Remazol Black B is shown in Figure 8.8. During the initial steady stage, a concentration of immobilized biomass is reached constant values of 1.3 x 10^9 CFU/g of alginate bead and increased only slightly for the longer operation time (Figure 8.8).

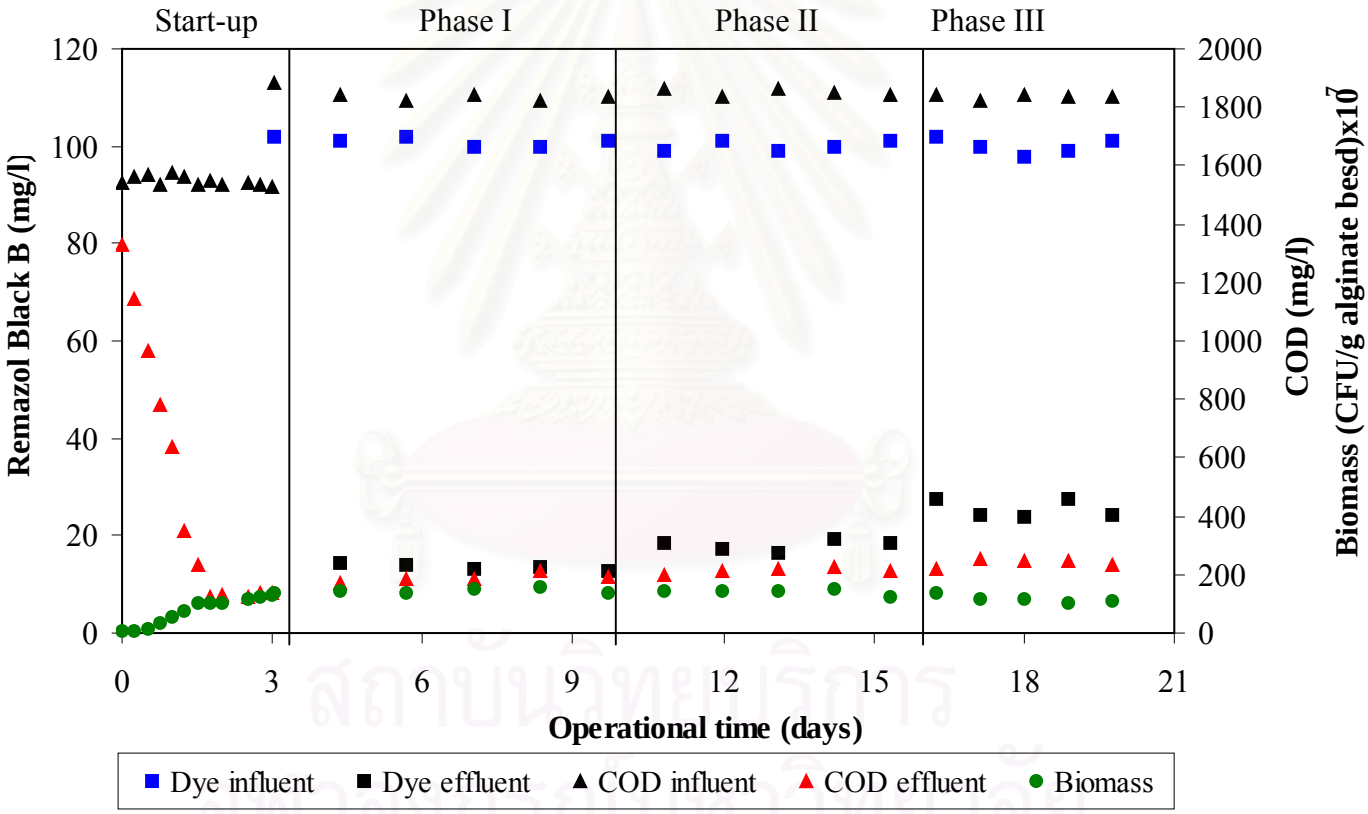


Figure 8.8 Performance of the reactor treating the Remazol Black B

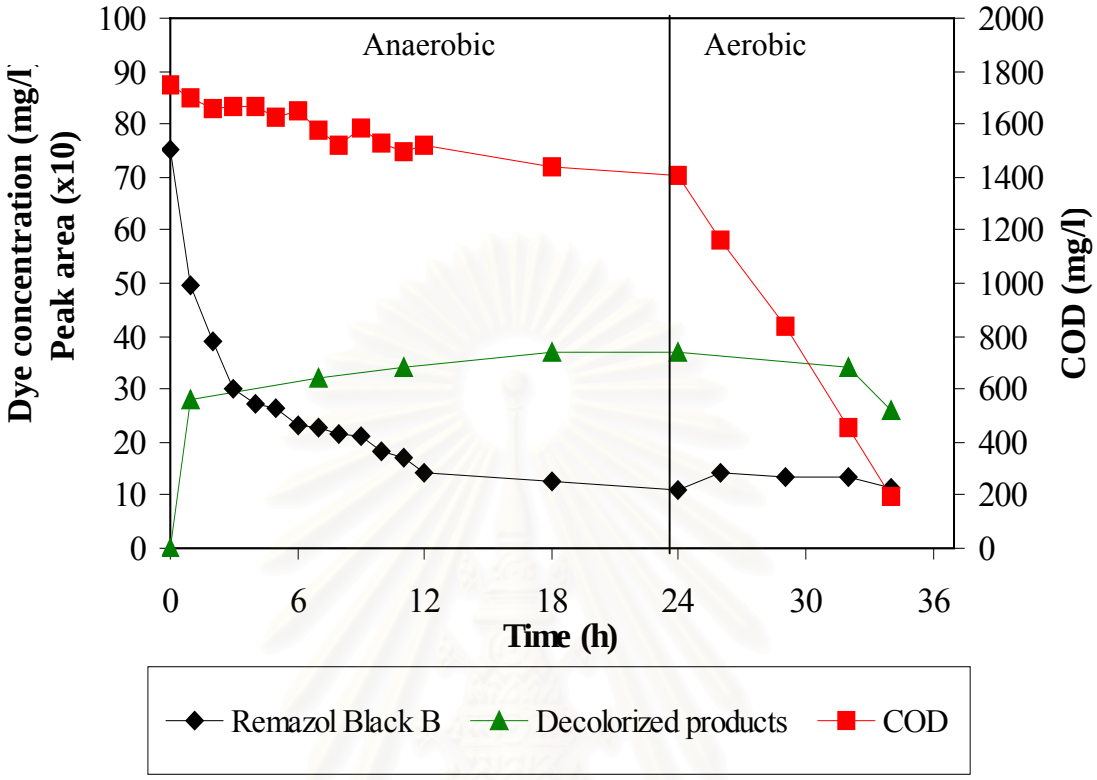


Figure 8.9 Profile of Remazol Black B, COD and decolorized products in an operational cycle at day 7

As shown in Figure 8.9, during the anaerobic phase, Remazol Black B concentration decreased from 76 to 15.6 mg/l within 12 h. The black dye was continuously decolorized with a very slightly decolorization rate until the end of anaerobic phase (24 h). Similar to the decolorization experiment of Remazol Brilliant Orange 3R, the concentration of COD sharply decreased form 1420 to 194 mg/l within 12 h of the following aerobic phase. Moreover, the major decolorized products (R_t = 5.5 min) seemed to decrease in the subsequent aerobic phase.

The characterization of the fluidized bed reactor before and after treatment of synthetic textile wastewater containing Remazol Black B by anaerobic-aerobic treatment system are shown in Figure 8.10A and 8.10B. The characteristics of synthetic textile wastewater obtained at different operational stages of the sequencing batchwise fluidized bed reactor are also shown in Figure 8.11.

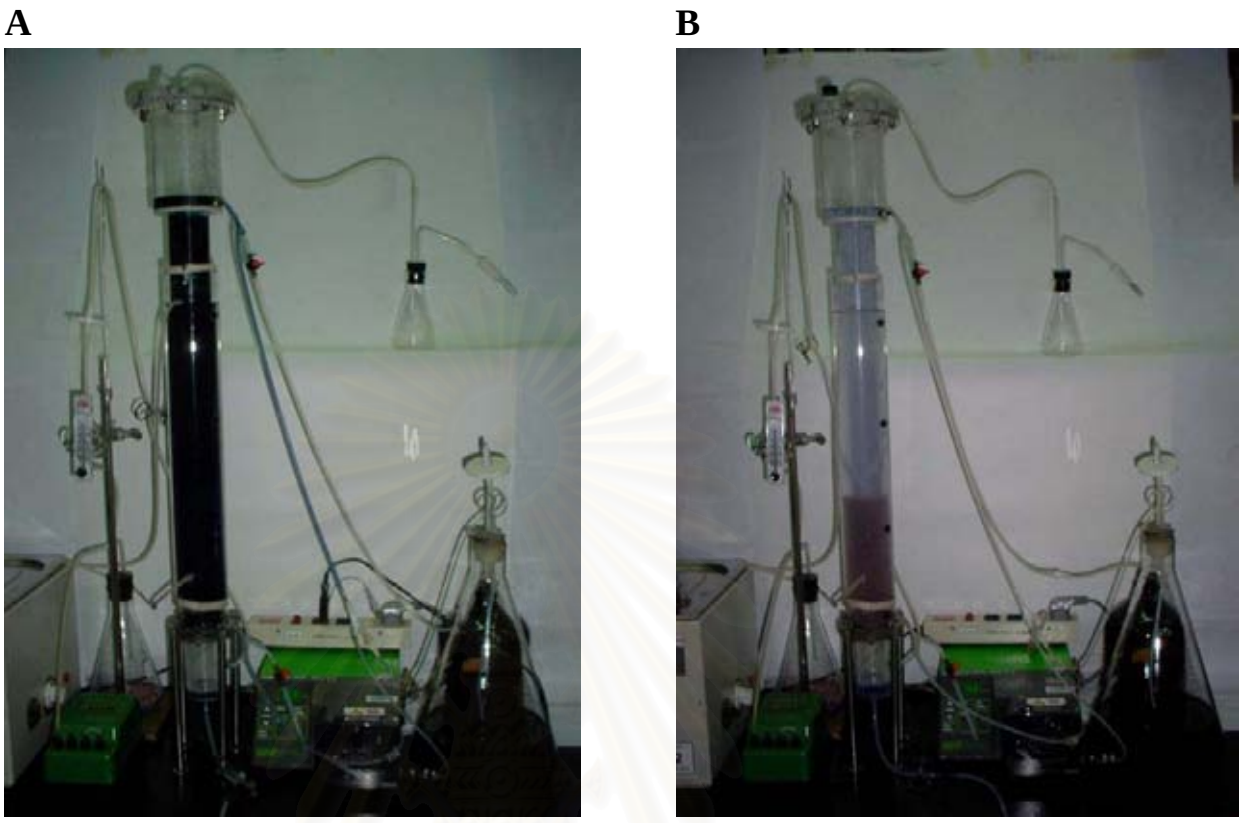
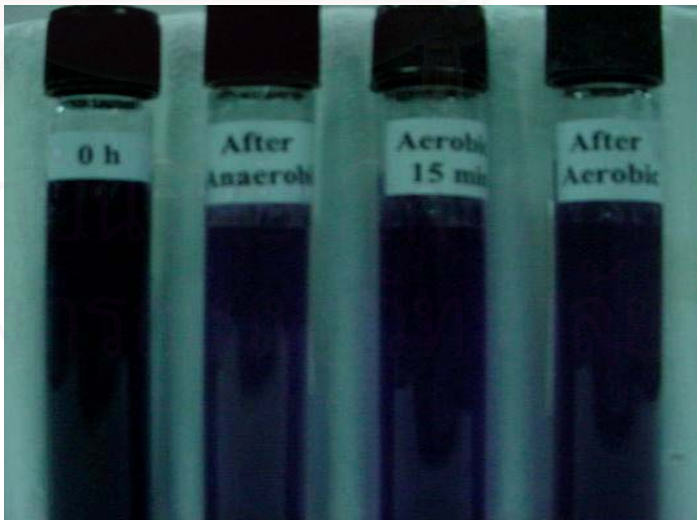


Figure 8.10. Fluidized bed reactor containing synthetic textile wastewater and Remazol Black B (A) before treatment with anaerobic-aerobic system, and (B) after treatment with anaerobic-aerobic system



A B C

Figure 8.11 Characteristics of synthetic textile wastewater containing Remazol Black B at (A) the beginning of anaerobic phase, (B) after anaerobic phase (C) 15 min after aerobic phase and (D) after aerobic phase.

8.2.4.3.3 Remazol Brilliant Violet 5R

Average results obtained after 20-day operation period are presented in Table 8.7. The contributions of anaerobic and aerobic phase to the total decolorization of Remazol Brilliant Violet 5R in the system are 64-93% and 3.5-4.7%, and to COD removal, 15-18% and 69-75%, respectively. These results indicate that color removal of the Remazol Brilliant Violet 5R occurs under anaerobic conditions and COD removal occurs under aerobic conditions in anaerobic-aerobic sequential treatment of textile wastewater.

Table 8.7 Average sequencing batch reactor performance parameter values for Remazol Brilliant Violet 5R decolorization with different anaerobic retention times of 12, 18 and 24 h, corresponding to operational phase I, II and III (Table 8.4), respectively

Parameters	Sampling	Phase I	Phase II	Phase III
Color (mg/l)	Influent	99.5	100.4	99.8
	Anaerobic	7.1	19.5	35.6
	Aerobic	3.6	14.8	32.8
	Total removal (%)	96.4	85.3	67.1
COD (mg/l)	Influent	1680	1622	1635
	Anaerobic	1366	1339	1378
	Aerobic	129	208	224
	Total removal (%)	92.3	87.1	86.3
HPLC (area)	Influent	0	0	0
	Anaerobic	2260	2055	1886
	Aerobic	771	745	718
DO (mg/l)	Anaerobic	0.38	0.54	0.47
	Aerobic	5.24	5.27	5.34

The performance of the fluidized bed reactor on the decolorization of Remazol Brilliant Violet 5R is shown in the Figure 8.12. The COD removal efficiency in the aerobic phase of fluidized bed reactor did not show significant change with varying of anaerobic retention time (Figure 8.12). Decolorization efficiency was over 90% for anaerobic retention time of 24 h (corresponding to phase I). However, efficiency decreased with decreasing in an anaerobic retention time to 18 and 12 h which 80 and 64% color removal efficiency was obtained, respectively. This result indicates that the decolorization of Remazol Brilliant Violet 5R need longer anaerobic retention time than the decolorization of Remazol Brilliant Orange 3R and Remazol Black B did.

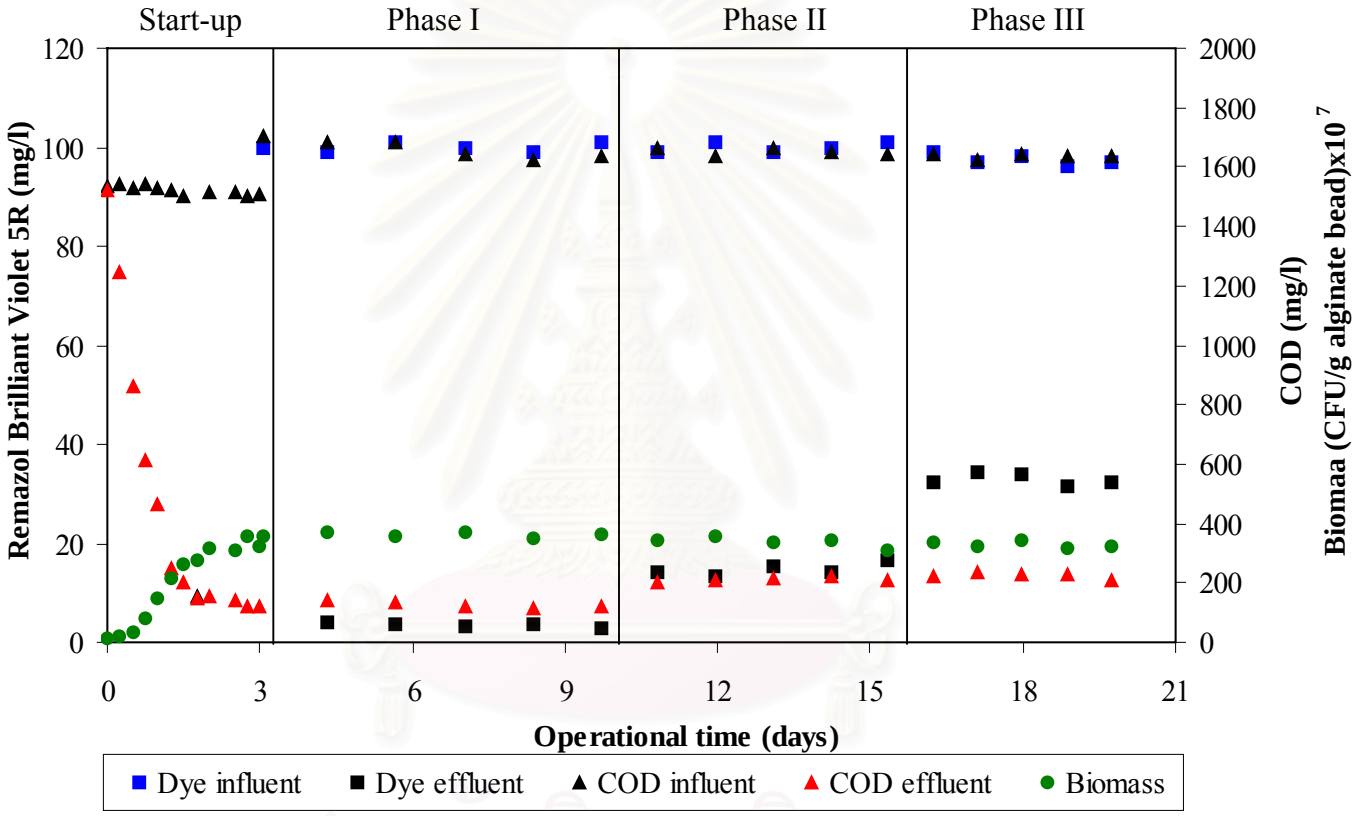


Figure 8.12 Performance of reactor treating Remazol Brilliant Violet 5R

The decolorization efficiency of Remazol Brilliant Violet 5R in this study is comparable to the decolorization of the same dye which reported by Lourenco and co-workers (2001). Lourenco et al. (2001) used sequencing batch reactor to decolorize Remazol Brilliant Violet 5R and found that 90% color removal was obtained in a 24-h cycle with a Sludge Retention Time (SRT) of 15 days and an aerated reaction phase of 10 h.

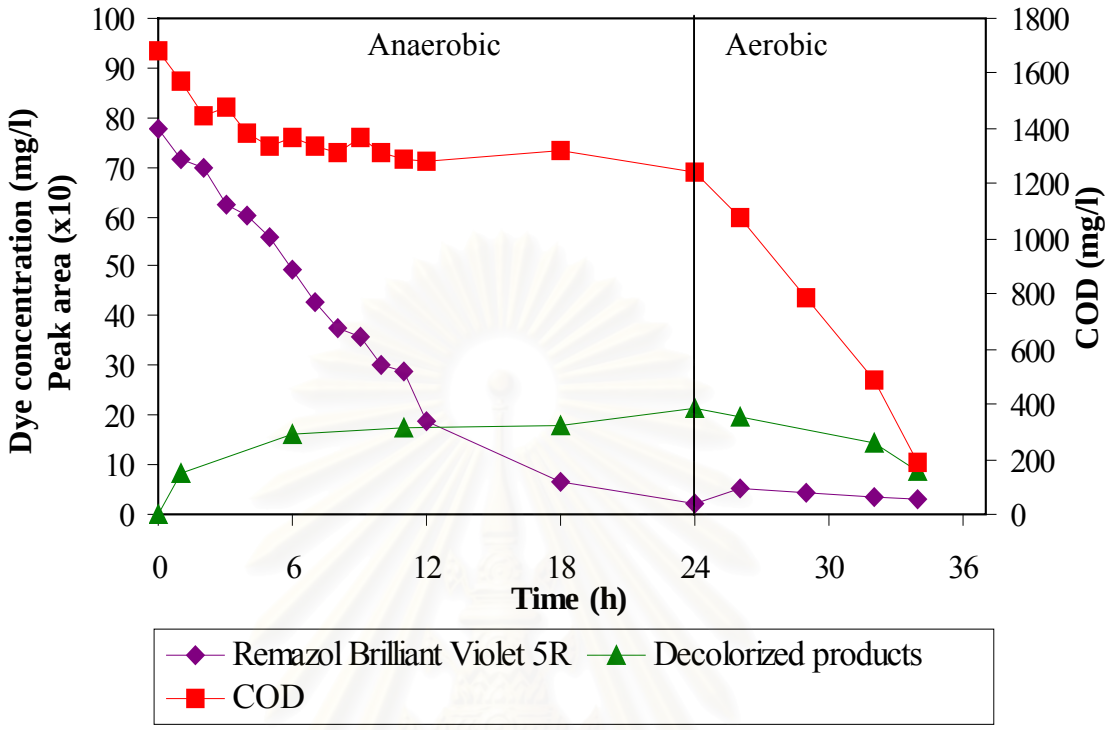


Figure 8.13 Profile of Remazol Brilliant Violet 5R, COD and decolorized products in an operational cycle at day 8

Typical examples of sequencing batch performance in terms of color, COD and decolorizing-products removal during 24-h cycles of the anaerobic reaction phase and 10-h of aerobic reaction phase is presented in Figure 8.13. Remazol Brilliant Violet 5R was nearly complete decolorized within 24 h of anaerobic phase. The COD was dramatically decreased from 1248 to 176 mg/l within 10 h of the subsequent aerobic phase. For the treatment of the copper-containing azo dye Remazol Brilliant Violet 5R in an anaerobic-aerobic system with three rotating-disc reactors, no indications for a mineralization of the decolorized products (aromatic amines) were detected by Sosath and Libra (1997). In contrast, the analysis of the fate of decolorized products of the violet dye in this study found that the major decolorized product is seemed to be removed within the subsequent aerobic phase (Figure 8.13).

The characterization of the fluidized bed reactor before and after treatment of synthetic textile wastewater containing Remazol Brilliant Violet 5R by anaerobic-aerobic treatment system are shown in Figure 8.14A and 8.14B. The characteristics of synthetic textile wastewater obtained at different operational stages of the sequencing batchwise fluidized bed reactor are also shown in Figure 8.15.

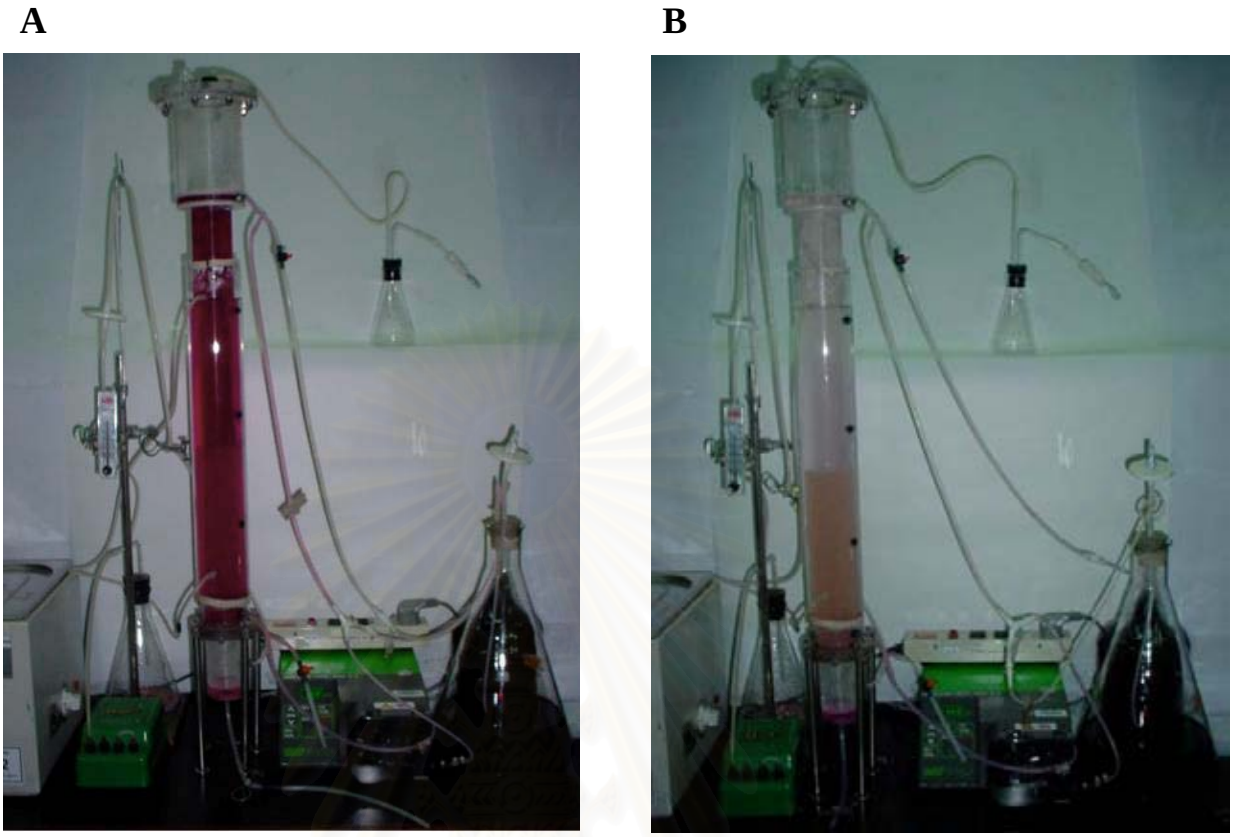


Figure 8.14. Fluidized bed reactor containing synthetic textile wastewater and Remazol Brilliant Violet 5R (A) before treatment with anaerobic-aerobic system, and (B) after treatment with anaerobic-aerobic system

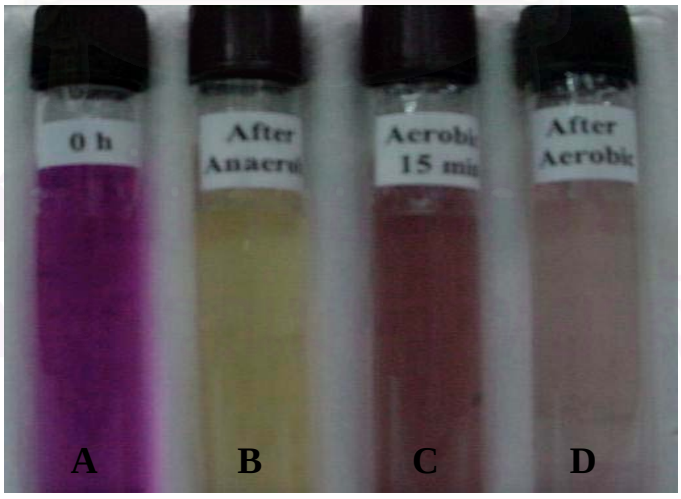


Figure 8.15 Characteristics of synthetic textile wastewater containing Remazol Brilliant Violet 5R at (A) the beginning of anaerobic phase, (B) after anaerobic phase (C) 15 min after aerobic phase and (D) after aerobic phase

8.2.4.3.4 Remazol Brilliant Blue R.

The results of this study show that the Remazol Brilliant Blue R can ultimate decolorize with rather high COD and color removal efficiencies in a sequencing batchwise fluidized bed reactor system. The COD, color removal efficiencies, and other operation parameters in an anaerobic and aerobic operational phase are summarized in Table 8.8.

Table 8.8 Average sequencing batch reactor performance parameter values for Remazol Brilliant Blue R decolorization with different anaerobic retention times of 12, 18 and 24 h, corresponding to operational phase I, II and III (Table 8.4), respectively.

Parameters	Sampling	Phase I	Phase II	Phase III
Color (mg/l)	Influent	100.4	100.2	100.6
	Anaerobic	42.9	65.0	68.4
	Aerobic	21.5	34.8	44.2
	Total removal (%)	78.6	65.2	58.0
COD (mg/l)	Influent	1810	1854	1794
	Anaerobic	1368	1452	1437
	Aerobic	102.8	114	118
	Total removal (%)	94.3	93.9	93.4
HPLC (area)	Influent	-	-	-
	Anaerobic	-	-	-
	Aerobic	-	-	-
DO (mg/l)	Anaerobic	0.28	0.34	0.48
	Aerobic	5.34	5.28	5.54

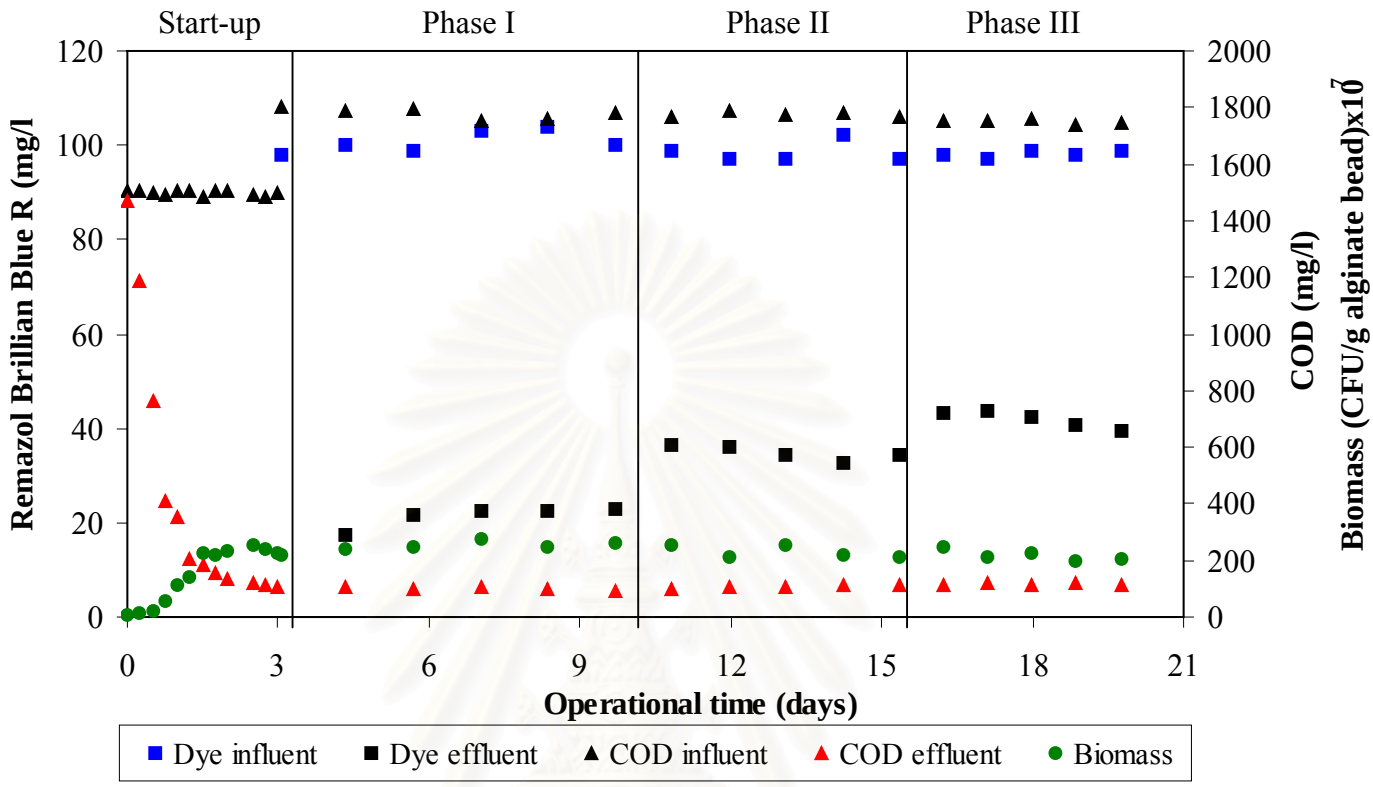


Figure 8.16 Performance of reactor treating Remazol Brilliant Blue R

Figure. 8.16 shows the behaviour of the system operated at different anaerobic retention times, in terms of influent and effluent concentration values of color, COD and immobilized biomass. Within the range of anaerobic retention time of 24 h (corresponding to phase I), the system was rather stable and the color concentration in the effluent was always lower than 20 mg/l. For anaerobic retention time of 18 h (phase II), the concentration of color slightly fluctuate over and although the system recovered its stability after 2 cycles, residual color (34 mg/l) was found in the effluent. For lower anaerobic retention time (12h), the reactor became unstable and the concentration of Remazol Brilliant Blue R in the effluent increased progressively to 43 mg/l.

In contrast to the decolorization trend, the COD removal efficiency did not affected by the varying of anaerobic retention time. The average overall COD removal efficiencies of operational phase I, II and III were 94.3, 93.9 and 93.4%, respectively (Table 8.8).

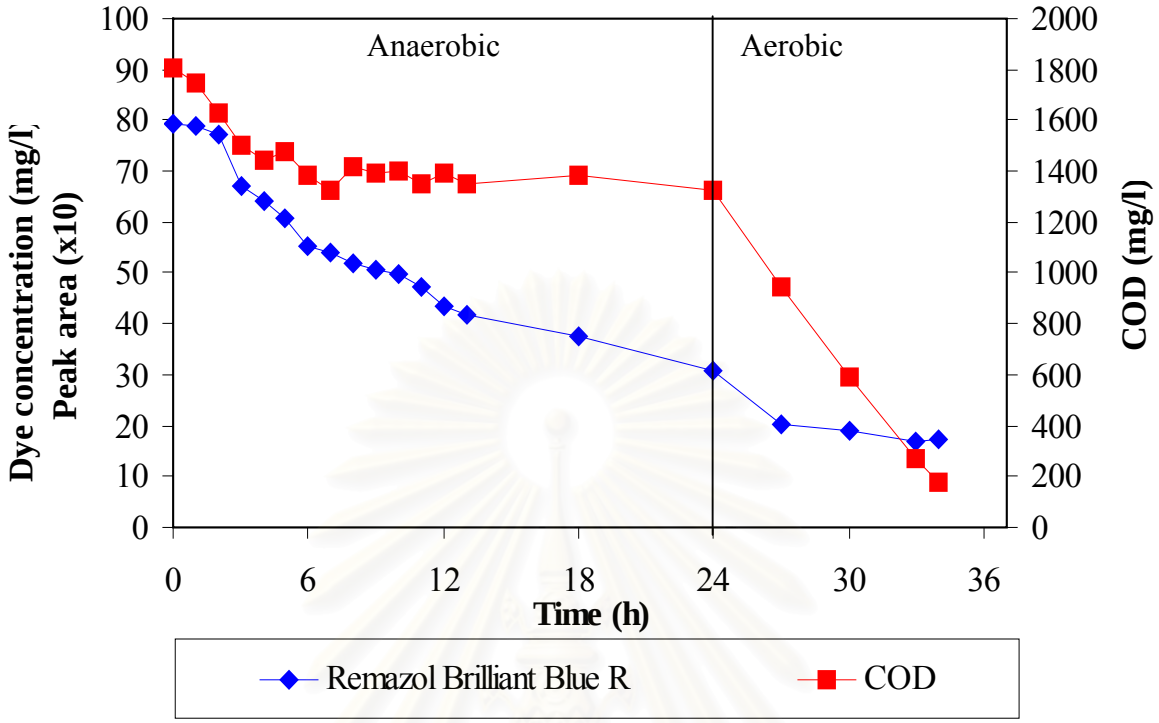


Figure 8.17 Profile of Remazol Brilliant Blue R, COD and decolorized products in an operational cycle at day 7

As shown in Figure 8.17, during the anaerobic phase, Remazol Brilliant Blue R concentration decreased from 80 to 29.5 mg/l within 24 h. During the following aerobic phase the concentration of COD sharply decreased form 1378 to 176 mg/l within 10 h. In contrast to the azo dyes, no decolorized products were found during anaerobic decolorization of the anthraquinone Remazol Brilliant Blue R. This phenomenon can be explained by the decolorization of this anthraquinone dye is mainly due to the biosorption rather than the anaerobic reduction of the dye. See chapter 7 for the details of decolorization mechanisms of anthraquinone and azo dyes.

The characterization of the fluidized bed reactor before and after treatment of synthetic textile wastewater containing Remazol Brilliant Blue R by anaerobic-aerobic treatment system are shown in Figure 8.18A and 8.18B. The characteristics of synthetic textile wastewater obtained at different operational stages of the sequencing batchwise fluidized bed reactor are also shown in Figure 8.19.

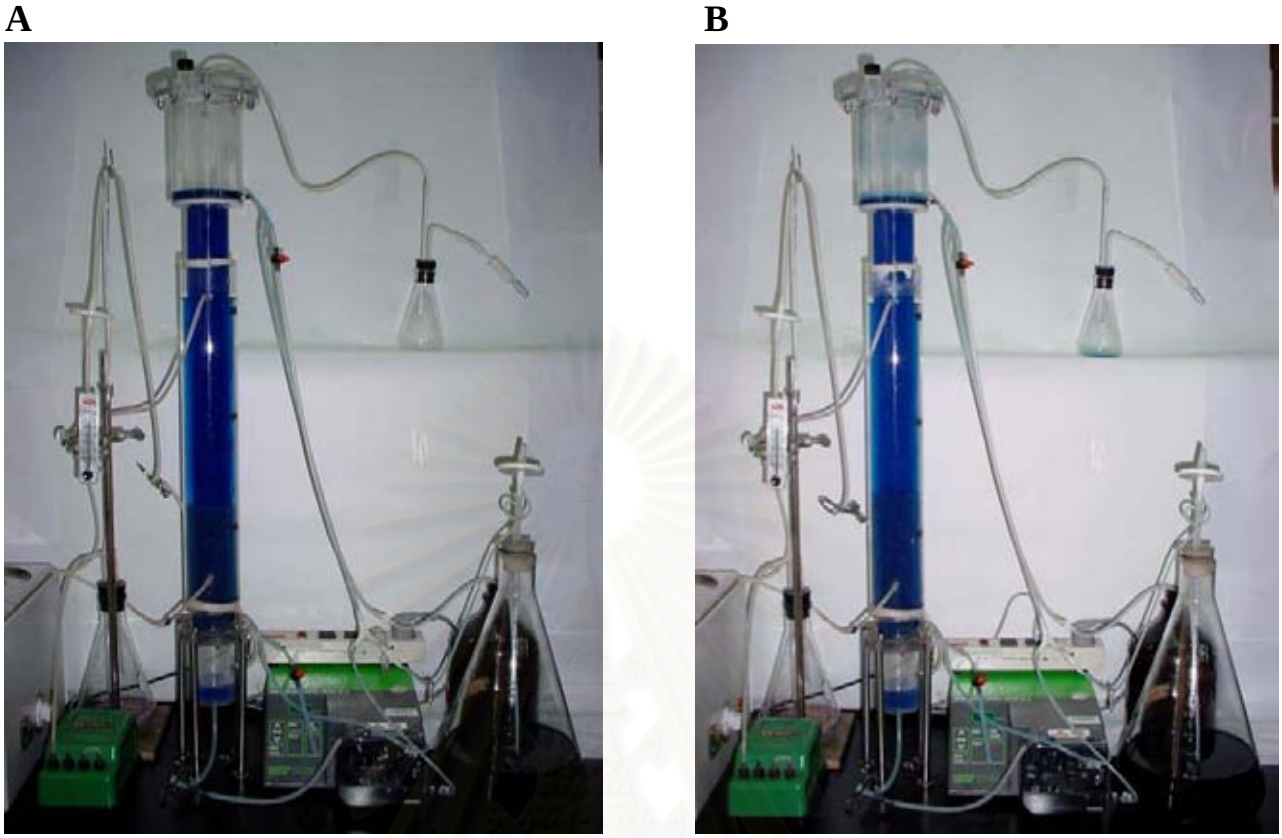
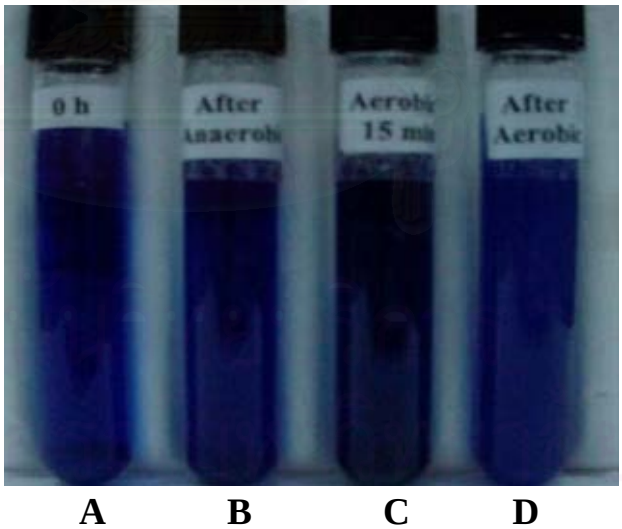


Figure 8.18. Fluidized bed reactor containing synthetic textile wastewater and Remazol Brilliant Blue R (A) before treatment with anaerobic-aerobic system, and



(B) after treatment with anaerobic-aerobic system.

Figure 8.19 Characteristics of synthetic textile wastewater containing Remazol BrilliantBlue R at (A) the beginning of anaerobic phase, (B) after anaerobic phase (C) 15 min after aerobic phase and (D) after aerobic phase

8.2.5 Conclusions

Lab-scale sequencing batch reactor systems were used in the present study with sequenced anaerobic and aerobic phases for color biodecolorization in a simulated textile effluent containing sulfonated azo reactive dyes and anthraquinone dye. The studied of sequencing batchwise fluidized bed reactor system proved to be potentially efficient for color removal from azo dye containing feeds when operated in steady-state conditions in what concerns the immobilized bacterial biomass concentration and relative duration of anaerobic reaction phases.

Azo dyes, Remazol Brilliant Orange 3R, Remazol Black B and Remazol Brilliant Violet 5R were gratuitously decolorized under experimental conditions employed. The color removal yield with the more complex diazo dye Remazol Black B and metal complex Remazol Brilliant Violet 5R were, however, much lower than that obtained with the monoazo Remazol Brilliant Violet 5R, even at higher anaerobic retention time values. For the diazo black dye and metal complex monoazo violet dye it was observed that the increase of the duration of the anaerobic phase could promote the biodecolorization .

Maximum overall color removal of all reactive dyes was achieved at highest anaerobic retention time (24 h) and most azo dyes removal occurred anaerobically. In the other hand, the majority of COD and decolorized products occurred in the subsequent aerobic phase. The summary of color and COD removal efficiencies under anaerobic and aerobic phase of the different operational phases are shown in Figure 8.20 and 8.21, respectively.

The aromatic amine metabolites produced by azo dye reduction in the anaerobic phase were seemed to remove under subsequent aerobic phase.

The depletion of oxygen is easily accomplished in anaerobic phase and enables the immobilized *Paenibacillus* sp. strain A5 and S1 to reduce reactive azo dyes. The establishment of low dissolved oxygen values (under 0.5 mg/l) in the anaerobic phase seems to be required for high decolorization efficiency for all azo dyes.

In the case of the anthraquinone dye Remazol Brilliant Blue R, COD removal did not affected by the varying of anaerobic retention time meanwhile, the decolorization of this dye was strongly depended on the retention time of overall anaerobic-aerobic treatment system.

The calcium alginate matrix adequately encapsulated the *Paenibacillus* sp. cells to the immobilization material and fugitive cells were not detectable for several days.

Paenibacillus sp. strain A5 and S1 grew densely at the surface area of gel beads because the mass-transfer limitation of oxygen, substrate and products presented in the gel matrix.

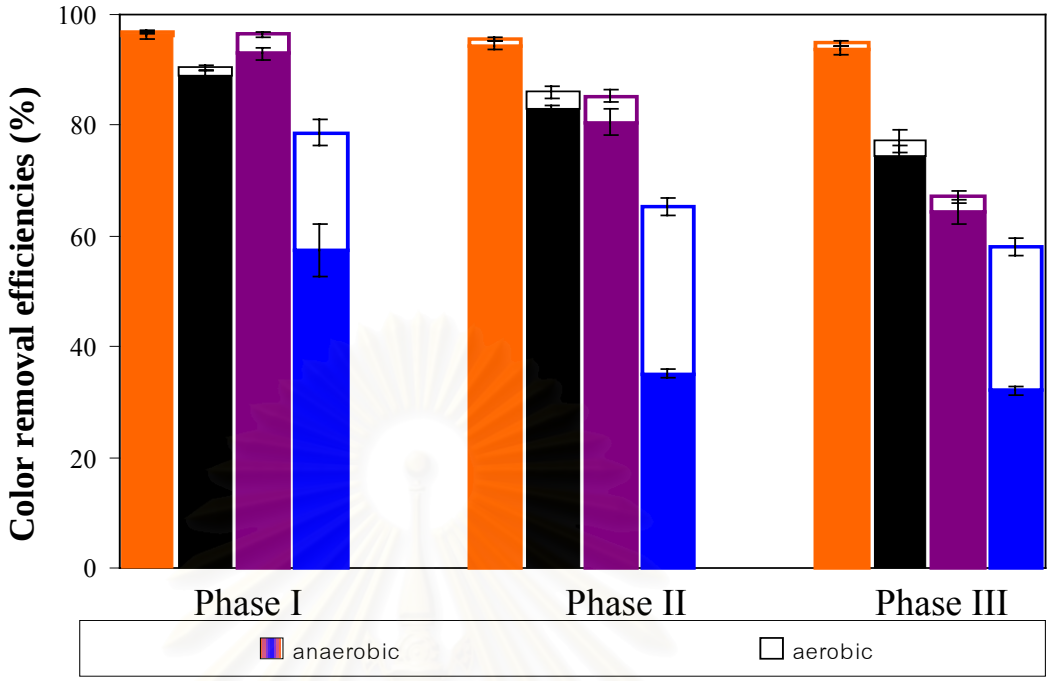


Figure 8.20. Summary of color removal efficiency under anaerobic and aerobic phase of the different operational phases. (orange) Remazol Brilliant Orange 3R; (black) Remazol Black B; (violet) Remazol Brilliant Violet 5R and (blue) Remazol Brilliant Blue R

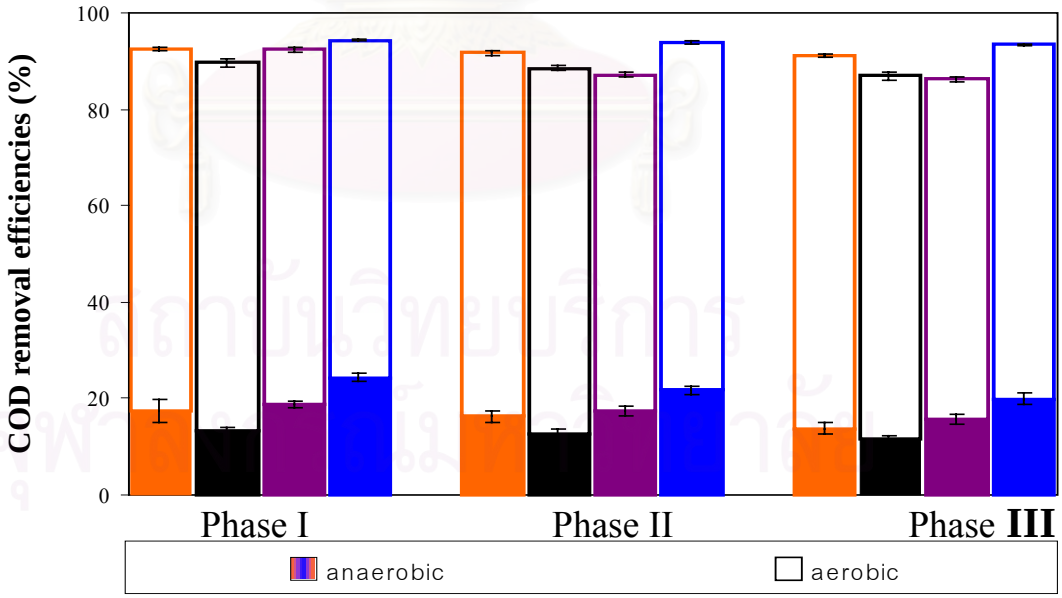


Figure 8.21. Summary of COD removal efficiency under anaerobic and aerobic phase of the different operational phases. (orange) Remazol Brilliant Orange 3R; (black) Remazol Black B; (violet) Remazol Brilliant Violet 5R and (blue) Remazol Brilliant Blue R

The drawback of the alginate beads in wastewater treatment in their mechanical instability. Nevertheless it seems reasonable to assume that the structure of the alginate polymer resembles the biofilms found ubiquitously on solid surfaces or soil particles in aqueous systems in nature (Beer et al., 1994). The observation that a matrix with immobilized aerobic bacteria and a thickness of less than 1 mm is sufficient to allow the mineralization of sulfonated azo dyes suggest that similar processes will also take place in nature. Thus bacteria that are ble to mineralize the reductive products of azo dyes aerobically will perform this reductive processes in flocs or biofilms, even heavily aerated sewage treatment plants. This process may explain the observation that certain azo compounds seem to be biodegraded in the activated sludge process (Shaul et al., 1991). For the development of a technical process in the present study, a more rigid material for immobilization of the decolorizing-bacteria cells, must be found before the application of sequential anaerobic-aerobic treatment.

This results obtained in the fluidized bed reactor suggest that it is possible to regulate aerobic and anaerobic metabolisms by controlling the air flow and then by maintaining a coexistence of the anaerobic and aerobic communities under air-limiting conditions. Up to now, no culture of associated anaerobic and aerobic microorganisms has previously been run for such a long time in continuous mode. Thus, it could be interesting to study the evolution of the relationship between these communities from the start-up of the culture (microorganisms co-immobilized in gel beads) to the main culture (microorganisms associated as a homogenous sludge bed). Fluidized-bed type decolorizing bioreactor has many advantages which are: reduced reactor volume, high throughput capacity, ideal mixing condition, and reduced secondary pollution problem.

Chapter 9

General conclusions

Two bacterial strains having a high capacity for rapid decolorization of reactive dyes, including Remazol Brilliant Orange 3R, Remazol Black B and Remazol Brilliant Violet 5R and Remazol Brilliant Blue R, were isolated from wastewater from a dyeing wastewater treatment plant. Both isolates were identified as *Paenibacillus* sp. strain A5 and S1

Although *Paenibacillus* sp. strain A5 and S1 played good growth in an aerobic condition, color removal of azo dyes was best in anoxic static cultures. The consortium of *Paenibacillus* sp. strain A5 and S1 showed the highest specific decolorization rate for the decolorization of all azo dyes. The lag phase before decolorization onset presented in the decolorization of azo dyes by the single strain was not observed in the decolorization by the mixed culture.

The course of the decolorization process approximates first-order kinetics with respect to dye concentration have also been reported by Wuhrmann et al. (1980), Weber and Wolfe (1987) and Carliell et al. (1994).

During aerobic post-treatment of anaerobically treated, azo dye containing wastewater, there will be competition between biodegradation and autooxidation of aromatic amines in a subsequent aerobic post-treatment step may be problematic, not only because the formed products are colored but also because some of these compounds, e.g., azoxy compounds, may cause toxicity (Field et al., 1995). It may as well be possible, however, that autoxidation leads to the formation of large, bulky, non-toxic, humic polymers which can easily be separated from the water phase.

The effects of influencing factors on the decolorization of reactive dyes were investigated in greatly details. For color removal, the optimal pH and temperature were 7 –10 and 30-44 ^{0}C, respectively. Additionally, to ensure an effective azo dye decolorization with *Paenibacillus* sp. strain A5 required a rigorous control of the DO concentration (below 0.5 mg/l) in the biological process. The presence of nitrate and copper in the anaerobic system were found to inhibited the anaerobic decolorization of azo dyes.

The Michaelis–Menten model can satisfactorily describe the dependence of specific decolorization rate on the concentration of reactive dyes. The exhibited a remarkable color removal capability, even at a high concentration of azo dye.

The mixed culture of strain A5 and S1 could also decolorize synthetic effluent containing a mixture of different reactive dyes. That is applicable to a wide variety of individual dyes and mixture of dyes. Decolorization of azo dyes is faster than anthraquinone dye in the dye mixtures under anaerobic condition.

Examination of the mechanism of the decolorization process of all reactive azo dyes by *Paenibacillus* sp. strain A5 indicated that it proceeded mostly by anaerobic reduction of azo chromophore present in the azo dyes molecules. The rate of decolorization of azo dyes are not dependent on the molecular weight of the dye indicating that cell permeation is probably not an important issue in the reductive mechanism. This observation combined with the non-enzymatic extracellular reduction mechanism involving reduced reactive azo compounds.

In contrast, decolorization of Remazol Brilliant Blue R, an anthraquinone dye, appears to proceed mainly by biosorption to bacterial cells. Remazol Brilliant Blue R is anthraquinoid-based chromophore combined with vinylsulfone as its reactive group which interact with the active groups on the cell surface of bacteria such as peptidoglycan, acidic polysaccharides, lipids, amino acids and other cellular components of the microorganism. In the present study, the Remazol Brilliant Blue R uptake by biomass of *Paenibacillus* sp. strain A5 may be due to the electrostatic attractions between the negatively charged dye anions and positively charged cell surface. Hydrogen ion also acts as a bridging ligand between the bacterial cell wall and the dye molecule.

The Langmuir model makes several assuptions, such as monolayer coverage and constant adsorption energy while the Freundlich equation deals with physicochemical adsorption on heterogeneous surfaces. The applicability of both Langmuir and Freundlich isotherms to the dye-*Paenibacillus* sp. cell systems implies that both monolayer adsorption and heterogeneous surface conditions used. The sorption of dye on the bacteria is thus likely to be complex, involving more than one mechanism.

The effects of anaerobic hydraulic retention time (12-24 h) on COD removal and decolorization of Remazol Brilliant Orange 3R, Remazol Black B, Remazol Brilliant Violet 5R and Remazol Brilliant Blue R in a sequential anaerobic-aerobic treatment system were investigated in this study. Coimmobilized cells of *Paenibacillus* sp. strain A5 and S1 in calcium alginate beads used as a bioparticle shown the very high decolorization ability over the reactive dyes used. Experimental results indicated that the system could be operated at the anaerobic retention time 24 h (Phase III) to obtained over 90% decolorization efficiency of all azo dyes in an anaerobic phase of the operational system. In the case of azo dyes, no significant color removal were observed under aerobic condition while COD removal was mainly obtained in the aerobic phase with an average value of 75% efficiency for all dye used.

The total color removal efficiencies at the minimum anaerobic retention time (12-h) were around 95, 77, 67 and 58% for the decolorization of Remazol Brilliant Orange 3R, Remazol Black B, Remazol Brilliant Violet 5R and Remazol Brilliant Blue R, respectively. Meanwhile, the total COD removal efficiencies at same anaerobic retention

time were around 91, 87, 86, 93% for the decolorization of Remazol Brilliant Orange 3R, Remazol Black B, Remazol Brilliant Violet 5R and Remazol Brilliant Blue R, respectively. Thus, it can be concluded that the anaerobic retention time rather affected the color removal potentiality than the COD removal potentiality of the treatment system.

The ability of coimmobilization of *Paenibacillus* sp. strain A5 and S1 to reduce a broad spectrum of reactive dyes holds promise for the application of high rate anaerobic systems as feasible first stage in the complete removal of azo dyes from textile wastewater. However, the kinetic data predicted that the complicated structure azo dyes (e.g., diazo and metal-complex azo dye) are reduced slowly. Long residence time would be necessary to reach a satisfying extent of decolorization. However, this problem may be overcome, as the results presented here indicate that redox mediators can be used to accelerate the anaerobic reduction of azo dyes.

Aromatic amines from the anaerobic breakdown of the azo dyes were not completely removed within the retention time at 10-h of the subsequent aerobic phase. Further analytical work on the complete removal of these byproducts is desirable.

It may be concluded that a biosorption process could be adopted as a cost effective and efficient approach for decolorization of effluents containing non-degradable dyes (e. g., anthraquinone dye) and it may be an alternative to more costly materials such as activated carbon. Biosorption technologies are still being developed and much more work is required.

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APPENDIXES

Appendix 1

Calibration curve for calculation of dye concentrations

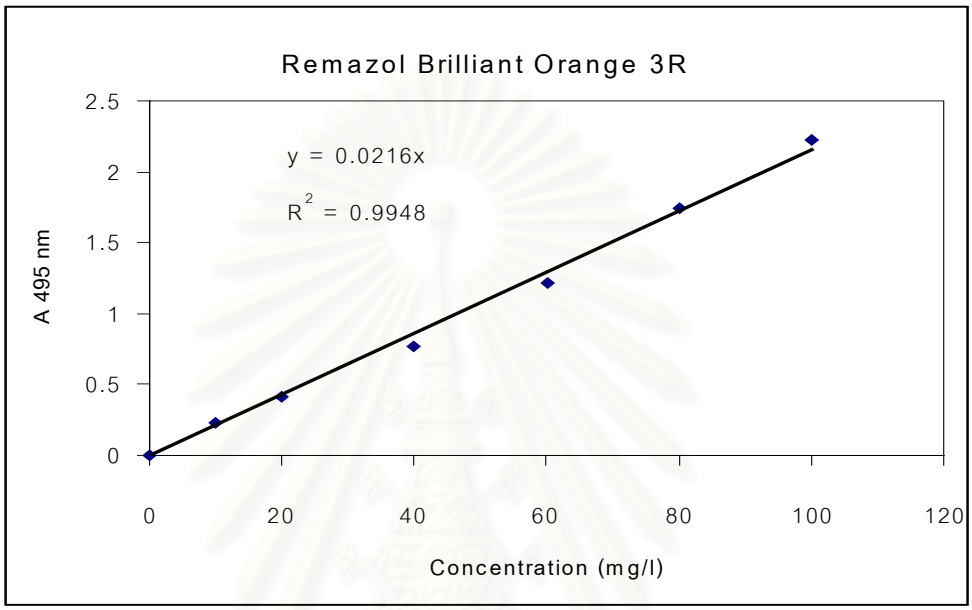

Figure 10.1 Calibration curve of Remazol Brilliant Orange 3R

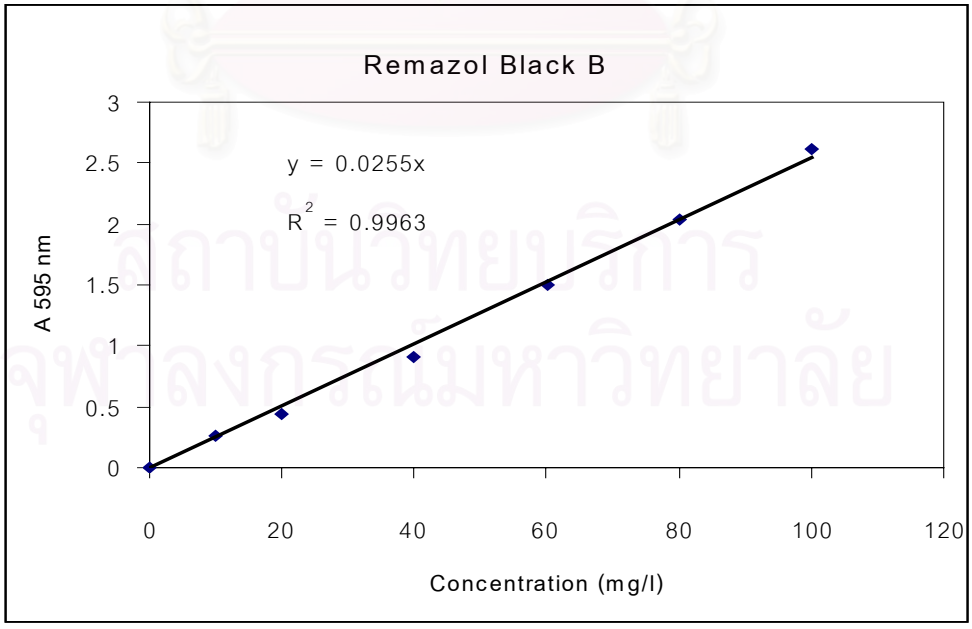

Figure 10.2 Calibration curve of Remazol Black B

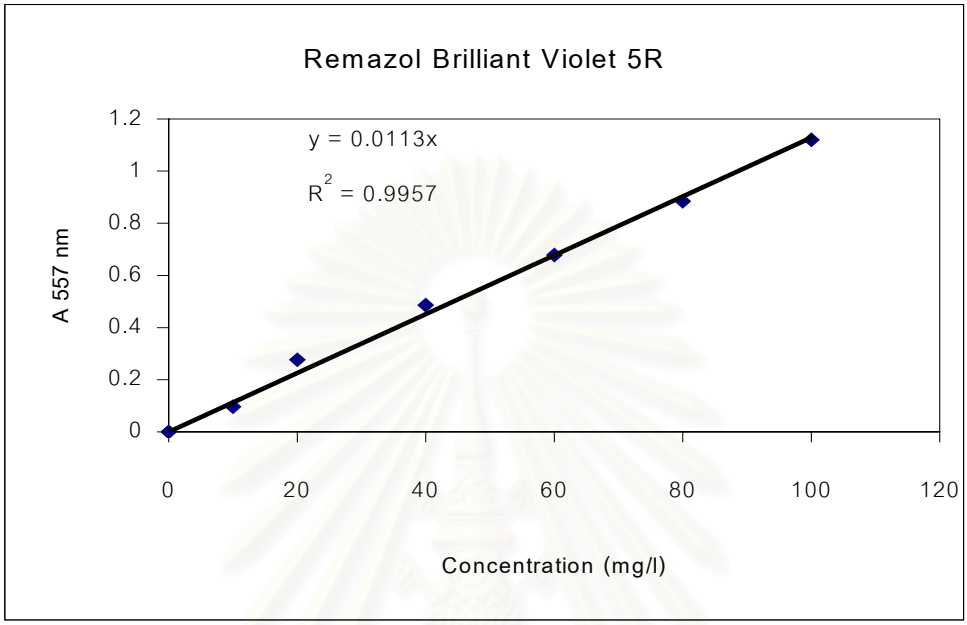

Figure 10.3 Calibration curve of Remazol Brilliant Violet 5R

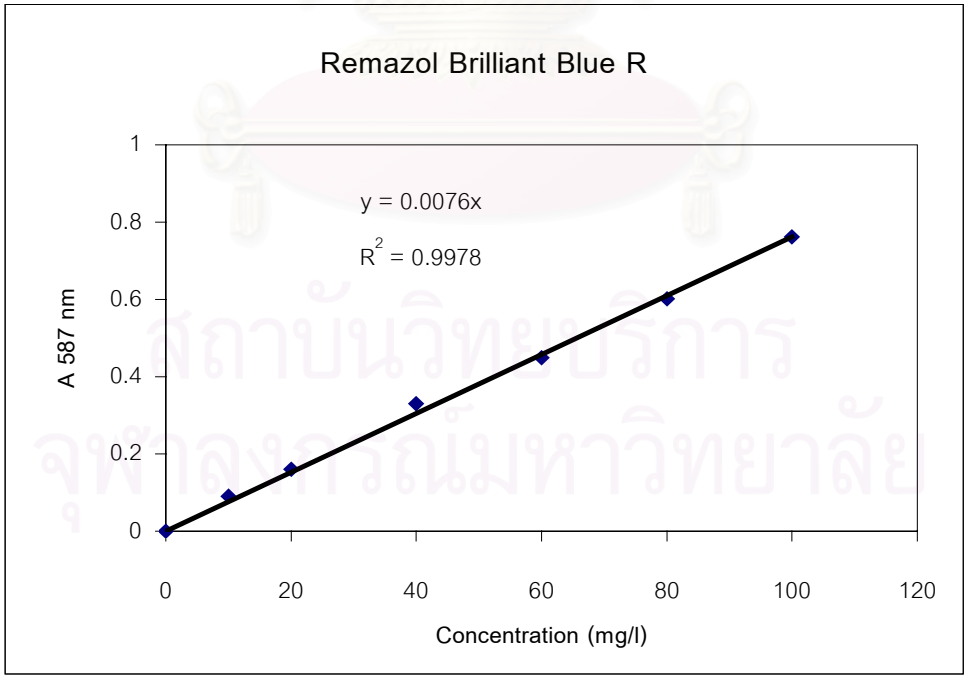

Figure 10.4 Calibration curve of Remazol Brilliant Blue R

Appendix 2

Alternate ADMI method

The ADMI color value may be determined spectrophotometrically, using a spectrophotometer with narrow (10 nm or less) spectral band and an effective operating range of 400 to 700 nm. The tristimulus values (X, Y and Z) can be calculated from the transmittance measurements multiply by the factors of individual transmittance at every wavelength. The factors are showed in Table 6.1.

Table: 10.1 Factors for percent transmittance at different wavelength (Allen et al., 1973).

Wavelength (nm)	X		%T Y		Z	
	Factor	%T x Factor	Factor	%T x Factor	Factor	%T x Factor
400		0.00108		0.00002		0.00513
410		0.00329		0.00009		0.01570
420		0.01238		0.00003		0.05949
430		0.02997		0.00122		0.14628
440		0.03975		0.00262		0.19938
450		0.03915		0.00443		0.20638
460		0.03362		0.00694		0.19299
470		0.02272		0.01058		0.14972
480		0.01112		0.01618		0.09461
490		0.00363		0.02358		0.05274
500		0.00052		0.03401		0.02864
510		0.00089		0.04833		0.01520
520		0.00579		0.06462		0.00712
530		0.01523		0.07934		0.00388
540		0.02785		0.09149		0.00195
550		0.04282		0.09832		0.00086
560		0.05880		0.09841		0.00039
570		0.07322		0.07992		0.00020
580		0.08417		0.06627		0.00016
590		0.08984		0.05316		0.00010
600		0.08949		0.04176		0.00007
610		0.08325		0.03153		0.00002
620		0.07070		0.02190		0.00002
630		0.05309		0.01443		0.00000 0.00
640		0.03693		0.00886		0.00000 0.00
650		0.02349		0.00504		0.00000 0.00
660		0.01361		0.00259		0.00000 0.00
670		0.00708		0.00134		0.00000 0.00

680	0.00369	0.00062	0.00000	0.00
690	0.00171	0.00062	0.00000	0.00
700	0.00156	0.00056	0.00000	0.00
	X = Sum =	Y = Sum =	Z = Sum =	

After get the three tristimulus values, convert the three value (X, Y, Z) to the corresponding Munsell values (V_x, V_y V_z) using published tables, 2, 3, 4 or by the Adams-Nickerson formula provided by Mclaren (1967) as follow:

$$X = 0.98071\ (1.2219\ V_x - 0.23111\ V^2_x + 0.23951\ V^3_x - 0.021009\ V^4_x = 0.000840\ V^5_x)$$

$$Y = (1.2219\ V_y - 0.23111\ V^2_y + 0.23951\ V^3_y - 0.021009\ V^4_y + 0.0008404\ V^5_y$$

$$Z = 1.18225\ (1.2219\ V_z - 0.23111\ V^2_z + 0.23951\ V^3_z - 0.0201009\ V^4_z + 0.0008404\ V^5_z)$$

Calculate the intermediate value of DE from the equation:

$$DE = [\{0.23(9.902 - V_y)\}^2 + \{V_y - V_x - 0.002\}^2 + \{0.4(V_z - V_y - 0.008)\}^2\}^{1/2}$$

Calculate the final ADMI color value as follow:

$$\text{ADMI value} = \frac{(F)\ (DE)}{b}$$

Where:

b = absorption cell light path, cm.

F = calibration factor of the standard calibration curve of DE value.

Appendix 3

Multiple components determination

Multivariate calibration consists of the establishment of an association between matrices of dye data. The process is composed of two steps: calibration and prediction. In this case, the calibration step consist of the development of a mathematical model which can reproduce a concentration matrix C_{STD} (contains '*m*' lines which correspond to samples and '*l*' columns of different dye concentrations) from a matrix E_{STD} (with '*m*' lines of samples and '*n*' column of selected wavelength values).

The determination of multiple components includes the calibration matrix acquired through the measurement of standard samples and the calculation for acquiring the concentration of unknown samples.

1 Calculation of calibration matrix

(a) Values which should be manually input as calculations condition (C_{STD})

<Standard m>

C*m*1 : Concentration of dye 1 contained in standard *m*
C*m*2 : Concentration of dye 2 contained in standard *m*
.C*ml* : Concentration of dye l contained in standard *m*

$$C_{STD} = \begin{bmatrix} C11 & C12 \ldots\ldots C1l \\ C21 & . \\ . & . \\ . & . \\ Cml & \ldots\ldots\ldots\ldots Cml \end{bmatrix}$$

where, *m* : number of standards
l : number of dyes

(b) Values acquired though measurement of Standard (E_{STD})

<Standard m>

E*m*1 : Absorbance at wavelength λ1 of standard *m*
E*m*2 : Absorbance at wavelength λ2 of standard *m*
E*mn* : Absorbance at wavelength λ*n* of standard *m*

$$E_{STD} = \begin{bmatrix} E11 & E12\ldots. & E1n \\ E21 & & . \\ . & & . \\ . & & . \\ Eml\ldots\ldots & & .Emn \end{bmatrix}$$

(c) Absorbance per unit concentration at wavelengths λ1, λ2,--- λ*m* of dye 1---- *l* (S)

<Dye *m*>

S*l*1 : Absorbance per unit concentration at wavelength λ1 of dye *l*
S*l*2 : Absorbance per unit concentration at wavelength λ2 of dye *l*
S*ln* : Absorbance per unit concentration at wavelength λ*n* of dye *l*

Where, *n* : number of wavelengths to be sampled

$$S = \begin{bmatrix} S11 & S12\ldots. & S1n \\ S21 & & . \\ . & & . \\ . & & . \\ Sl\ldots\ldots & & .Sln \end{bmatrix}$$

(d) Relational expressions

E11 = C11 S11 + C12 S21 + ---- + C1*l* S*l*1
E12 = C11 S12 + C12 S22 + ---- + C1*l* S*l*2
E*ml* = C11 Sl*m* + C12 S2*m* + ---- + C1*l* Sl*m*

$$\left[\begin{bmatrix} E11 & E12\ldots\ldots Eln \\ E21 & . \\ . & . \\ . & . \\ Eml\ldots\ldots\ldots\ldots & Emn \end{bmatrix} - \begin{bmatrix} C11 & \\ C12\ldots\ldots. & C1l \\ C21 & . \\ . & . \\ . & . \\ Cml\ldots\ldots\ldots\ldots & Cm \end{bmatrix} \begin{bmatrix} S11 & S12\ldots\ldots S1n \\ S21 & . \\ . & . \\ . & . \\ Sl\ldots\ldots\ldots\ldots & Sln \end{bmatrix}\right] \longrightarrow \text{minimum}$$

or

$$(E_{STD} - C_{STD}S)^2 \longrightarrow \text{minimum}$$

(**e) From the expression above, the following expression can be developed for the determining a calibration matrix.**

$$S = (C_{STD}{}^t\, C_{STD})^{-1} \bullet C_{STD}{}^t \bullet E_{STD}$$

(2) Calculation of unknown sample

(a) Measured values

The following values are obtained through measurement of unknown sample X.

e1 : Absorbance at λ1 of sample X
e2 : Absorbance at λ2 of sample X
.
e*n* : Absorbance at λ*n* of sample X

$$\textbf{Where, } \mathbf{E_X} = \begin{bmatrix} \mathbf{e1} \\ \mathbf{e2} \\ \bullet \\ \mathbf{e}\boldsymbol{n} \end{bmatrix}$$

(b) Using the multivariate analysis/least squares method, the concentration of each component can be determined by equation below.

$$C_X = (S^t\, S)^{-1} \bullet S^t \bullet E_X$$

$$\textbf{Where, } \mathbf{C_X} = \begin{bmatrix} \mathbf{C1} \\ \mathbf{C2} \\ \bullet \\ \mathbf{C}\boldsymbol{l} \end{bmatrix}$$

C1 : Concentration of dye 1
C2 : Concentration of dye 2.
C*l* : Concentration of dye *l*

Appendix 4

Chemical oxygen demand (COD)

COD was measured by closed reflux titrimetric method following Standard methods (5220 C).

Reagents

a. **Standard potassium dichromate digestion solution, 0.0167 M**:

Add to about 500 ml. Distilled water 4.913 g $K_2Cr_2O_7$, primary standard grade, previously dried at 103 ^{0}C for 2 h, 167 ml conc. H_2SO_4. Dissolve, cool to room temperature, and dilute to 1,000 ml.

b. Sulfuric acid reagents:

Add Ag_2SO_4, reagent or technical grade, crystals or powder, to conc. H_2SO_4 at the rate of 5.5 g Ag_2SO_4/kg H_2SO_4. Let stand 1 to 2 days to dissolve Ag_2SO_4.

c. **Ferroin indicator solution:**

Dissolve 1.485 g 1,10-phenanthroline monohydrate and 695 mg $FeSO_4 \bullet 7H_2O$ in distilled water and diluted to 100 ml. This indicator solution may be perchased already prepared.

d. Standard ferrous ammonium sulfate titrant (FAS), approximately 0.10 M:

Dissolve 39.2 g $Fe(NH_4)_2(SO_4)_2 \bullet 6H_2O$ in distilled water. Add 20 ml. conc. H_2SO_4, cool, and dilute to 1000 ml. Standardize solution daily against standard $K_2Cr_2O_7$ digestion solution as follows:

Add reagents according to Table 3.2 to a culture tube containing the correct volume of distilled water substituted for sample. Cool tube to room temperature and add 0.05 to 0.10 ml (1 to 2 drops) ferroin indicator and titrate with FAS titrant.

Molarity of FAS solution

$$= \frac{\text{Volume 0.0167 M } K_2Cr_2O_7 \text{ solution titrated, ml} \quad \text{x } 0.10}{\text{Volume FAS used in titration, ml}}$$

Procedure

Wash culture tubes and caps with 20% H_2SO_4 before first use to prevent contamination. Refer to table 3.2 for proper sample and reagent volumes. Place sample in culture tube and add digestion solution. Carefully run sulfuric acid reagent down inside of vessel so an acid layer is formed under the sample digestion solution layer. Tightly cap tubes and invert each several times to mix completely.

Place tubes in oven preheated to 150 ^{0}C and reflux for 2 h. Cool to room temperature and place vessel in test tube rack. Remove culture tube caps and transfer contents in larger container for titrating. Add 0.05 to 0.10 ml ferroin indicator and stir rapidly on magnetic stirrer while titrating with 0.10 M FAS. The end point is sharp color change from blue-green to reddish brown, although the blue-green may reappear within minutes. Use distilled water as a blank for titration.

$$\text{COD as mg } O_2\, l^{-1} = \frac{(A-B) \times M \times 8000}{\text{ml. sample}}$$

where:

A = ml FAS used for blank
B = ml FAS used for sample, and
M = molarity of FAS.

Table 10.2 Sample and reagent quantities for various digestion vessels

Digestion Vessel	Sample (ml)	Digestion solution (ml)	Sulfuric acid reagent (ml).	Total final volume (ml)
Culture tubes:				
16 x 100 mm	2.5	1.5	3.5	7.5
20 x 150 mm	5.0	3.0	7.0	15.0
25 x 150 mm	10.0	6.0	14.0	30.0

BIOGRAPHY

Mr. Nuttapun Supaka was born on May 31, 1974 at Bangkok, Thailand. He has graduated and holds a B.Sc. in Microbiology from Department of Microbiology Faculty of Science Chulalongkorn University in 1996. He has graduated and holds a M.Sc. in Industrial Microbiology from Department of Microbiology Faculty of Science Chulalongkorn University in 1999. He has applied to study Ph.D. in Biotechnology at the Program of Biotechnology Faculty of Science Chulalongkorn University in 2000. The Royal Golden Jubilee Program of the Thailand Research Fund financially supported his Ph.D. program.