



Humic Substance Enhanced Anaerobic Reduction of Sulfonated Azo Dyes by *Paenibacillus* sp. Strain A5

Somsak Damronglerd^a

Fellow of the Academy of Science, The Royal Institute, Thailand

Nuttapun Supaka^b

Kanchana Juntongjin^m

Supat Chareonpornwattana^m

Marie-Line Delia^c

Pierre Strehaiano^c

Abstract

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. Possible applications of AQDS stimulated anaerobic reduction of azo dyes for the treatment of dye-containing wastewaters are also discussed.

Key words : sulfonated azo dyes, azo reductase, anthraquinone-2,6-disulfonate, *Paenibacillus* sp.

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 (62). 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 (12). 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 (1). Sulfonated azo dyes are known to be resistant to

^a Department of Chemical Technology, Faculty of Science, Chulalongkorn University

^b Program of Biotechnology, Faculty of Science, Chulalongkorn University

^m Department of Microbiology, Faculty of Science, Chulalongkorn University

^c Laboratoire de Génie Chimique, INP-ENSIACET, 5, rue Paulin Talabot Site Basso Combo-BP 1301, Toulouse 31106, France



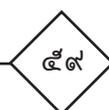
degradation by aerobic bacteria due to the strong electron-withdrawing property of the azo group thought to protect against attack by oxygenases (28) so that there generally pass biodegradative processes in conventional sewage treatment systems untreated (40, 48). The azo dyes-containing effluents from 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 (23).

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 (9, 13, 19, 22, 44, 45). 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 (17). 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 (56) and therefore, intracellular reductases do not function (46). Thus, an extracellular nonspecific biological process may be vital for treatment of

the textile effluents. From the currently known biological systems, the required unspecificity 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 (14, 26, 29, 42, 43, 50). 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 (14, 42, 50). 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 (7, 11, 31, 47).

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 (49). 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 (10, 15, 31). 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 (47) accepting the electrons. Anthraquinone-2,6-disulfonate (AQDS) have been used as a defined model for such moieties (10, 11, 31). Most AQDS-respiring microorganisms are capable of transferring electrons to AQDS, reducing it to anthraquinone-2,6-disulfonate (AH₂QDS). 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 (31). The anaerobic microbial oxidation of phenol and *p*-cresol in granular sludge was recently found to be coupled to the reduction of AQDS (11). 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 (7). The rates of azo dye decolorization are also enhanced in the presence of different quinoid redox mediators, especially anthraquinone-2-sulfonate. (29, 42, 59). 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 which is responsible for the reduction of sulfonated azo dyes by whole cells of strain A5 in the presence of humic





substance was also determined.

MATERIALS AND METHODS

Bacteria strain and culture condition.

Paenibacillus sp. strain A5, a facultative anaerobic and endospore-forming bacteria, was originally isolated from textile effluent treatment plant. The isolation and characterization of this strain have been described before (51). Cell suspensions of strain A5 are able to anaerobically reduce various sulfonated azo dyes to corresponding aromatic amines (52). Strain A5 was routinely grown at 30°C on minimal medium containing glucose as a sole carbon and energy source. The basic composition of minimal medium (MMG) was (in g l⁻¹): glucose 0.9, (NH₄)₂SO₄ 0.28, NH₄Cl 0.23, KH₂PO₄ 0.067, MgSO₄ • 7H₂O 0.04, CaCl₂•2H₂O 0.022, FeCl₃•6H₂O 0.005, NaCl 0.15, NaHCO₃ 1.0 and 1 ml l⁻¹ of a trace element solution containing (in g l⁻¹) ZnSO₄•7H₂O 0.01, MnCl₂•4H₂O 0.1, CuSO₄•5H₂O 0.392, CoCl₂•6H₂O 0.248, NaB₄O₇•10H₂O 0.177 and NiCl₂•6H₂O 0.02.

Anaerobic reduction of sulfonated azo dyes with whole cells.

Cells of strain A5 were grown aerobically in MMG to an optical density at 600 nm (OD₆₀₀) 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⁻¹) 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 × g, 10 min), and the concentration of sulfonated azo dyes in supernatant was determined spectrophotometrically at λ_{max} of each azo dye.

Standard assay for determination of azo reductase 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. (29) and Russ et al. (46). For the standard assay, the anaerobically prepared reaction mixtures contained in 800 μl of 50 μM of Tris-HCl buffer (pH 7.5),

25 μM of the respective sulfonated 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°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 these azo dyes, which are summarized in Table 1.

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 × g for 10 min, washed twice, and resuspended in Na-K phosphate buffer (50 mM, pH 7.5) to an OD₆₀₀ 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⁻¹) by ultrasonication using an ultrasonic processor (Sonicator[®] W-385, Heat systems-Ultrasonics, Inc.). After removing the cell debris by centrifugation at 10,000 × g for 15 min at 4°C, the supernatant was ultracentrifuged at 100,000 × g for 30 min at 4°C (29, 43). 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” (29, 43). For the isolation of the membrane-bound azo reductase, 1 ml of this preparation was incubated on ice for 5 min with 950 μ l of 50 mM Tris-HCl buffer (pH 7.5) and 50 μ l of a solution of Triton X-100 (20%, v/v). Finally, 200 μ l of this mixture was used for the azo dye reduction as described above. The remaining membrane-bound protein was stored at -20°C until used.

Enzyme assays.

(i) NADH : quinone oxidoreductase.

The NADH: quinone oxidoreductase was measured spectrophotometrically by a modification of the method given by Matsushita et al. (32). 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, 1 ml) containing 50 mM of Tris-HCl (pH 7.5), 50 μ M of menaquinone dissolved in dimethyl sulfoxide, and 2.5 mM of NaCN. The reaction was started by the addition of 200 μ M 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 (16), thus, the reduction rate of NADH was measured at 348 nm (molar extinction coefficient = $5.9 \text{ mM}^{-1} \text{ cm}^{-1}$), where reduced and oxidized

forms of menaquinone have identical absorbivity.

(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 (25) and Russ et al (46). 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 μ M of riboflavin. The reaction was started by the addition of 250 μ M 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 $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$.

Determination of protein content.

The protein content of cell extracts and soluble cell membrane were determined by the method of Bradford (6) 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.

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^{-1}) 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) from 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 from 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).

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 $10,000 \times g$ 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 through 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) 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 (33).

Analytical techniques

The concentration of sulfonated azo dyes was determined spectrophotometrically (UV WINLAB, Perkin Elmer). Sulfonated azo dyes and the corresponding reduction products were analyzed by high performance liquid chromatography (HPLC; Shimadzu model LC-3A chromatograph (Shimadzu Corp., Kyoto, Japan) equipped with Shimadzu model SPD-2A detector). A reverse-phase column was Pegasil ODS, (4.6 mm x 150 mm [inside diameter] column, Senshu Scientific Co., Ltd., Tokyo, Japan). A mobile phase composed of 50% methanol, 0.3% H_3PO_4 , and 49.7% water was used with the flow rate of 0.5 ml min^{-1} . The eluates were monitored by UV absorption at 275 nm. Concentra-

tion of AH_2QDS were determined spectrophotometrically by monitoring the absorbance at 450 nm by using a molar extinction coefficient of $2.7 \text{ mM}^{-1} \text{ cm}^{-1}$ obtained from a calibration curve of AQDS chemically reduced by dithionite as previously described (10).

Chemicals.

Remazol Brilliant Orange 3R, Remazol Black B, and Remazol Brilliant Violet 5R were kindly supplied by Dystar, Thailand, Ltd. NADH, FAD, ubiquinone (Q1), and menaquinone (MK) were purchased from 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.).

RESULTS

AQDS stimulation of different 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 (for structural formulas see Fig. 1) 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 (Fig. 2). 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 (31). Abiotic reduction of sulfonated azo dyes did not occur in controls without cells or added electron donor (Fig. 2).

Quinone component(s) in cell membrane of strain A5.

Newman and Kolter have recently shown that there is a common biochemical basis for AQDS and humic acid respiration involving the biosynthesis of menaquinone, 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 cell membrane (34). Every *Bacillus* sp. so far examined contains menaquinone as the sole quinone component and general absence of ubiquinone (33). Like all the other bacilli, lipid extraction of culture grown aerobically showed that only menaquinone was found in *Paenibacillus* sp. strain A5. (as assayed by thin layer chromatography). Thin layer chromatography also revealed that menaquinone was not present in the supernatants of strain A5.



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 (26, 29, 30, 42, 43). 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 (Fig. 3). Concentration of AQDS as low as 50 μ M significantly stimulated all sulfonated azo dyes reduction by cell suspensions of *Paenibacillus* sp. strain A5 (Fig. 3). The rate of azo dyes reduction was also dependent on the concentration of AQDS added.

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 2).

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 (45, 56). 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 (14, 26, 29, 42, 43, 50). 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 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 reductase 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 cytoplasmic fraction, however, this externally added quinoid mediator had rather lower stimulating effect on azo dyes reduction than the addition of FAD (Table 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.

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 (4, 5, 32, 58). Furthermore, it was previously suggested that in *Shingomonas 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) (29). 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 (22, 45, 46). 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 3).

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 (31, 42, 50). Therefore, both reactions were analyzed separately (Fig. 4). 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, AH₂QDS) as evidenced by the orange color formation and increased

absorbance at 450 nm (10)) of the culture medium within 10-h incubation period (Fig. 4). At the end of anaerobic incubation, the buffer turned orange owing to the accumulation of AH₂QDS. 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 (data not shown).

To evaluate the second reaction in proposed mechanism, sulfonated azo dyes were added into cell-free culture filtrates containing of AH₂QDS, it was found that the orange color of AH₂QDS 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 AH₂QDS (≈ 0.25 mM) demonstrated that the chemical reduction of orange and violet dye required two moles of AH₂QDS to produce the corresponding aromatic amines and by four

moles of AH₂QDS to produce the corresponding aromatic amines of black dye. The stoichiometry suggested a complete reduction of sulfonated azo dyes to the corresponding aromatic amines.

Characterization of products formed from chemical reduction of various sulfonated azo dyes.

The reduction products of sulfonated azo dyes present in the reaction mixtures were analyzed by HPLC as described in Materials and Methods. HPLC chromatogram of reduction products of individual azo dyes by AH₂QDS was compared with the HPLC chromatogram of completely reduction products of each azo dye obtained by reduction the dye with sodium dithionite, a strong chemical reducing agent (2). Form comparison results, the complete reduction of each azo dye to its corresponding amines by AH₂QDS was proposed in this study (data not shown).

DISCUSSION

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 (29, 41). 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 (Fig. 2). 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 FADH₂], 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 (46).

In many intestinal bacterial isolates, a flavin compound (riboflavin, flavin adenine dinucleotide, or flavin mononucleotide (FMN)) is required for azoreductases activity (19, 22, 45, 46). The most generally accepted hypothesis for this phenomenon is that many bacterial cells possess a rather unspecific cytoplasmic flavin-dependent reductases (flavin reductases) which transfers electron under anaerobic conditions via (soluble) flavins to the azo dyes (46, 50). 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 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 (FADH₂) can then spontaneously reduce the three sulfonated azo dyes to the corresponding amines (19, 22, 45, 46). 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 2). Thus, this has generally been explained by the low permeability of the cell membranes for the highly polar sulfonated azo compounds (56). 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 (56). In addition, it was clearly demonstrated in our 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 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 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 (29, 46, 50).

Thus, in the intact cells, other enzyme systems, which does not require transport of the azo dyes through the cell membrane, are presumably responsible for the

unspecific reduction of various sulfonated azo dyes by *Paenibacillus* sp. strain A5. Several quinoid redox mediator compounds, for example; anthraquinone-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 (26, 27, 29, 30, 42, 43). In this article we report that 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 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, indicating that the cells reduced the AQDS to the corresponding hydroquinone (AH₂QDS). 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 (18, 29, 42, 43, 50, 57). 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 (16). NDH-1 is an energy-transducing enzyme meanwhile NDH-2 appears to have no role in energy transduction (16). Some gram-positive bacteria such as *Bacillus subtilis* have non-energy generating NDH 2 but not NDH 1 (3).

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 (60). 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) (61), may be also able to function under anaerobic conditions in the appropriate conditions as quinone-dependent azo reductases in many bacterial genera (43, 60). 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 quinoid mediators.

In biological systems, quinones were also shown to accelerate azo dye

reduction by anaerobically incubated aerobic biomass as well as granular sludge (29, 42, 43). Theoretically, feasible quinoid 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_o') 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 (19, 42). For bacterial azo dye reduction, i.e., coupled to the oxidation of organic primary electron donors by anaerobically incubated bacteria, the E_o' value of NAD(P)H, the cellular redox cofactors with the lowest electron potential (-320 mV), can be taken into account (54). It was recently suggested that quinoid redox mediators with standard redox potentials (E_o') between approximately -320 mV and -50 mV could in general function as effective redox mediators in the bacterial reduction of sulfonated azo dyes (42). Thus quinones with a rather negative redox potential such as AQDS are suitable as redox mediators for the anaerobic treatment of azo dyes.

Kudlich et al. (29) localized a quinoid redox mediator-dependent azo reductase activity in the membrane of a gram-negative bacterium, *Sphingomonas* sp. strain BN6. Therefore, in the present study, the AH₂QDS is suggested to shuttle reduction

equivalents in the cells to extracellular sulfonated azo dye and reduce the azo compound in a purely chemical reaction (Fig. 5). 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 Fig. 5 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 (AH₂QDS) with the sulfonated azo dyes allows for extremely unspecific reduction processes which are mainly governed by the redox potentials of the AH₂QDS 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 (8, 20, 37, 35, 36), it has been repeatedly suggested to combined the anaerobic reduction of the azo dyes with an aerobic treatment system for the amines formed (21, 24, 38, 39, 52, 53).

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* (30). 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 (54).

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 (7, 10, 11). Whether microbially produced extracellular molecules have a similar role remains an important question. Another possibility for the reduction of extracellular quinoid 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* (34). 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 (34). 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 (55). 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 (34).

Because the rather high reduction rate of sulfonated azo dyes in the absence of any exogenous redox mediators found in whole cells experiment (Fig. 2 and Table 3), we cannot eliminate the possibility that some unknown enzymatic activities are involved in the anaerobic

reduction of sulfonated azo dyes by whole cells of strain A5. We are currently attempting to explore the other extracellular electron transferring mechanisms of strain A5 which are involved for the reduction of the AQDS to clarify this mechanism.

ACKNOWLEDGEMENTS

This work was funded by the Thailand Research Fund. We gratefully acknowledge Dystar Thai Ltd., for providing the dyes used in this study.

REFERENCES

1. Anliker, R. 1979. Ecotoxicology of dyestuffs: a joint effort by industry. *Ecotox. Environ. Safety* 3 : 59-74.
2. Bechtold, T., E. Burtcher, A. Turcanu, and O. Bobleter. 1994. The reduction of vat dye by indirect electrolysis, *J. Soc. Dyers Color.* 110 : 14-19.
3. Bergsma, J., R. Strijker, J.Y. Alkema, H.G. Seijen, and W.N. Konings. 1981. NADH dehydrogenase and NADH oxidation in membrane vesicle from *Bacillus subtilis*. *Eur. J. Biochem.* 120 : 599-606.
4. Bertsova, Y.V., A.V. Bogachev, and V.P. Skulachev. 1998. Two NADH : ubiquinone oxidoreductases of *Azotobacter inelandii* and their role in the respiratory protection. *Biochem. Biophys. Acta.* 1363 : 125-133.
5. Bjorklof, K., V. Zickermann, and M. Finel. 2000. Purification of the 45 kDa, membrane bound NADH dehydrogenase of *Escherichia coli* (NDH-2) and analysis of its interaction with ubiquinone analogues. *FEBS Lett.* 467 : 105-110.
6. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of



- microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72** : 248-254.
7. **Bradley, P.M., F.H. Chapelle, and D.R. Lovley.** 1998. Humic acids as electron acceptors for anaerobic microbial oxidation of vinyl chloride and dichloroethene. *Appl. Environ. Microbiol.* **64** : 3102-3105.
 8. **Brown, D., and P. Laboureur.** 1983. The aerobic biodegradability of primary aromatic amines. *Chemosphere* **12** : 405-414.
 9. **Carliell, C.M., S.J. Barelay, N. Naidoo, C.A. Buckley, D.A. Mulholland, and E. Senior.** 1995. Microbial decolorization of reactive azo dye under anaerobic conditions. *water SA.* **21** : 61-69.
 10. **Cervantes, F.J., S.van der Velde, G. Lettinga, and J.A. Field.** 2000. Competitive between methanogenesis and quinone respiration for ecological important substrates in anaerobic consortia. *FEMS Microbiol. Ecol.* **34** : 161-171.
 11. **Cervantes, F.J., S. van der Velde, G. Lettinga, and J.A. Field.** 2000. Quinone as terminal electron acceptors for anaerobic microbial oxidation of phenolic compounds. *Biodegradation* **11** : 313-321.
 12. **Chudgar, R.J.** 1985. Azo dyes, p. 821-875. In J.I. Kroschwitz (ed.), *Kirk-Othmer encyclopedia of chemical technology*, 4th ed., vol. 3. John Wiley, New York, N.Y.
 13. **Chung, K.-T., G.E. Fulk, and M. Egan.** 1978. Reduction of azo dyes by intestinal anaerobes. *Appl. Environ. Microbiol.* **35** : 558-562.
 14. **Chung, K.-T., S.E. Stevens, and C.E. Cerniglia.** 1992. The reduction of azo dyes by the intestinal microflora. *Crit. Rev. Microbiol.* **18** : 175-190.
 15. **Coates, J.D., D.J. Ellis, E. Roden, K. Gaw, E.L. Blunt-Harris, and D.R. Lovley.** 1998. Recovery of humic-reducing bacteria from a diversity of sedimentary environments. *Appl. Environ. Microbiol.* **64** : 1504-1509.
 16. **Cook, S.A., and A.K. Shiemke.** 2002. Evidence that a type-2 NADH : quinone oxidoreductase mediates electron transfer to particulate methane monooxygenase in *Methylococcus capsulatus* *Arch. Biochem. Biophys.* **398** : 32-40.
 17. **Correia, V.M., T. Stephenson, and S.J. Judd.** 1994. Characteristics of textile wastewaters - a review. *Environ. Technol.* **15** : 917-929.
 18. **Davis, K.J., and J.H. Doroshov.** 1986. Redox cyclic of anthracyclines by cardiac mitochondria. I. Anthracycline radical formation by NADH dehydrogenase. *J. Biol. Chem.* **261** : 3060-3067.
 19. **Dubin, P., and K.L. Wright.** 1975. Reduction of azo food dyes in cultures of *Proteus vulgaris*. *Xenobiotica.* **5** : 563-571.
 20. **Feigel, B.J., and H.J. Knackmuss.** 1993. Syntrophic interactions during degradation of 4-aminobenzenesulfonic acid by a two species bacterial culture. *Arch. Microbiol.* **159** : 124-130.
 21. **Fitzgerald, S.W., and P.L. Bishop.** 1995. Two stage anaerobic/aerobic treatment of sulfonated azo dyes. *J. Environ. Sci. Health A.* **30** : 1251-1276.
 22. **Gingell, R., and R. Walker.** 1971. Mechanism of azo reduction by *Streptococcus faecalis*. II. The role of soluble flavins. *Xenobiotica* **1** : 231-239.
 23. **Hao, O.J., H. Kim, and P.-C. Chiang.** 2000. Decolorization of wastewater. *Crit. Rev. Environ. Sci. Technol.* **30** : 449-505.
 24. **Haug, W., A. Schmidt, B. Nortemann, D.C. Hempel, A. Stolz, and H.J. Knackmuss.** 1991. Mineralization of the sulfonated azo dye Mordant Yellow 3 by a 6 aminonaphthalene-2-sulfonate-degrading bacterial consortium. *Appl. Environ. Microbiol.* **57** : 3144-3149.
 25. **Izumi, Y., and T. Ohshiro.** 2001. Purification and characterization of enzymes involved in desulfurization of dibenzothiophene in fossil fuels. *J. Mol. Cat.B Enz.* **11** : 1061-1064.
 26. **Keck, A., J. Klein, M. Kudlich, A. Stolz, H.-J. Knackmuss, and R. Mattes.** 1997. Reduction of azo dyes by mediators originating in the naphthalenesulfonic acid degradation pathway of *Sphingomonas* sp. strain BN6. *Appl. Environ. Microbiol.* **63** : 3684-3690.
 27. **Keck, A., J. Rau, T. Reemtsma, R. Mattes, A. Stolz, and J. Klein.** 2002. Identification of quinoid redox mediators that are formed during the degradation of naphthalene-2-sulfonate by *Sphingomonas xenophaga* BN6. *Appl. Environ. Microbiol.* **68** : 4341-4349.
 28. **Knackmuss, H.** 1996. Basic knowledge and perspectives of bioelimination of xenobiotic compounds. *J. Biotechnol.* **51** : 287-295.
 29. **Kudlich, M., A. Keck, J. Klein, and A. Stolz.** 1997. Localization of the enzyme system involved in the anaerobic reduction of azo dyes by *Sphingomonas* sp. strain BN6 and effect of artificial redox mediators on the rate of azo dye reduction. *Appl. Environ. Microbiol.* **63** : 3691-3694.
 30. **Laszlo, J.A.** 2000. Regeneration of azo-dye-saturated cellulosic anion exchange resin by *Burkholderia cepacia* anaerobic dye reduction. *Environ. Sci. Technol.* **34**:167-172.
 31. **Lovley, D.R., J.D. Coates, E.L. Blunt-Harris, E.J. P. Phillips, and J.C. Woodward.** 1996. Humic substances as electron acceptors for microbial respiration. *Nature.* **382** : 288-291.
 32. **Matsushita, K., A. Otofujii, M. Iwahashi, H. Toyama, and O. Adachi.** 2001. NADH dehydrogenase of *Corynebacterium glutamicum*. Purification of an NADH



- dehydrogenase II homolog able to oxidize NADPH. FEMS Microbiol. Lett. **204** : 271-276.
33. **Meganathan, R., and R. Coffell.** 1985. Identity of the quinone in *Bacillus alcalophilus*. J. Bacteriol. **164** : 911-913.
34. **Newman, D.K., and R. Kolter.** 2000. A role for excreted quinones in extracellular electron transfer. Nature **405** : 94-97.
35. **Nortemann, B., J. Baumgarten, H.G. Rast, and H.J. Knackmuss.** 1986. Bacterial communities degrading amino and hydroxynaphthalenesulfonates. Appl. Environ. Microbiol. **52** : 1195-1202.
36. **Nortemann, B., A.E. Kuhm, H.J. Knackmuss, and A. Stolz.** 1994. Conversion of substituted naphthalenesulfonates by *Pseudomonas* sp. BN6. Arch. Microbiol. **161** : 320-327.
37. **Ohe, T., and Y. Watanabe.** 1986. Degradation of 2-naphthylamine-1-sulfonic acid by *Pseudomonas* strain TA-1. Agric. Biol. Chem. **50** : 1419-1426.
38. **O'Neill, C., A. Lopez, S. Esteves, F.R. Hawkes, D.L. Hawkes, and S. Wilcox.** 2000. Azo-dye degradation in an anaerobic-aerobic treatment system operating on simulated textile effluent. Appl. Microbiol. Biotechnol. **53** : 249-254.
39. **O'Neill, C., F.R. Hawkes, D.L. Hawkes, S. Esteves, and S.J. Wilcox.** 2000. Anaerobic-aerobic biotreatment of simulated textile effluent containing varied ratios of starch and azo dye. Wat. Res. **34** : 2355-2361.
40. **Pagga, U., and D. Brown.** 1986. The degradation of dyestuffs: part II-behaviour of dyestuffs in aerobic biodegradation tests. Chemosphere **15** : 479-491.
41. **Raffi, F., and T. Coleman.** 1999. Cloning and expression in *Escherichia coli* of an azoreductase gene from *Clostridium perfringens* and comparison with azoreductase genes from other bacteria. J. Basic Microbiol. **39** : 29-35.
42. **Rau, J., H-J. Knackmuss, and A. Stolz.** 2002. Effects of different quinoid redox mediators on the anaerobic reduction of azo dyes by bacteria. Environ. Sci. Technol. **36** : 1497-1504.
43. **Rau, J. and A. Stolz.** 2003. Oxygen-insensitive nitroreductases NfsA and NfsB of *Escherichia coli* function under anaerobic conditions as lawsone-dependent azo reductases. Appl. Environ. Microbiol. **69** : 3448-3455.
44. **Razo-Flores, E., M. Luijten, B. Donlon, G. Lettinga, and J. Field.** 1997. Biodegradation of selected azo dyes under methanogenic conditions. Water Sci. Technol. **36** : 65-72.
45. **Roxon, J.J., A.J. Ryan, and S.E. Wright.** 1967. Enzymatic reduction of tartrazine by *Proteus vulgaris* from rats. Fd. Cosmet. Toxicol. **5** : 645-656.
46. **Russ, R., J. Rau, and A. Stolz.** 2000. The function of cytoplasmatic flavin reductases in the bacterial reduction of azo dyes. Appl. Environ. Microbiol. **66** : 1429-1434.
47. **Scott, D.T., D.M. McKnight, E.L. Blunt-Harris, S.E. Kolesar, and D.R. Lovley.** 1998. Quinone moieties act as electron acceptors in the reduction of humic substances by humics-reducing microorganisms. Environ. Sci. Technol. **32** : 2984-2989.
48. **Shaul, G.M., T.J. Holdsworth, C.R. Dempsey, and K.A. Dostal.** 1991. Fate of water soluble azo dyes in the activated sludge process. Chemosphere **22** : 107-119.
49. **Stevenson, F. J.** 1994. Humus chemistry: genesis, composition, reactions, 2nd ed. John Wiley & Sons, Inc., New York, N.Y.
50. **Stolz, A.** 2001. Basic and applied aspects in the microbial degradation of azo dyes. Appl. Microbiol. Biotechnol. **56** : 69-80.
51. **Supaka, N.** 2003. Microbial decolorization of reactive dyes in an anaerobic-aerobic treatment system, Ph.D. thesis. Chulalongkorn University, Bangkok, Thailand.
52. **Supaka, N., K. Juntongjin, M.L. Delia, P. Strehaiano, and S. Damronglerd.** Microbial decolorization of reactive azo dyes in a sequential anaerobic-aerobic system. Chem. Eng. J., in press.
53. **Tan, N., F.X. Prenafeta-Bold, J.L. Opsteeg, G. Lettinga, and J.A. Field.** 1999. Biodegradation of azo dyes in cocultures of anaerobic granular sludge with aerobic aromatic amine degrading enrichment cultures. Appl. Microbiol. Biotechnol. **51** : 865-871.
54. **Van der Zee, F.P., I.A.E. Bisschops, and G. Lettinga.** 2003. Activated carbon as an electron acceptor and redox mediator during the anaerobic biotransformation of azo dyes. Environ. Sci. Technol. **37** : 402-408.
55. **Ward, M.J., Q.S. Fu, K.R. Rhoads, C.H. J. Yeung, A.M. Spormann, and C.S. Criddle.** 2003. A derivative of the menaquinone precursor 1,4-dihydroxy-2-naphthoate is involved in the reductive transformation of carbon tetrachloride by aerobically grown *Shewanella oneidensis* MR-1. Appl. Microbiol. Biotechnol., in press.
56. **Wuhrmann, K., K. Mechsner, and T. Kappeler.** 1980. Investigation on rate-determining factors in the microbial reduction of azo dyes. Eur. J. Appl. Microbiol. **9** : 325-338.
57. **Yagi, T.** 1991. Bacterial NADH-quinone oxidoreductase. J. Bioenerg. Biomembr. **23** : 211-225.
58. **Yano, T.** 2002. The energy-transducing NADH: quinone oxidoreductase, complex I. Mol. Asp. Med. **23** : 345-368.
59. **Zee, V.F.P., G. Lettinga, and J.A. Field.**



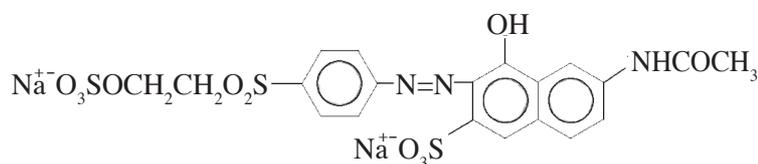
2000. The role of (auto) catalysis in the mechanism of an anaerobic azo reduction. *Water Sci. Technol.* **42** : 301-308.
60. **Zenno, S.H.K. Koike, A.N. Kumar, R. Jayaraman, M. tanokura, and K. Saigo.** 1996. Biochemical characterization of NfsA, the *Escherichia coli* majornitrore ductase exhibiting a high amino acid sequence homology to Frp, a *Vibrio harveyi* flavin oxidoreductase. *J. Bacteriol.* **178** : 4508-4514.
61. **Zenno, S., H. Koike, M. Tanokura, and K. Saigo.** 1996. Gene cloning, purification, and characterization of NfsB, a minor oxygen-insensitive nitroreductase from *E. coli*, similar in biochemical properties to FraseI, the major flavin reductase in *Vibrio fischeri*. *J. Biochem.* **120** : 736-744.
62. **Zollinger, H.** 1987. *Color Chemistry*, VCH Verlagsgesellschaft mbH, Weinham, FDR.

TABLE 1. Properties of sulfonated azo dyes used in this study.

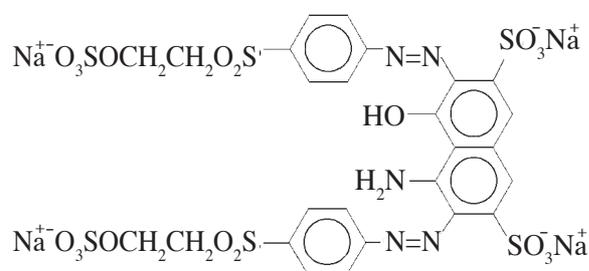
Azo dye	Generic name	C.I. number	λ_{\max} (nm)	Extinction coefficient ($\text{mM}^{-1} \text{cm}^{-1}$)
Remazol Brilliant Orange 3R ^a	reactive orange 16	17757	495	15.4
Remazol Brilliant Violet 5R ^a	reactive violet 5	18097	557	6.8
Remazol Black B ^a	reactive black 5	20505	595	29.1

^aThe dyes were provided by Dystar Thai Ltd., and not further purified. The absorption maxima and molar extinction coefficients were determined in Na-K phosphate buffer (50 mM; pH 7.5).

A. Remazol Brilliant Orange 3R



B. Remazol Black B



C. Remazol Brilliant Violet 5R

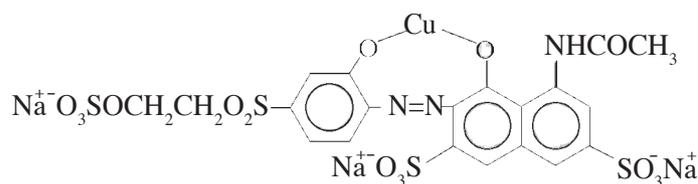


FIG. 1. Chemical structure of the sulfonated azo dyes used in this study.



TABLE 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 ($\mu\text{mol min}^{-1} \text{g of protein}^{-1}$) 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	2.4
	FAD	1.3	1.9	3.4
Remazol Brilliant Violet 5R	None	0.6	5.9	3.3
	AQDS	1.0	10.2	5.0
	FAD	0.7	6.4	7.0

^aThe 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.

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

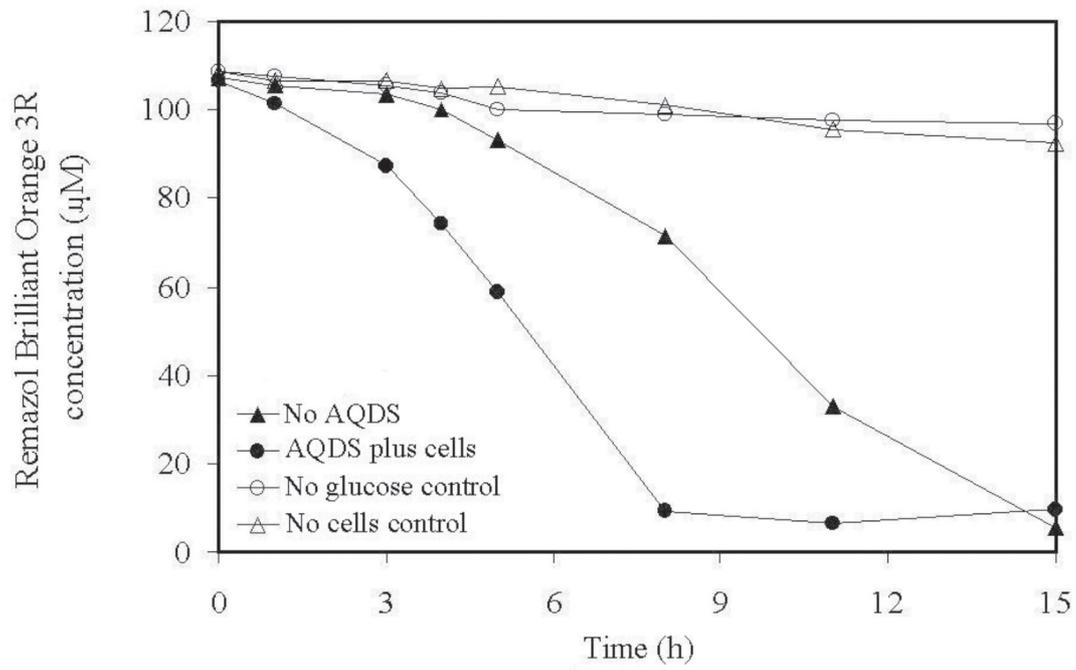
TABLE 3. Specific activities of quinone reductase and flavin reductase in membrane-bound and cell extract of *Paenibacillus* sp. strain A5.

Enzyme	Specific activity ($\mu\text{mol min}^{-1} \text{g of protein}^{-1}$) ^a	
	Membrane	Cell extract
Quinone reductase	81.8	8.8
Flavin reductase	6.8	72.1

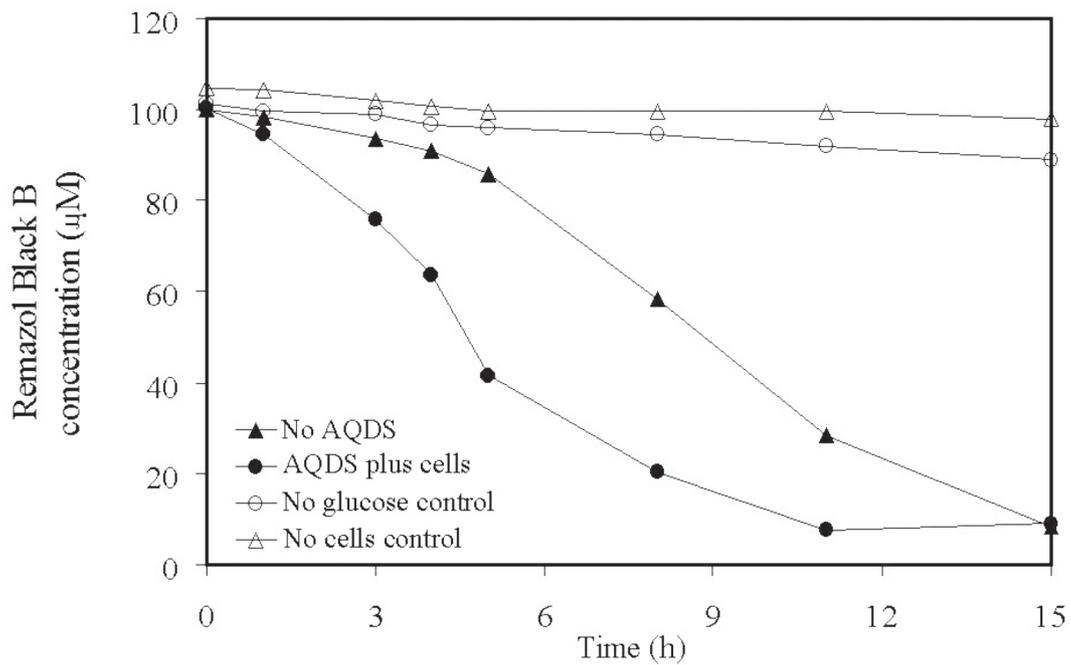
^aThe enzyme activities were determined spectrophotometrically as described in Materials and Methods.



A



B



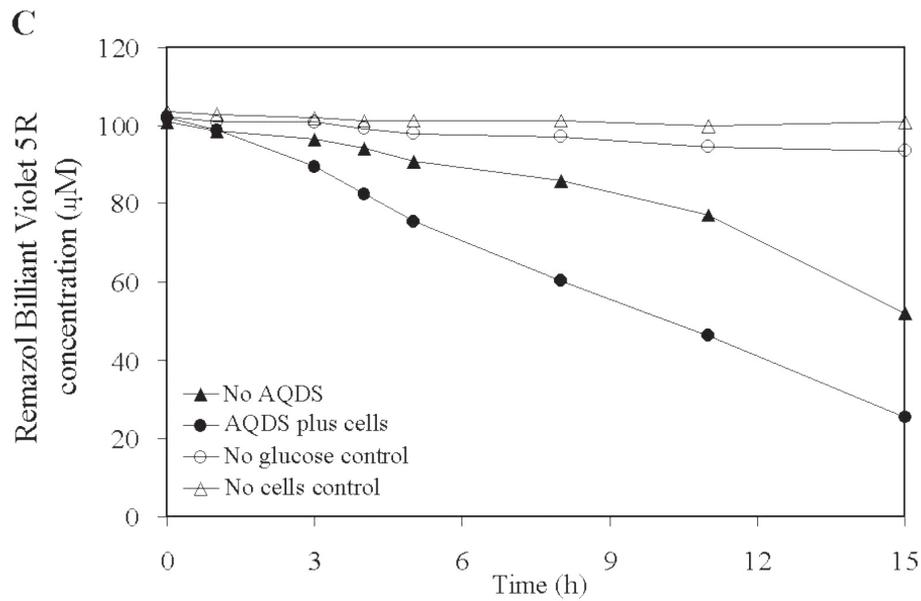


FIG. 2. Reduction of sulfonated azo dyes by *Paenibacillus* sp. strain A5 in the presence or absence of AQDS. The reduction of Remazol Brilliant Orange 3R (A), Remazol Black B (B), and Remazol Brilliant Violet 5R (C).

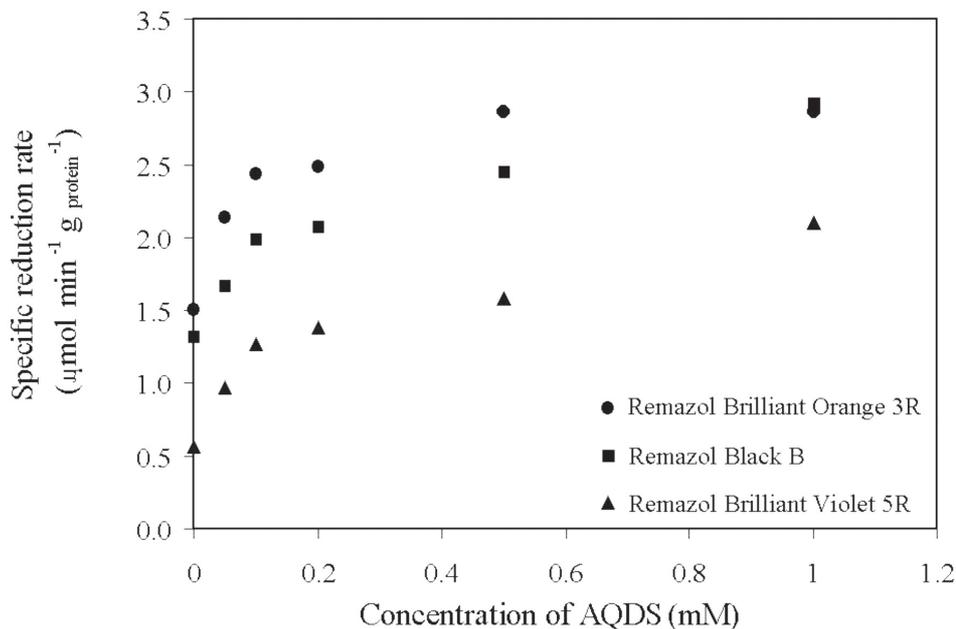


FIG. 3. Anaerobic reduction of sulfonated azo dyes in the presence of different concentrations of AQDS by whole cells of *Paenibacillus* strain A5. The cells of strain A5 were grown aerobically in a minimal medium with glucose (5 mM). When cell reached the lated-exponential growth phase, cells were harvested by centrifugation and resuspended anaerobically in Na-K phosphate buffer (pH 7.5; 50 mM). This cell suspension (protein content, 0.09 g l^{-1}) 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.

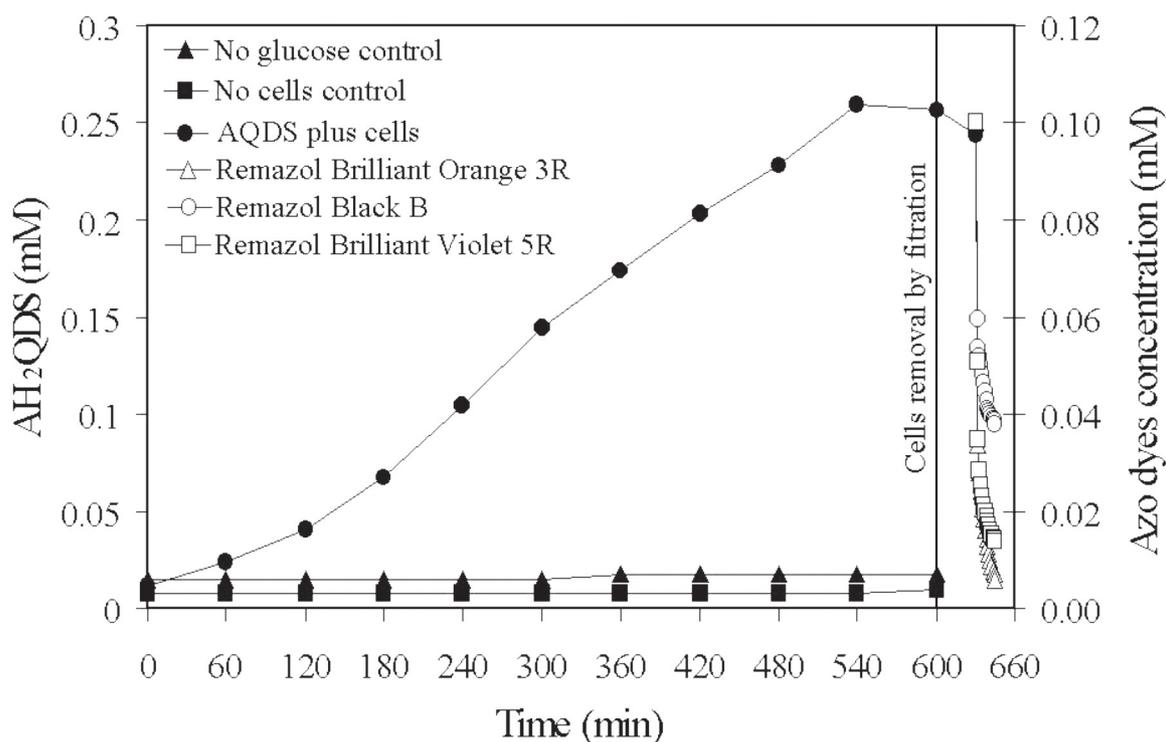


FIG. 4. Enzymatic reduction of AQDS to AH₂QDS by *Paenibacillus* strain A5 and subsequent chemical reduction of sulfonated azo dyes by AH₂QDS. Washed cells of *Paenibacillus* sp. strain A5 (0.09 g of protein l⁻¹) were suspended in 10 ml. Na-K phosphate buffer (50 mM; pH 7.5) containing glucose (5 mM) as the electron donor and AQDS (250 μM) as electron acceptor. The concentration of reduced AQDS (AH₂QDS) 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 100μM 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.

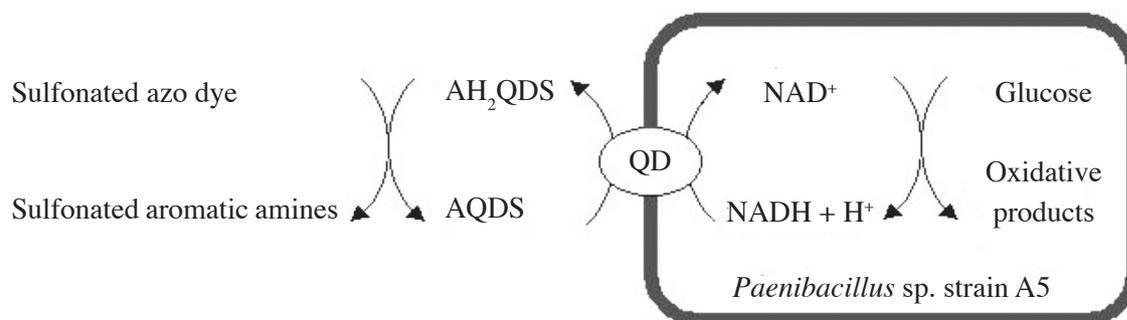


FIG. 5. Model for mechanism by which AQDS, a humic analogue stimulate sulfonated azo dyes reduction by *Paenibacillus* sp. strain A5

**บทคัดย่อ**

สารชีวмикช่วยเพิ่มความสามารถลดสีประเภทเอโซซัลโฟเนตในกระบวนการไร้อากาศด้วย *Paenibacillus* sp. Strain A5

สมศักดิ์ ดำรงค์เลิศ^๑, ณัฐพันธ์ ศุภกา^๒, กาญจนา จันทองจีน^๒, สุพัฒน์ เจริญพรวัฒนา^๒, มารีย์-ลีเนอ ดิลีญา และปีแอร์ สเตรเออาโน^๓

^๑ราชบัณฑิต สำนักวิทยาศาสตร์ ราชบัณฑิตยสถาน, ภาควิชาเคมีเทคนิค คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

^๒ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

^๓ Laboratoire de Génie Chimique, INP-ENSIACET, 5 rue Paulin Talabot Site Basso Combo-BP 1301, Toulouse 31106, France

สารชีวмикควิโนน แอนทราควิโนน ๒, ๖-ไดซัลโฟเนต (AQDS) สามารถทำหน้าที่เป็นสารรีดอกซ์ที่คล้ายกับสารที่ผลิตโดยแบคทีเรียชนิดไม่ใช้ออกซิเจน *Paenibacillus* sp. strain A5 ซึ่งสามารถทำลายสารสีประเภทเอโซ สารสีชนิดนี้ใช้มากในอุตสาหกรรมการย้อมผ้า จากการศึกษาการแบ่งเซลล์ของแบคทีเรีย strain A5 พบว่า สารเอนไซม์ที่ผลิตได้มีอยู่ที่ผนังเซลล์และที่ไซโทพลาสซึม สารที่ติดผนังเซลล์สามารถย่อยสารซัลโฟเนตเอโซได้เมื่อมีสารจำพวกควิโนนร่วมอยู่ด้วย ดังนั้น การประยุกต์ใช้สาร AQDS เพื่อย่อยสีเอโซที่มีอยู่ในน้ำเสียด้วยระบบไร้อากาศจึงมีความเป็นไปได้สูง

คำสำคัญ : ซัลโฟเนตเอโซ, รีดักเทสเอโซ, แอนทราควิโนน ๒, ๖-ไดซัลโฟเนต, *Paenibacillus* sp.