Biological and chemical mechanisms of reductive decolorization of azo dyes

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A completion indicates only a new beginning.

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This thesis is dedicated to Mi Kyung.

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Biologische und chemische Mechanismen der reduktiven Entfärbung von Azofarbstoffen

Abstract:

Die Entfärbungsmechanismen von Azofarbstoffen wurden unter anaeroben Bedingungen untersucht. Sulfatreduzierende Bakterien in anaerober Mischkultur sind zum großen Teil an der Entfärbung von C. I. Reactive Orange 96 beteiligt, da diese, wie auch andere Reaktivfarbstoffe, nach der Hydrolyse einen hohen Sulfatgehalt aufweisen. Methanogene Bakterien hatten hingegen keinen signifikanten Anteil an der Entfärbung. Die Reinkultur von *Desulfovibrio desulfuricans* entfärbte C. I. Reactive Orange 96 und C. I. Reactive Red 120 sowohl bei Anwesenheit von Sulfat als auch bei Mangel an Sulfat. Die sulfatreduzierenden Bakterien produzieren bei der Sulfat-Atmung Sulfid. Das Sulfid spaltet die Azobrücke rein chemisch. Dabei überwiegt je nach pH-Wert die Reaktion durch H_2S, HS^- oder S^{2-}. Bei Verwendung von Pyruvat und unter sulfatarmen Bedingungen werden vermutlich die bei der Pyruvat-Fermentation freigesetzten Elektronen durch Coenzyme zu Azofarbstoffen als terminale Elektronakzeptoren übertragen. Dies führt zur Entfärbung der Farbstoffe. Die chemische Entfärbung von C. I. Reactive Orange 96 durch Sulfid zeigte eine Kinetik *erster* Ordnung hinsichtlich der Farbstoff- und Sulfidkonzentration. Sie erfolgt bei neutralem oder basischem pH schneller als bei sauerem pH. Dies ist auf eine Zunahme des Anteils von HS^- bei neutralem oder basischem pH zurückzuführen. Die Aufdeckung der biologischen und chemischen Reaktionsmechanismen sowie die Beschreibung ihrer Kinetik können hilfreich sein bei der Entwicklung von Verfahren zur Behandlung farbstoffhaltiger Abwasserteilströme der Textilveredelung.

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NOMENCLATURE

Symbol	Meaning	Unit
c_B	bacteria concentration as total suspended solids (TSS)	g/L TSS
c_D	dye concentration	mmol/L, mg/L
c_{DOC}	DOC concentration	mg/L
c_{H+}	proton concentration	mol/L
c_{H2S}	concentration of H_2S	mmol/L, mol/L
c_{HS-}	concentration of HS^-	mmol/L, mol/L
c_i	concentration of inhibitor	mmol/L
c_{MoO4}	molybdate concentration	mmol/L
c_{ox}	concentration of oxidated form of a redox pair in solution	mol/L
c_{RA}	concentration of reducing agent	mmol/L
c_{RE}	concentration of reduction equivalents	mmol/L
c_{red}	concentration of reduced form of a redox pair in solution	mol/L
c_S	auxiliary substrate concentration	mmol/L
c_{S2-}	concentration of S^{2-}	mmol/L, mol/L
$c_{S(II)}$	concentration of sulfide species; $c_{S2-} + c_{HS-} + c_{H2S}$	mmol/L, mol/L
c_{SO4}	sulfate concentration	mmol/L, mg/L
E	redox potential of a redox pair	V, mV
ΔE	difference of redox potentials of two redox pairs building a redox reaction	V
E_0	standard redox potential of a redox pair	V, mV
E_0'	redox potential for a redox pair at pH 7 and 25°C	V, mV
F	Faraday constant; $9.648 \cdot 10^4$	C mol^{-1}
ΔG	difference of free energy of two redox pairs	J/mol, kJ/mol
$\Delta G_o'$	difference of free energy of two redox pairs at pH 7 and 25°C	kJ/mol
HRT	hydraulic retention time in continuous-flow reactor	h
k	rate constant for decolorization	L $mmol^{-1}$ min^{-1}, L $mmol^{-1}$ h^{-1}
k_0	rate constant of H_2S for decolorization	L $mmol^{-1}$ min^{-1}
k_1	rate constant of HS^- for decolorization	L $mmol^{-1}$ min^{-1}
k_2	rate constant of S^{2-} for decolorization	L $mmol^{-1}$ min^{-1}
K_1	dissociation constant of sulfide	mol/L
K_2	dissociation constant of sulfide	mol/L
k´	rate constant for decolorization	min^{-1}, h^{-1}
k´´	rate constant for decolorization	min^{-1}, h^{-1}
k_{asc}	rate constant for decolorization with ascorbate	L $mmol^{-1}$ h^{-1}

Symbol	Meaning	Unit
k_{cys}	rate constant for decolorization with cysteine	L $mmol^{-1}$ h^{-1}
K_i	coefficient for inhibition by molybdate	$mmol^{-1}$ L
K_{Lac}	saturation coefficient for lactate as substrate	mmol/L, µmol/L
K_{pyr}	saturation coefficient for pyruvate	mg/L
K_S	saturation coefficient for substrate	mmol/L
K_{SO4}	saturation coefficient for sulfate	mmol/L, µmol/L
m	number of protons	
m	mean value	
n	number of measured values	
Q	$k \mu_{max} Y_{S2-/S} c_B Y_{B/S}^{-1}$	
R	gas constant; 8.314	J mol^{-1} K^{-1}, atm L mol^{-1} K^{-1}
r_D	decolorization rate	mmol L^{-1} h^{-1}, mmol L^{-1} min^{-1}
$r_{D,max}$	maximal decolorization rate witout molybdate	mg L^{-1} h^{-1}, mmol h^{-1} L^{-1}
r_{DM}	decolorization rate under influence of molybdate	mg L^{-1} h^{-1}, mmol h^{-1} L^{-1}
r_S	rate of substrate degradation	mmol h^{-1} L^{-1}
S	standard deviation	
t	time	h, min
t	coefficient of Student´s *t*-distribution	
T	temperature	K, °C
V_R	reactor volume in liquid phase	L
$Y_{Ac/SO4}$	yield coefficients of acetate on sulfate	mol/mol
$Y_{B/S}$	yield coefficient of bacteria on substrate	mol/mol, g/g
$Y_{B/Lac}$	yield coefficient of bacteria on lactate	g-cell/g
$Y_{B/Pyr}$	yield coefficient of bacteria on pyruvate	g-TSS/g, g-cell/g
$Y_{B/SO4}$	yield coefficient of bacteria on sulfate	g-TSS/mol, g-TSS/g, g-cell/g
$Y_{D/asc}$	yield coefficient of dye (reduction) on ascorbate	mol/mol
$Y_{D/cys}$	yield coefficient of dye (reduction) on cysteine	mol/mol
$Y_{D/RE}$	yield coefficient of dye (reduction) on reduction equivalent	mol/mol
$Y_{D/S(II)}$	yield coefficient of dye (reduction) on sulfide	mmol/mmol

Symbol	Meaning	Unit
$Y_{D/S}$	yield coefficient of dye (reduction) on substrate	mmol/mmol, mg/mg
$Y_{D/S2-}$	yield coefficient of dye (reduction) on sulfide	mol/mol
$Y_{RE/S}$	yield coefficient of reduction equivalent on substrate	mol/mol
$Y_{S2-/S}$	yield coefficient of sulfide on substrate	mol/mol
$Y_{SO4/Lac}$	yield coefficient of sulfate (reduction) on lactate	mol/mol
$Y°_{Ac/S}$	real yield coefficient of acetate on lactate as substrate	mol/mol
$Y°_{B/S}$	real yield coefficient of bacteria on lactate	mol/mol
$Y°_{CO2/S}$	real yield coefficient of CO_2 on lactate	mol/mol
$Y°_{H2O/S}$	real yield coefficient of H_2O on lactate	mol/mol
$Y°_{NH3/S}$	real yield coefficient of NH_3 (degradation) on lactate	mol/mol
$Y°_{S2-/S}$	real yield coefficient of S^{2-} on lactate	mol/mol
$Y°_{SO4/S}$	real yield coefficient of SO_4^{2-} (reduction) on lactate as substrate	mol/mol
z	number of exchanged electrons	
α	$100 \cdot (1 - c_D/c_{D,0})$, decolorization degree	%
α_0	fraction of H_2S of the total sulfide species; $(1 + K_1 c_{H+}^{-1} + K_1 K_2 c_{H+}^{-2})^{-1}$	
α_1	fraction of HS^- of the total sulfide species; $(c_{H+} K_1^{-1} + 1 + K_2 c_{H+}^{-1})^{-1}$	
α_2	fraction of S^{2-} of the total sulfide species; $(c_{H+}^2 K_1^{-1} K_2^{-1} + c_{H+} K_2^{-1} + 1)^{-1}$	
β	$(c_{RE,0} - Y_{D/RE}^{-1} c_{D,0})^{-1} \ln\{(c_{D,0} c_{RE})/(c_D c_{RE,0})\}$; equation for evaluation of kinetic model	
γ	$(c_{D,0} - c_D)/(c_D c_{RE,0})$; equation for evaluation of kinetic model	
μ_{max}	maximum specific growth rate	h^{-1}
μ	specific growth rate	h^{-1}

Index

a	exponent indicating reaction order with respect to concentration of dye
b	exponent indicating reaction order with respect to concentration of reduction equivalent
0	index indicating the starting condition with respect to concentration or the influent concentration
1	index indicating effluent concentration

Abbreviation

AMP	adenosine-5´-monophosphate	
ATP	adenosine-5´-triphosphate	
BES	2-bromoethanesulfonic acid	
c_3	tetraheme cytochrome	
COD	chemical oxygen demand	
COD/S ratio	ratio of COD of substrate to sulfate-sulfur concentration	(g/g)
D. desulfuricans	*Desulfovibrio desulfuricans*	
D. vulgaris	*Desulfovibrio vulgaris*	
DOC	dissolved organic carbon	
FAD	flavin adenine dinucleotide	
Fd	ferredoxin	
Fd^-	reduced form of ferredoxin	
$Fe(OH)_2$	iron dihydroxide	
FMN	flavin mononucleotide	
LFB	lactate-utilizing fermentative bacteria	
MPB	methane producing bacteria	
$NAD(P)^+$	nicotinamide adenine dinucleotide (phosphate)	
RA	reducing agent (candidate)	
RE	reduction equivalent	
R-N=N-R´	a mono-azo dye	
$R\text{-}NH_2$	a reduction product of a mono-azo dye R-N=N-R´	
$R´\text{-}NH_2$	a reduction product of a mono-azo dye R-N=N-R´	
RO 96	C. I. Reactive Orange 96	
RR 120	C.I. Reactive Red 120	
S(II)	sulfide species including H_2S, HS^-, and S^{2-}	
SRB	sulfate reducing bacteria	
TSS	total suspended solids	

1. INTRODUCTION

Textile finishing has a strong impact on the aquatic environment. The consumption of water by the textile finishing industry in Germany was reported as approximately 65 million m^3 for the year 1992 (TVI 1994). The average specific discharge of wastewater in the German textile finishing industry in 1993 amounted to 115 m^3/ton-textile material, in which the wastewater contained high contents of inorganic and organic substances as dyes, textile additives, and basic chemicals (Schönberger and Kaps 1994). Azo dyes are used to a great extent in textile finishing, and have become of concern in wastewater treatment because of their color, bio-recalcitrance, and potential toxicity to animals and humans (Levine 1991). Thus the wastewater with azo dyes must be decolorized and furthermore mineralized in appropriate systems combining biological and chemical processes.

Biological treatment of textile wastewater has the advantage of cost-effectiveness. The anaerobic treatment of textile wastewater can reduce the high level of COD of the wastewater (Schönberger and Kaps 1994) and decolorize a variety of dyes (Brown and Laboureur 1983). Treatment schemes for colored wastewater based on the anaerobic bacterial reduction of the azo bond are being used by the textile industry, as well as being studied by many groups (Walker 1970; Wuhrmann et al. 1980; Chung and Stevens, 1993). Azo dyes can also be reduced chemically, e.g. by dithionite (Weber and Adams 1995). Treatment based on chemical reduction, however, usually leads to an unwanted increase in salinity of the wastewater, or an increase in sludge production. Moreover, the major drawback of both bacterial and chemical reduction is that only azo dyes can be reduced and the dye metabolites, the aromatic amines, are not removed. This has led to the development of two-stage (anaerobic and subsequent aerobic) bacterial treatment, in which the dye metabolites are hopefully mineralized in the aerobic stage.

Analogous to the reductive decolorization, the oxidative decolorization of azo dyes can also be carried out both biologically and chemically. Chemical oxidants, i.e. ozone or hydrogen peroxide with UV-light, or Fenton´s reagent, can decolorize most classes of textile dyes, and, depending on the dose, can mineralize them. The photocatalysis using UV-light with TiO_2 or ZnO as catalysts enables decolorization, and transformation or mineralization of several azo dyes (Peralta-Zamora et al. 1999; Hu and Wang 1999). Azo dyes have rarely been decolorized under aerobic conditions by bacteria (Zimmermann et al. 1982; Idaka et al. 1978, 1987). The aerobic decolorization of a number of textile dyes, mainly azo dyes, by different species of white rot fungi has been reported (Ollikka et al. 1993; Swamy and Ramsay 1999a, 1999b). Decolorization of some azo dyes by white rot fungi cultivated under sterile conditions produces metabolites, which can partly be mineralized by aerobic bacteria. However, the

establishment of an unsterile treatment system utilizing the nonspecific enzymes of white rot fungi fails due to a higher growth rate of bacteria (Borchert et al., in preparation).

This research presents an overview of the known bacterial mechanisms for decolorization, and then concentrates on disclosing new mechanisms active in the decolorization of azo dyes, describing the role of diverse groups of bacteria and the chemical kinetic aspects of decolorization. From the engineering point of view, this research hopefully serves in developing effective means to control and improve the decolorization process in textile wastewater treatment systems.

2. BASICS OF DECOLORIZATION OF AZO DYES AND OBJECTIVES OF RESEARCH

2.1. Redox Potential and Decolorization

Redox processes describe a process of electron exchange. With donating electrons an oxidation occurs, in turn a reduction takes place by accepting electrons, as seen in the equation (Eq.)(1) for a redox half reaction of a redox pair.

$$\text{Reduced Form (Red)} \underset{\text{Reduction}}{\overset{\text{Oxidation}}{\rightleftharpoons}} \text{Oxidized Form (Ox)} + ze^- \qquad (1)$$

where z indicates the number of exchanged electrons; the charge balance is assumed in Eq.(1), in that the Oxidized Form has higher oxidation state than Reduced Form via electron donation.

An oxidation or a reduction cannot occur in isolation. A redox reaction, i.e. a reaction with coupled oxidation and reduction, must necessarily involve two redox pairs. The redox potential is an electrochemical measuring degree which determines the capability of a redox pair either to donor or accept electrons. The hydrogen electrode serves as a standard for zero point. The redox potential E for a redox pair is described with the following Nernst´s equation (Riedel 1994):

$$E = E_0 + \frac{R\,T}{z\,F} \ln (c_{ox}/c_{red}) \qquad (2)$$

where z number of exchanged electrons

c_{ox} concentration of oxidized form of a redox pair in solution (mol/L)

c_{red} concentration of reduced form of a redox pair in solution (mol/L)
E_0 standard redox potential of a redox pair (V)
E redox potential at T K dependent on the concentrations of oxidized and reduced form of a redox pair (V)
R 8.314 J mol^{-1} K^{-1}; gas constant
T absolute temperature (K)
F $9.648 \cdot 10^4$ C mol^{-1}; Faraday constant

From Eq.(2), $E=E_0$ follows for $c_{ox}=c_{red}$. The redox potential E at 25 °C can be calculated practically using the following Eq. with decadic logarithm (Riedel 1994):

$$E = E_0 + \frac{0.059}{z} \log (c_{ox}/c_{red}) \quad (3)$$

The free energy change ΔG of a redox half reaction is related to the redox potential E according to the following Eq. (Zehnder and Stumm 1988):

$$\Delta G = - z F E \quad (4)$$

Logically the difference of redox potentials ΔE of two redox pairs can be associated with the difference of free energy ΔG of the two pairs.

$$\Delta G = - z F \Delta E \quad (5)$$

where ΔE difference of redox potentials of two redox pairs building a redox reaction (V)
ΔG difference of free energy of the two redox pairs (J/mol), assuming ΔG = ΔG (for one redox pair) −ΔG (for the other redox pair)

The difference of the redox potentials is authoritative for the degree of free energy to be gained between two redox half reactions. A chemical redox reaction occurs spontaneously, if $\Delta G<0$. This indicates that a redox process is thermodynamically feasible, if a redox pair with a more negative redox potential reduces another redox pair. The free energy gainable between the two redox half reactions is the same, irrespective of the intermediate courses e.g. in the case of bacterial metabolisms. In various redox processes pH gets involved. The redox half reaction of pH-dependent equilibrium can be expressed in general as following:

$$\text{Reduced Form (Red)} \longrightarrow \text{Oxidized Form (Ox)} + mH^+ + ze^- \quad (6)$$

where m number of protons (H^+)

The redox potential E at 25 °C for Eq.(6) involving pH-influence can be estimated as below (Riedel 1994):

$$E = E_0 + \frac{0.059}{z} \log (c_{ox} \cdot c_{H+}{}^{m} / c_{red}) \quad (7)$$

where c_{H+} concentration of proton (mol/L)

The redox potential at pH 7 (i.e. $c_{H+}=10^{-7}$ mol/L) and 25 °C for $c_{ox}=c_{red}$, i.e. E_0', is significant as a standard for biological systems, and can be defined from Eq.(7) as follows:

$$E_0' = E_0 + \frac{0.059}{z} \log (10^{-7m}) \quad (8)$$

where E_0' redox potential at pH 7 and 25 °C for $c_{ox}=c_{red}$ (V)

From Eq.(8), it follows that $E_0'=E_0$ for m=0 (i.e. without pH-influence). The difference of redox potentials of two redox pairs $\Delta E_0'$ can be linked with the difference of free energy $\Delta G_0'$ of the two pairs.

$$\Delta G_0' = - z \; F \; \Delta E_0' \quad (9)$$

where $\Delta E_0'$ difference of redox potentials of two redox pairs at pH 7 and 25 °C (V)
$\Delta G_0'$ difference of free energy of the two redox pairs at pH 7 and 25 °C (J/mol)

The redox potential also depends on the complex-building of a redox pair with other inorganics or organics.

The redox potential sequence is significant for decolorization of azo dyes. That both electron carriers (coenzymes) FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide) in reduced form are capable of reducing an azo dye, e.g. C. I. Reactive Orange 96 (RO 96) can be derived from the order of the redox potentials (Table 1). If the chemical reduction hypothesis for the decolorization of azo dyes in biological systems (Dubin and Wright 1975; Gingel and Walker 1971) is valid, the reaction of $FADH_2$ or $FMNH_2$ with the dye RO 96 has no advantage from the viewpoint of thermodynamics in comparison with some reducing agents (e.g. HS^-). That is, E_0'=-270 mV for S^0/HS^- and E_0'=-220 mV for $FAD/FADH_2$ and $FMN/FMNH_2$ (Table 1). Therefore, the chemical decolorization of azo dyes may also be expected from sulfide (HS^-, H_2S) and other reducing agents with a large negative redox potential, e.g. cysteine, titanium(III) citrate, and dithionite (Table 1). Logically, not only the

thermodynamics, but also the availability of the reducing agents and the reduction kinetic coefficients must be considered as the rate controlling factor in the decolorization.

Table 1. Redox half potentials for several biochemical redox reactions at pH 7 and 25 °C

Half reaction (oxidized form/ reduced form)	E_0' (mV)	Transfer of
$2SO_3^{2-}$/ $S_2O_4^{2-}+2H_2O$ [a]	-574	$2e^- + 4\ H^+$
Ti(IV)·citrate/ Ti(III)·citrate [d]	-480	e^-
cystine/ 2 cysteine [b]	-340	$2e^- + 2\ H^+$
NAD^+/ $NADH+H^+$ [b]	-320	$2e^- + 2\ H^+$
S°/ HS^- [b]	-270	$2e^- + H^+$
S°/ H_2S [c]	-250	$2e^- + 2\ H^+$
FAD/ $FADH_2$ [b]	-220	$2e^- + 2\ H^+$
FMN/ $FMNH_2$ [b]	-220	$2e^- + 2\ H^+$
RO 96 [e]/ two aromatic amines	-133	$4e^- + 4\ H^+$

[a] Calculated for pH 7 from Huheey (1988).
[b] Karlson (1994).
[c] Gottschalk (1986).
[d] Zehnder and Wuhrmann (1976).
[e] RO 96: C.I. Reactive Orange 96; The redox potential for the dye was measured with a pulspolarography and is listed as mean value (Schmid 1994).

2.2. Decolorization Mechanisms of Azo Dyes

According to the existing hypotheses for anaerobic decolorization of azo dyes by bacteria, the reduction equivalents generated by the oxidation of auxiliary substrates, i.e. organic carbon complexes, as electron donor via $NAD(P)^+$ [nicotinamide adenine dinucleotide (phosphate)] reduce the azo bond, to form aromatic amines as colorless metabolites (Roxon et al 1967; Chung and Stevens 1993). These hypotheses vary in respect to the emphasis placed on the involvement of azo reductase (cytoplasmic enzyme) or on the final reduction mechanism. Recent studies have introduced some extracellular non-enzymatic redox mediators into a possible anaerobic reduction scheme for organic or inorganic molecules such as dioxins, Fe(III)-oxide, or azo dyes (Adriaens et al. 1996; Lovley et al. 1996; Keck et al. 1997). An overview of known bacterial mechanisms for decolorization is summarized in Table 2.

Table 2. Summary of known bacterial mechanisms for the decolorization of azo dyes

Culture		Cond.*	Hypothesis	Sulfate	Author
Species	Classification				
Clostridium sp. *Butyrivibrio* *Eubacterium*	obligate anaerobe (isolated from human intestinal microflora) fermentative	An	Azo reductase with/without cofactors♥ (extracellular)	–**	Rafii et al. (1990)
Proteus vulgaris	facultative anaerobe (isolated from rat-intestinal microflora) probably fermentative	An	Azo reductase ($NADPH+H^+$-dependent FMN-flavoprotein) with cofactors	–	Roxon et al. (1967)
diverse bacteria	facultative anaerobe (fermentative) etc.	An	Azo reductase with cofactors	–	Chung and Stevens (1993)♣
Pseudomonas KF46	aerobe	Ae	Azo reductase; $NAD(P)H+H^+$	–	Zimmermann et al. (1982)
Bacillus cereus *Sphaerotilus natans* activated sludge	facultative anaerobe obligate aerobe	An Anox An	$FADH_2$ (intracellular)	–	Wuhrmann et al. (1980)
Mixed culture from an anaerobic digester		An	$FADH_2$ or $FMNH_2$	0/5/10 mM	Carliell et al. (1995)
Proteus vulgaris	facultative anaerobe	An	electron carrier supposed as $FADH_2$ or $FMNH_2$ (extracellular)	–	Dubin and Wright (1975)
Streptococcus faecalis	facultative anaerobe fermentative	An	$FMNH_2$ $FMNH_2$ riboflavin (coupled with azoreductase; probably intracellular)	–	Gingell and Walker (1971)
Sphingomonas BN6	probably facultative anaerobe	An	Redox mediators (1,2-dihydroxy-naphtalene or its decomposition products)	–	Keck et al. (1997)
Mixed culture	facultative anaerobes included	An	intracellular (probably $NADH+H^+$; $FADH_2$)	Yes	Liebelt (1997)
Mixed culture	facultative anaerobes included	An Anox	$NADH+H^+$; $FADH_2$	0/10/20 g/L	Gläser (1992)

* An: anaerobic conditions; Anox: anoxic conditions; Ae: Aerobic conditions.

** not available, or not relevant to the culture.

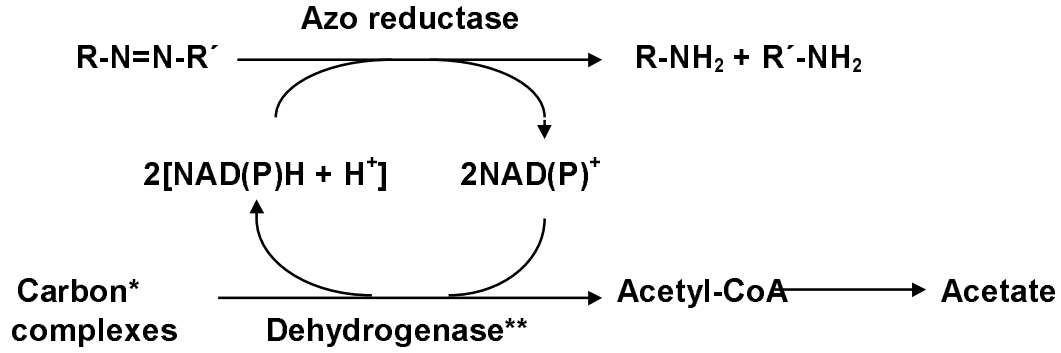
♥ cofactors: FAD, FMN, and riboflavin.

♣ Literature review; the aerobic reduction of azo dyes by some bacteria was also reported.

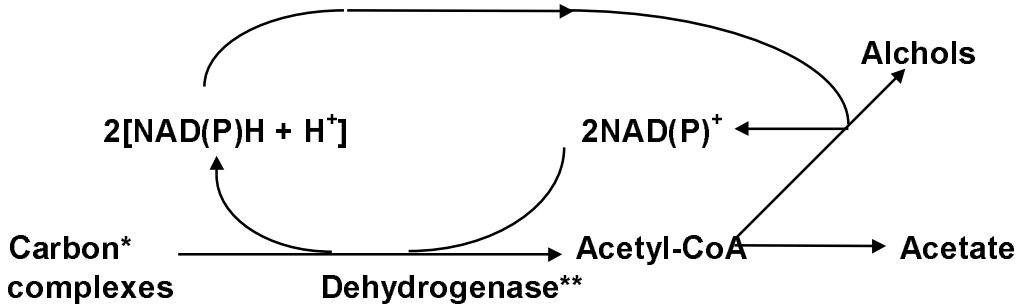
2.2.1. Hypothesis of decolorization of azo dyes by azo reductase or coenzymes

Hypothesis of decolorization of azo dyes by azo reductase

Various authors (Rafii et al. 1990; Roxon et al. 1967; Chung and Stevens 1993) have emphasized the necessity of the involvement of azo reductase in the decolorization of azo dyes [Fig. 1(a)], chiefly assuming electron carriers (coenzymes) flavin nucleotides (FMN, FAD) or riboflavin as cofactors. Although a lot of the microorganisms reported to produce the azo reductase are facultative anaerobic bacteria (Chung and Stevens 1993), some obligate anaerobic bacteria (e.g. *Butyrivbrio* sp., *Clostridium* sp.) producing azo reductase have been isolated from human intestinal microflora (Rafii et al. 1990). Notably, Zimmermann et al. (1982) purified an oxygen-insensitive azo reductase from an aerobic bacterium *Pseudomonas* KF46. The azo reductase reduction hypotheses are based mostly on an intracellular reduction process, whereas Rafii et al. (1990) found an extracellular release of azo reductases by some strains of human intestinal microflora.



(a)



(b)

Fig. 1. Hypothesis for anaerobic decolorization of azo dyes (R-N=N-R´: mono-azo dye) by azo reductase (modified from Roxon et al 1967) **(a)** and general fermentation pathway without azo dyes (modified from Dofling 1988) **(b)**: $R\text{-}NH_2$ and $R´\text{-}NH_2$ are metabolites as the reduction products of the azo dyes. *For example, glucose. **Enzyme liberating (transmitting) electrons (or $e^- + H^+$) from the carbon complexes.

A question what advantages bacteria could obtain from anaerobic decolorization is of significance. Roxon et al. (1967) suggested, on the basis of studies of reduction of an azo food dye with facultative anaerobic bacteria *Proteus vulgaris*, that azo dyes act under anaerobic conditions as alternative terminal electron acceptors for $NAD(P)^+$-dependent flavoenzymes which are normally involved in cellular electron transport [Fig. 1(a)]. Fermentative bacteria (e.g. degrading glucose) usually use a part of organic compounds (e.g. acetyl-CoA) generated during the fermentation processes as terminal electron acceptors, reducing the organics to e.g. ethanol [Fig. 1(b)]. Azo dyes are expected to be reduced instead of e.g. acetyl-CoA, leading to more oxidized catabolic products (e.g. acetate) coupled to an ATP (adenosine-5´-triphosphate)-gain. In summary, bacteria can possibly utilize azo dyes as electron acceptors in order to maximize the energy gain.

Hypothesis of simple chemical reduction of azo dyes by coenzymes

Other works have focused on the final reaction consisting of a simple chemical reduction of azo dyes by electron carriers (coenzymes) (Wuhrmann et al. 1980; Dubin and Wright 1975; Gingell and Walker 1971) (Fig. 2). Wuhrmann et al. (1980) proposed an intracellular chemical reduction of azo dyes by reduced flavin nucleotides ($FADH_2$), stressing that the rate of permeation of the dyes through the cell membrane might be an important rate-controlling step in the intracellular reduction, and the sulfonic acid substitution of the azo dye structure seemed to be an effective inhibitor of permeation. Furthermore Dubin and Wright (1975) hypothesized a chemical decolorization involving an extracellular electron carrier. In view of the mechanism of electron transport in bacterial metabolism, it is difficult to separate the function of enzyme and coenzyme. Although decolorization may be realized by the final chemical reduction with reduced coenzymes ($FADH_2$, $FMNH_2$), the coenzymes depend on cytoplasmic reducing enzymes to supply electrons. This is supported by Gingell and Walker (1971) who regarded the soluble flavin as an electron shuttle between a dye and a NADH-dependent azo reductase. In addition, it does not seem that FAD or FMN functions extracellularly in bacterial systems with intact cells.

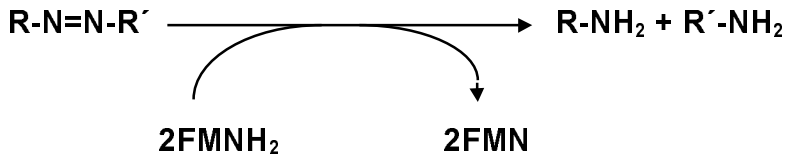


Fig. 2. Hypothesis for simple chemical decolorization of azo dyes (R-N=N-R´: mono-azo dye) by coenzymes ($FMNH_2$; $FADH_2$) under anaerobic conditions (modified from Gingell and Walker 1971)

Little has been reported about the potential for sulfate reducing bacteria (SRB) to decolorize azo dyes. While a direct study of decolorization with SRB was lacking, some authors delivered indirect information about the influence of sulfate level on anaerobic decolorization of azo dyes (Carliell et al. 1995; Glässer 1992). Carliell et al. (1995) found no significant difference in the decolorization rate of the azo dye C.I. Reactive Red 141 for two sulfate concentrations (5, 10 mmol/L) with a mixed population of bacteria from an anaerobic digestor. Glässer (1992) reported the inhibition of decolorization of C.I. Mordant Yellow 3 in the presence of 10 and 20 g/L Na_2SO_4 in the bacterium consortium with facultative anaerobic bacteria.

2.2.2. Hypothesis of decolorization of azo dyes by extracellular non-enzymatic redox mediators

An extracellular redox mediator in the form of humic substances (no coenzyme) can be used as an electron shuttle by Fe(III)-reducing bacteria to reduce Fe(III)-oxide (Lovley et al. 1996). That is, the microbially reduced humic substances can abiotically transfer electrons to Fe(III) oxides. The authors also suggested that the humic substances permits Fe(III)-reducing microorganisms to indirectly reduce Fe(III) oxides faster than Fe(III) is reduced in absence of humic substances because it alleviates the need for the microorganisms to come into direct physical contact with Fe(III) oxides in order to reduce them. Although this is not directly applicable to the azo dye reduction, the principal mechanism of the redox mediator is worthy of remark.

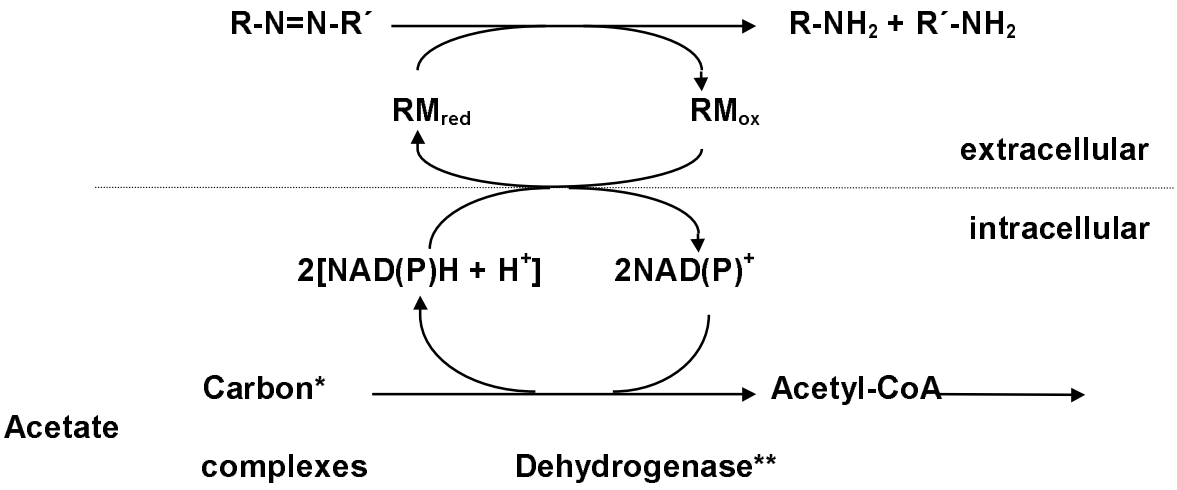


Fig. 3. Hypothesis for anaerobic decolorization of azo dyes (R-N=N-R´: mono-azo dye) by extracellular non-enzymatic redox mediators (modified from Keck et al. 1997): *Auxiliary substrate (glucose). RM_{red} and RM_{ox} indicate a redox mediator in reduced and oxidized form respectively. **Enzyme liberating electrons (or $e^- + H^+$) from the carbon complexes.

Keck et al. (1997) proposed an anaerobic non-enzymatic cleavage of azo bonds by extracellular redox mediators (no coenzymes) produced naturally during the aerobic metabolism of a xenobiotic compound (2-naphthalenesulfonate) by a strain of *Sphingomonas* sp. (Fig. 3). Thus other possible redox mediators or reduction equivalents formed gratuitously during various metabolisms of different bacteria may be involved in the azo dye reduction. Analogous to the azo reductase reduction hypothesis [Fig. 1(a)], redox mediators are expected to contribute to an ATP-gain e.g. from acetate production by bacteria (Fig. 3).

2.2.3. Electron Donor and Decolorization of Azo Dyes

The intracellular reduction hypothesis assumes that an azo dye will act as an oxidant inside the cell or at the cell membrane. The extracellular reduction schemes often assume electron shuttles between azo dyes and intermediate electron donors. Thus the decolorization rate should improve with an increased concentration of auxiliary substrate (original electron donor) (Carliell et al. 1995; Glässer 1992) controlling the rate of formation of the reduction equivalents or intermediate electron donors. The lysis products of dead cells of a bacterial consortium with facultative anaerobes can function as an auxiliary substrate for the anaerobic azo dye reduction, with the active cells metabolizing the lysis products (Liebelt 1997). On the other hand, some reduction equivalents (e.g. sulfide- or iron-containing enzymes) released from dead cells may also be expected to contribute chemically to decolorization (Dubin and Wright 1975).

2.2.4. Methane Producing Bacteria and Possibility of Decolorization

Methane is the most reduced organic compound and its formation is the terminal step of the anaerobic food chain (Gottschalk 1986). Representatives of various genera of methane producing bacteria (MPB) and the substrates utilized can be divided into two following groups (Gottschalk 1986):

1) Obligate chemolithotrophic MPB that grow on $CO_2 + H_2$ according to the equation:

$$CO_2 + 4H_2 \longrightarrow CH_4 + 2H_2O \qquad (10)$$

2) Methylotrophic MPB that grow on methyl-group-containing substrates (acetate, methanol, methylamines). The degradation equation for acetate is:

$$CH_3COOH \longrightarrow CH_4 + CO_2 \qquad (11)$$

Some of the first group of microorganisms also grow on substrates such as HCOOH and CO by producing the intermediates CO_2 + H_2 leading to CH_4 (Gottschalk 1986). The first group of MPB are hydrogen consuming bacteria.

The possibility for MPB to decolorize azo dyes has been rarely reported. Razo-Flores et al. (1997) suggested that the decolorization of a pharmaceutical azo dye is carried out by methanogenic granular sludge by catabolizing the dye itself as an electron donor. However there was no evidence that MPB were responsible for the decolorization.

2.3. Metabolism of Sulfate Reducing Bacteria

SRB have been shown to contribute to anaerobic transformation or mineralization of xenobiotics. For example, a strain of SRB has been implicated in the reductive dehalogenation of a chlorinated organic compound as terminal electron acceptor coupled to energy conservation (Louie and Mohn 1999), and an isolate of SRB co-metabolizes a contaminant TNT (2,4,6-trinitroltoluene) as the sole nitrogen source (Preuss et al. 1993). The transformation of xenobiotics can possibly facilitate the subsequent aerobic degradation of them. In line with these metabolic capabilities of SRB, the anaerobic treatment of azo dyes can be viewed to have positive potential.

2.3.1 Kinetic coefficients and real stoichiometric coefficients for sulfate respiration

Kinetic coefficients for sulfate respiration

Plenty of studies of kinetic coefficients for sulfate respiration have been reported in particular for *Desulfovibrio* (the most extensively studied genus of SRB) (Table 3). Okabe et al. (1992), and Okabe and Characklis (1992) studied the effects of temperature, sulfate-, nitrogen- and phosphate-concentrations on kinetic coefficients for sulfate respiration of *Desulfovibrio desulfuricans* (*D. desulfuricans*) grown on lactate and sulfate. Konish et al. (1996) showed the pH-optimum as 7.0 (from pH 6.0-8.5) for the maximum specific growth rate μ_{max} in a batch reactor with *D. desulfuricans* growing on lactate plus sulfate. Nethe-Jaenchen and Thauer (1984), and Brandis and Thauer (1981) investigated the kinetic coefficients for sulfate respiration with H_2 by some *Desulfovibrio* species. Tucker et al. (1996) studied the kinetic coefficients of sulfate respiration with pyruvate in the presence of uranium U(IV). In sulfate respiration with lactate by *D. desulfuricans*, μ_{max} were in the range of 0.34-0.37 h^{-1} for T=35-37 °C and pH=7.0-7.2 (Okabe et al. 1992; Okabe and Characklis 1992; Konish et al. 1996).

Real stoichiometric coefficients for sulfate respiration by sulfate reducing bacteria

The reaction equation for the degradation of lactate to acetate in sulfate respiration can be described as follows, taking into consideration both catabolism and anabolism of SRB:

$$CH_3CHOHCOOH + Y°_{SO4/S}\, SO_4^{2-} + Y°_{NH3/S}\, NH_3 \rightarrow Y°_{B/S}\, CH_xN_yO_z + Y°_{Ac/S}\, CH_3COOH + Y°_{CO2/S}\, CO_2 + Y°_{H2O/S}\, H_2O + Y°_{S2-/S}\, S^{2-} \quad (12)$$

where $CH_xN_yO_z$ empirical formulation for bacteria in C-normed expression [e.g. $CH_{1.4}N_{0.2}O_{0.4}$ from Characklis (1990)]

- $Y°_{SO4/S}$ real yield coefficient of SO_4^{2-} (reduction) on lactate as substrate(mol/mol)
- $Y°_{NH3/S}$ real yield coefficient of NH_3 (degradation) on lactate (mol/mol)
- $Y°_{B/S}$ real yield coefficient of bacteria on lactate (mol/mol)
- $Y°_{Ac/S}$ real yield coefficient of acetate on lactate (mol/mol)
- $Y°_{CO2/S}$ real yield coefficient of CO_2 on lactate (mol/mol)
- $Y°_{H2O/S}$ real yield coefficient of H_2O on lactate (mol/mol)
- $Y°_{S2-/S}$ real yield coefficient of S^{2-} on lactate (mol/mol)

It is assumed in Eq.(12) that the sole nitrogen source is NH_3. In general, if three of the above yield coefficients (stoichiometric coefficients) are measured, four other coefficients can be estimated via elemental C-, H-, N-, and O-balance of Eq.(12). Using Eq.(12), the growth of bacteria can quantitatively be estimated according to the degraded amount of substrate. Okabe et al. (1992) determined the stoichiometric coefficients for sulfate respiration with lactate by *D. desulfuricans* in a chemostat as follows:

$$CH_3CHOHCOOH + 0.42\pm0.012\, SO_4^{2-} + 0.019\, NH_3 \rightarrow 0.094\pm0.007\, CH_{1.4}N_{0.2}O_{0.4} + 0.95\pm0.03\, CH_3COOH + 1.00\pm0.03\, CO_2 + 0.74\, H_2O + 0.38\pm0.04\, S^{2-} \quad (13)$$

where T=35 °C; pH=7.0; $c_{SO4}>K_{SO4}$ (c_{SO4}: concentration of sulfate; K_{SO4}: saturation coefficient for sulfate). The stoichiometric coefficients for lactate, sulfate, bacterial cells, and acetate were obtained from experimental data. The stoichiometric coefficient for CO_2 was calculated by the measurement of TOC (total organic carbon).

The anabolism of *Desulfovibrio* is explained by a reductive carboxylation of the activated acetate (acetyl-CoA) to pyruvate as the first step in cell synthesis, which is observed in many anaerobic bacteria (Badziong et al. 1979). In this regard, it is understandable that $Y°_{Ac/S}<1$ in Eq.(13) due to the utilization of substrate for the biosynthesis. Interesting is the case of catabolism of H_2 plus SO_4 by *Desulfovibrio.* In that case, at least an organic C_2-compound such as acetate in addition to CO_2 was required for cell synthesis. One third of the cell material

was derived from CO_2; two-thirds were derived from acetate (Badziong et al. 1978). Although autotrophic growth was reported for other hydrogen-utilizing SRB (e.g. *Desulfococcus niacini*; Imhoff-Stuckle and Pfennig 1983), growth was rather slow.

Table 3. Kinetic coefficients for sulfate respiration of SRB using lactate, pyruvate, H_2, or acetate

Culture	Reactor/ Condition	Energy source	Kinetic coefficient	Literature
D. desulfuricans	chemostat T=35°C pH=7.0	lactate SO_4^{2-}	μ_{max} =0.344 h^{-1}, K_{SO4}=1.8 mg/L, $Y_{B/Lac}$=0.020 g-cell/g $Y_{B/SO4}$=0.047 g-cell/g, $Y_{SO4/Lac}$ =0.38-0.42 M/M (sulfate limited) $Y_{SO4/Lac}$ =0.48-0.68 M/M (excess in sulfate)	Okabe et al. (1992)
	T=43°C pH=7.0		μ_{max} =0.352 h^{-1}, K_{SO4}=1.0 mg/L,$Y_{B/SO4}$=0.017 g-cell/g $Y_{B/Lac}$=0.043 g-cell/g	
D. desulfuricans	chemostat T=35°C	lactate SO_4^{2-}	μ_{max} =0.37 h^{-1}, K_{Lac}= 2.2 mg/L,$Y_{B/SO4}$=0.051 g-cell/g, $Y_{B/Lac}$=0.024 g-cell/g	Okabe and Characklis (1992)
	T=43°C pH=7.0		μ_{max} =0.55 h^{-1}, K_{Lac}= 10.0 mg/L,$Y_{B/SO4}$=0.071 g-cell/g, $Y_{B/Lac}$=0.032 g-cell/g	
D. vulgaris *D. sapovorans* *D.salexigens*	batch T=20/30°C pH=7.1	lactate SO_4^{2-}	K_{SO4}=0.5 mg/L at 20 °C, *$Y_{B/SO4}$=0.141 g-TSS/g at 30°C K_{SO4}=0.7 mg/L at 20 °C, $Y_{B/SO4}$=0.115 g-TSS/g at 30°C K_{SO4}=7.4 mg/L at 20 °C, $Y_{B/SO4}$=0.125 g-TSS/g at 30°C	Ingvorsen and Jorgensen (1984)
D. vulgaris *Desulfotomaculum orients*	batch	lactate SO_4^{2-}	$Y_{B/SO4}$=0.122 g-TSS/g $Y_{B/SO4}$=0.040 g-TSS/g	Liu and Peck (1981)
D. vulgaris	chemostat T=35°C pH=6.8	H_2, SO_4^{2-}	μ_{max} =0.23 h^{-1}, K_{SO4}= 1.0 mg/L,$Y_{B/SO4}$=0.135 g-TSS/g	Nethe-Jaenchen and Thauer (1984)
D. desulfuricans	batch T=37°C pH=7.0	lactate SO_4^{2-}	μ_{max} =0.367 h^{-1},$Y_{B/SO4}$=6.28 10^{13} cells/mol	Konishi et al. (1996)
Desulfovibio sp.	batch/conti T=37°C pH=7.0-7.2	lactate SO_4^{2-}	μ_{max} =0.25 h^{-1}, K_{Lac}= 1.5 mmol/L	Zellner et al. (1994)
D. vulgaris *D. desulfuricans* *D. gigas*	T=34-37°C pH=6.2	H_2, SO_4^{2-}	$Y_{B/SO4}$=0.104 g-TSS/g $Y_{B/SO4}$=0.078 g-TSS/g $Y_{B/SO4}$=0.078 g-TSS/g	Brandis and Thauer (1981)
D. desulfuricans	chemostat T=28°C pH=7.4	pyruvate SO_4^{2-}	μ_{max} =0.196 h^{-1}, K_{Pyr}=127 mg/L, $Y_{B/Pyr}$=0.021 g-cell/g In presence of U(VI)	Tucker et al. (1996)
Desulfobactor postgatei	batch T=30°C pH=7	acetate SO_4^{2-}	μ_{max} = 0.03 h^{-1}, K_S=~0.2 mmol/L	Schönheit et al. (1982); Thauer (1982)
Desulfobactor postgatei	batch T=30 °C pH=7.2	acetate SO_4^{2-}	μ_{max} = 0.030 h^{-1}, K_{SO4}=16.3 mg/L (0.17 mmol/L)	Ingvorsen et al. (1984)
Desulfobactor postgatei	batch T=29-32°C pH=7.1-7.3	acetate SO_4^{2-}	μ_{max} = 0.035 h^{-1}, $Y_{B/SO4}$=4.8 g-TSS/M (0.081 g-TSS/g)	Widdel and Pfennig (1981)

*Overall yield on sulfate, lactate, or pyruvate (g-TSS/g) refers to cells + extracellular polymeric substance + precipitates
Note: The symbols used are explained in nomenclature.

2.3.2. Models for conservation of energy

Two following models to account for the conservation of energy in *Desulfovibrio* via the electron transport phosphorylation (chemiosmotic process) have been suggested: 1) hydrogen cycling hypothesis (Odom and Peck 1981); 2) trace hydrogen transformation model (Lupton et al. 1984). The models deal with a question whether molecular hydrogen is an obligatory residual product in substrate amount (i.e. not in trace amount) during growth on substrate (e.g. lactate, pyruvate) with sulfate. Logically, the enzyme hydrogenase involved in the production of H_2 plays a central role (Legall and Fauque 1988) in the explanation of catabolism of these SRB.

Hydrogen cycling hypothesis

The hydrogen cycling scheme is illustrated by Legall and Fauque (1988) as follows (Fig. 4): The formation of molecular hydrogen from lactate and pyruvate in the cytoplasm or on the cytoplasm surface of the cell membrane takes place, and H_2 is rapidly diffused across the cytoplasmic membrane. On the external surface of the membrane, H_2 is oxidized by the periplasmic hydrogenase, which requires tetraheme cytochrome c_3, and the electrons produced from this oxidation are transferred across the membrane, leaving the protons at the external surface of the membrane. The electrons are used in cytoplasm for the reduction of sulfate to sulfide, resulting in the consumption of eight electrons. The net effect is the transfer of eight protons across the cytoplasmic membrane without direct coupling of proton translocation to electron transfer. The proton gradient via the vectorial electron transfer will then drive the synthesis of ATP in the conventional fashion via a reversible ATPase. To sum up, the periplasmic hydrogen itself generates the proton motive force.

The hydrogen cycling model is supported by the following results: the growth on H_2 as the sole energy source plus sulfate by some *Desulfovibrio* (Nethe-Jaenchen and Thauer 1984; Brandis and Thauer 1981) and the H_2-production in substrate amount in sulfate respiration with pyruvate monitored with a membrane in-let massenspectrometer (Peck et al. 1987). The growth on H_2 and sulfate is explained by chemiosmotic ATP-gain via proton gradient generated across the cytoplasmic membrane by the periplasmic oxidation of hydrogen, because of no possibility of energy gain with substrate-level phosporylation. The localization of hydrogenase in the periplasmic space (Steenkamp and Peck 1981) backs up this explanation. Peck et al. (1987) demonstrated that during the metabolism of pyruvate plus sulfate, substrate amounts (not trace amounts) of hydrogen were produced and consumed simultaneously, in which periplasmic hydrogenase are active.

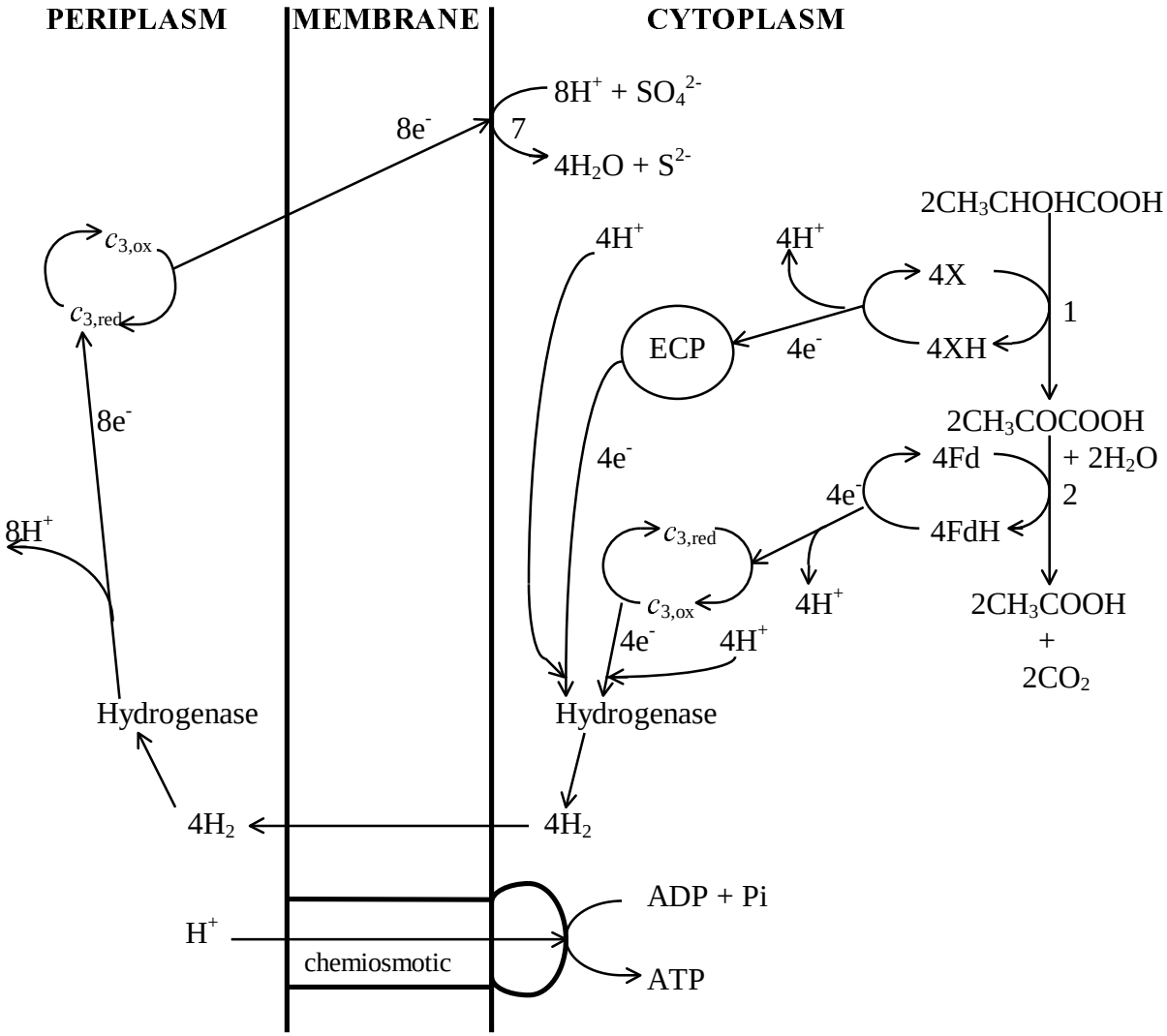


Fig. 4. Hypothetical and schematic physiological function of hydrogen metabolism for cells grown on lactate and sulfate after the hydrogen cycling model (modified from Lupton et al. 1984 and Gottschalk 1986): Hydrogen is an obligate intermediate in energy metabolism as hydrogen production via the cytoplasmic hydrogenase is coupled to hydrogen consumption by a periplasmic hydrogenase, which generates a proton motive force.

1=lactate dehydrogenase (membrane-bound); 2=pyruvate:Fd oxidoreductase; 7=enzymes for sulfate reduction; ECP= Electron carrier protein; Fd=ferredoxin; X=H-acceptor (unknown).

The reaction equation for the respective step is as follows:

1:	$2CH_3CHOHCOOH + 4X$	$\rightarrow$	$2CH_3COCOOH + 4XH$
2:	$2CH_3COCOOH + 2H_2O + 4Fd$	$\rightarrow$	$2CH_3COOH + 2CO_2 + 4FdH$
3:	$4XH$	$\rightarrow$	$4X + 4[H]$
4:	$4FdH$	$\rightarrow$	$4Fd + 4[H]$
5:	$8[H]$	$\rightarrow$	$4H_2$ (via cytoplasmic hydrogenase)
6:	$4H_2$	$\rightarrow$	$8[H]$ (via periplasmic hydrogenase)
7:	$SO_4^{2-} + 8H^+ + 8e^-$	$\rightarrow$	$S^{2-} + 4H_2O$
Total:	$2CH_3CHOHCOOH + SO_4^{2-}$	$\rightarrow$	$2CH_3COOH + 2CO_2 + S^{2-} + 2H_2O$

Note: The reactions for step 5 and 6 are performed together with cytochrome c_3.

Hypothesis of trace hydrogen transfer

As an alternative, the concept of trace hydrogen transformation (Fig. 5) has been proposed in which hydrogen production, mainly in trace amounts, is regarded as controlling the redox state of internal electron carriers linked to energy conservation or as adjusting the catabolic process to an external situation (e.g. presence of toxic substances). In this model, hydrogen production occurs as a side reaction of regulating the levels of reduced electron carriers which link to electron transport phosphorylation via sulfate reduction (Lupton et al. 1984). Logically, the functions of hydrogenase significantly differ from those in the hydrogen cycling hypothesis. The cytoplasmic hydrogenase which indirectly couples to ferredoxin oxidation maintains the redox state of ferredoxin so as not to overreduce the electron carriers involved in other reactions (e.g. lactate oxidation) (Lupton et al. 1984). The energy conservation for this concept is attained via a typical Mitchell loop (Mitchell 1975), i.e. a membrane-bound vectorial electron-hydrogen transfer linked to cytoplasmic substrate oxidation reactions. In line with the trace hydrogen transformation model, hydrogen utilization by the peripasmic hydrogenase was suggested as preventing the loss of energy in the form of hydrogen (Tsuji and Yagi 1980). The production of 1 mol/L H_2 from the oxidation of 2 mol/L lactate despite excess in sulfate has been interpreted in terms of the regulation of electron flow by the electron carrier tetraheme cytochrome c_3 during growth (Traore et al. 1981).

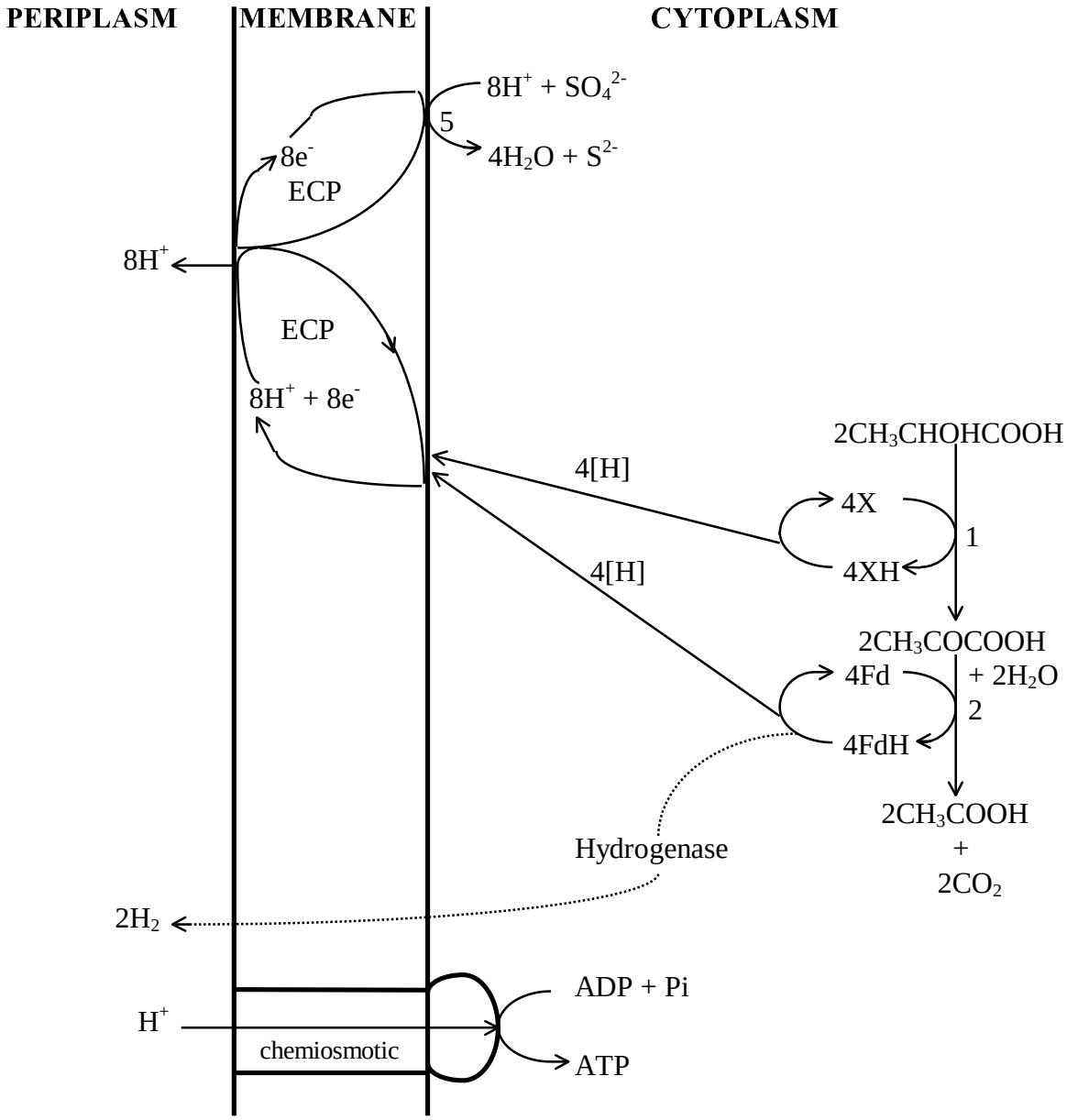


Fig. 5. Hypothetical schema of hydrogen transformation model for growth on lactate and sulfate (modified from Lupton et al. 1984): A cytoplasmic hydrogenase acts to control the redox level of ferredoxin (Fd) to regulate vectorial electron and proton transfer which generates a membrane-bound proton motive force. Dotted line indicates alternative pathway.

1=lactate dehydrogenase (membrane-bound); 2=pyruvate:Fd oxidoreductase; 5=enzymes for sulfate reduction; ECP= Electron carrier protein; Fd=ferredoxin; X=H-acceptor (unknown). The electron transfer from Fd to H_2 may be mediated by other electron carriers (e.g. cytochrome c_3) and enzymes (i.e. oxidoreductases) in addition to hydrogenase.

The reaction equation for the respective step is as follows:

1:	$2CH_3CHOHCOOH + 4X$	$\rightarrow$	$2CH_3COCOOH + 4XH$
2:	$2CH_3COCOOH + 2H_2O + 4Fd$	$\rightarrow$	$2CH_3COOH + 2CO_2 + 4FdH$
3:	$4XH$	$\rightarrow$	$4X + 4[H]$
4:	$4FdH$	$\rightarrow$	$4Fd + 4[H]$
5:	$SO_4^{2-} + 8H^+ + 8e^-$	$\rightarrow$	$S^{2-} + 4H_2O$
Total:	$2CH_3CHOHCOOH + SO_4^{2-}$	$\rightarrow$	$2CH_3COOH + 2CO_2 + S^{2-} + 2H_2O$

2.3.3. Influence of sulfate on catabolism of sulfate reducing bacteria

In the presence of sulfate some SRB (e.g. *Desulfovibrio*) oxidize pyruvate or lactate as electron donor via sulfate respiration into acetate and CO_2, which constitute substrate for methane producing bacteria (MPB). The sulfide produced during the sulfate respiration is of interest because of its potential to decolorize azo dyes with its high reducing power. If lactate is available as substrate in sulfate respiration, the oxidation of 2 mol/L lactate should be coupled with the reduction of 1 mol/L sulfate (s. Fig. 4). Moreover it is derivable from Fig. 4 that in the presence of pyruvate as substrate in sulfate respiration, the oxidation of 4 mol/L pyruvate should be associated with the reduction of 1 mol/L sulfate, since 8 electrons are indispensable to the reduction of 1 mol/L sulfate.

In the absence of sulfate many sulfate reducers (e.g. *Desulfovibrio* species) are capable of fermenting pyruvate even in pure cultures (Postgate and Campbell 1966; Peck et al. 1987; Bryant et al. 1977). More notably, some SRB belonging to the genus *Desulfovibrio* could not grow on lactate without sulfate in pure cultures, but grow via interspecies hydrogen transfer when cocultured with MPB (Bryant et al. 1977). From a thermodynamic point of view the reaction of lactate fermentation to acetate, CO_2, and H_2 by the SRB is endergonic unless the partial hydrogen pressure is kept low. For the maintenance of low partial pressure of hydrogen the MPB will serve by H_2-uptake to produce methane. It is interesting whether analogous to the role of H_2-utilizing MPB azo dyes could possibly function as an external electron (or hydrogen) scavenging system in fermentation of SRB, leading to the reduction of azo bonds (i.e. decolorization). If this is possible, a further question remains to be answered, whether the function of azo dyes as the electron scavenger in fermentation of SRB could possibly result in more oxidized catabolic products (e.g. acetate) coupled to ATP gain, compared to the syntrophy of H_2-utilizing MPB for the fermentation of SRB. The existing hypotheses for anaerobic decolorization of azo dyes often suggested the azo bond reduction by cytoplasmic enzymes with electron carriers such as $NAD(P)^+$, FMN, FAD, or riboflavin at fermentative bacteria (s. Table 2). The determination of a possible decolorization mechanism in fermentation of SRB can serve in transferring these existing hypotheses to the special case of SRB or otherwise in differentiating the decolorization mechanism of the fermentative bacteria and the SRB at fermentation.

2.4. Kinetic Models for Chemical Decolorization

In general, a decolorization process of azo dyes based on the reduction of azo bonds is influenced by the concentrations of azo dye and reduction equivalent. The reduction equivalent can directly be generated in biological metabolisms, or can abiotically be available in the

system. In the case of chemical decolorization, the following equation can be proposed for the decolorization rate in a batch reactor:

$$r_D = -\frac{dc_D}{dt} = k\, c_D^{\,a}\, c_{RE}^{\,b} \qquad (14)$$

where r_D decolorization rate (mmol h^{-1} L^{-1})
k rate constant (unit dependent on the reaction orders)
c_D concentration of dye at time t (mmol/L)
c_{RE} concentration of reduction equivalent at time t (mmol/L)
a reaction order with respect to dye concentration
b reaction order with respect to concentration of reduction equivalent

In order to determine the rate constant k, the reaction orders must be obtained via measurements. Assuming the first order with respect to both concentration of dye and reduction equivalent (a=1, b=1), it follows from Eq.(14):

$$-\frac{dc_D}{dt} = k\, c_D\, c_{RE} \qquad (15)$$

where k rate constant (L $mmol^{-1}$ h^{-1})

2.4.1. Limitation of chemical decolorization by dye concentration

The model of chemical decolorization after Eq.(15) can be examined for the case of great excess in the reduction equivalent in comparison with dye concentration. Then, the change of the reduction equivalent concentration during decolorization can be negligible. Eq.(15) is transformed to:

$$\frac{dc_D}{dt} = -k' c_D \qquad (16)$$

where $k' = k\, c_{RE}$
rate constant (h^{-1})

For $c_D=c_{D,0}$ at t=0, it follows from integration of Eq.(16):

$$\ln(c_D/c_{D,0}) = -k' t \qquad (17)$$

where $c_{D,0}$ concentration of dye at starting point (mmol/L)

Plotting the values of $\ln(c_D/c_{D,0})$ vs. time t, the first order with respect to dye concentration can be verified, if a straight line can be obtained. Similarly, the first order with respect to the concentration of the reduction equivalent can be investigated in the case of excess in the dye concentration.

2.4.2. Limitation of chemical decolorization by concentration of dye and reduction equivalent

In order to inspect the model of Eq.(15) under the influence of both the reduction equivalent and dye, a derivation of an evaluation equation from Eq.(15) is required. If the reduction equivalent is abiotically available, the coupling condition between the dye and reduction equivalent concentration can be assumed as below:

$$\frac{dc_D}{dc_{RE}} = Y_{D/RE} \qquad (18)$$

where $Y_{D/RE}$ yield coefficient of dye (reduction) on reduction equivalent (mmol/mmol)

It follows from Eq.(18) via integration:

$$c_{RE} = c_{RE,0} - Y_{D/RE}^{-1} (c_{D,0} - c_D) \qquad (19)$$

To make the derivation easier, c_D and c_{RE} of Eq.(15) are substituted using x_D and P as follows:

$$\frac{dx_D}{dt} = k\, c_{D,0} (1 - x_D) (P - Y_{D/RE}^{-1} x_D) \qquad (20)$$

$$\text{since } x_D = 1 - (c_D/c_{D,0}); \quad P = c_{RE,0}/c_{D,0} = \text{constant};$$
$$P - Y_{D/RE}^{-1} x_D = c_{RE}/c_{D,0} \text{ [using Eq.(19)]} \qquad (21)$$

Eq.(20) is transformed via the method of partial fraction into:

$$\frac{dx_D}{(1 - x_D) (P - Y_{D/RE}^{-1} x_D)} = k\, c_{D,0}\, dt \qquad (22)$$

If $P - Y_{D/RE}^{-1} \neq 0$, i.e. $c_{RE,0}/c_{D,0} \neq Y_{D/RE}^{-1}$, Eq.(22) is transformed via integration into:

$$\frac{1}{(P - Y_{D/RE}^{-1})} \left[\ln \frac{P - Y_{D/RE}^{-1} x_D}{1 - x_D} \right]_{x_{D,0}}^{x_D} = k\, c_{D,0}\, t \qquad (23)$$

Solving Eq.(23) and resubstituting x_D and P with Eq.(21), it follows:

$$\frac{1}{(c_{RE,0}-Y_{D/RE}^{-1}\, c_{D,0})}\left[\ln\frac{c_{D,0}\{c_{RE,0}-Y_{D/RE}^{-1}(c_{D,0}-c_D)\}}{c_D\, c_{RE,0}}\right] = k\,t \qquad (24)$$

Assuming that β is equal to the left-hand side of Eq.(24) as following:

$$\frac{1}{(c_{RE,0}-Y_{D/RE}^{-1}c_{D,0})}\left[\ln\frac{c_{D,0}\{c_{RE,0}-Y_{D/RE}^{-1}(c_{D,0}-c_D)\}}{c_D\, c_{RE,0}}\right] = \beta \qquad (25)$$

and plotting the values of β vs. time t, the decolorization model with the first order with respect to both concentration of dye and reduction equivalent can be validated, to obtain the rate constant k as the slope.

If $P - Y_{D/RE}^{-1} = 0$, i.e. $c_{RE,0}/c_{D,0} = Y_{D/RE}^{-1}$, Eq.(20) is transformed via the method of partial fraction into:

$$\frac{dx_D}{(1-x_D)^2} = k\, c_{RE,0}\, dt \qquad (26)$$

Integrating Eq.(26) by using the starting condition $x_D=x_{D,0}$ at t=0, it follows:

$$\left[\frac{1}{(1-x_D)}\right]_{x_{D,0}}^{x_D} = k\, c_{RE,0}\, t \qquad (27)$$

After solving Eq.(27) and substituting $(1- x_D)$ with $(c_D/c_{D,0})$, it follows:

$$\frac{c_{D,0}-c_D}{c_D\, c_{RE,0}} = k\,t \qquad (28)$$

Assuming that γ is equal to the left-hand side of Eq.(28) as following:

$$\frac{c_{D,0}-c_D}{c_D\, c_{RE,0}} = \gamma \qquad (29)$$

and plotting the values of γ vs. time t, the decolorization model with the first order with respect to both concentration of dye and reduction equivalent can be verified, to attain the rate constant k as the slope.

The decolorization degree α in % is defined as follows:

$$\alpha = 100 \cdot \left[1 - \frac{c_D}{c_{D,0}}\right] \qquad (30)$$

2.4.3. Limitation of chemical decolorization by biological degradation of substrate

If reduction equivalents are generated from the bacterial catabolisms, the decolorization kinetics is to be combined with the kinetics of the substrate degradation. In general, the following relation can be proposed for the bacterial substrate degradation (Monod 1942) in a batch reactor:

$$r_S = -\frac{dc_S}{dt} = \frac{\mu_{max}\ c_S}{Y_{B/S}\ (K_S + c_S)}\ c_B \qquad (31)$$

where r_S rate of substrate degradation (mmol h^{-1} L^{-1})
μ_{max} maximum specific growth rate (h^{-1})
K_S saturation coefficient for substrate (mmol/L)
$Y_{B/S}$ yield coefficient of bacteria on substrate (mmol/mmol or g/g)
c_B concentration of bacteria at time t (mmol/L or g/L)
c_S concentration of substrate at time t (mmol/L)

Via the method of partial fraction and integration of Eq.(31) in the assumption of $c_B = c_{B,0}$=constant, it follows for $c_S = c_{S,0}$ at t=0:

$$K_S\ \ln\frac{c_S}{c_{S,0}} - (c_{S,0} - c_S) = \frac{\mu_{max}\, c_{B,0}}{Y_{B/S}}\ t \qquad (32)$$

Eq.(32) is transformed to:

$$(c_{S,0} - c_S) = K_S\ \ln\frac{c_S}{c_{S,0}} - \frac{\mu_{max}\, c_{B,0}}{Y_{B/S}}\ t \qquad (33)$$

The production of reduction equivalent can be coupled with the degradation of substrate via the yield coefficient of the reduction equivalent on substrate ($Y_{RE/S}$) as following:

$$Y_{RE/S} = \frac{c_{RE} - c_{RE,0}}{c_{S,0} - c_S} \qquad (34)$$

where c_{RE} concentration of reduction equivalent (mmol/L)
$Y_{RE/S}$ yield coefficient of reduction equivalent on substrate (mmol/mmol)

Eq.(34) is transformed to:

$$c_{RE} = c_{RE,0} + Y_{RE/S}\,(c_{S,0} - c_S) \qquad (35)$$

Putting Eq.(35) in Eq.(15), in the assumption that Eq.(15) describes a chemical decolorization rate as a function of the concentrations of dye c_D and biologically generated reduction equivalent c_{RE}, it follows:

$$r_D = k\, c_D\, c_{RE} = k\, c_D\, [c_{RE,0} + Y_{RE/S}\,(c_{S,0} - c_S)] \qquad (36)$$

Putting Eq.(33) in Eq.(36), the decolorization rate as a function of substrate concentration can be obtained as follows, so far as $c_B = c_{B,0}$=constant:

$$r_D = k\, c_D \left[c_{RE,0} + Y_{RE/S} \left\{ K_S \ln \frac{c_S}{c_{S,0}} - \frac{\mu_{max}\, c_{B,0}}{Y_{B/S}}\, t \right\} \right] \qquad (37)$$

Eq.(37) shows that the decolorization rate can be affected not only by the concentration of dye and the initial concentration of the reduction equivalent, but also by the bacterial catabolism of substrate as electron source. This influence is represented by the kinetic coefficients of the bacterial metabolism (μ_{max}, $Y_{B/S}$, K_S, etc.). For example a higher maximum specific growth rate μ_{max} will enhance the decolorization rate.

2.4.4. Limitation of chemical decolorization by sulfate respiration

If SRB get involved in chemical decolorization of azo dyes, the substrate degradation by SRB can affect the decolorization process. For SRB the substrate degradation is coupled with sulfate reduction. A double Monod-kinetic model can be proposed with respect to substrate and sulfate concentration, modified from the Eq.(31) of Monod kinetics as follows (Nedwell 1982; Ramm and Bella 1974):

$$r_S = -\frac{dc_S}{dt} = \frac{\mu_{max}}{Y_{B/S}} \frac{c_S}{(K_S + c_S)} \frac{c_{SO4}}{(K_{SO4} + c_{SO4})}\, c_B \qquad (38)$$

where K_{SO4} saturation coefficient for sulfate (mmol/L)

c_{SO4} concentration of sulfate (mmol/L)

It is probable that the decolorization rate through SRB is affected by the bacterial catabolism of both substrate (electron donor) and sulfate (electron acceptor). For $K_{SO4} << c_{SO4}$, Eq.(38) is simplified to:

$$r_S = -\frac{dc_S}{dt} = \frac{\mu_{max}}{Y_{B/S}} \frac{c_S}{(K_S + c_S)}\, c_B \qquad (39)$$

That is, the substrate degradation undergoes the Monod-Kinetics like Eq.(31) because the high concentrations of sulfate for $K_{SO4} << c_{SO4}$ do not enhance the rate of substrate degradation r_S any more. In the case of $c_B=c_{B,0}$=constant, Eq.(37) is also applicable to the chemical decolorization through SRB for $K_{SO4} << c_{SO4}$.

2.5. Objectives of Research

Putting the existing hypotheses for the azo dye reduction (Chap. 2.2.1) into the perspective of the classical three-step anaerobic degradation of organic carbon complexes, the anaerobic decolorization of azo dyes is thought to take place in the first and second of the three steps by the acidogenic and acetogenic bacteria. However, differentiation as to which group of bacteria play a prevailing role in the decolorization of azo dyes has not been reported. Furthermore, the study of decolorization mechanisms with respect to bacterial metabolism or bioenergetics has been rarely reported. As can be seen from the discussion of enzymatic/non-enzymatic, intracellular/extracellular reduction presented in Chap. 2.2, the chemical reduction of azo dyes is not always far from the biological processes.

The following questions arise from the literature review:

1. Can SRB in a mixed culture contribute to the decolorization of azo dyes in the presence of sulfate (Chap. 4)?
2. Can MPB in a mixed culture play a role in the decolorization of azo dyes (Chap. 4)?
3. How does the concentration of sulfate affect the decolorization process of a pure culture of SRB? Can azo dyes possibly function as an electron accepting system in bacterial catabolisms, leading to ATP gain (Chap. 5)?
4. Are there any possibilities of the chemical (abiotic) decolorization of azo dyes in the environment? If so, what can the kinetics of the related chemical decolorizations be like (Chap. 6)?

Therefore, this research concentrates on disclosing new mechanisms active in the decolorization of azo dyes, describing the role of various groups of bacteria. In addition, this research elucidates the correlation between the bacterial catabolisms and the possible accompanying decolorization of azo dyes, employing the pure culture of *Desulfovibrio desulfuricans* (*D. desulfuricans*). Finally, the chemical kinetic aspects in relation to the decolorization of azo dyes are studied.

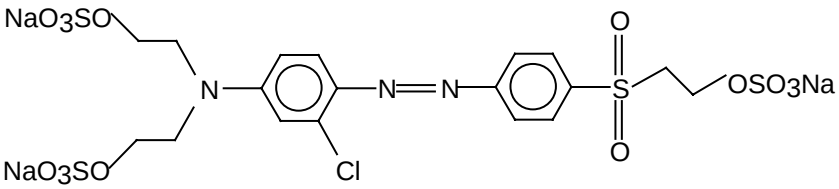
This research deals with synthetic wastewater to deliver basic information for the treatments of real textile waste water, covering the following three topics: 1) decolorization tests in the anaerobic mixed culture, 2) decolorization tests in the pure culture of SRB, 3) kinetic tests for

chemical decolorization of azo dyes. The first topic was handled in the batch and continuous experiments; the rest of the topics were only in batch tests. Information on the experimental implementations (Chap. 3) is described divided into these three topics.

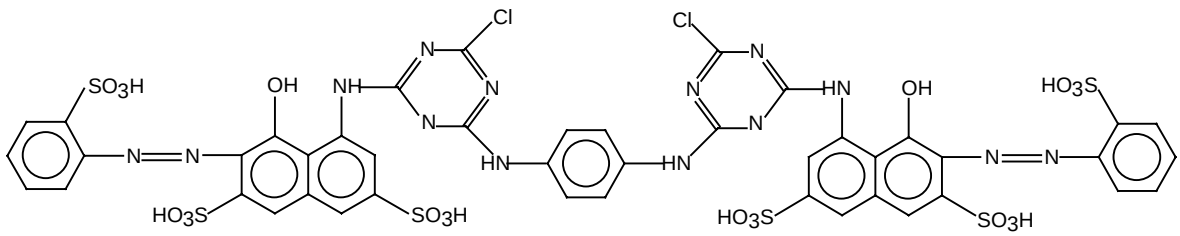
3. MATERIAL AND METHODS

3.1. Material

The reactive mono- and di-azo dyes C.I. Reactive Orange 96 (RO 96) and C.I. Reactive Red 120 (RR 120) were used as commercial products (DyStar, Frankfurt, Germany; Ciba Spezialitätenchemie, Basel, Switzerland) (Fig. 6).



C. I. Reactive Orange 96 (RO 96)



C. I. Reactive Red 120 (RR 120)

Fig. 6. Textile azo dyes used: RO 96 and RR 120

RO 96, more precisely its substituent in form of $-OSO_3^-$, was hydrolyzed at pH 11.3 with NaOH and subsequently adjusted to pH 7.0 with 2 mol/L HCl, because dyes are commonly hydrolyzed during the textile finishing process and this form is mainly found in real textile wastewater. RR 120 was employed without hydrolysis owing to its lack of the substituent ($-OSO_3^-$). The commercial product RO 96 contained approximately 60 % dye, 20 % Na_2SO_4 and 20% other additives (including 5% hexylene glycol) by weight, while that of RR 120 contains approximately 70 % dye and 30 % additives (Table 4). Only for the decolorization tests in the pure culture except for a control test with lactate in the mixed culture, an ion

exchanger (Amberlite IRA-420, Merck, Darmstadt, Germany) exchanging sulfate ion in the dye solution with chloride ion was used to eliminate sulfate from the commercial product of RO 96, then achieving less than 0.38 mmol/L sulfate per 1 mmol/L RO 96. RR 120 was not prepared with the ion exchanger because of its low sulfate content (≤ 0.42 mmol/L sulfate per 1 mmol/L RR 120). Each dye solution was sterilized with a 0.2 μm sterile cellulose acetate filter (Sartorius, Göttingen, Germany) prior to use.

Table 4. Composition of commercial product of RO 96 and RR 120

	RO 96		RR 120	
Pure dye stuff in %[1]		60		70
Additives in %		40		30
	-Na_2SO_4	20	-Dispergator[2]	17
	-KCl	10	- H_2O	7
	-Hexylene glycol	5	- Na_2HPO_3	5
	-H_2O etc.	5	- Oil	1

[1] by weight

[2] condensation of naphthalinesulfonic acid-formaldehyde

The chemicals used were of the highest quality available from Merck or Sigma (St. Louis, USA). The gases used were of 99.999%-purity (N_2) or 99.8%-purity (CO_2) (vol/vol) (Linde, Berlin, Germany), trace oxygen was removed by a reducing column (Ochs, Göttingen, Germany; Hewlett-Packard, Avondale, USA). The gas was sterilized by a cotton filter.

3.2. Inoculum

Inoculum for decolorization tests in anaerobic mixed culture

A continuous-flow fixed bed anaerobic lab reactor (Fig. 8) had been seeded originally with anaerobic sludge from digestors of the municipal sewage plant Falkenberg in Berlin. The lab scale reactor has decolorized RO 96 over two years anaerobically in the presence of peptone or yeast extract and acetic acid as auxiliary substrate at 33 °C. The bacteria in the lab reactor were immobilized on polyurethane foam cubes (length =1 cm). These bacteria-filled cubes were removed from the lab reactor, shortly preserved in the incubation bottle completely filled with the Medium I and directly used for the inoculation for batch tests. The influence of a possible carry-over of sulfide from the foam cube into the batch reactors at inoculation was

negligible for decolorization tests. The maximal sulfide concentration in the batch reactors due to a water-filled foam cube would be 0.01 mmol/L by estimation. The real concentration of sulfide should be much lower due to the reaction of sulfide with the traces of dissolved oxygen in the Medium II and III (Table 5).

Inoculum for decolorization tests in anaerobic pure culture

Desulfovibrio desulfuricans (*D. desulfuricans*) (DSM 642^T) was obtained from the collection of Fachgebiet Ökologie der Microorganismen at the Technische Universität Berlin, grown on 19 mmol/L lactate and 9.5 mmol/L sulfate at 37 °C in 55 mL screw cap bottles completely filled with the medium III (Table 5) amended with 1 mmol/L sulfide, and after 3 d of growth shortly preserved below 18 °C for inoculation as stock culture.

3.3. Experimental Systems

The decolorization tests in the anaerobic mixed culture were conducted in sterile 100 mL bottles as batch reactors, where bacteria were immobilized in the polyurethane foam [Fig. 7(a)]. The batch reactors were filled with 55 mL of sterile medium (s. Chap. 3.5) leaving approximately 45 mL gas space. The batch reactors were sealed with thick butyl rubber stoppers or teflon-backed butyl rubber septen plus aluminum-crimp caps. The batch reactors were also used to investigate decolorization in pure culture of SRB, but as suspended cells [Fig. 7(b)].

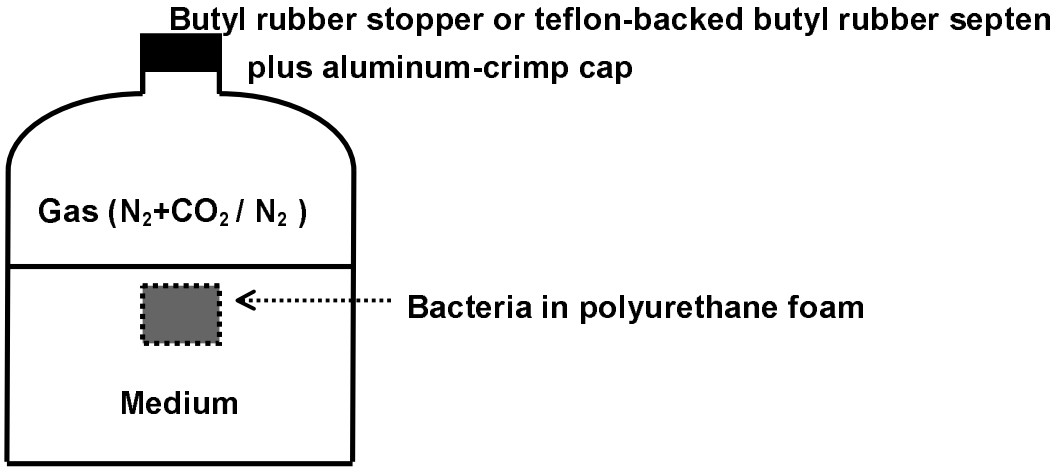


Fig. 7(a). Anaerobic batch reactors for decolorization tests with the anaerobic mixed culture: V_R=55 mL. The medium was not reduced.

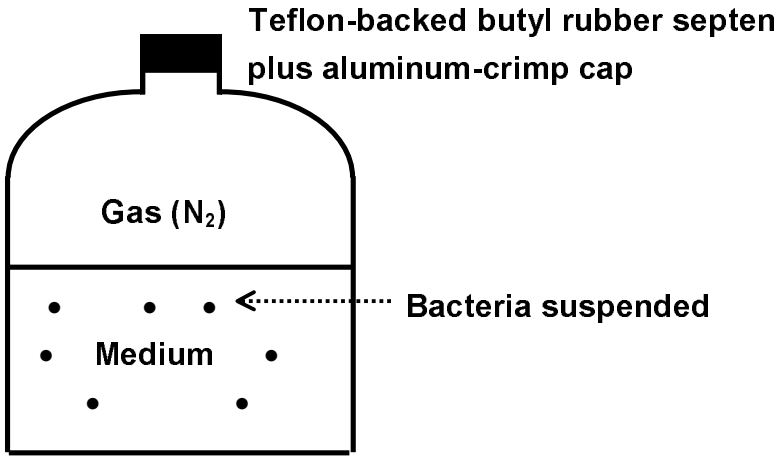


Fig. 7(b). Anaerobic batch reactors for decolorization tests with the pure culture of SRB: V_R=55 mL. The medium was not reduced.

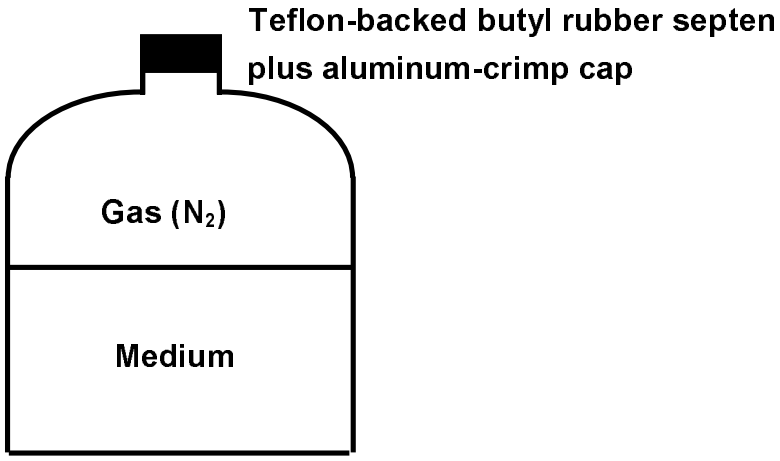


Fig. 7(c). Anaerobic batch reactors for chemical decolorization with yeast extract and reducing agents (ascoribc acid, cysteine, etc.) besides sulfide: V_R=55 mL. The medium was not reduced.

The same batch reactors were employed to study the chemical decolorization with yeast extract and reducing agents (ascoribc acid, cysteine, etc.) besides sulfide, but without bacteria [Fig. 7(c)]. The continuous-flow fixed bed anaerobic lab reactor with the mixed culture (Fig. 8) was employed to inspect the influence of sulfate level on decolorization. The modified Erlenmeyer bottles with a side-leg (d = 12 mm) (Fig. 9) were used to track the kinetics of chemical decolorization with sulfide. The batch reactors were sealed with a double septen-silicon plus a hole screw cap. The reactors were helpful because otherwise the monitoring of the dye concentrations which changed very rapidly would have been difficult.

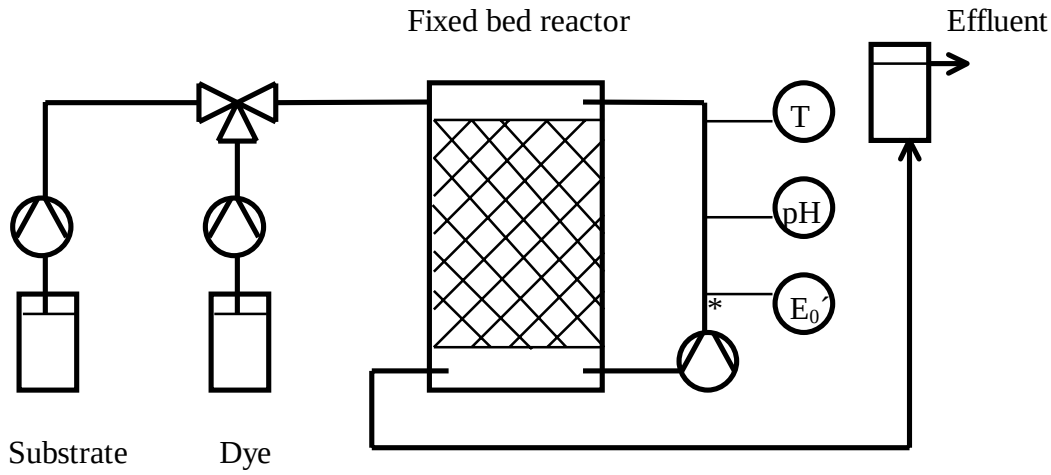


Fig. 8. Anaerobic continuous-flow fixed bed lab reactor, V_R = 10.5 L: * place for sampling

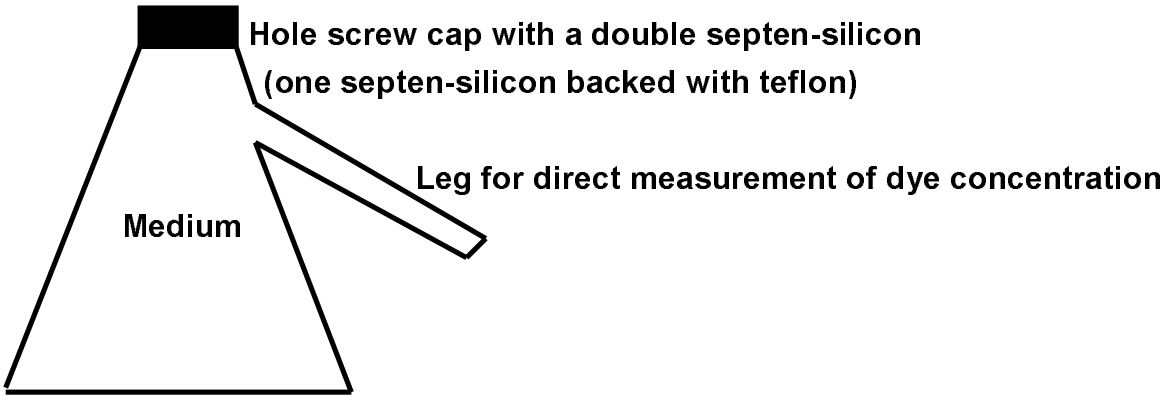


Fig. 9. Batch reactor for kinetic study of decolorzation with sulfide: V_R = 140 mL.

3.4. Analytical Methods

The concentration of DOC was determined by a difference measurement between total dissolved carbon and dissolved inorganic carbon with a DIMA TOC-100 (DIMATEC, Essen, Germany). The concentration of the dyes RO 96 and RR 120 was measured at 460 and 580 nm respectively with a UV-VIS spectrophotometer with a measuring range of 200-1000 nm (model UV-1202, Shimadzu, Kyoto, Japan). The low detection limit for RO 96 was 0.002 ± 0.0000 mmol/L. A shift of the wave length of the maximum absorption of the dyes during decolorization was scarcely observed. The shift was so small that the dye concentration was monitored at the same wave lengths. To study the kinetics of chemical decolorization with sulfide, the side-leg of the batch reactor (Fig. 9) was inserted in a measuring hole of a spectrophotometer (model Lasa 20, Dr Bruno Lange, Düsseldorf, Germany), in which the dye concentration could be measured directly at 440 nm without sampling. For the determination

of dye concentration a calibration for each reactor had been made via the direct measurement of extinction vs. the standard concentrations (Fig. 10). The reactor was shaken cautiously by hand prior to the direct measurement of dye content to avoid possible dead volume in the leg.

The dissolved sulfate concentration for the batch tests in the anaerobic mixed culture was determined according to the turbidity of precipitated $BaSO_4$ at 670 nm on the UV-VIS spectrophotometer (Madsen and Aamand 1991). This measurement, however, was only made before the addition of the dye, due to the precipitation of the dye by barium ion. Thus, the start concentration of total sulfate in the batch reactor was calculated from the measured concentration of sulfate provided in the medium and the theoretical sulfate concentration originating from the dye itself. The sulfate concentration in the continuous-flow fixed bed reactor with the anaerobic mixed culture and in the batch reactors with the pure culture was monitored using an ion chromatograph (advanced chromatography module, Dionex, Sunnyvale, USA) with an analytical column (Ionpac AS 14, Dionex). The measurement is based on conductivity detection, in which eight components (fluoride, acetate, chloride, nitrite, bromide, nitrate, phosphate, sulfate) were identified together according to the respective retention time. The eluent composed of 3.5 mmol/L Na_2CO_3 and 1.0 mmol/L $NaHCO_3$ was used at a flow rate of 1.2 mL/min. The eluent was dosed by diluting the concentrate of Na_2CO_3 and $NaHCO_3$ in degassed, deionized water with a specific resistance of 17.8 megohm-cm or greater. The detection limit for sulfate was 0.01 ± 0.001 mmol/L, based on the injection volume of 10 µL. The acetate concentration was analyzed using a gas chromatograph (model 5890, Hewlett-Packard) with a flame ionization detector at 250 °C under following conditions: carrier gas, N_2; column, fused silica capillary column [25 m x 0.25 mm (ID)] (Permabond®-FFAP-DF-0.25, Macherey-Nagel, Düren, Germany); column temperature, 75 °C→140 °C at 20 °C/min, →220°C at 60 °C/min. Samples were acidified with 50 % formic acid. The detection limit for acetate was 0.04 ± 0.002 mmol/L, based on the injection volume of 1 µL.

The dissolved sulfide concentration was determined colorimetrically as colloidal CuS at 480 nm with the spectrophotometer, modified from Cord-Ruwisch (1985). 100 µL of samples were added to 4 mL reagent composed of 50 mmol/L HCl and 5 mmol/L CuSO4 in deionized water, immediately mixed strongly 10 s with a swirling machine (Type: Reax 1, Heidolph). The detection limit for sulfide was 0.25 mmol/L, based on the sample volume of 100 µL. Sulfide was only measured in colorfree samples due to interference in the colorimetrical sulfide-measurement by the dyes. The bacteria concentration for the decolorization tests in the anaerobic mixed culture was reported as total suspended solids (TSS), where the sample, suspended by squeezing the foam cube, was centrifuged 15 min at 4000 rpm, decanted and dried 12 h at 105 °C. For the decolorization tests in the pure culture because of the low starting bacteria concentration in the batch cultures, the sample for measurement of TSS was withdrawn directly from the stock cultures used for inoculation and centrifuged 15 min at 4000

rpm, decanted and dried 12 h at 105 °C. The final bacteria concentration for the batch tests was corrected for the dilution at inoculation. Each analytical measurement was carried out at least three times for the respective sample. The data points in the figures indicate measured mean values. The error bar in the figures shows the 95 or 99 % confidence interval using Student´s *t*-distribution, determined with the following formula: $m \pm (t \ S \ n^{-1/2})$, where n = number of measured values; m = mean value; S = standard deviation; t = coefficient of Student´s *t*-distribution (95 or 99 % confidence). The error bar is not shown for points where intervals are too small.

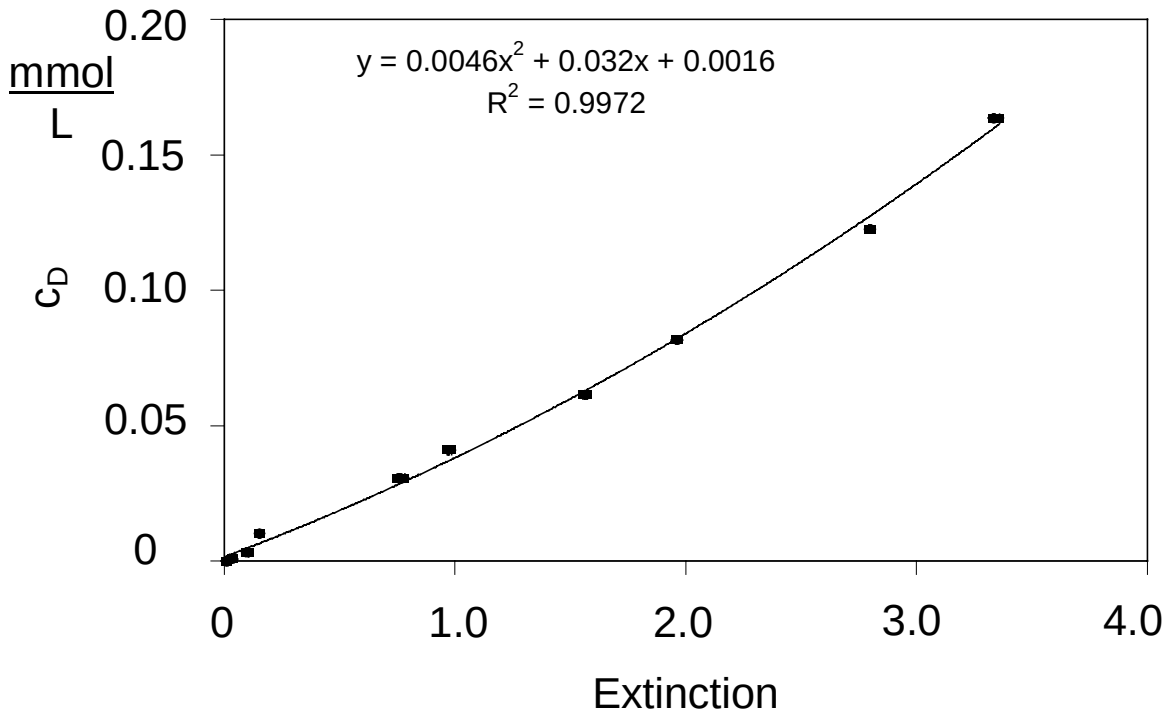


Fig. 10. Calibration of extinction vs. concentration of RO 96 at 440 nm directly using a batch reactor without sampling: The data points are average values (n=8).

3.5. Experimental Implementations

3.5.1. Decolorization tests in anaerobic mixed culture

Batch tests

Variations in the composition of assay medium were made according to the objectives, in that Medium I contained 1.1 mmol/L Na_2S (if not otherwise noted, mmol/L or g/L refers to the final concentration in the medium), subject to the short-term preservation of bacteria; Medium II and III contained no reducing agent for the decolorization tests and the investigation of

degradation of dissolved organic carbon (DOC) (Table 5). Each sterile assay medium consisted of inorganic nutrients, buffer components, trace elements SL 9 (Tschech and Pfennig 1984), and vitamin. The Medium I and II contained bicarbonate buffer, whereas the Medium III was phosphate-buffered.

Trace element solution, vitamin solution, bicarbonate solution or phosphate solution and, if necessary, sulfide solution were added to the autoclaved, cooled basal medium containing the inorganic nutrients (Table 5). The medium was adjusted to pH 7.3 with sterile 2 mol/L HCl. The stock sulfide solution had been prepared by dissolving the appropriate amount of $Na_2S \cdot 9H_2O$ in distilled, deaerated water, and was sterilized with the 0.2 µm sterile cellulose acetate filter under N_2 atmosphere.

Table 5. Composition of assay media

Composition	unit	Medium I	Medium II	Medium III
Inorganic nutrients and buffer components	g/L			
NaCl		1.2	1.2	1.2
KCl		0.28	0.28	0.28
NH_4Cl		0.3	0.3	0.3
$MgCl_2 \cdot 6H_2O$		0.4	0.4	0.4
$CaCl_2 \cdot 2H_2O$		0.15	0.15	0.15
$NaHCO_3$		2.5	2.5	0.8
KH_2PO_4		0.2	0.2	1.6**
Na_2HPO_4		0	0	1.4**
Trace element solution*	mL/L	1	1	1
Vitamin B_{12}	µg/L	25	25	25
Reducing agent Na_2S	mmol/L	1.1	0	0

All concentrations refer to 1L of the medium composed of distilled water.

* Composition of trace element solution (SL9) in g/L: nitrilotriacetic acid, 12.8; $FeCl_2 \cdot 4H_2O$, 2.0; $ZnCl_2$, 0.07; $MnCl_2 \cdot 2H_2O$, 0.08; H_3BO_3, 0.006; $CoCl_2 \cdot 6H_2O$, 0.19; $CuCl_2 \cdot 2H_2O$, 0.002; $NiCl_2 \cdot 6H_2O$, 0.024; $Na_2MoO_4 \cdot 2H_2O$, 0.036 (Tschech and Pfennig 1984).

** Exceptionally, 6.4 g/L KH_2PO_4 and 5.6 g/L Na_2HPO_4 were used for $c_{MoO4} \geq 55$ mmol/L in the experiments of Fig. 11.

The batch reactors were filled with 55 mL of the fresh sterile medium. The following components were added from sterile stock solutions to the medium before the inoculation: 15 or 20 mmol/L of auxiliary substrate - either lactate (DL-$CH_3CHOHCOONa$) or acetate (CH_3COONa), sulfate (Na_2SO_4) as electron acceptor, dye solution and if necessary selective inhibitors 2-bromoethanesulfonic acid (BES; $C_2H_4BrO_3SNa$) to hinder MPB or molybdate (MoO4; $Na_2MoO_4 \cdot 2H_2O$) to inhibit SRB. The reactors were flushed with oxygen-free mixture of N_2-CO_2 (4:1 volume) or N_2 to remove oxygen, bacteria were added, and the reactors were sealed with thick butyl rubber stoppers or teflon-backed butyl rubber septen plus aluminum-crimp caps, in which a low excess pressure (0.05-0.1 bar) of the gases was maintained to avoid penetration of oxygen. The reactors were shaken continuously in a shaking machine (model 1083, GFL, Burgwedel, Germany) or only hand-shaken before sampling. All tests were carried out at 33°C in duplicate or triplicate. The samples (0.5-1 mL) were taken aseptically under oxygen-free mixture of N_2-CO_2 (4:1 volume) or N_2 atmosphere, filtered with a 0.2 µm glass fiber filter (Sartorius). In addition, for a control experiment the bacteria were sterilized by autoclaving at 120 °C and 1.3 bar for 2 h.

Continuous tests

The experimental conditions for the continuous tests are summarized in Table 6.

Table 6. Experimental conditions in the anaerobic continuous-flow fixed bed reactor: phase I: $c_{SO4,0}$ = 160 mg/L; phase II: $c_{SO4,0}$ =75 mg/L.

Parameter	Symbol	Unit	Amount
pH			7.1
Temperature	T	°C	33
Redox potential	E'_0	mV	-200
Reactor volume	V_R	L	10.5
Hydraulic retention time	HRT	h	12.1
Dye concentration (RO 96)	$c_{D,0}$	mg/L	100
Concentration of substrate as yeast extract and acetic acid	$c_{S,0}$	mg/L DOC	106
- concentration of yeast extract	$c_{yeast,0}$	mg/L DOC	37
- concentration of acetic acid	$c_{Ac,0}$	mg/L DOC	69
Sulfate concentration	$c_{SO4,0}$	mg/L	75 -160

Cosubstrate (acetic acid and yeast extract) and the dye RO 96 as influent were added from the separate tanks. The cosubstrate in the tank was acidified with HCl below pH 3 to avoid its possible bacterial predegradation. To vary the sulfate concentration in the influent, the dye was

diluted for phase I in tap water, whereas the dye was diluted for phase II in deionized water amended with the trace element solution SL 9 and an appropriate amount of sulfate. The constant pH and temperature in the reactor were maintained using a pH control system with NaOH and a thermoregulator (NK 22, Haake, Germany) respectively. The sampling (40 mL) was conducted from the circulation pipe, assuming the complete mixing. The samples were centrifuged 15 min at 4000 rpm (model 302K, Sigma, Harz, Germany), filtered with the 0.2 μm glass fiber filter.

3.5.2. Decolorization tests in pure culture of sulfate reducing bacteria

Similar to the preparation of the medium for the decolorization tests in the anaerobic mixed culture, the medium III which contained no reducing agent (Table 5) was prepared. The pH of the medium was adjusted to pH 7.1 with sterile 2 mol/L HCl. Sterile 100 mL bottles as batch reactors were filled with 55 mL of the fresh sterile medium. The following components were added from sterile stock solutions to the medium before the inoculation: substrate - either pyruvate ($CH_3COCOONa$) or lactate ($DL\text{-}CH_3CHOHCOONa$), if necessary sulfate (Na_2SO_4) as terminal electron acceptor. The dye addition time was varied according to the experimental goal. A portion (1-2.5 mL) of the stock culture was inoculated as suspended cells, and the reactors were flushed with sterile N_2 to remove oxygen, sealed with teflon-backed butyl rubber septen plus aluminum-crimp caps, in which a low excess pressure (0.05-0.1 bar) of the gas was maintained. The influence of a possible carry-over of sulfide produced at sulfate reduction from the stock culture into the batch reactors at inoculation was not observed for decolorization tests because of their low concentrations caused by stripping with N_2 in the stock culture. The batch reactors were shaken continuously in a shaking machine (model 1083, GFL, Burgwedel, Germany). All tests were undertaken at 37 °C in duplicate or triplicate. The samples (0.25 or 0.5 mL) were taken aseptically with a sterile 1 mL syringe (Ochs, Göttingen, Germany) under N_2 atmosphere, filtered with the 0.2 μm glass fiber filter.

3.5.3. Kinetic tests of chemical decolorization of azo dyes

Sulfide ($Na_2S{\cdot}9H_2O$), L-ascorbic acid sodium salt ($C_6H_7NaO_6$) (Aldrich), L-cysteine ($C_3H_7NO_2S$), $Fe(II)SO_4$, Ti(III)-citrate, sodium dithionite, and yeast extract were employed for the tests of the chemical decolorization of azo dyes. These batch tests were kept strictly sterile by autoclaving or filtering all the materials. Ti(III)-citrate stock solution (0.1 mol/L) was prepared under the exclusion of oxygen by dissolving sodium citrate in $TiCl_3$ solution (15 %), and adjusted to pH 7.3 with Na_2CO_3. The Ti(III)-citrate stock solution was sealed under N_2 atmosphere, and stored in the dark at room temperature. Other stock solutions were prepared

by dissolving appropriate concentrations in the deaerated distilled water, and sealed under N_2 atmosphere.

To study the chemical decolorization with yeast extract and the reducing agents besides sulfide, sterile 100 mL bottles as batch reactors were filled with 55 mL of the medium composed of phosphate buffer (KH_2PO_4, 4.53-22.65 g/L; Na_2HPO_4, 6.63-33.15 g/L) in deaerated (by boiling) distilled water. The pH was maintained for 7.05-7.10. Dye, and yeast extract or a reducing agent from sterile stock solutions were added to the medium. Exceptionally, dithionite was added as powder to the medium. The reactors were flushed with N_2 to remove oxygen, sealed with teflon-backed butyl rubber septen plus aluminum-crimp caps, in which a low excess pressure (0.05-0.1 bar) of the gas was maintained. The batch reactors were shaken continuously in the shaking machine at 33 °C. The samples (1 mL) were taken under oxygen-free N_2 atmosphere, filtered with the 0.2 μm glass fiber filter.

To investigate the kinetics of decolorization with sulfide, 140 mL batch reactors were filled with approximately 135 mL of the medium composed of pH-buffer in deaerated (by boiling) distilled water. Sulfide and dye from the sterile stock solutions were added to the medium. The reactors were completely filled with the medium and sealed with a double septen-silicon plus a hole screw cap. For pH 7.05-7.10, the medium was phosphate-buffered (KH_2PO_4, 4.53 g/L; Na_2HPO_4, 6.63 g/L) without adjustment. For tests of the pH influence on decolorization kinetics the pH (6.3; 6.5) was maintained, in which 24.3 g/L of KH_2PO_4 and 6.6 g/L of Na_2HPO_4 were employed as buffer for pH 6.25-6.30; 23.0 g/L of KH_2PO_4 and 6.1 g/L of Na_2HPO_4 were used for pH 6.48-6.50. The pH 4.1 was kept with the buffer of 2.30 g/L CH_3COONa and 3.12 g/L CH_3COOH (99.8 %). For the control of pH 9.0 the buffer solution of 1.60 g/L NaOH and 2.48 g/L H_3BO_3 was used amended with 1 mol/L HCl. The sampling (0.25 mL) for sulfide measurement, if necessary, was conducted through the double septen with the sterile syringe under N_2 atmosphere. The batch reactors were shaken continuously in the shaking machine at 37 °C.

4. DECOLORIZATION MECHANISMS OF AZO DYES IN ANAEROBIC MIXED CULTURE

Decolorization tests in the anaerobic mixed culture were carried out in the batch reactors [Fig. 7(a)] in order to investigate the role of diverse groups of bacteria (sulfate reducing bacteria, methane producing bacteria, and fermentative bacteria) in the decolorization of the azo dye RO 96.

4.1. Influence of Sulfate Reducing Bacteria on Decolorization

The reducing agent sulfide is a commonly used substance in methanogenic research to scavenge oxygen from the medium and to establish low redox potential, ensuring anaerobic conditions. Initial abiotic experiments revealed that sulfide chemically decolorized RO 96 under exclusion of oxygen (s. Chap. 6). All further experiments were carried without addition of a reducing agent.

Deduced from the chemical reduction of RO 96 by sulfide, the role of SRB in the decolorization of azo dyes came into question. Thus, the SRB inhibitor molybdate was used to look into the role of SRB in decolorization. The selective effect of molybdate to inhibit SRB has been widely reported (Oremland and Taylor 1978; Newport and Nedwell 1988; Oremland and Capone 1988; Castro et al. 1999), although molybdate, or more precisely a molybdate-sulfide complex, has been reported to inhibit hydrogenase synthesis in the fermentation of glucose by the anaerobic bacteria *Ruminococcus albus* (Wolin and Miller 1980). Molybdate inhibits the enzymatic formation of a stable intermediate APS (adenosine-5´-phosphosulfate) required for the sulfate reduction, causing a rapid depletion of ATP pools in SRB (Oremland and Capone 1988).

In the batch reactor with sterilized bacteria, nearly no decolorization took place, pointing out that active bacteria are indispensable to decolorization (Fig. 11). The usage of acetate as the sole energy source, disregarding the organic dye additive, i.e. hexylene glycol, due to its negligible quantity (only 0.5 % of RO 96 on mole base) and the dye because of its almost non-biodegradability in the anaerobic mixed culture (s. Fig. 15), was to rule out fermentative bacteria and acetogenic bacteria (syntrophic hydrogen producing acetogenic bacteria; homoacetogenic bacteria) from the decolorization process in Fig. 11. A thermophilic obligately syntrophic anaerobe reported to utilize acetate (Zinder and Koch 1984) was not considered, due to its thermophilic growth condition at 60 °C. Sulfur reducing bacteria capable of utilizing acetate were also out of the question because there was no availability of elemental sulfur in the medium at the beginning. That is, the acetate could be degraded anaerobically primarily by MPB or by SRB in the presence of sulfate. With 8 mmol/L molybdate, over 95% of

decolorization of RO 96 took approximately three times longer than without the inhibitor (~120 h vs. 40 h). This demonstrates that SRB were necessary for a faster decolorization. The higher concentration (20-80 mmol/L) of the SRB inhibitor molybdate led to significantly slower decolorization of RO 96, verifying the contribution of SRB to the decolorization.

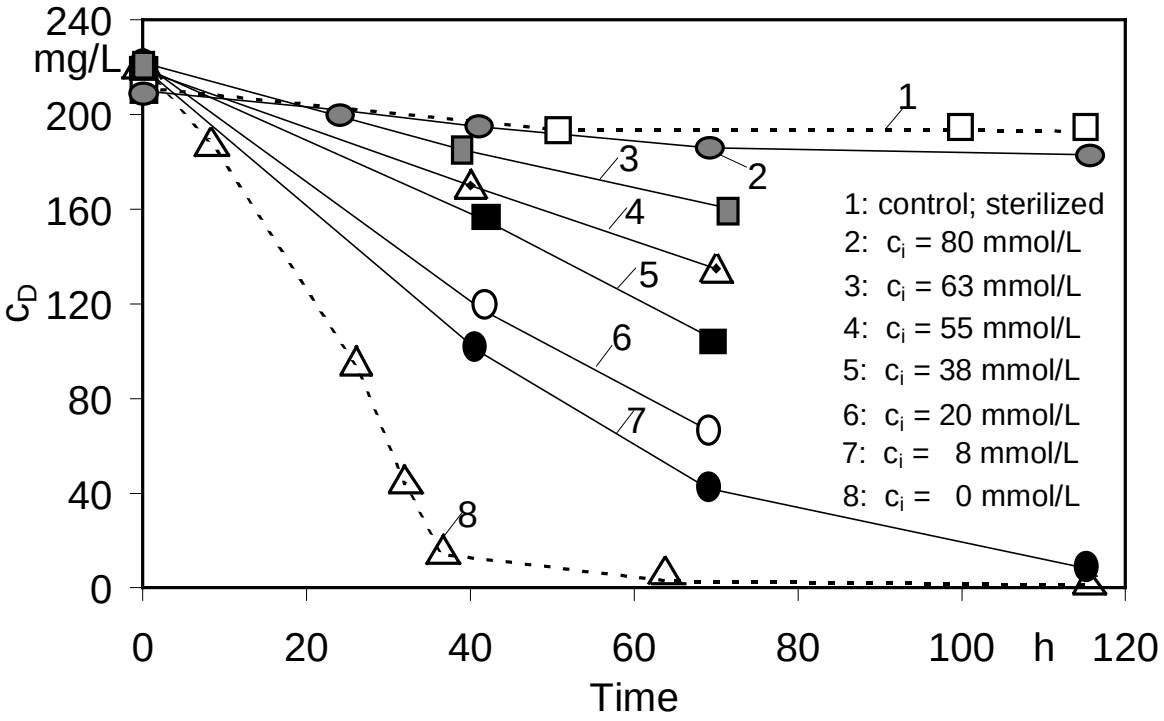


Fig. 11. Decolorization of RO 96 as a function of concentration c_i of the SRB inhibitor molybdate (MoO_4) in the presence of acetate and sulfate: $c_{S,0}$=20 mmol/L acetate, $c_{SO4,0}$=2.4 mmol/L, $c_{D,0}$=200 mg/L (0.18 mmol/L), $c_{B,0}$=0.8 g/L TSS, pH=7.3, T=33 °C. Medium II/III. Each data point is averaged (n=3).

The reason why the relatively high concentrations of molybdate (≤ 80 mmol/L) were required for an almost complete inhibition of decolorization, may be that the biofilm in the foam cube protected some of the SRB from the inhibitor. In addition, a question is interesting whether molybdate might scavenge sulfide ions produced by SRB, possibly resulting in inhibition of the decolorization with sulfide. This question was cleared up with a control experiment with 0.3 mmol/L RO 96, 1.3 mmol/L sulfide, and 20 mmol/L molybdate but without bacteria. The dye was abiotically decolorized by sulfide, which was not hampered by molybdate (data not shown).

A model was tried in order to describe the influence of molybdate concentration on the decolorization. Based on the double Monod-kinetics of substrate degradation by SRB, it follows:

$$r_S = \frac{\mu_{max}}{Y_{B/S}} c_B \frac{c_S}{(K_S + c_S)} \frac{c_{SO4}}{(K_{SO4} + c_{SO4})} \quad (38)$$

where r_S rate of substrate degradation (mmol h^{-1} L^{-1})
μ_{max} maximum specific growth rate (h^{-1})
K_S saturation coefficient for substrate (mmol/L)
$Y_{B/S}$ yield coefficient of bacteria on substrate (mmol/mmol, g/g)
c_B concentration of bacteria at time t (mmol/L, g/L)
c_S concentration of substrate at time t (mmol/L)
K_{SO4} saturation coefficient for sulfate (mmol/L)

Assuming that the decolorization kinetics can be coupled with the substrate degradation kinetics via the yield coefficient of dye (reduction) on substrate during decolorization, it follows:

$$r_D = Y_{D/S} r_S \quad (40)$$

where r_D decolorization rate (mmol h^{-1} L^{-1}, mmol h^{-1} L^{-1})
$Y_{D/S}$ yield coefficient of dye (reduction) on substrate (mmol/mmol, mg/mg)

The maximal decolorization rate $r_{D,max}$ can be defined from Eq.(40) as follows, in that $c_S >> K_S$, $c_{SO4} >> K_{SO4}$, and $c_B = c_{B,0}$ (approximately constant) in Eq.(38):

$$r_{D,max} = Y_{D/S} \frac{\mu_{max}}{Y_{B/S}} c_{B,0} \quad (41)$$

where $r_{D,max}$: maximal decolorization rate without molybdate (mg L^{-1} h^{-1}, mmol h^{-1} L^{-1})

The decolorization rate under the influence of molybdate r_{DM} may be described by non-competitive inhibition as follows:

$$r_{DM} = r_{D,max} \frac{1}{1 + c_{MoO4}/K_i} \quad (42)$$

where K_i coefficient for inhibition by molybdate (mmol/L)
c_{MoO4} concentration of molybdate (mmol/L)
r_{DM} decolorization rate under influence of molybdate (mg L^{-1} h^{-1}, mmol h^{-1} L^{-1})

For $c_{MoO4}=0$, $r_{DM}=r_{D,max}$ follows from Eq.(42); for $c_{MoO4}=K_i$, $r_{DM}=0.5r_{D,max}$. A higher concentration of molybdate should lead to a decrease in the decolorization rate. K_i may reflect

the composition of bacterial consortium, and the conditions of population, etc. Eq.(42) is transformed to:

$$\frac{r_{D,max}}{r_{DM}} = 1 + \frac{c_{MoO4}}{K_i} \qquad (43)$$

If a plot of ($r_{D,max}$ / r_{DM}) vs. c_{MoO4} represents a straight line, this model can be verified and K_i can be attained from the slope.

The model of Eq.(43) was applied to the assessment of the results of Fig. 11, since the concentration of substrate, sulfate, and bacteria were relatively high (i.e. $c_{S,0}$=20 mmol/L acetate, $c_{SO4,0}$=2.4 mmol/L, $c_{B,0}$=0.8 g/L TSS) in comparison with K_S=~0.2 mmol/L acetate (Schönheit et al. 1982; Thauer 1982), K_{SO4} =0.17 mmol/L (Ingoversen et al. 1984), and $Y_{B/SO4}$=4.8 g-TSS/mol-SO_4 (Widdel and Pfennig 1981; $Y_{B/SO4}$ indicates yield coefficient of bacteria on sulfate) (s. Table 3), disregarding the small differences of temperature or pH. The decolorization of Fig. 11 occurred in the correlation with sulfate respiration. Thus, if 2.4 mmol/L sulfate were degraded during the decolorization, 2.4 mmol/L sulfate could probably contribute to the bacterial growth of only approximately 11.5 mg-TSS/L (calculated using the $Y_{B/SO4}$). The maximal decolorization rate without molybdate $r_{D,max}$ was estimated with the decolorization course without molybdate of Fig. 11 (from 0 to approximately 35 h) showing a pseudo-zero-order kinetics with respect to the dye concentration. Similarly, the decolorization rates affected by the concentrations of molybdate r_{DM} were estimated with the respective decolorization courses from 0 to approximately 70 h of Fig. 11 (Table 7).

Fig. 12 shows that the data points ($r_{D,max}$/ r_{DM}) matched a straight line within approximately 63 mmol/L of molybdate added, but over the concentration the data point went aside from the line. The decolorization rate at 80 mmol/L molybdate is too small, thereby sensitively affecting the ratio of $r_{D,max}$/ r_{DM}. The coefficient for inhibition by molybdate K_i was obtained to be 18.2 mmol/L for the range of 0-63 mmol/L molybdate. This value of K_i is consistent to the result that the decolorization rate at ~20 mmol/L molybdate revealed the half of that without molybdate (s. Table 7). This quantified dependence of decolorization on the molybdate concentration supports the fact that the decolorization through SRB in the presence of sulfate resulted from the chemical reduction with sulfide, not from a reduction with reduced (co)-enzymes (of catabolic pathway). It should be noted that the inhibition kinetics is restricted to the given experimental conditions (bacterial consortium; immobilization method, etc.).

Table 7. Correlation of decolorization rate of RO 96 as a function of concentration of molybdate in the presence of acetate and sulfate, based on the results of Fig. 11

c_{MoO4} (mmol L^{-1})	r_{DM} (mg L^{-1} h^{-1})	standard deviation of r_{DM}	$r_{D,max}/ r_{DM}$ $\left[\frac{\text{mg } L^{-1} h^{-1}}{\text{mg } L^{-1} h^{-1}}\right]$
0	4.70	0.71	1.0
8	2.80	0.17	1.7
20	2.40	0.14	2.0
38	1.60	0.05	2.9
55	1.30	0.03	3.6
63	0.95	0.05	5.0
80	0.59	0.13	8.0

r_{DM}: decolorization rate affected by molybdate (mg L^{-1} h^{-1});
$r_{D,max}$: maximal decolorization rate without molybdate (mg L^{-1} h^{-1})

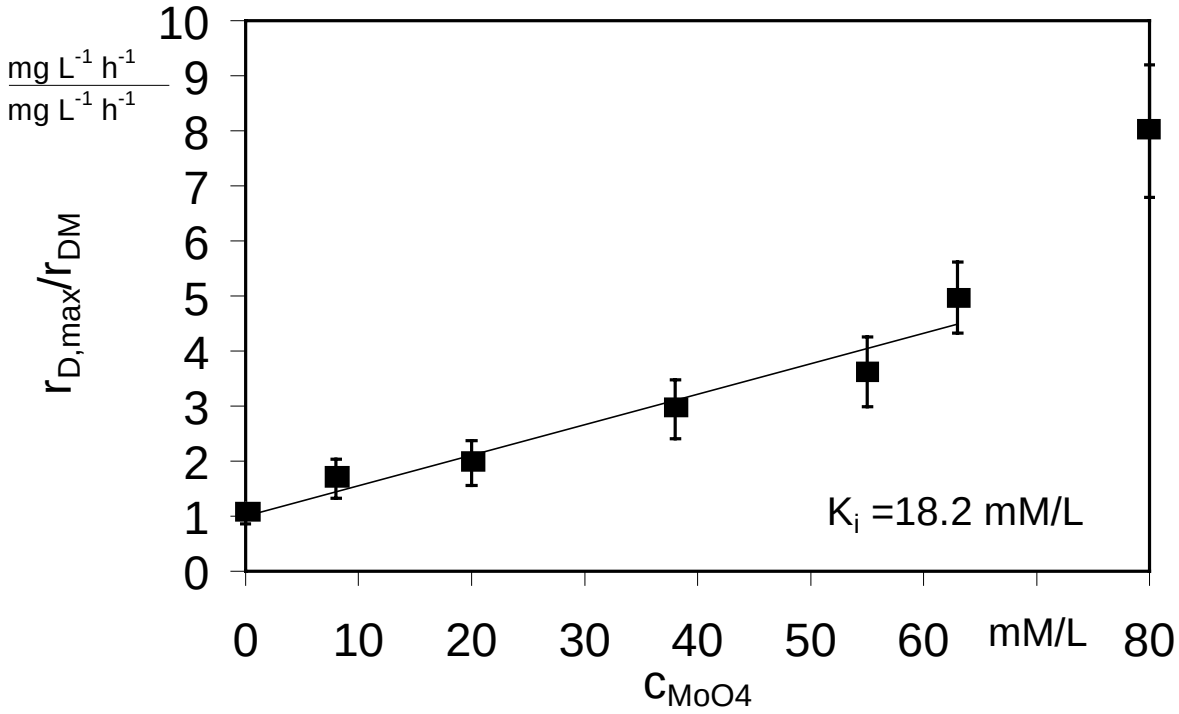


Fig. 12. Correlation of decolorization rate of RO 96 as a function of concentration of molybdate in the presence of acetate and sulfate: $c_{D,0}$=200 mg/L (0.18 mmol/L), c_{MoO4}= 0-80 mmol/L, $c_{S,0}$=20 mmol/L acetate, $c_{SO4,0}$=2.4 mmol/L, $c_{B,0}$=0.8 g/L TSS, pH=7.3, T=33 °C. Each data point is an average value (n=3). The error bar is the 95 % confidence interval using the *t*-distribution.

4.2. Influence of Methane Producing Bacteria on Decolorization

2-bromoethanesulfonic acid (BES) was utilized to identify the role of methane producing bacteria (MPB) in decolorization of RO 96. BES functions as a selective inhibitor hindering MPB because it acts as a competitive inhibitor of the methylcoenzyme M reductase complex of MPB (Oremland and Capone 1988). BES is an analog of methylcoenzyme M. The inhibition of methanogenesis from acetate or CO_2 at low concentrations of BES (0.6-7 mmol/L) in anaerobic bacterial consortiums was reported by many authors (Bouwer and McCarty 1983; Healy et al. 1980; Zehnder and Brock 1980). Zinder et al. (1984) showed that a possible differential susceptibility to BES by the various MPB could exist. Inhibition of methanogenesis from acetate required a much lower concentration than that from CO_2 (1 mmol/L vs. 50 mmol/L BES).

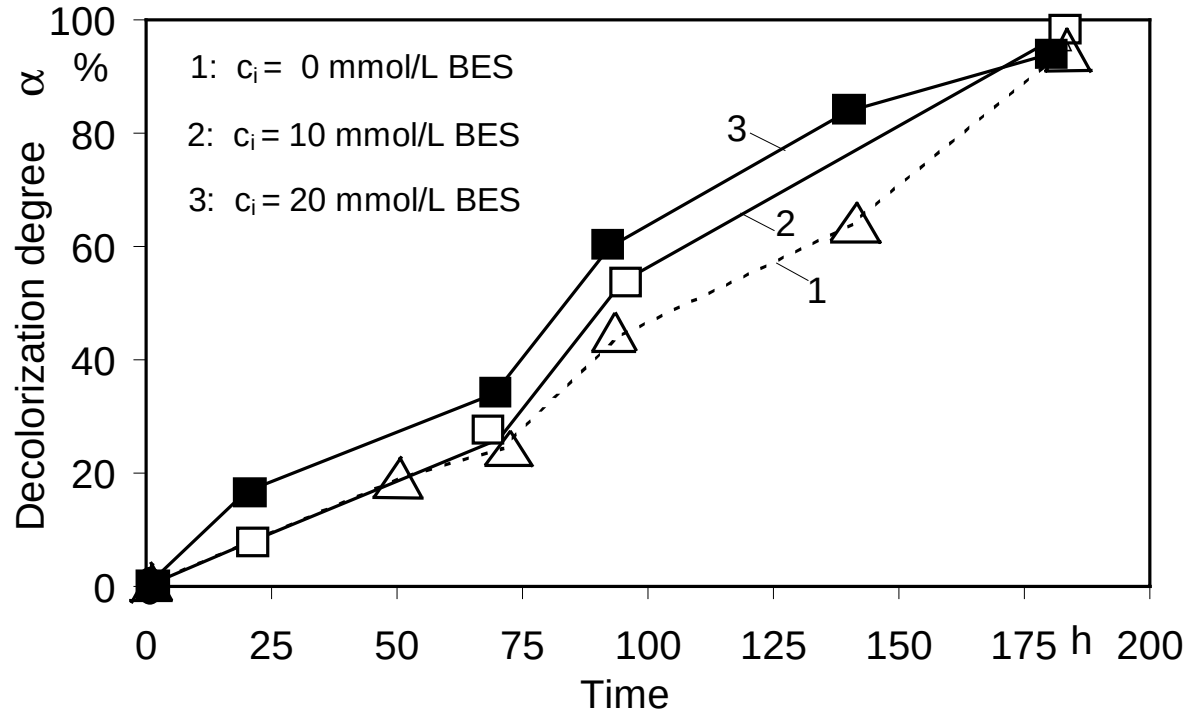


Fig. 13. Decolorization degree of RO 96 as a function of concentration c_i of the MPB inhibitor BES in the presence of acetate and sulfate: $c_{S,0}$=15 mmol/L acetate, $c_{SO4,0}$=1.1 mmol/L, $c_{D,0}$=70 mg/L (0.06 mmol/L), $c_{B,0}$=0.3 g/L TSS, pH=7.3, T=33 °C, Medium III. Each data point is an average value (n=3).

In the presence of acetate and sulfate, inhibition of MPB by BES (10, 20 mmol/L) did not significantly affect the decolorization of RO 96 (Fig. 13). Thus, MPB took no part in decolorizing RO 96. It is interesting that with the BES the decolorization of RO 96 occurred even slightly faster than without inhibitor. One possible explanation for this phenomenon is that

the sulfonate moiety of BES might be used by SRB as electron acceptor. Sulfide possibly produced in the reduction of the sulfonate moiety might improve the decolorization. However Lie et al. (1996) showed that a strain of *Desulfovibrio* utilized some sulfonates (isethionate, cysteate) as electron acceptor in place of sulfate, whereas BES did not function as electron acceptor for the strain. In particular, sulfate-grown SRB are not thought to use BES as an electron acceptor in preference to sulfate. Thus the more probable explanation is the competition between the SRB and MPB in the mixed population. That is, while SRB and MPB competed for the common substrate acetate, the inhibition of MPB possibly led to a more rapid degradation of acetate by SRB, bringing prompter sulfide development for the decolorization.

4.3. Influence of Fermentative Bacteria on Decolorization

Lactate is a common substrate for a large number of species of SRB (Postgate and Campbell 1966; Widdel 1988) and fermentative bacteria. The pathway of lactate degradation in a mixed culture is differentiated, depending on e.g. sulfate and substrate concentration or partial pressure of hydrogen (Widdel 1988). In general, the degradation of lactate to acetate by SRB in the presence of sulfate, accompanied by acetate conversion by acetate-utilizing SRB and MPB, is proposed to be favored over the degradation of lactate by lactate-utilizing fermentative bacteria (LFB) (Widdel 1988). The decolorization of RO 96 in presence of 1.3 mmol/L sulfate but without an inhibitor took place 2.4 times faster (ca. 90 % decolorization within 25 h) than that in the absence of sulfate and an inhibitor (ca. 90 % decolorization within 60 h) (Fig. 14). In the absence of sulfate primarily LFB could be active in decolorizing RO 96. This result indicates that also in the presence of lactate and sulfate as the energy source SRB gave a significant contribution to the decolorization of RO 96. When both inhibitors of MPB and SRB were provided in the presence of lactate and sulfate, the decolorization rate of RO 96 decreased to a great extent (ca. 30 % decolorization within 90 h). This may be ascribed to the fact that the LFB were at an additional disadvantage due to the increase in the hydrogen partial pressure caused by the inhibition of MPB with BES. A possible function of some SRB as a kind of LFB, converting lactate to acetate, CO_2, and hydrogen (Bryant et al. 1977; Zellner et al. 1994) might be expected due to the inhibition of sulfate reduction with molybdate. However the simultaneous inhibition of MPB with BES may prevent this possibility. Bryant et al. (1977) revealed the impossibility of the lactate fermentation by some strains of *Desulfovibrio* without a syntrophy of MPB.

That the SRB were able to produce sulfide leading to a decolorization under a relatively low sulfate concentration (1.3 mmol/L) can be explained by the high affinity of SRB to sulfate (Table 3). With a pure culture of *D. desulfiricans* Okabe et al. (1992) showed the saturation coefficients for sulfate and lactate K_{SO4}, K_{Lac} of 19 and 25 µmol/L respectively. It has to be

investigated further, whether SRB gave a large contribution to the decolorization, in comparison with that of the LFB, which is influenced by kinetic coefficients (μ_{max}, $Y_{B/S}$, K_{Lac}, etc) for both bacterial groups.

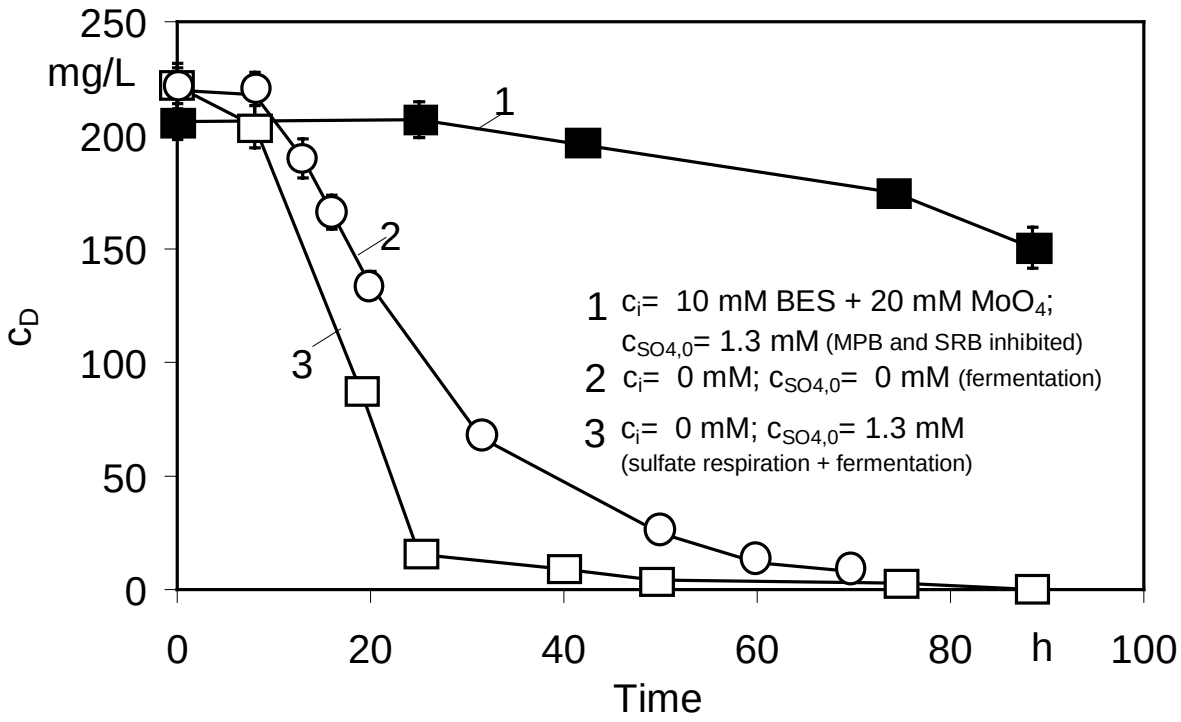


Fig. 14. Comparison of decolorization of RO 96 with and without inhibitors for both SRB and MPB in the presence of lactate and sulfate: $c_{S,0}$=20 mmol/L lactate, $c_{SO4,0}$=0-1.3 mmol/L, $c_{D,0}$= 200-220 mg/L (0.16-0.18 mmol/L), $c_{B,0}$=0.8 g/L TSS, pH=7.3, T=33 °C, Medium III. Each data point is an average value (n=6). The error bar is the 95 % confidence interval using the *t*-distribution.

4.4. Influence of Inhibitors on Substrate Degradation

Fig. 15 shows the influence of inhibitors on substrate degradation with respect to DOC (as mg/L) in the presence of acetate, sulfate and RO 96 in the anaerobic batch reactors. The start concentrations of dye and acetate were equal for the three reactors. The higher start concentration in the reactor with the inhibitor BES is due to the DOC of the inhibitor itself (4 mmol/L BES = 96 mg/L DOC, that were not thought to be used as energy and carbon source).

In the reactor with BES only 90 mg/L DOC was degraded within 100 h, thereafter no further change occurred, whereas 440 mg/L DOC was removed within 200 h in the other two reactors without BES. This blockage of DOC degradation with BES can be explained by the inhibition of MPB. That is, as long as sulfate was available (2.4 mmol/L), ~2.4 mmol/L acetate (58

DOC-mg/L) was degraded by SRB (s. Table 8), after which further degradation of the remaining acetate was blocked due to the inhibition of MPB by BES. In the reactor with molybdate, however, acetate was degraded mostly by MPB owing to the inhibition of SRB. Thus, the DOC was degraded slightly slower than in the reactor without inhibitor, but was removed to a low level in both. The DOC of acetate plus RO 96 was not completely degraded in both because of the almost non-degradability of the dye (65 mg-DOC/L).

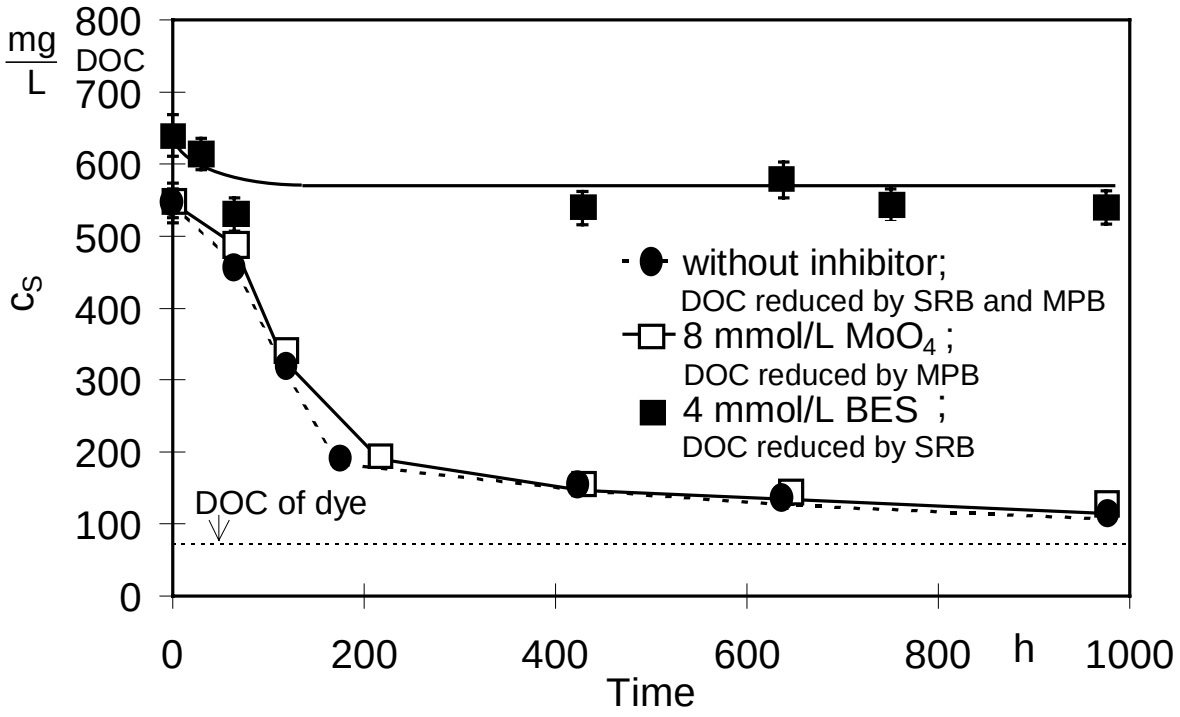
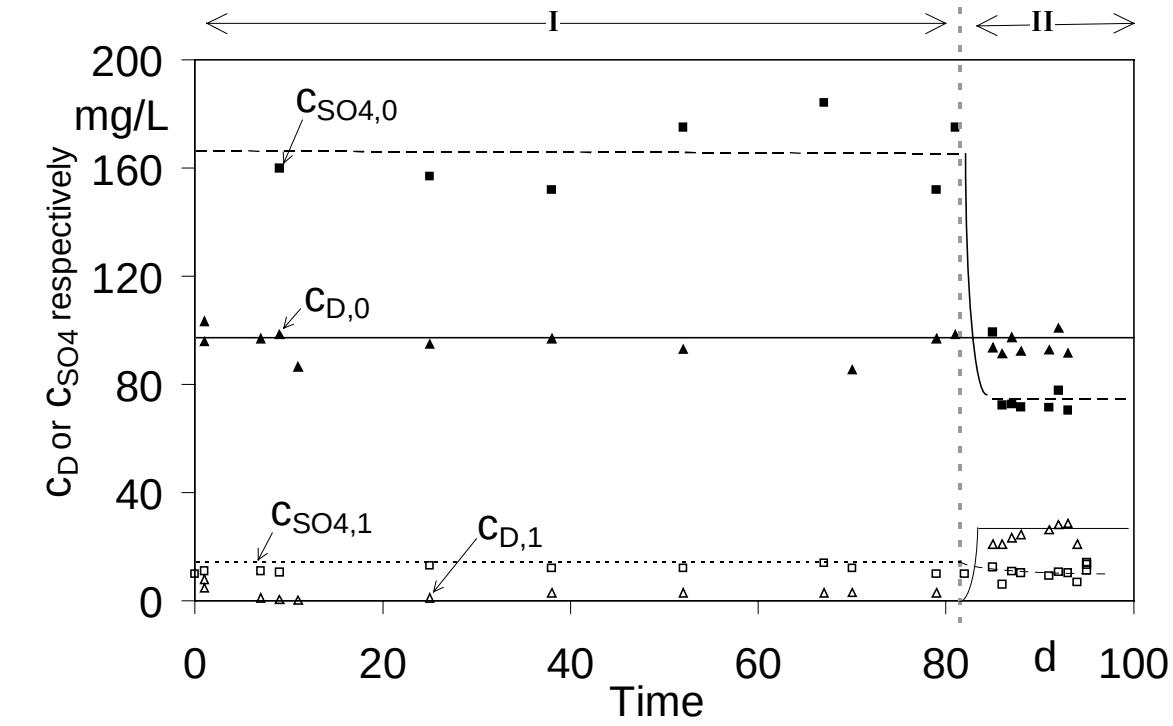


Fig. 15. DOC-degradation with and without inhibitors in the presence of acetate, sulfate and RO 96: $c_{S,0}$=20 mmol/L acetate (480 mg/L DOC), $c_{SO4,0}$=2.4 mmol/L, $c_{D,0}$=0.18 mmol/L RO 96 (65 mg/L DOC), $c_{B,0}$=0.8 g/L TSS, pH=7.3, T=33 °C, Medium II. Each data point is an average value (n=3). The error bar is the 95 % confidence interval using the *t*-distribution.

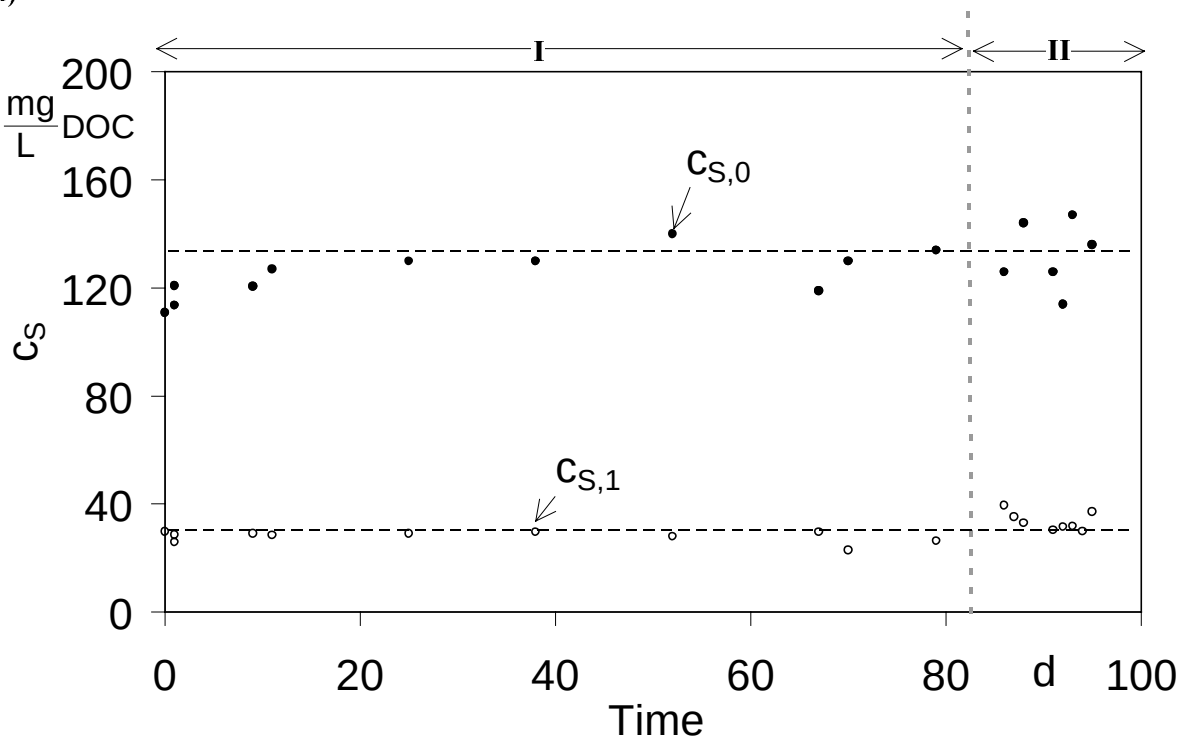
Although the inhibition of methanation through molybdate was reported in wastewater sludge degrading glucose (Puhakka et al. 1990), their results are not consistent with those of Ueki et al. (1988) and Oremland and Taylor (1978). Ueki et al. (1988) reported with municipal sewage sludge that the inhibition of SRB with 5 mmol/L molybdate completely blocked the sulfate reduction, but did not retard the methane production. The addition of molybdate to the marine sediment sludges increases the rate of methane formation over the uninhibited sludges (Oremland and Taylor 1978; Banat et al. 1983). Also in these experiments inhibition of MPB by molybdate was not observed, as can be seen from the DOC-degradation in the reactor with molybdate.

4.5. Influence of Sulfate Concentration on Decolorization

To confirm the contribution of SRB to the decolorization of azo dyes, a variation of sulfate concentration was made in the continuous-flow fixed bed reactor (s. Fig. 8). While the substrate (acetic acid and yeast extract) and dye concentrations in influent remained nearly constant, the sulfate concentration in the influent was varied from 160 mg/L (phase I) to 75 mg/L (phase II) (Fig. 16). In phase I the complete decolorization of RO 96 occurred; in phase II the decolorization stayed at approximately 74 % at the hydraulic retention time HRT=12.1 h. This indicates that the decrease in the sulfate concentration led to a decrease in the decolorization rate. That is, the presence of a lower sulfate concentration led to a lower production of sulfide capable of decolorizing the dye, which probably determined the decolorization rate. This result may also be explained by the aspect of electron partition in catabolisms of bacterial consortium. At the lower sulfate level more flow of electron probably shifted toward fermentation or methane production which possibly had less or no contribution to decolorization compared with the sulfate reduction of SRB. The incomplete sulfate reduction in both phases (approximately 90 %) is probably related to competition with methanation or fermentation for the common substrates (acetic acid, yeast extract).



(a)



(b)

Fig. 16. Influence of sulfate level on decolorization of RO 96 in anaerobic continuous-flow reactor at 33°C, HRT=12 h and pH =7.1: **(a)** influent- and effluent-concentration of sulfate and RO 96; **(b)** influent- and effluent-concentration of DOC.

Phase I: $c_{D,0}$=100 mg/L (0.08 mmol/L), $c_{SO4,0}$=160 mg/L (1.7 mmol/L), $c_{S,0}$=106 mg DOC/L (acetic acid and yeast extract).

Phase II: $c_{D,0}$=100 mg/L (0.08 mmol/L), $c_{SO4,0}$= 75 mg/L (0.8 mmol/L), $c_{S,0}$= 106 mg DOC/L (acetic acid and yeast extract).

4.6. Postulated Mechanism of Decolorization of Azo Dyes

4.6.1. Postulated mechanism

These experiments with the anaerobic mixed culture in the presence of selective inhibitors have proved that SRB are involved in the decolorization of the mono-azo dye RO 96. The decolorization mechanism of a mono-azo dye through SRB can be postulated as follows (s. Table 8 and 9): SRB catabolize the electron donor (e.g. acetate; lactate) in tandem with sulfate reduction (step I). This takes place biologically in the cells. The sulfides generated from sulfate reduction by SRB diffuse out of the cells, forming S^{2-}, HS^-, or H_2S (e.g. HS^- and H_2S, ~50 % : 50 % according to the dissociation constant at pH 7; s. Fig. 40) (step II). The sulfides chemically reduce the azo bond of the mono-azo dye, producing two aromatic amines and elemental sulfur as the reduction products (step III).

The decolorization reaction of a mono-azo dye resulting from degradation of acetate, or lactate by SRB can be estimated stoichiometrically as following (s. Table 8 and 9):

1 mol acetate-degradation → 1 mol sulfide-production →0.5 mol RO 96-decolorization (44)
2 mol lactate-degradation → 1 mol sulfide-production →0.5 mol RO 96-decolorization (45)

TABLE 8. Postulated mechanism for the decolorization of a mono-azo dye in the degradation of acetate by SRB

Step	Reaction
I biological	$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + H^+ + S^{2-}$ (Widdel 1988; Thauer et al. 1977; Widdel and Pfennig 1981)
II chemical	$S^{2-} + H^+ \longleftrightarrow HS^-$ $HS^- + H^+ \longleftrightarrow H_2S$
III chemical	$0.5H_2S + 0.25R\text{-}N{=}N\text{-}R' \rightarrow 0.5S + 0.25R\text{-}NH_2 + 0.25R'\text{-}NH_2$ or $0.5HS^- + 0.25R\text{-}N{=}N\text{-}R' + 0.5H^+ \rightarrow 0.5S + 0.25R\text{-}NH_2 + 0.25R'\text{-}NH_2$ or $0.5S^{2-} + 0.25R\text{-}N{=}N\text{-}R' + H^+ \rightarrow 0.5S + 0.25R\text{-}NH_2 + 0.25R'\text{-}NH_2$

R-N=N-R´ symbolizes a mono-azo dye;
R - NH_2 and R´- NH_2 indicate the aromatic amines.

TABLE 9. Postulated mechanism for the decolorization of a mono-azo dye in the degradation of lactate by SRB

Step	Reaction
I biological	$2CH_3CHOHCOO^- + SO_4^{2-} \rightarrow 2CH_3COO^- + 2HCO_3^- + 2H^+ + S^{2-}$ (Gottschalk 1986)
II chemical	$S^{2-} + H^+ \longleftrightarrow HS^-$ $HS^- + H^+ \longleftrightarrow H_2S$
III chemical	$0.5H_2S + 0.25R\text{-}N{=}N\text{-}R' \rightarrow 0.5S + 0.25R\text{-}NH_2 + 0.25R'\text{-}NH_2$ or $0.5HS^- + 0.25R\text{-}N{=}N\text{-}R' + 0.5H^+ \rightarrow 0.5S + 0.25R\text{-}NH_2 + 0.25R'\text{-}NH_2$ or $0.5S^{2-} + 0.25R\text{-}N{=}N\text{-}R' + H^+ \rightarrow 0.5S + 0.25R\text{-}NH_2 + 0.25R'\text{-}NH_2$

R-N=N-R´ symbolizes a mono-azo dye;
R - NH_2 and R´- NH_2 indicate the aromatic amines.

This mechanism can obtain a practical meaning in decolorization processes, since SRB have general advantages of thermodynamics and kinetics in the presence of sulfate over other bacteria (e.g. MPB, fermentative bacteria) competing for common substrates (Widdel 1988; Colleran et al. 1995), and real waste water from the textile finishing usually contains high sulfate concentrations.

4.6.2. Factors influencing decolorization of azo dyes through sulfate reducing bacteria

In order to design engineering systems for a complete decolorization in a real and complex system, an understanding of the correlation between the possible decolorization of azo dyes and the diverse catabolisms of different bacteria is necessary (s. Fig. 17 in combination with Table 10). The presence of competitive electron acceptors in the biological system is a rate controlling factor for the decolorization. The sequence of electron accepting processes is differentiated according to the free energy gained in the respective catabolism, which depends on the redox potential difference of the electron donors and acceptors, e.g. aerobic respiration, nitrate-, sulfate-, and CO_2-respiration in that order (Fig. 17). The redox potential level of fermentation is close to that of sulfate- or CO_2-respiration (Characklis 1990). Fig. 17 shows that under the various catabolisms, the fermentation and sulfate respiration can contribute to

decolorization, whereas aerobic respiration, nitrate- and CO_2-respiration cannot be involved in decolorization. The inability of MPB to decolorize azo dyes can be deduced from the fact that the methanogenesis from CO_2 or acetate is a terminal electron accepting step in anaerobic catabolism of organic carbon complexes in mixed cultures. Sulfate respiration by SRB with H_2 or acetate is also the terminal step, but sulfide produced in the sulfate respiration is able to decolorize azo dyes. Some SRB have a catabolic capability of respiration also with other sulfur oxides (e.g. sulfite, thiosulfate) as terminal electron acceptor than sulfate, producing sulfide (Widdel 1988). Also sulfur reducing bacteria (e.g. *Desulfuromonas actoxidans*) are expected to possibly take part in the decolorization process through anaerobic sulfur reduction to sulfide (Schlegel 1992). Another possibility for sufide to be generated in anaerobic bacterial systems is expected from the degradation of sulfur-containing amino acids in proteins. Aulenbach and Heukelekian (1955) revealed the formation of sulfide in the anaerobic degradation of sulfur-containing amino acids such as cystine and methionine. Thus sulfide originating from the anaerobic degradation of proteins may contribute to decolorization. A contribution of Fe(III) reducing bacteria to decolorization at basic pH can also be possible, with $Fe(OH)_2$ developed from the reduction of Fe(III).

Interpretation of experimental results showing that denitrification occurred preferentially to the reduction of azo dyes, thereby causing a lag phase or inhibition of the decolorization (Wuhrmann et al. 1980; Carliell et al. 1995; Glässer 1992) falls in line with the redox potential sequence of Fig. 17. Naturally, the activities of diverse metabolisms in a system, in accordance with the availability of substrates, are not excluded. This is well illustrated by the work of Lens et al. (1995), in which the authors found the presence of MPB and SRB in a variety of aerobic biofilms and (probably flocks of) activated sludges. Partial dye decolorization was found in aerobic biofilm reactors (Harmer and Bishop 1992).

In relation to the influence of sulfate level on the decolorization of azo dyes, Carliell et al. (1995) found no significant difference in the decolorization rate of the azo dye C.I. Reactive Red 141 for two sulfate concentrations (5, 10 mmol/L) with bacteria from an anaerobic digestor. Assuming that SRB are involved in the decolorization, this lack of a significant difference in decolorization rate may be attributed to the presence of a sufficient amount of sulfate (originating probably from the dye additives) in the initial solution to decolorize the dye without a need for additional sulfate. Glässer (1992) showed the inhibition of decolorization of C.I. Mordant Yellow 3 in the presence of 10 and 20 g/L Na_2SO_4 in the bacterium consortium with facultative bacteria. The reason for this inhibition is probably not only the toxicity of H_2S developed by the sulfate reduction, but also the increase of the osmotic pressure caused by the high salinity (Isa et al. 1986).

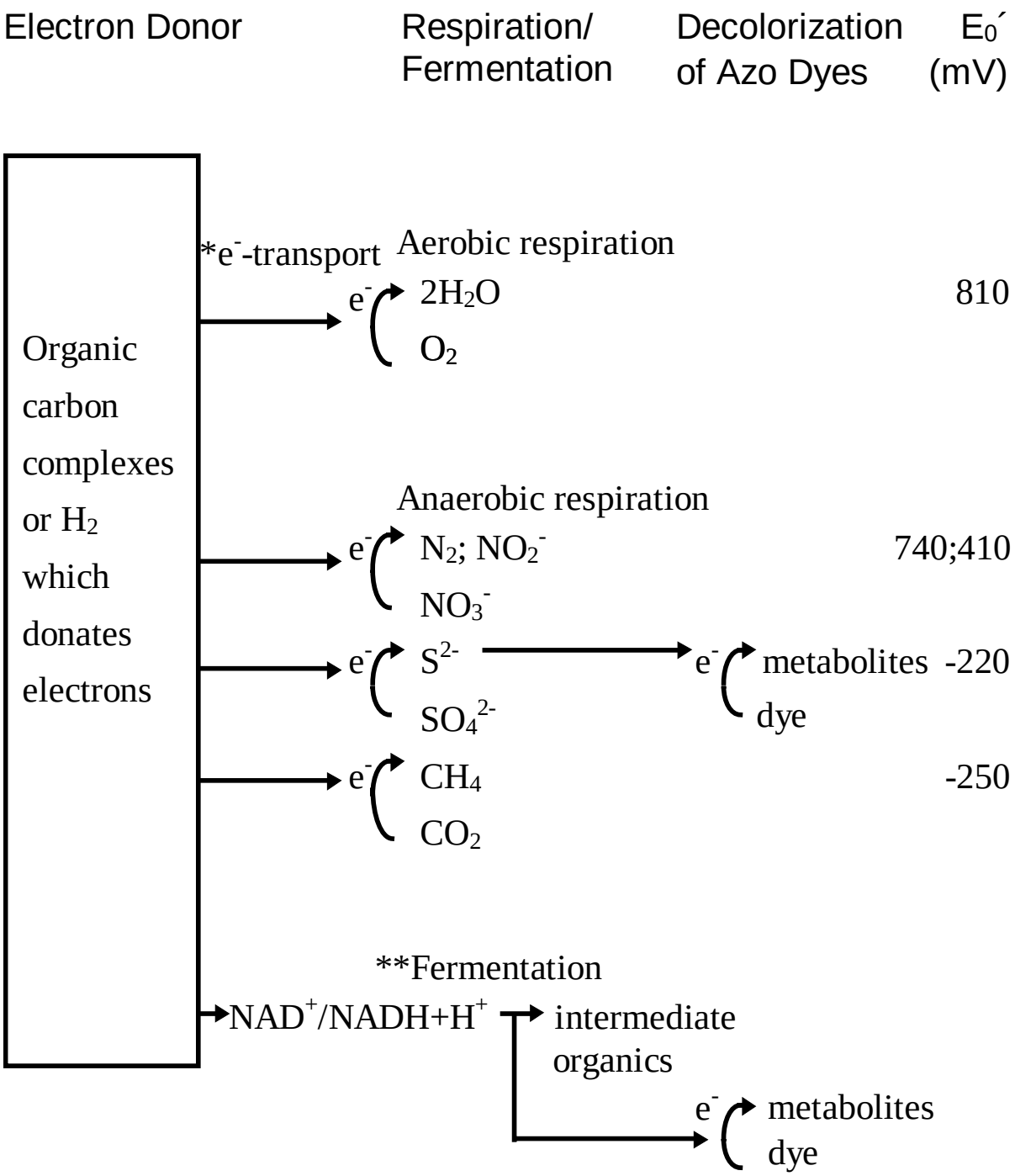


Fig. 17. Correlation between bacterial catabolisms (aerobic/anaerobic respiration, fermentation; modified from Schlegel 1992) and possible decolorization of azo dyes: Redox half potentials (E_0´) for the related catabolisms at pH 7 and 25 °C (Characklis, 1990). *Number of electrons transported is not considered. **It is hypothesized that azo dyes can possibly function as alternative terminal electron acceptors.

Table 10. Examples of the catabolism of aerobic/ anaerobic respiration and fermentation with organic carbon (CH_2O; formaldehyde) or H_2 according to Fig. 17

Reaction	Note
$CH_2O + O_2 \longrightarrow CO_2 + H_2O$	aerobic respiration[1]
$5CH_2O + 4NO_3^- + 4H^+ \longrightarrow 5CO_2 + 2N_2 + 7H_2O$	nitrate respiration[1]
$2CH_2O + SO_4^{2-} \longrightarrow 2CO_2 + S^{2-} + 2H_2O$	sulfate respiration[1]
$4H_2 + CO_2 \longrightarrow CH_4 + 2H_2O$	CO_2 respiration[2]
$3CH_2O + H_2O \longrightarrow 2CH_3OH + CO_2$	fermentation[1]

[1]Stumm and Morgan (1996); the reaction of sulfate respiration is modified.

[2]Thauer et al. (1977); Schlegel (1992)

Analogous to the proposed chemical extracellular decolorization mechanism of Dubin and Wright (1975), the redox potential of the dyes is an important factor in the decolorization through SRB. The reduction of azo dyes by sulfide, conditioned with a redox potential difference, is thought to be unspecific, similar to that by extracellular non-enzymatic organic redox mediators (electron shuttles) (Keck et al. 1997). The decolorization mechanism of azo dyes with sulfide is differentiated from that with the redox mediators, in that sulfide is principally not recycled because of its oxidation to elemental sulfur during decolorization; the redox mediators are recycled. The COD/S ratio [ratio of COD of substrate to sulfate-sulfur concentration (g/g)] may also be a rate controlling factor in the decolorization through SRB, since the COD/S ratio was proposed to be the most significant factor in determining the partitioning of the electron flow between SRB and MPB (Mizuno et al. 1994; Colleran et al. 1995; Omil et al. 1998).

Sulfide toxicity may significantly affect the competition outcome between SRB and other bacteria, and thus influence the decolorization through SRB. Uberoi and Bhattacharya (1995) proposed a relatively low level of the sulfide toxicity, showing that in degradation of propionate at pH 7.3 the sulfide inhibition limit was near 14.5 mmol/L of the total sulfide. Over this limit, sulfide was more toxic to SRB than to acetogenic bacteria or MPB. Analogous to this result, fifty percent inhibition of the lactate utilization of *D. desulfuricans* occurred at a total sulfide concentration of approximately 16 mmol/L at pH 7.0, with sulfide inhibition being reversible (Okabe et al. 1992). In contrast, Isa et al. (1986) reported a more optimistic result concerning the sulfide toxicity. Free H_2S, which is thought to be the most toxic species of sulfide, up to 23.5 mmol/L caused less than 20% inhibition of methane production from acetate. At the same level of free H_2S, less than 30% inhibition of sulfate reduction occurred with acetate and ethanol as substrate. The authors attributed this to acclimation of MPB and

SRB to the high sulfide concentrations. It is logical to assume that pH is an influential factor to detoxify the sulfide inhibition, since it determines the distribution of sulfide species (Oleszkiewicz et al. 1989).

To eliminate the sulfide toxicity, there have been various trials, e.g., the coculturing SRB with photosynthetic sulfur bacteria which oxidize sulfide to sulfur (Maree and Strydom 1987), the coupled anaerobic/aerobic treatment with sulfate reduction and sulfide oxidation to elemental sulfur by an aerobic *Thiobacillus* sp. (Fox and Venkatasubbiah 1996), and the precipitation of hydrogen sulfide by iron added in the sulfate-reducing chemostats (Gupta et al. 1994a). Lawrence and McCarty (1965) viewed another aspect of the precipitation of sulfides by heavy metals that sulfides played a significant role in preventing toxicity of most heavy metals in anaerobic treatment.

4.6.3. Possibilities of mineralization of azo dyes

Dyes must not only be decolorized but also mineralized, since the aromatic amines, the reduction products of the azo dyes, can be mutagenic and/or possibly carcinogenic (Levine 1991). Thus the development of various forms of two-stage (anaerobic decolorization and subsequent aerobic degradation of metabolites) bacterial treatment has been tried by many groups (Haug et al. 1991; Soewondo 1997; Brown and Hamburger 1987). The two-stage treatment seems to attain a meaning also in light of detoxifying sulfide via its reaction with oxygen. Haug et al. (1991) obtained complete anaerobic decolorization of the azo dye C.I. Mordant Yellow 3 and the complete aerobic mineralization of the metabolites. In the two stage continuous-flow reactors treating RO 96, only ~30 % DOC of the dye could be mineralized aerobically (Soewondo 1997). Brown and Hamburger (1987) showed in the screening tests that the aromatic amines produced by the anaerobic reductive cleavage of the azo bonds of a large number of dyes (e.g. C.I. Acid Yellow 36, C.I. Acid Yellow 25, C.I. Direct Red 7) could be degraded with a range from 25 to 75 % by activated sludge, which was controlled by DOC and/or specific analysis for particular metabolites. The authors suggested that lipophilic aromatic primary amines are aerobically degradable, but depending on their precise structure, e.g. some sulfonated aromatic amines are not degradable. This is also consistent with the results of Pagga and Brown (1986). Zeyer et al. (1985) showed that a strain of *Moraxella* sp. was able to utilize as sole source of carbon and nitrogen some ring-substituted anilines (e.g. 4-chloroaniline) which are used as educts in the synthesis of many azo dyes.

One potential strategy to increase mineralization may be to foster an adaptation of the anaerobic bacteria to azo dyes or the metabolites as the sole C-, N-, or energy source. Possible mineralization of azo dyes may be supported by the participation of MPB or SRB. A small

degradation (approximately 15 %) of a metabolite (reduction product) of RO 96 as the sole N-, C-, or energy source was shown at pH 7.3 and 33 °C under the anaerobic mixed culture in the presence of sulfate (data not shown). This partial degradation of the metabolite corresponded to DOC-degradation coupled to sulfate reduction, suggesting a possible participation of SRB utilizing the metabolite as N-, C- or energy source. Razo-Flores et al. (1997) showed a possibility to mineralize a pharmaceutical azo dye under methanogenic conditions with granular sludge, in which the bacteria had been adapted to the lack of an auxiliary substrate.

4.7. Summary

1. In the presence of acetate and sulfate, SRB significantly contributed to the decolorization of RO 96 in the anaerobic mixed culture.
2. MPB played no role in the decolorization of RO 96 in the anaerobic mixed culture.
3. Also in the presence of lactate and sulfate, SRB gave a significant contribution to the decolorization of RO 96.
4. The mechanism of decolorization of azo dyes based on the extracelluar chemical reduction with sulfide was postulated. Sulfide produced via sulfate respiration by SRB chemically decolorizes azo dyes.

Based on these results, the next chapter enters into the study of decolorization mechanism in a pure culture of SRB.

5. MECHANISMS OF DECOLORIZATION OF AZO DYES BY *DESULFOVIBRIO DESULFURICANS*

In order to elucidate the correlation between the catabolism of SRB and the accompanying decolorization of azo dyes, batch experiments were made employing the pure culture of *D. desulfuricans* in the presence of lactate or pyruvate as energy and carbon source with varying sulfate levels. The purpose behind the use of pure cultures was to rule out possible influences of other bacteria on decolorization tests. *D. desulfuricans* is an incomplete oxidizer degrading pyruvate or lactate only to acetate and CO_2 with sulfate reduction (Widdel 1988). Acetate production is an indispensable process for such incomplete oxidizers, coupled to ATP gain via the substrate-level phosphorylation.

5.1. Inhibition by C. I. Reactive Orange 96 of Activation of Catabolism of *Desulfovibrio desulfuricans*

In initial tests, the azo dye RO 96 (0.26-0.33 mmol/L) was added at the starting point (t=0), in which the activation of catabolism via pyruvate or lactate as substrate (electron donor) was lacking irrespective of the presence of sulfate, revealing no significant production of acetate from lactate or pyruvate (Fig. 18). No decolorization occurred. This lack of catabolism is probably attributed to the inhibition of the catabolic activation by the dye, since in the absence of a dye the activation of a catabolic process with lactate or pyruvate was successful (Fig. 19).

At the starting stage in the lag phase, even without the dye, the bacteria already had difficulty in overcoming an unadvantageous redox state in the assay media due to no use of a reducing agent, showing a relatively long lag phase (over 20 h) (Fig. 19). The double load with the unadvantageous redox state and inhibition of the organisms with the dye could probably retard the activation of catabolism of *D. desulfuricans* (Fig. 18). Moreover the bacteria had not been acclimated to azo dyes. In further experiments azo dyes were added after the activation of catabolism (i. e. after pyruvate or lactate had been degraded).

From Fig. 19(a) and (b), it is derivable that the yield coefficients of acetate on sulfate $Y_{Ac/SO4}$ from lactate and pyruvate were approximately 2 and 4 mol/mol respectively. 18.5 mmol/L of acetate from the lactate degradation were produced coupling to the reduction of 9.2 mmol/L sulfate, while 16 mmol/L of acetate from the pyruvate degradation were produced coupling to the reduction of 4 mmol/L sulfate.

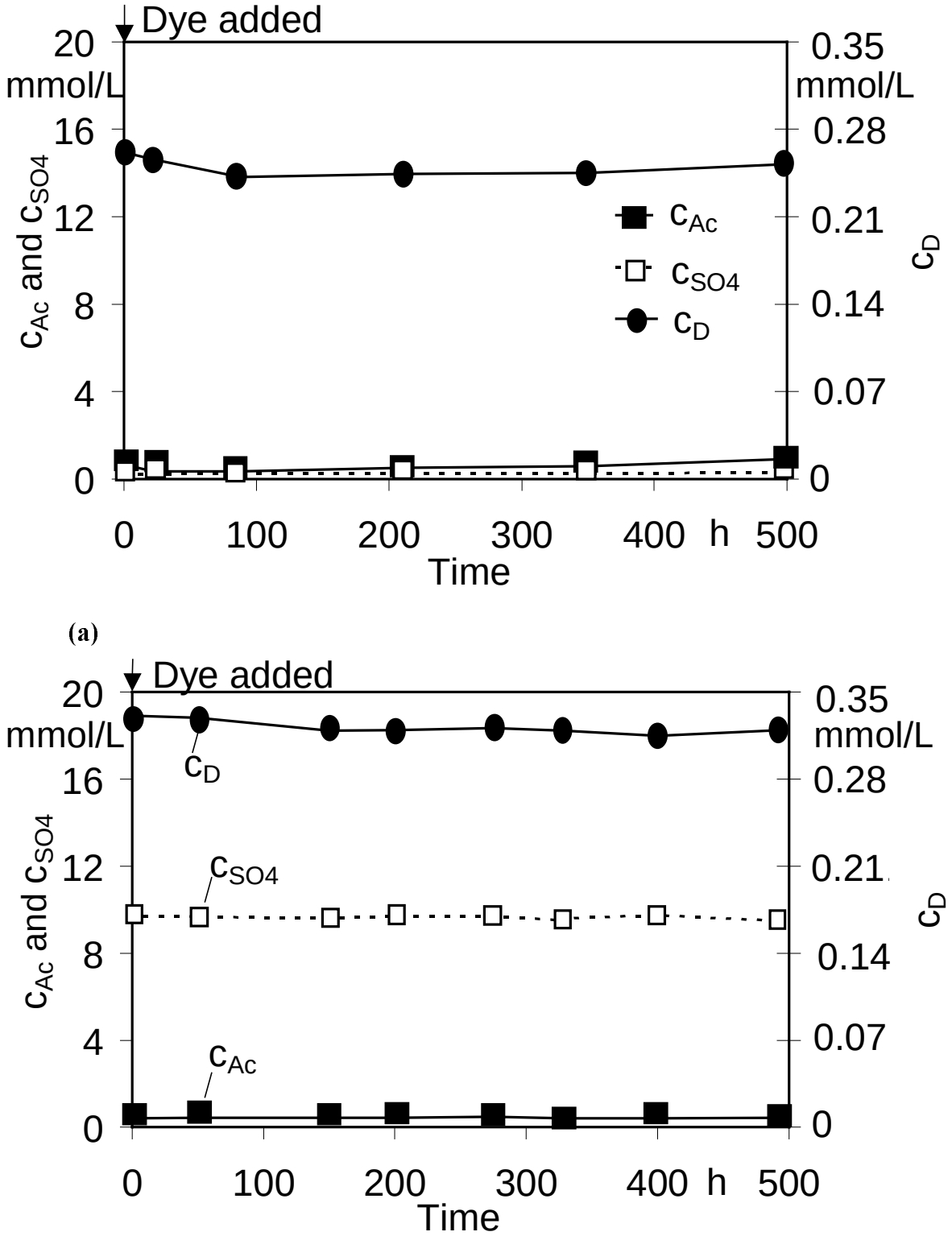
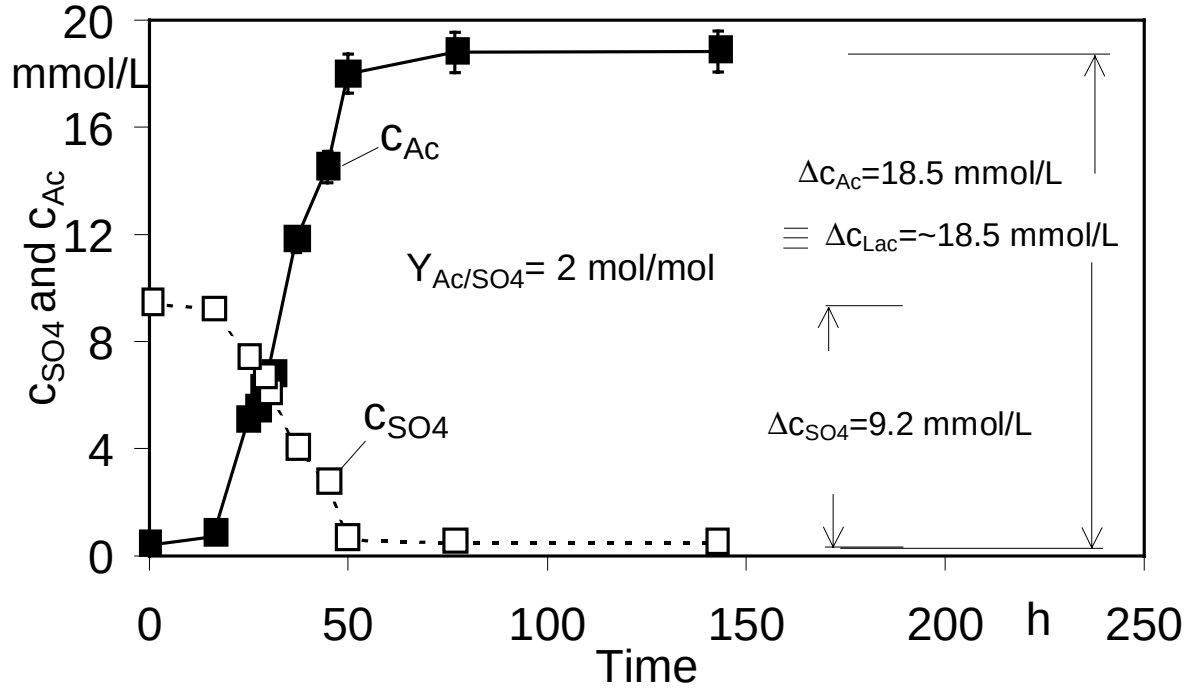


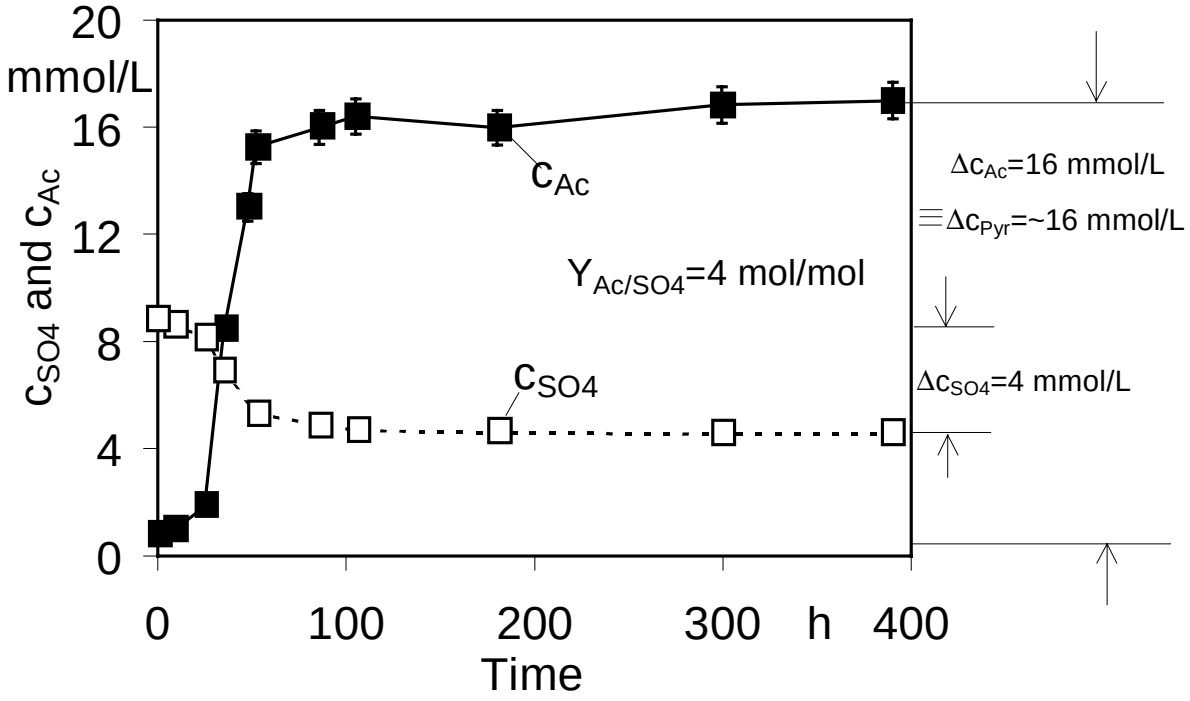
Fig. 18. Inhibition by RO 96 of catabolic activation of *D. desulfuricans* with pyruvate or lactate: pH = 7.1 and T=37 °C.

(a) $c_{D,0}$ =0.26 mmol/L, $c_{SO4,0}$ =0.2 mmol/L, $c_{S,0}$=19.1 mmol/L pyruvate, $c_{B,0}$=10 mg/L TSS

(b) $c_{D,0}$ =0.33 mmol/L, $c_{SO4,0}$ =9.7 mmol/L, $c_{S,0}$=19.2 mmol/L lactate, $c_{B,0}$=11 mg/L TSS



(a)



(b)

Fig. 19. Acetate production in sulfate respiration with lactate (a) and pyruvate (b) by *D. desulfuricans* in absence of an azo dye: T=37 °C, pH=7.1.

(a) $c_{SO4,0}$=9.4 mmol/L, $c_{S,0}$=19.4 mmol/L lactate, $c_{B,0}$=11 mg/L TSS,

(b) $c_{SO4,0}$ = 8.9 mmol/L; $c_{S,0}$=18.2 mmol/L pyruvate, $c_{B,0}$= 24 mg/L TSS,

Each data point is averaged. The error bar is the 99 % confidence interval using the *t*-distribution (n=3).

5.2. Decolorization of Azo Dyes in Sulfate Respiration of *Desulfovibrio desulfuricans*

To investigate the possibility of decolorization of azo dyes in sulfate respiration with pyruvate or lactate by *D. desulfuricans*, the acetate production and sulfate reduction were followed. Pyruvate ($c_{S,0}$=18.2 mmol/L at start) as electron donor was limited compared with sulfate ($c_{SO4,0}$=9.5 mmol/L) as electron acceptor, thereby making possible only sulfate respiration, not fermentation (Fig. 20). The production of 17 mmol/L acetate was coupled to the reduction of 4.2 mmol/L sulfate within the first 100 h (s. stoichiometry of Table 11), after that the acetate production and the sulfate reduction were almost finished, indicating exhaustion of the electron donor. The dissolved sulfide concentration was measured at ~3.3 mmol/L. RO 96 was added at exhaustion of the electron donor (360 h), to allow decolorization only with the accumulated catabolic product, i.e. sulfide. RO 96 (0.21 mmol/L) was completely decolorized at a high rate (over 95 % decolorization within 2 h from 360 to 362 h), demonstrating the chemical reduction of RO 96 with sulfide. The small discrepancy between the produced acetate concentration (~17 mmol/L) and the initial concentration of pyruvate (18.2 mmol/L) may be explained by the use of substrate for the bio-synthesis (anabolism) (Badziong *et al*. 1979).

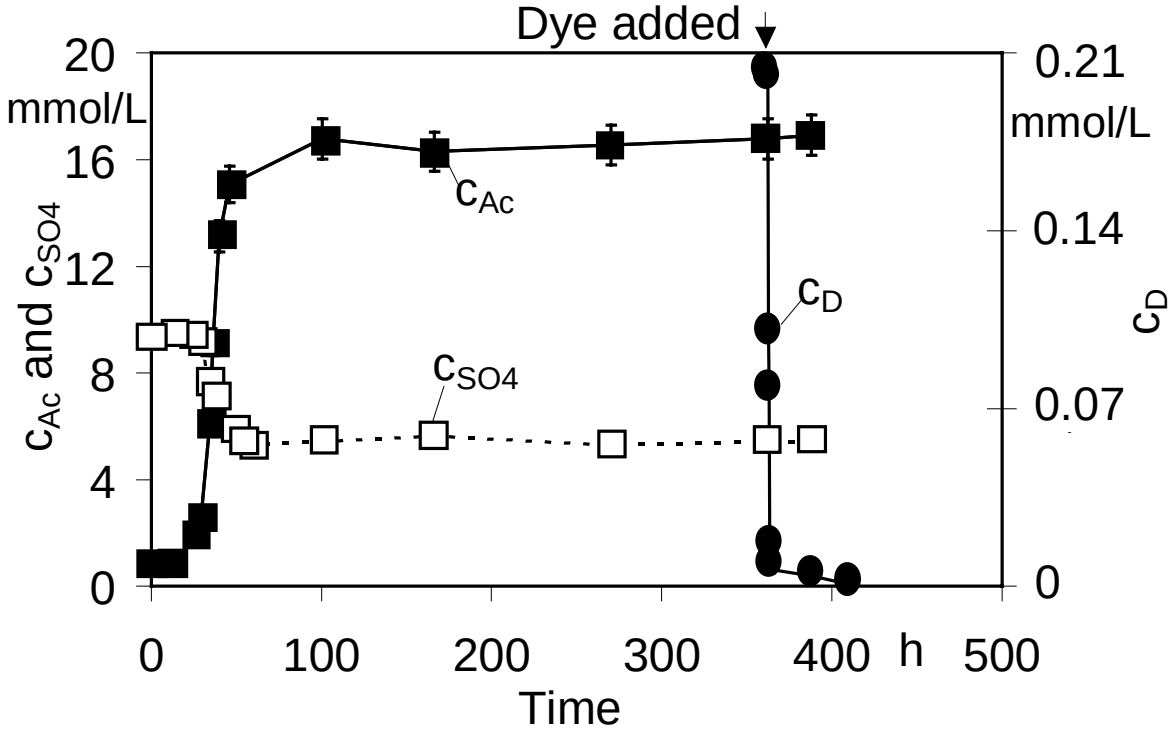


Fig. 20. Anaerobic decolorization of RO 96 in sulfate respiration with pyruvate by *D. desulfuricans* at excess in sulfate: $c_{SO4,0}$=9.5 mmol/L, $c_{S,0}$=18.2 mmol/L pyruvate, $c_{D,0}$=0.21 mmol/L, $c_{B,0}$=24 mg/L TSS, $c_{S(II)}$ =~3.3 mmol/L at 360 h (concentration of dissolved sulfide), T=37 °C, pH=7.1. Each data point is averaged. The error bar is the 99 % confidence interval using the *t*-distribution (n=3).

The decolorization mechanism of a mono-azo dye in sulfate respiration with pyruvate as electron donor by *D. desulfuricans* can be postulated as follows (Fig. 21, Table 11): Electrons (or $e^- + H^+$) are liberated from the oxidation of pyruvate to acetate plus CO_2, coupled to ATP gain via acetyl-CoA. The electrons are utilized for the reduction of sulfate as the terminal electron acceptor to sulfide, yielding ATP via chemiosmotic process (Gottschalk 1986) (step I of Table 11). Sulfides diffuse out of the cells, forming S^{2-}, HS^-, or H_2S (dependent on pH) (step II). The sulfides chemically reduce the azo bond of the dye, producing two aromatic amines (i.e. dye metabolites) and elemental sulfur as the reduction products (step III). This chemical decolorization is not linked to an ATP-gain via sulfate respiration by *D. Desulfuricans*.

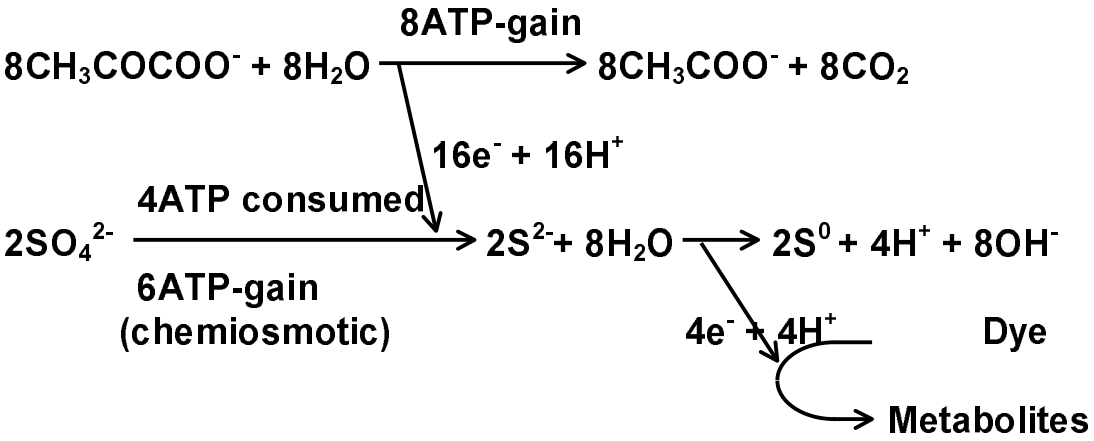


Fig. 21. Schematic pathway of decolorization of a mono-azo dye in sulfate respiration with pyruvate by *D. desulfuricans*: Electron transfer from pyruvate oxidation to sulfate reduction occurs biologically. The azo dye reduction proceeds chemically. The balance of ATP is from Gottschalk (1986).

Analogous to the sulfate respiration with pyruvate, the sulfate respiration with lactate also led to the production of acetate and the reduction of sulfate (Fig. 22). The production of approximately 18.5 mmol/L acetate was coupled to the reduction of approximately 9.1 mmol/L sulfate within the first 77 h (s. stoichiometry of Table 9), after which the acetate production was almost finished and sulfate was in depletion. The addition of RO 96 (0.21 mmol/L) at 143 h brought about a complete decolorization with a high rate (over 95 % decolorization within approximately 2 h), revealing the chemical reduction of RO 96 with the accumulated sulfide. The small discrepancy between the produced acetate concentration (approximately 18.5 mmol/L) and the initial concentration of lactate (19.4 mmol/L) may be reasoned by the bio-synthesis of *D. desulfuricans* with a reductive carboxylation of the activated acetate (acetyl-CoA) to pyruvate as the first step in cell synthesis (Badziong et al. 1979), approaching the

results of Okabe et al. (1992). Analogous to the decolorization of RO 96, biogenic sulfide chemically decolorized the di-azo dye RR 120 (data not shown).

Table 11. Postulated mechanism for decolorization of a mono-azo dye in sulfate respiration with pyruvate by *D. desulfuricans*, corresponding to Fig. 21:

Step	Reaction
I. biological	$8CH_3COCOO^- + 2SO_4^{2-} \rightarrow 8CH_3COO^- + 8CO_2 + 2S^{2-}$ (Peck et al. 1987)
II. chemical	$S^{2-} + H^+ \longleftrightarrow HS^-$ $HS^- + H \longleftrightarrow H_2S$
III. chemical	$H_2S + 0.5R\text{-}N{=}N\text{-}R' \rightarrow S + 0.5R\text{-}NH_2 + 0.5R'\text{-}NH_2$ or $HS^- + 0.5R\text{-}N{=}N\text{-}R' + H^+ \rightarrow S + 0.5R\text{-}NH_2 + 0.5R'\text{-}NH_2$ or $S^{2-} + 0.5R\text{-}N{=}N\text{-}R' + 2H^+ \rightarrow S + 0.5R\text{-}NH_2 + 0.5R'\text{-}NH_2$

R-N=N-R´ symbolizes a mono-azo dye;
R - NH_2 and R´- NH_2 indicate the aromatic amines.

A possibility that under sulfate-rich conditions the electrons liberated from substrate oxidation by SRB might be transferred to azo dyes in preference to sulfate, resulting in decolorization, is unlikely because of the enzyme specificity or regulation of the sulfate-grown SRB oriented to sulfate respiration. This is reasoned by a result that in the anaerobic mixed culture with sulfate the addition of molybdate hindering the sulfate reduction of SRB caused a significant decrease in the decolorization rate of RO 96, pointing out the decolorization of SRB coupled to sulfate reduction (Chap. 4.1).

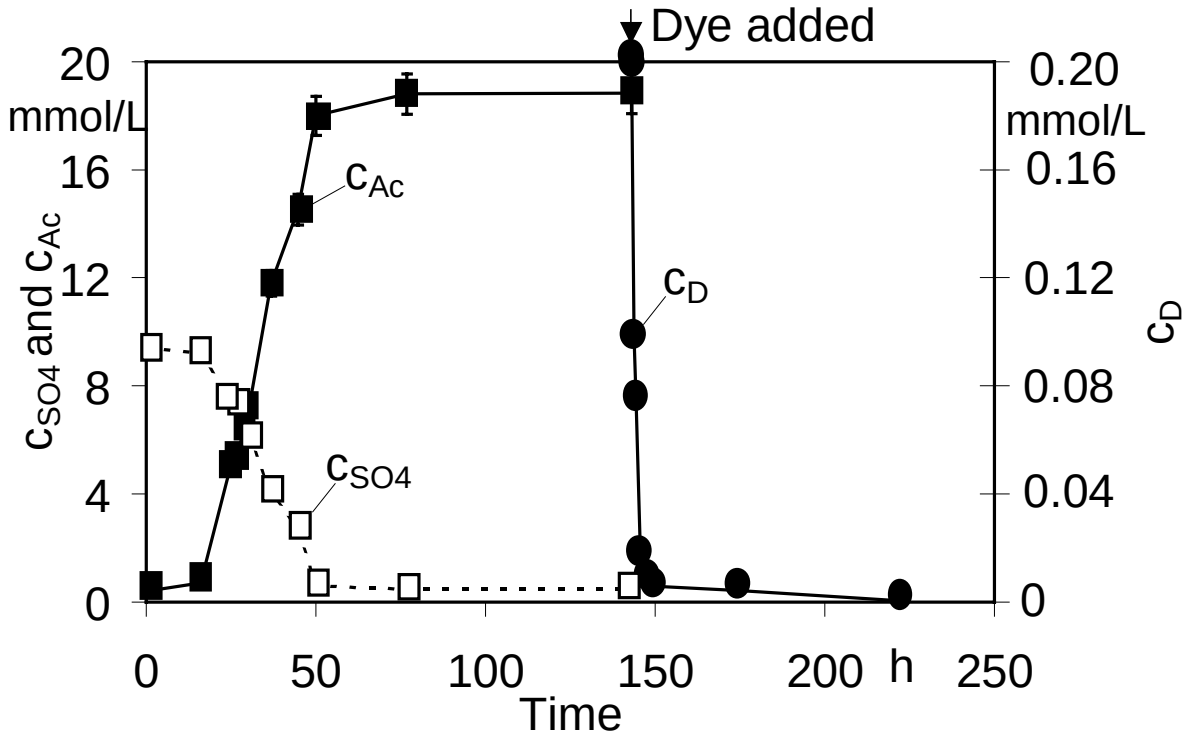


Fig. 22. Anaerobic decolorization of RO 96 in sulfate respiration with lactate by *D. desulfuricans*: $c_{SO4,0}$=9.4 mmol/L, $c_{S,0}$=19.4 mmol/L lactate, $c_{D,0}$=0.21 mmol/L, $c_{B,0}$=11 mg/L TSS, T=37 °C, pH=7.1 Each data point is averaged. The error bar is the 99 % confidence interval using the *t*-distribution (n=3).

In Fig. 23, the electron acceptor sulfate (2.4 mmol/L at start) was limited for the electron donor pyruvate (18.2 mmol/L). 9 mmol/L acetate were produced coupled with the reduction of 2.3 mmol/L sulfate within the first 42 h. At that time RO 96 (0.26 mmol/L) was added, bringing about a rapid decolorization of RO 96 with sulfide (over 95 % decolorization within 2 h). After 42 h, despite the depletion of sulfate, acetate was further produced (to a level of 15.3 mmol/L), indicating the immediate switch-over of catabolism from sulfate respiration to fermentation.

Peck et al. (1987) showed that the catabolism of *D. vulgaris* with pyruvate shifted from sulfate respiration to fermentation as a result of the inhibition of sulfate reduction with molybdate, leading to H_2-production. This switching possibility of some SRB may be extended to various conditions disabling sulfate respiration (e.g. sulfate depletion, inhibition of enzymes with some metabolic products, etc).

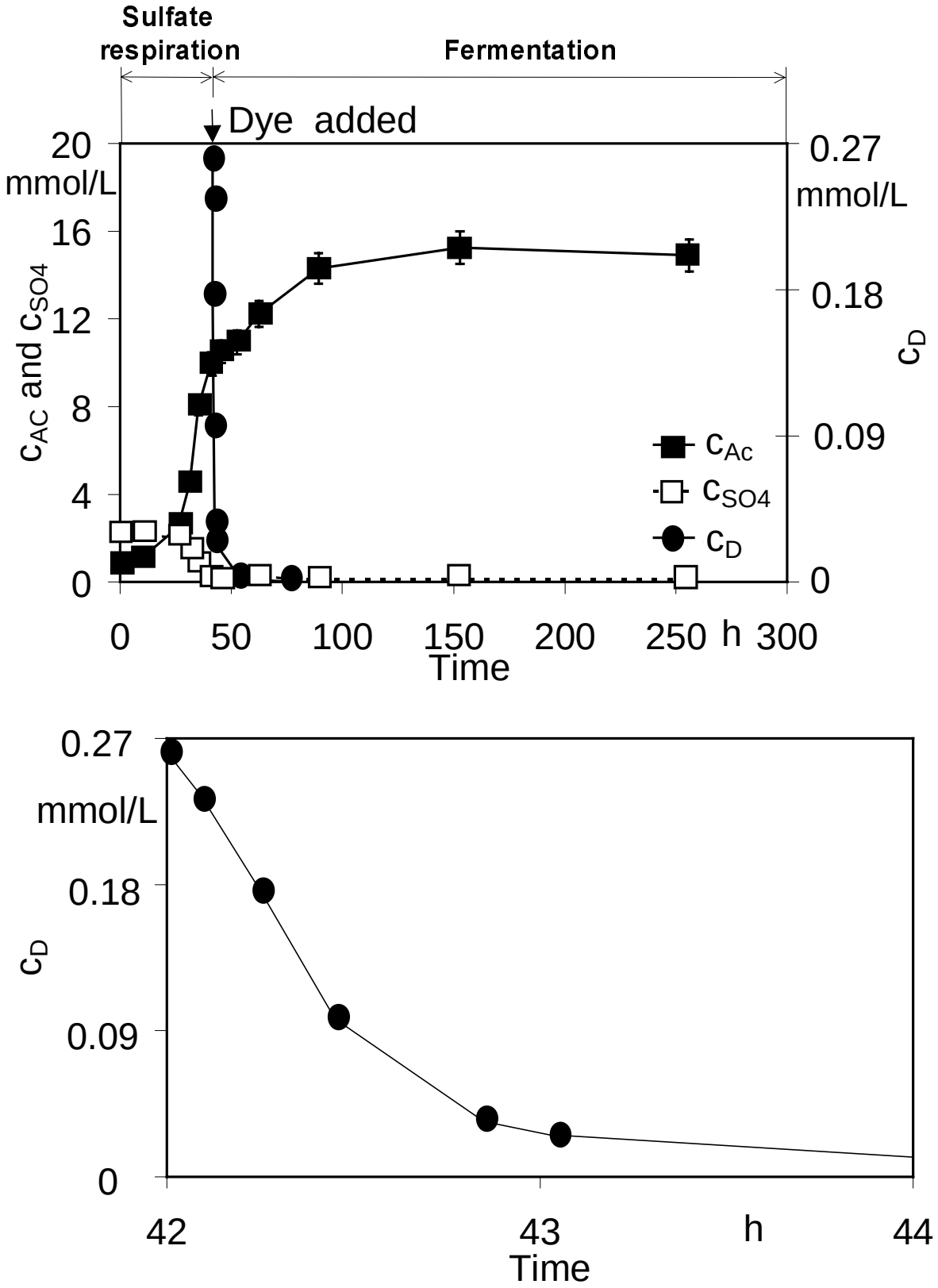
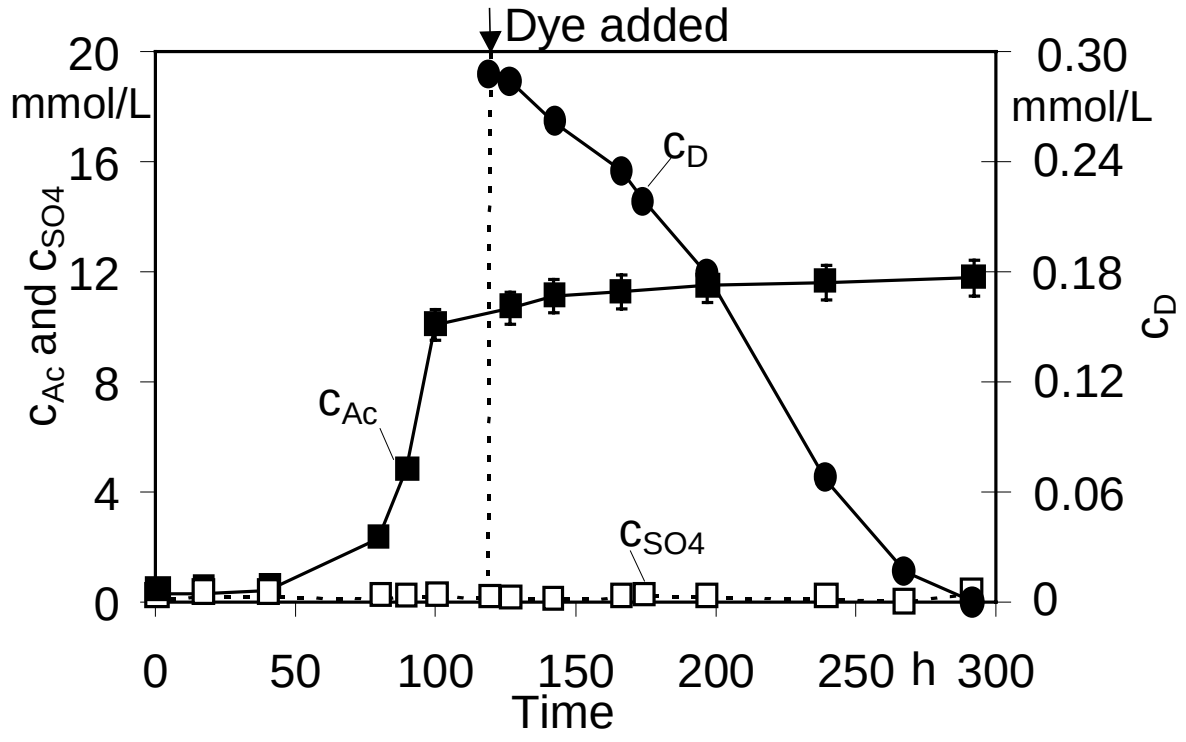


Fig. 23. Anaerobic decolorization of RO 96 in sulfate respiration with pyruvate by *D. desulfuricans* at limitation of sulfate: $c_{SO4,0}$ =2.4 mmol/L, $c_{S,0}$=18.2 mmol/L pyruvate, $c_{D,0}$= 0.26 mmol/L, $c_{B,0}$= 24 mg-TSS/L. T=37 °C, pH 7.1. Each data point is averaged. The error bar is the 99 % confidence interval using the *t*-distribution (n=3).

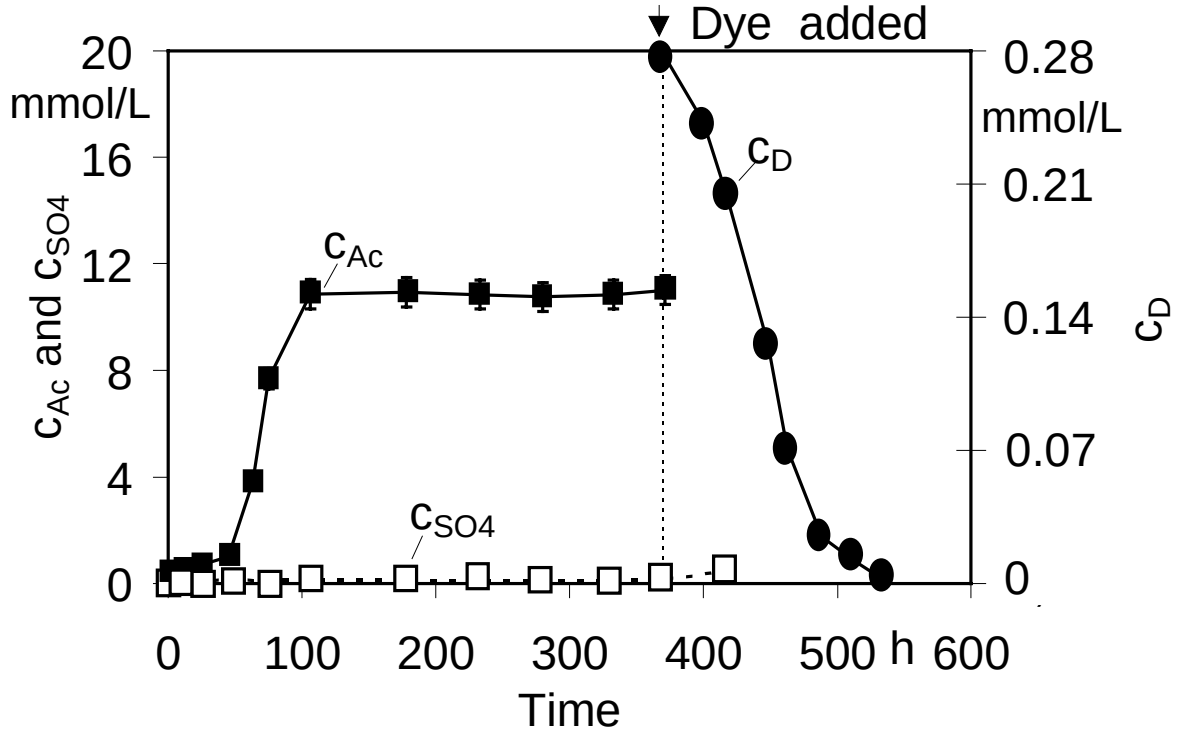
5.3. Decolorization of Azo Dyes in Fermentation of *Desulfovibrio desulfuricans*

The fermentation products of pyruvate by *D. desulfuricans* have been reported as acetate, CO_2 and H_2 (Senez, 1954; Postgate, 1984). To investigate the possibility that azo dyes can be decolorized in the fermentation of pyruvate, acetate production was tracked under sulfate-depleted conditions (≤ 0.1 mmol/L). Acetate was produced from the fermentation of pyruvate (Fig. 24), while the fermentation of lactate failed (< 1 mmol/L within 500 h; data not shown). The difference in free energy ($\Delta G_0'$) gained from the oxidation of the respective substrate to acetate, CO_2, and H_2, i.e. $\Delta G_0'$= - 4.2 kJ/mol-lactate; $\Delta G_0'$= - 47.3 kJ/mol-pyruvate (Thauer et al. 1977) may be the reason. The dye RO 96 (0.29 mmol/L) was added at 120 h when the acetate production from the fermentation of pyruvate had slowed drastically [Fig. 24(a)]. The production of only 10.7 mmol/L acetate (before 120 h) compared with the starting concentration of pyruvate (19.1 mmol/L) may be attributed to the inhibition of catabolism by an increase in the hydrogen partial pressure (i.e. thermodynamical inhibition) (s. Table 12), corresponding to the remarks of the influence of partial hydrogen pressure on fatty acid oxidation by Zehnder and Stumm (1988). Molecular hydrogen H_2 cannot reduce azo dyes. The decolorization (95 %) of RO 96 took place within 150 h (from 120 to 270 h). The slow rate of decolorization was coupled with the low rate of acetate production during decolorization. The small amount of sulfate (≤ 0.1 mmol/L) was probably utilized for biosynthesis (i.e. assimilatory sulfate reduction). If the sulfate had dissimilatorily been reduced to sulfide via sulfate respiration at all, the sulfide could theoretically have decolorized at most 0.05 mmol/L of a mono-azo dye (s. stoichiometry of Table 11).

In the next experiment [Fig. 24(b)] the dye addition time was varied. The course of acetate production of Fig. 24(b) was almost similar to that of Fig. 24(a) due to the same initial conditions, in that 10.4 mmol/L aceate were produced within 106 h. The late addition of RO 96 (0.28 mmol/L) at 371 h, when the acetate concentration was almost constant (c_{Ac}=10.8 mmol/L at 371 h), led to a slow decolorization of RO 96 (96 % within 141 h from 371 to 512 h).



(a)



(b)

Fig. 24. Anaerobic decolorization of RO 96 via fermentation of pyruvate by *D. desulfuricans* in variation of addition time of RO 96 at 37 °C and pH = 7.1:
(a) $c_{D,0}$=0.29 mmol/L RO 96, $c_{SO4,0}$= 0.1 mmol/L, $c_{S,0}$=19.1 mmol/L pyruvate, $c_{B,0}$=10 mg/L TSS, (b) $c_{D,0}$=0.28mmol/L RO 96, $c_{SO4,0}$=0.1 mmol/L, $c_{S,0}$=19.1 mmol/L pyruvate, $c_{B,0}$=10 mg/L TSS. Each data point is averaged. The error bar is the 99 % confidence interval using the *t*-distribution (n=3).

The decolorization via fermentation of pyruvate by *D. desulfuricans* is thought to involve enzymes (e.g. pyruvate:ferredoxin-oxidoreductase, hydrogenase) and/or coenzymes [electron carriers: e.g. ferredoxin (Fd), tetraheme cytochrome c_3] active in the electron transport of *Desulfovibrio´*s catabolism of pyruvate (Bruschi et al. 1977; Guerlesquin et al. 1980; Legall and Fauque 1988). It is not yet clear which coenzymes or enzymes may be responsible for the final reduction of azo dyes. One possibility to show that related coenzymes can decolorize azo dyes may be usage of the coenzymes as commercial products in a system without intact cells (possibly with cell-extracts). An interesting variant at this try may be the increase of the concentration of coenzymes added, which can be significant both for abiotic and biological systems. It should be emphasized that a generalization as to the function and localization of electron transfer proteins cannot hold good for every species of *Desulfovibrio*. This was illustrated by a study of the balance of metabolism by different species of *Desulfovibrio* grown on lactate plus sulfate, producing H_2 and other intermediate organics besides acetate despite sulfate-rich conditions (Traore et al. 1981, 1982). The likely biological decolorization mechanism of a mono-azo dye via fermentation of pyruvate as electron donor may schematically be as follows (Fig. 25, Table 12): Electrons (or $e^- + H^+$) are liberated from the oxidation of pyruvate to acetate plus CO_2 via enzymes or coenzymes e.g. Fd (step I of Table 12). The reduced enzymes or coenzymes e.g. Fd^- transfer the electrons to the dye, giving rise to decolorization, instead of to the protons (H^+), resulting in the production of H_2 (step II). This implicates that the azo dye functions as a terminal electron acceptor for pyruvate fermentation, connected to a net ATP-gain from acetate production, instead of H^+.

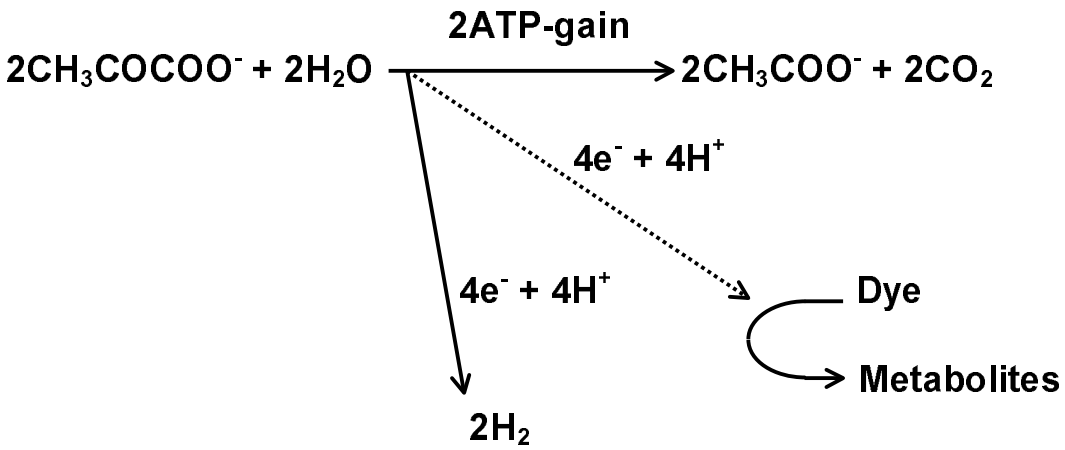


Fig. 25. Schematic pathway of decolorization of a mono-azo dye (R-N=N-R´) via fermentation of pyruvate by *D. desulfuricans*: Electron transfer is mediated by (co-)enzymes. The dotted line indicates an alternative electron transfer. The amount of ATP-gain for pyruvate degradation to acetate is from Gottschalk (1986).

Table 12. Likely biological pathway for decolorization of a mono-azo dye (R-N=N-R´) via fermentation of pyruvate by *D. desulfuricans*, corresponding to Fig. 25:

Step	Reaction
I. Pyruvate oxidation	$2CH_3COCOO^- + 2H_2O + 4Fd \rightarrow 2CH_3COO^- + 2CO_2 + 4Fd^- + 4H^+$ (Müller 1980)
II. H_2-production or Decolorization	$4Fd^- + 4H^+ \rightarrow 4Fd + 2H_2$ $4Fd^- + 4H^+ + R\text{-}N{=}N\text{-}R' \rightarrow 4Fd + R\text{-}NH_2 + R'\text{-}NH_2$

Note: Fd^- indicates the reduced form of the coenzyme Fd.
The H_2-production is mediated by other (co-)enzymes in addition to Fd.
A final reduction of the azo dye by Fd is assumed.

In light of the principal mechanism of scavenging electron or hydrogen, the possible role of azo dyes as alternative terminal electron acceptors for the fermentation of pyruvate by *D. desulfuricans*, avoiding H_2-production, may be comparable to that of H_2-utilizing MPB enabling some strains of *D. desulfuricans* to ferment lactate or ethanol via interspecies hydrogen transfer (Bryant et al. 1977). Lactate or ethanol was not fermentable in the pure cultures of *D. desulfuricans*. In addition to avoiding a possible increase in the partial pressure of hydrogen, the advantageous (more positive) redox potential of e.g. the RO 96/metabolites-pair (E_0´= -133 mV; Schmid, 1994) compared with that of the H^+/H_2-pair (E_0´= -420 mV) may facilitate electron transfer to the azo dye.

Another dye RR 120 with two azo bonds revealed similar results to RO 96 (Fig. 26). The addition of RR 120 at 391 h, when the acetate concentration was almost constant (near 11 mmol/L) probably owing to an increase in the partial pressure of hydrogen, led to a slow decolorization of RR 120 (90 % decolorization within 200 h from 391 to 591 h).

The localization of decolorizaion via the fermentation of *D. desulfuricans* is not yet clear. Cytoplasmic reduction of azo dyes is not probable for many highly charged (e.g. sulfonated) or polymeric azo dyes. Such highly charged dyes are not thought to be transported across the cell-membrane at the expense of energy for the transport, as noted by Wuhrmann et al. (1980) and Levine (1991). Cytoplasmic reduction may be feasible with uncharged azo dyes. Assuming the cytoplasmic reduction of azo dyes, a further question of whether the decolorization may be valid as a respiratory dye reduction yielding additional ATP via electron transport phosphorylation is interesting. This question falls in line with a result that nitrate could be utilized as terminal electron acceptors for respiration by *D. desulfuricans* (Steenkamp and

Peck 1981). A periplasmic decolorization model of azo dyes may also be rationalized by the probable difficulty of dye transport across the cell membrane and the localization of hydrogenase in periplasmic space of *D. desulfuricans* (Steenkamp and Peck, 1981). This assumption can possibly be deduced from the reduction of a widely used redox dye, methyl viologen (MV) with formate hydrogenase in the periplasmic space of the facultative anaerobic bacteria *Escherichia coli* (Jones and Garland 1977). Methyl viologen was regarded as unable to cross the cytoplasmic membranes in both oxidation states, i.e. MV^{++}.

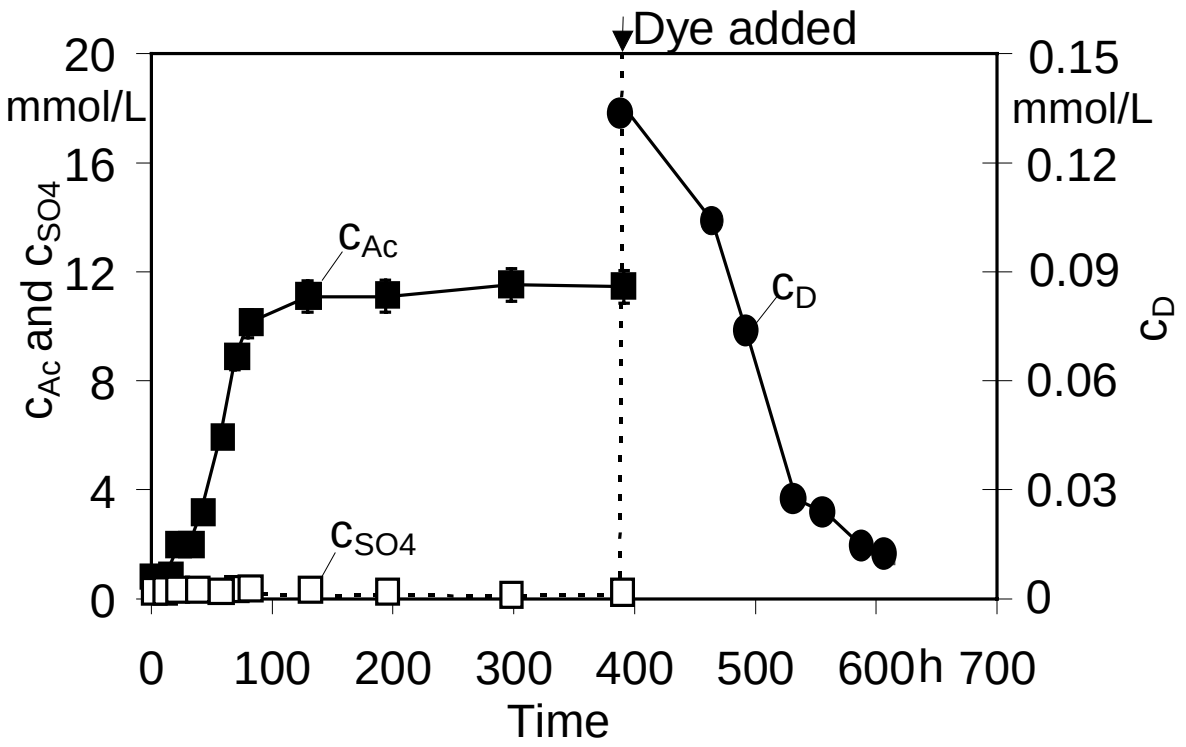


Fig. 26. Anaerobic decolorization of the di-azo dye RR 120 via fermentation of *D. desulfuricans*: $c_{D,0}$= 0.14 mmol/L RR 120, $c_{SO4,0}$ = 0.1 mmol/L, $c_{S,0}$=18.2 mmol/L pyruvate, $c_{B,0}$= 24 mg-TSS/L, pH 7.1, T=37 °C. Each data point is averaged. The error bar is the 99 % confidence interval using the *t*-distribution (n=3).

5.4. Comparison of Various Decolorization Pathways and Engineering Aspects

Unlike *D. desulfuricans* which uses H^+ as terminal electron acceptor for the fermentation, fermentative bacteria (e.g. degrading glucose) usually use a part of organic compounds (e.g. acetyl-CoA or pyruvate) generated during the fermentation processes as terminal electron acceptors, reducing the organics to e.g. ethanol or lactate. Based on the role of azo dyes in fermentation of *D. desulfuricans*, azo dyes can probably be expected to act as alternative terminal electron acceptors also for the fermentative bacteria. That is, azo dyes may be reduced instead of e.g. acetyl-CoA, leading to more oxidized catabolic products (e.g. acetate) coupled to an ATP-gain. This possible role of azo dyes may, in principle, correspond to the coupling

relationship of H_2-utilizing bacteria for fermentative bacteria (Iannotti et al. 1973). Roxon et al. (1967) proposed that azo dyes act under anaerobic conditions as alternative terminal electron acceptors for a facultative anaerobic bacterium (*Proteus vulgaris*) fermenting glucose.

The fermentation pathway of SRB producing H_2 can be active in some methanogenic ecosystems that are low in sulfate. In a sulfate-depleted complex system with anaerobic mixed populations the transfer pathway of electrons liberated from the oxidation of substrate by SRB is affected by possible competition among H_2-utilizers (e.g. MPB, homoacetogens) and other external electron scavengers such as azo dyes, some organics or inorganics. Under sulfate-rich conditions, the decolorization process with sulfide can be effective also in anaerobic bacterial consortiums with sulfate (Chap. 4). The sulfide can be accumulated as the reduction product of sulfate outside the cells for subsequent decolorization. This brings practical aspects. For example, the effluent of anaerobic reactors producing sulfide can be fed back to a buffer tank of textile wastewater under exclusion of oxygen in order to achieve abiotic pre-decolorization with the biogenic sulfide. In contrast, the reduction equivalents in form of reduced enzymes or coenzymes (electron carriers) probably involved in decolorization via fermentation of *D. desulfuricans* do not appear to be accumulated or stored in bulk. An electron trap by electron carriers (e.g. as Fd^-) in the cells may take place to a small extent. However, the electron carriers are present in only limited amounts in the cells, and thus have to be regenerated (reoxidized) via the reduction of H^+ to H_2 to keep the process of substrate oxidation running. This can be true for other fermentative bacteria using enzymes or coenzymes such as NAD^+, FAD, or FMN in decolorization (Rafii et al. 1990; Chung and Stevens 1993). In this regard, the decolorization of azo dyes with sulfide can be more efficient for technical processes than that with (co-)enzymes of the fermentation pathway of *D. desulfuricans* or other fermentative bacteria.

In summary, it is probable that two pathways for decolorization of azo dyes are available in bacterial systems: 1) a direct electron transfer to azo dyes as terminal electron acceptors via (co-)enzymes during bacterial catabolism (e.g. fermentation), connected to ATP-gain; 2) a gratuitous reduction of azo dyes by some end products of bacterial catabolism (e.g. sulfate respiration), not linked to ATP-gain in catabolism. Possibly, some organics (Keck et al. 1997) or inorganics may get involved in both pathways, by acting as electron shuttles between the reduction equivalents generated in catabolism and the azo dyes.

5.5. Summary

1. Under sulfate-rich conditions, the sulfide produced from sulfate respiration with pyruvate or lactate by *D. desulfuricans* caused a chemical decolorization of the azo dyes RO 96 and RR 120.
2. In depletion of sulfate (≤ 0.1 mmol/L), the decolorization of RO 96 and RR 120 occurred in correlation with fermentation of pyruvate by *D. desulfuricans*. It is suggested that the electrons liberated from the pyruvate oxidation to acetate and CO_2 were transferred via enzymes and/or coenzymes (electron carriers) to the dyes as alternative terminal electron acceptors, giving rise to decolorization, instead of to the H^+, resulting in the production of H_2.

Based on the results of Chap. 4 and 5, the kinetics of chemical decolorization with sulfide becomes of interest.

6. KINETICS OF CHEMICAL AZO DYE REDUCTION

6.1. Chemical Azo Dye Reduction by Various Organic/Inorganic Substances

Various organic/inorganic substances (cysteine, ascorbate, dithionite, etc.) were tried in order to disclose the possibility of chemical decolorization of azo dyes. Such compounds or their derivatives can possibly be generated during bacterial metabolism. Thus the batch experiments aimed at broadening the spectrum of the decolorization mechanism of azo dyes.

6.1.1. Chemical decolorization of C. I. Reactve Orange 96 with cysteine

The amino acid cysteine ($C_3H_7NO_2S$) is involved in bacterial metabolism in the form of a part of a variety of enzymes (e.g. Fd; Gottschalk 1986). Cysteine itself comprises sulfur-complex, and the pair of 2-cysteine/cystine possesses a large negative redox potential (E_0´= -340 mV, Table 1).

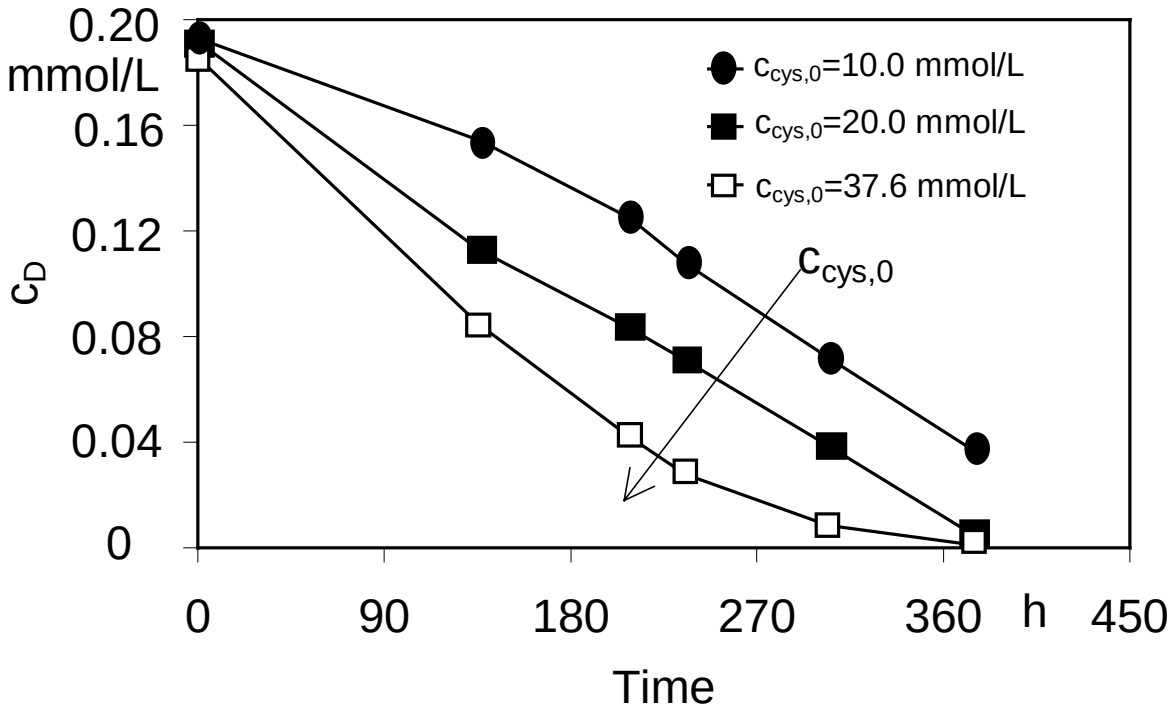


Fig. 27. Chemical decolorization of RO 96 in variation of cysteine concentration under sterile conditions excluding O_2: $c_{D,0}$=0.19 mmol/L RO 96, T=33 °C, pH= 7.1

Cysteine (10 mmol/L) chemically decolorized the dye RO 96 (0.19 mM) (81 % decolorization within 376 h) (Fig. 27). A more rapid decolorization occurred at the higher starting concentrations of cysteine (20, 37.6 mM) (80 % within 305 h; 96 % within 304 h respectively).

The stoichiometry of the decolorization of a mono-azo dye (R-N=N-R´) with cysteine can be proposed to follow Eq.(47):

$$4C_3H_7NO_2S \longrightarrow 2C_6H_{12}N_2O_4S_2 + 4H^+ + 4e^- \quad \text{(Karlson 1994; Table 1)} \quad (46)$$

$$4H^+ + 4e^- + R\text{-}N{=}N\text{-}R' \longrightarrow R\text{-}NH_2 + R'\text{-}NH_2 \quad (46\text{-}1)$$

$$\text{Total: } 4C_3H_7NO_2S + R\text{-}N{=}N\text{-}R' \longrightarrow 2C_6H_{12}N_2O_4S_2 + R\text{-}NH_2 + R'\text{-}NH_2 \quad (47)$$

The oxidation of 4 mol/L cysteine to 2 mol/L cystine should be coupled to the reduction of 1 mol/L mono-azo dye. The yield coefficient of dye (reduction) on cysteine $Y_{D/cys}$ may be described as follows:

$$\frac{dc_D}{dc_{cys}} = Y_{D/cys} = 0.25 \text{ mol/mol} \quad (48)$$

It is also interesting whether cysteine is degradable by bacteria at all, possibly resulting in liberation of sulfide. Stams et al. (1985) reported that a marine *Desulfoviobrio* strain degraded cysteine (partially from 20 mmol/L), producing 2.8 mmol/L NH_4^+, 2.3 mmol/L acetate, and 3.0 mmol/L sulfide. Thus cysteine may also indirectly contribute to the decolorization of azo dyes in biological systems, when degraded by bacteria, bringing about the release of sulfide.

6.1.2. Chemical decolorization of C. I. Reactve Orange 96 with ascorbate

Ascorbate ($C_6H_7O_6^-$) (or ascorbic acid) is a widely used anti-oxidation compound in the food industry. In a lot of photosynthetic organisms (e.g. plants) ascorbate is an essential substance of oxidation security systems. Fig. 28 shows that the dye RO 96 (0.19 mmol/L) was decolorized with ascorbate (10 mmol/L as the initial concentration) (88 % within 149 h). The addition of a higher concentration of ascorbate (20, 37.7 mmol/L) enhanced the decolorization rate of RO 96 (over 95 % within 118 h; within 71 h respectively).

The stoichiometry of the decolorization of a mono-azo dye (R-N=N-R´) with ascorbate can be suggested to undergo Eq.(50):

$$2C_6H_7O_6^- \longrightarrow 2C_6H_5O_6^- + 4H^+ + 4e^- \quad \text{(Karlson 1988)} \quad (49)$$

$$4H^+ + 4e^- + R\text{-}N{=}N\text{-}R' \longrightarrow R\text{-}NH_2 + R'\text{-}NH_2 \quad (49\text{-}1)$$

$$\text{Total: } 2C_6H_7O_6^- + R\text{-}N{=}N\text{-}R' \longrightarrow 2C_6H_5O_6^- + R\text{-}NH_2 + R'\text{-}NH_2 \quad (50)$$

The oxidation of 2 mol/L ascorbate to 2 mol/L dehydroascorbate should be linked to the decolorization of 1 mol/L mono-azo dye. The yield coefficient of dye (reduction) on ascorbate $Y_{D/asc}$ may be obtained as following:

$$\frac{dc_D}{dc_{asc}} = Y_{D/asc} = 0.5 \text{ mol/mol} \quad (51)$$

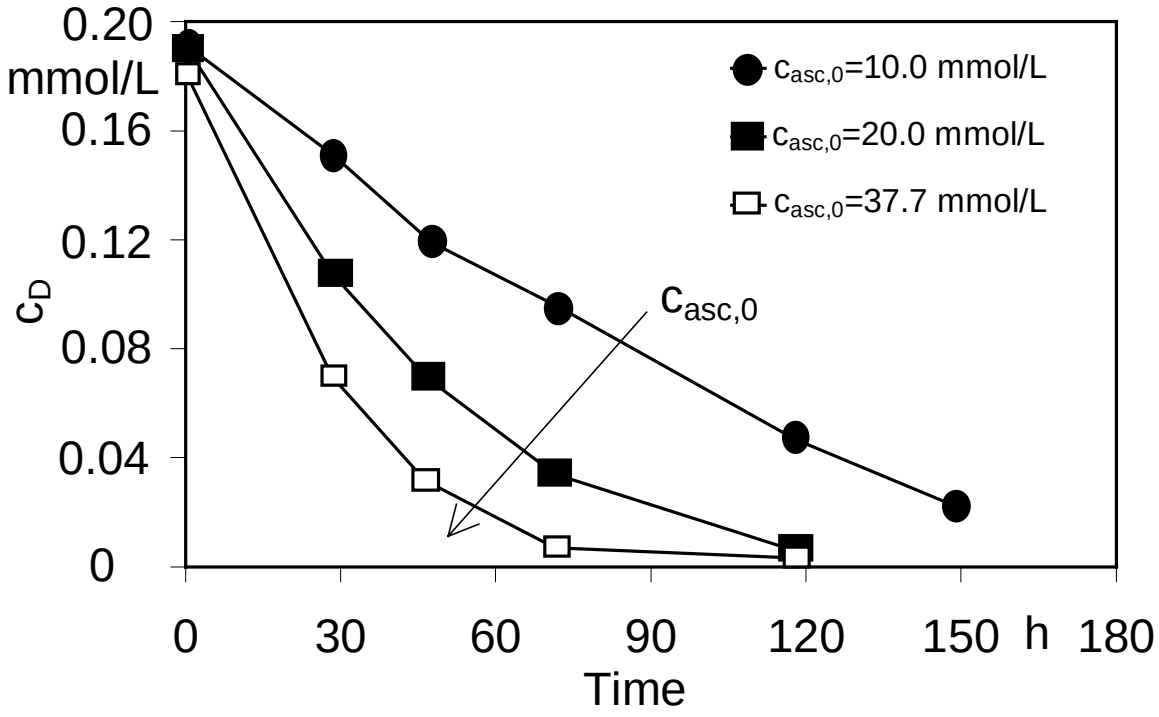


Fig. 28. Chemical decolorization of RO 96 in variation of level of ascorbate under sterile conditions excluding O_2: $c_{D,0}$=0.18-0.19 mmol/L RO 96,T=33 °C, pH= 7.1

From the thermodynamic point of view the decolorization of RO 96 by ascorbate appears to be infeasible, in that $E_0' = +60$ mV for the ascorbate/dehydroascoric acid-pair (Karlson 1988); $E_0' = -133$ mV for the RO 96/metabolites-pair (Table 1). On the one hand, the redox potential depends on the concentration of reactants [s. Eq.(2)]. That is, a shift of the redox potential for the ascorbate/dehydroascorbate-pair towards a more negative direction because of an excess in ascorbate (10, 20, 37.7 mmol/L) might enable the decolorization of RO 96 (0.18-0.19 mmol/L). On the other hand, the redox potential for the RO 96/metabolites-pair has to be investigated more precisely.

One possibility for other vitamins to decolorize azo dyes can be deduced from the discussion of the dechlorination (i.e. reduction) of dioxins (Adriaens et al. 1996). The authors suggested that vitamin B_{12} available in particular microbial communities (e.g. MPB) abiotically reduces the dioxins. Vitamin B_{12} may be expected to chemically decolorize azo dyes.

6.1.3. Decolorization kinetics of C. I. Reactive Orange 96 with cysteine and ascorbate

A kinetic model for the decolorization of RO 96 with cysteine or ascorbate respectively was assumed as the first order with respect to both dye and reducing agent concentration:

$$-\frac{dc_D}{dt} = k\, c_D\, c_{RA} \qquad (52)$$

where k rate constant (L $mmol^{-1}$ h^{-1})
c_D concentration of dye at time t (mmol/L)
c_{RA} concentration of reducing agent at time t (mmol/L)

Using the yield coefficient of dye reduction on the respective reducing agent of Eq.(48) and (51), the concentrations of cysteine and ascorbate (at any time t) during decolorization were estimated.

The model of Eq.(52) was examined based on the decolorization courses of Fig. 27 and 28, assuming that $k\, c_{RA}$ were almost constant owing to great excess in cysteine and ascorbate compared with the dye concentration ($c_{RA,0}$ vs. $c_{D,0}$). Eq.(52) was converted to:

$$\frac{dc_D}{dt} = -k'\, c_D \qquad (53)$$

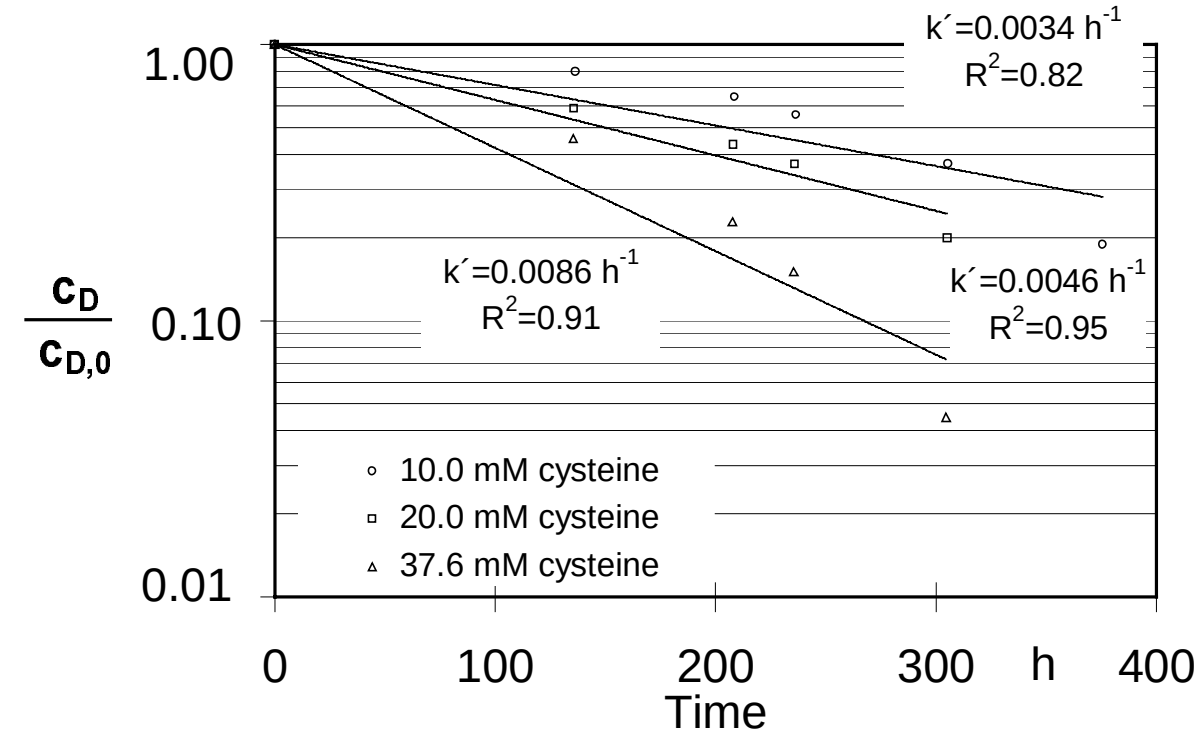
where $k' = k\, c_{RA}$;
rate constant (h^{-1})

For $c_D = c_{D,0}$ at t=0, it follows from integration of Eq.(53):

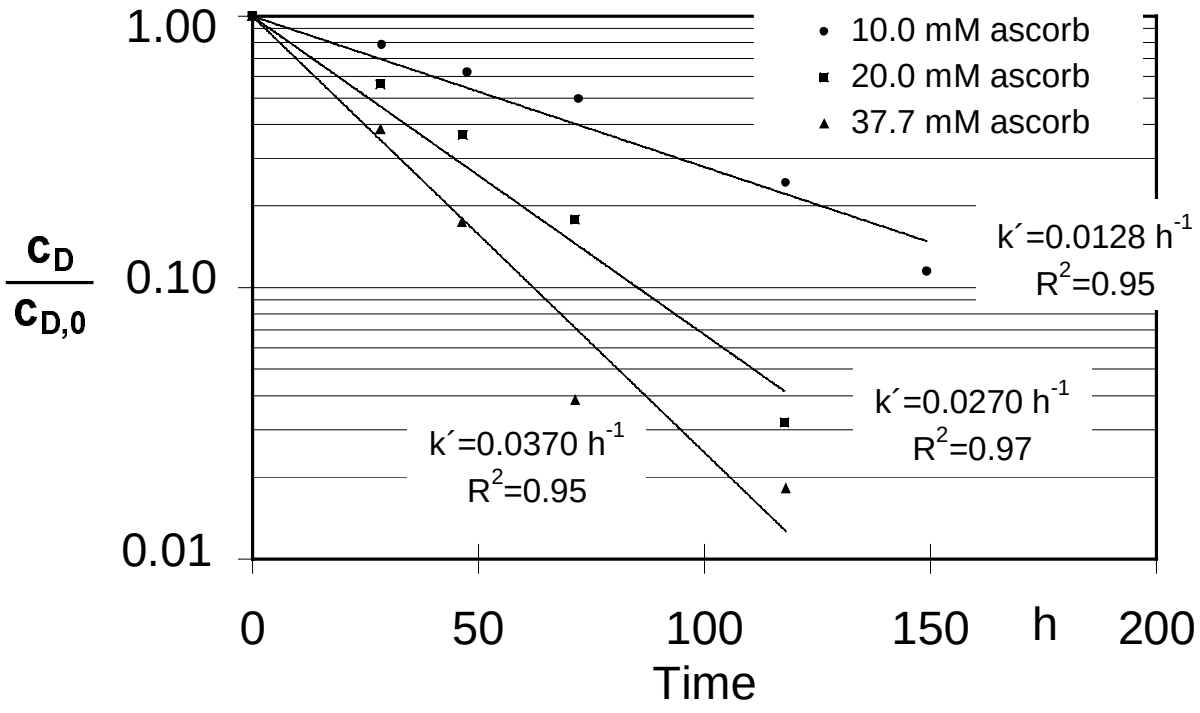
$$\ln(c_D / c_{D,0}) = -k'\, t \qquad (54)$$

where $c_{D,0}$ starting concentration of dye (mmol/L)

The plot of $\ln(c_D/c_{D,0})$ vs. time t for the respective initial concentration of ascorbate represented each straight line [Fig. 29(b)], verifying the first order with respect to the dye concentration. The rate constant k´ was enhanced at the higher concentration of ascorbate (20, 37.7 mmol/L), since k´ reflected the term of ascorbate concentration. The data points of $\ln(c_D/c_{D,0})$ vs. time t for the initial concentration of cysteine of 20 mmol/L approached a straight line, whereas those for 10 and 37.6 mmol/L cysteine were relatively scattered from each straight line [Fig. 29(a)].



(a)



(b)

Fig. 29. Determination of decolorization kinetics of RO 96 assuming nearly constant concentration of cysteine (a) and ascorbate (b): $c_{D,0}$= 0.18-0.19 mmol/L, T=33 °C, pH=7.1

To validate the model of Eq.(52) under the influence of both concentrations of the respective reducing agent and the dye RO 96 (in other words, to determine the reaction order with

respect to the concentration of the respective reducing agent), Eq.(25) was employed amended with the yield coefficient of dye on cysteine [Eq.(48)] and on ascorbate [Eq.(51)] respectively:

$$\beta = \frac{1}{(c_{RA,0} - 2\, c_{D,0})} \left[\ln \frac{c_{D,0} \{c_{RA,0} - Y_{D/RA}^{-1} (c_{D,0} - c_D)\}}{c_D\, c_{RA,0}} \right] = k\, t \qquad (55)$$

where $c_{RA,0}$ indicates the starting concentration of a reducing agent, i.e. either cysteine or ascorbate; $Y_{D/RA}$ = yield coefficient of dye (reduction) on the respective reducing agent.

Using Eq.(55) the decolorization courses of Fig. 27 and 28 were reevaluated (Fig. 30). The data points lay near each straight line, to give the rate constant for cysteine k_{cys}=0.0002 L mmol^{-1} h^{-1} and that for ascorbate k_{asc}=0.0010 L mmol^{-1} h^{-1}. This indicates that the chemical decolorization of RO 96 by cysteine or ascorbate respectively corresponded to the kinetics of the first order with respect to both dye and reducing agent concentration. The data points in the initial phase were relatively well fitted to the straight lines, whereas the data points were scattered in the end phase. The low concentrations of RO 96 in the end phase influenced the value β of the evaluation equation much more sensitively than the high concentrations in the initial phase.

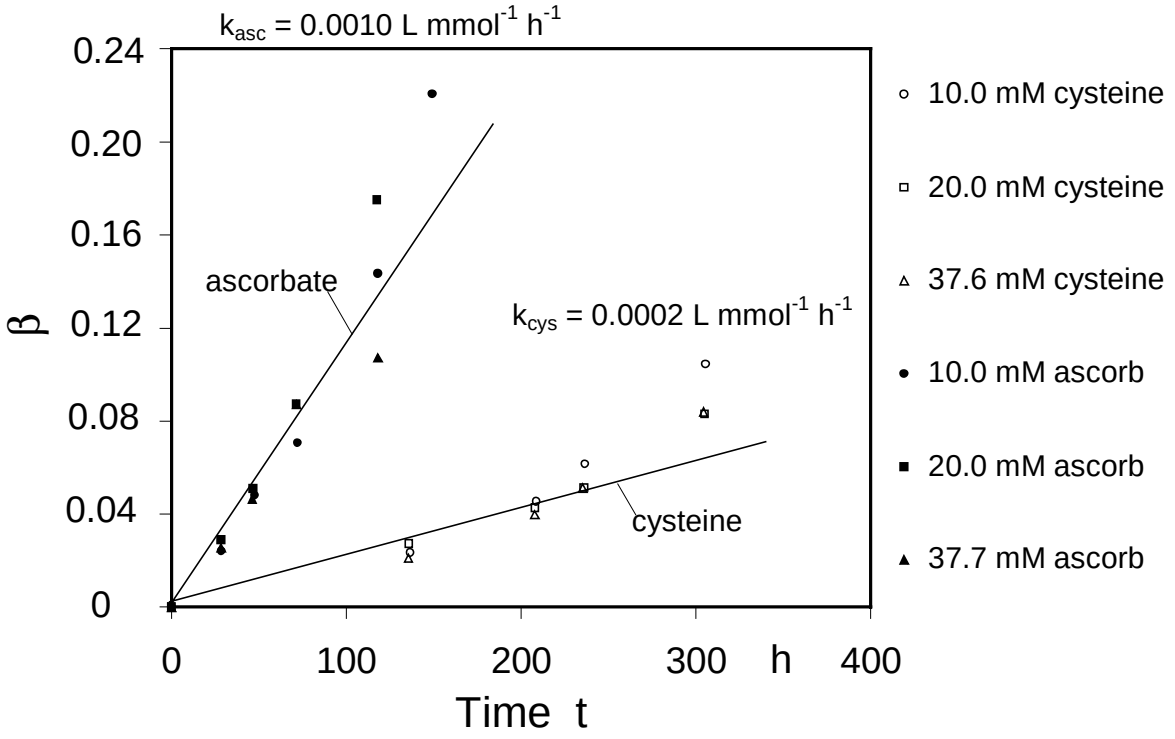


Fig. 30. Evaluation of the course of decolorization with ascorbate and cysteine to determine the rate constant k:

$$\beta = \frac{1}{(c_{RA,0} - Y_{D/RA}^{-1}\, c_{D,0})} \left[\ln \frac{c_{D,0} \{c_{RA,0} - Y_{D/RA}^{-1} (c_{D,0} - c_D)\}}{c_D\, c_{RA,0}} \right] \qquad (55)$$

The rate constant for ascorbate was approximately 5-times higher than that for cysteine. Detailed information about the respective rate constants for ascorbate and cysteine is summarized in Table 13. It should be noted that the regression degrees for the determination of k for cysteine (10, 37.6 mmol/L) were relatively low.

Table 13. Rate constant k for cysteine and ascorbate at 33 °C and pH 7.1

Reducing agent (RA)	$c_{RA,0}$ (mmol/L)	$c_{D,0}$ (mmol/L)	k (L $mmol^{-1}$ h^{-1})	R^2	$c_{RA,0}/c_{D,0}$
Ascorbate	10.0	0.19	0.0013	0.950	59
Ascorbate	20.0	0.19	0.0014	0.969	118
Ascorbate	37.7	0.18	0.0010	0.952	236
cysteine	10.0	0.19	0.0003	0.878	59
cysteine	20.0	0.19	0.0002	0.945	118
cysteine	37.6	0.19	0.0002	0.909	235

6.1.4. Chemical decolorization of C. I. Reactve Orange 96 with other substances

Other substances such as dithionite and Ti(III)-citrate with a large negative redox potential chemically decolorized RO 96 at a high rate (Table 14). The decolorization of RO 96 (0.2 mmol/L) with yeast extract (2.5 g/L) did not succeed (< 5 % within 150 h). Despite this failure a possible chemical decolorization of azo dyes with some products (e.g. cysteine) generated during the degradation of yeast extract by bacteria cannot be excluded.

Table 14. Occurrence of chemical decolorization of RO 96 with various organic/inorganic substances at 33 °C under sterile conditions excluding O_2

Substance (RA)	Decolorization degree α (in duration)	$c_{D,0}$ (mmol/L)	$c_{RA,0}$ (mmol/L)	pH
Ti(III)-citrate	97 % (≤ 5 min)	0.20	10	7
dithionite	97 % (≤ 5 min)	0.20	10	7
Fe(II)	88 % (≤ 5 min)	0.16	10	12
Fe(II)	16 % (115 h)	0.16	10	8
yeast extract	< 5 % (150 h)	0.20	2.5 g/L	7

Table 14 also shows the influence of pH on the decolorization of RO 96 by Fe(II) (i.e. $FeSO_4$). At pH=12 the decolorization occurred very rapidly (88 % decolorization within 5 min), whereas at pH=8 only a small amount of RO 96 was decolorized at a slow rate (only 16 % within 115 h), a part of which could possibly be due to the adsorption of RO 96 on the precipitation of Fe-complex. This pH-influence on the decolorization with Fe(II) may be understood in recognition of the fact that at basic pH Fe(II) leads to $Fe(OH)_2$ possessing a large negative potential (E = -632 mV at pH 12 and 25 °C for the pair of $Fe_2O_3 \cdot 3H_2O/2Fe(OH)_2+2OH^-$, calculated from Christen 1982). A drawback of the decolorization with Fe(II) is an unwanted sludge production of Fe-complex.

6.2. Kinetics of Chemical Azo Dye Reduction by Sulfide

6.2.1. Failure in decolorization with sulfide under exposure to oxygen

Before entering into the study of kinetics for decolorization of the mono-azo dye RO 96 with sulfide, the possibility that azo dyes can be decolorized by sulfide in the presence of O_2 was examined. Sulfide failed to decolorize RO 96 (Fig. 31), probably reacting preferentially with O_2 instead of the dye.

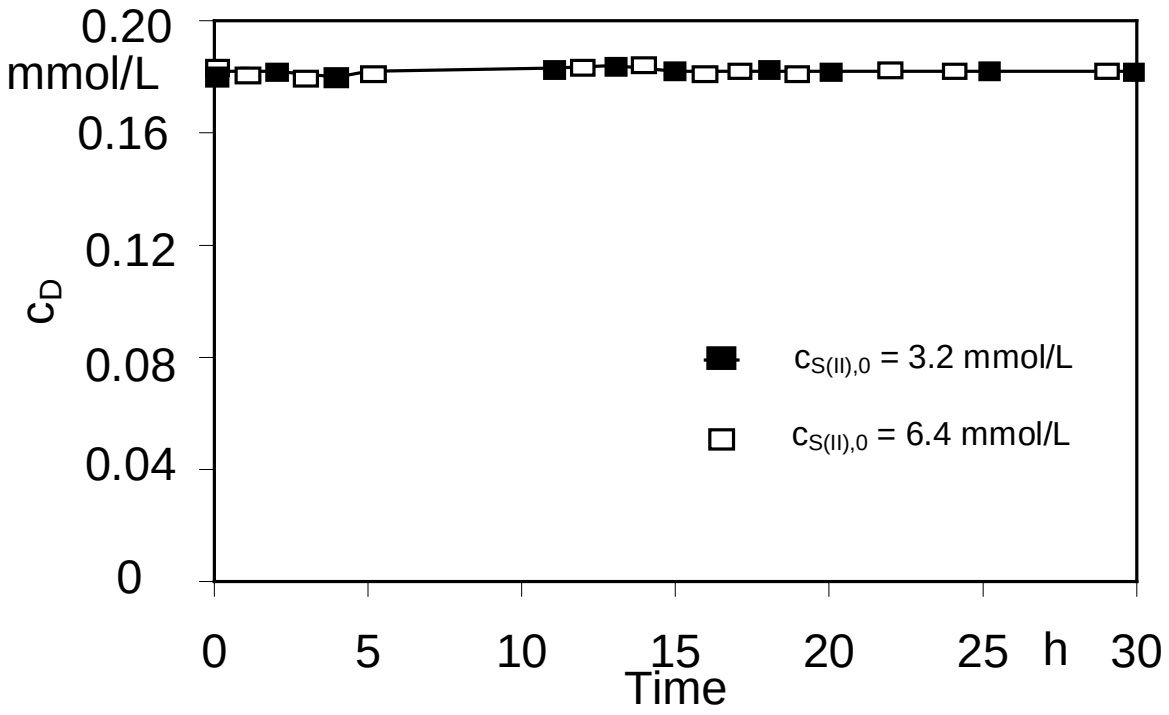


Fig. 31. Influence of oxygen on decolorization of RO 96 at excess in sulfide S(II) (~50 % HS^-, 50 % H_2S): $c_{D,0}$ = 0.18 mmol/L, T=20 °C, pH=7.1

This phenomenon is principally in line with the generally known inhibition of microbial azo reduction in the presence of oxygen. One explanation for this inhibition (Chung and Stevens 1993) was the preferential reduction of oxygen instead of the dye by the reduced flavin nucleotides ($FMNH_2$; $FADH_2$). Chen and Morris (1972) suggested that the reaction of sulfide with O_2 undergoes a sulfide-sulfur-polysulfide cycle (Fig. 32), showing the pH-influence on the oxygenation rate of sulfide.

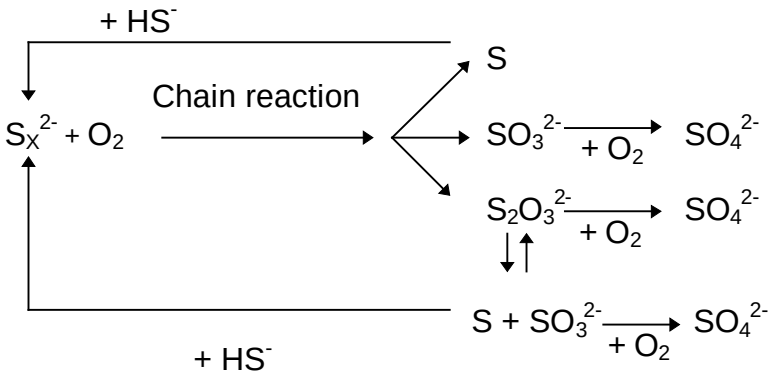


Fig. 32. Reaction pathway of oxygenation of sulfide (Chen and Morris 1972): polysulfide S_x^{2-} as intermediates, x can be 2-5.

6.2.2. Kinetics of azo dye reduction by sulfide under exclusion of oxygen

Batch experiments for the decolorization of RO 96 by sulfide were carried out under the exclusion of O_2 to establish a model for the decolorization kinetics. This study of the kinetic model with sulfide serves in understanding the decolorization of azo dyes through SRB. In all further experiments, sulfide S(II) indicated the sulfide species including S^{2-}, HS^-, and H_2S, in that

$$c_{S(II)} = c_{H2S} + c_{HS-} + c_{S2-} \quad (56)$$

as a function of pH (s. Fig. 40). For pH=7 ~50 % HS^- and 50% H_2S are available:

$$c_{S(II)} = c_{H2S} + c_{HS-} \quad (57)$$

The three same batch reactors (s. Fig. 9) were employed but identified differently as the reactor A, B, and C in order to track the successive decolorizations in the respective reactor (Fig. 33). The first decolorization of RO 96 (~0.06 mmol/L) at 1.2 mmol/L sulfide as a starting concentration had a delay (~2.5 h) in the reactor A. RO 96 (~0.05 mmol/L) was freshly added in the same reactor at 13 h, when 0.9 mmol/L of sulfide (i.e. the rest content of sulfide after the first decolorization) were available, leading to decolorization without delay. The same was true for the reactors B and C, independent of the addition time and concentration of RO 96. The

delay may be attributed to trace oxygens in the medium. This can indirectly be discerned by the sulfide balance during the first decolorizations. The declorization of ~0.05 mmol/L RO 96 should be coupled to the oxidation of ~0.1 mmol/L sulfide, but ~0.3 mmol/L of sulfide were consumed. The difference of ~0.2 mmol/L sulfide was probably made by the reaction with the trace oxygens. For the kinetic study of decolorization with sulfide, the following strategy was selected to avoid the delay phase of decolorizaiton caused by trace oxygens: The same concentration (0.05 mmol/L) of RO 96 was added at the start, the decolorization occurred with delay. After the first decolorization the dye was freshly added for the determination of kinetics, because a favorable redox state had been established leading to a decolorization without delay, in other words the trace oxygens had reacted with sulfide.

In addition, the second decolorizations appear to be incomplete particularly in reactor B and C. This was caused by elemental sulfur generated in the reaction of sulfide with the dye. The elemental sulfur exhibited a slight yellowish (white-yellow) color, to hinder the precise measurement of dye in low concentrations with the spectrophotometer. The elemental sulfur had been accumulated after the first and second decolorization in the respective reactor. Thus in all further experiments the dye concentration less than 0.01 mmol/L was not significantly evaluated for the decolorization kinetics.

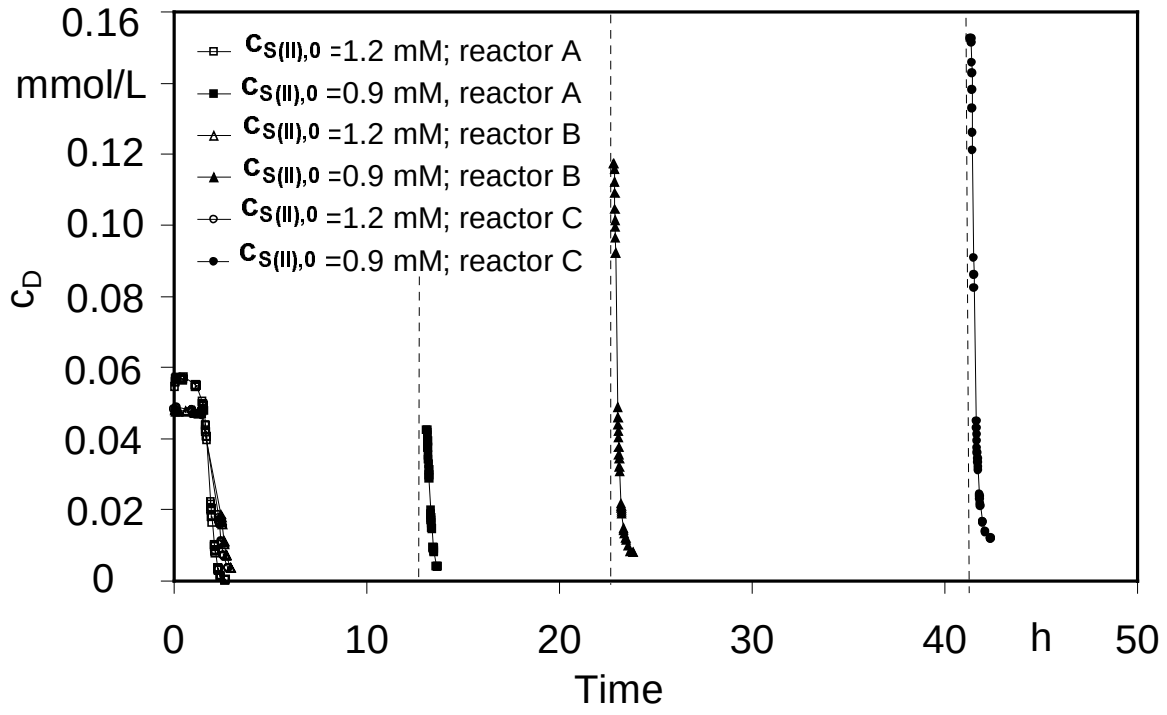


Fig. 33. Decolorization course of RO 96 by sulfide with/without delay phase in the batch reactor A, B, and C: at 37 °C, pH=7.1

Fig. 34 describes the decolorization course of RO 96 by sulfide in the variation of both levels of RO 96 and sulfide, revealing a proportionality between the decolorization rate and the concentration of sulfide. In general, the reaction equation for the decolorization of RO 96 was suggested as following:

$$2S^{2-} + 4H^{+} + R\text{-}N{=}N\text{-}R' \longrightarrow R\text{-}NH_2 + R'\text{-}NH_2 + 2S^0 \quad (58)$$

$$2HS^{-} + 2H^{+} + R\text{-}N{=}N\text{-}R' \longrightarrow R\text{-}NH_2 + R'\text{-}NH_2 + 2S^0 \quad (59)$$

$$2H_2S + R\text{-}N{=}N\text{-}R' \longrightarrow R\text{-}NH_2 + R'\text{-}NH_2 + 2S^0 \quad (60)$$

where R-N=N-R´ symbolize the mono-azo dye RO 96.

Sulfide dissolves in water, depending on pH as S^{2-}, HS^{-}, and H_2S. These species were assumed to be oxidized by the dye with different reaction rate. Thus the decolorization reactions should occur as parallel reaction steps in the form of Eq.(61) assuming the first order with respect to both the concentration of dye and the respective sulfide species:

$$-\frac{dc_D}{dt} = k_0\, c_{H2S}\, c_D + k_1\, c_{HS-}\, c_D + k_2\, c_{S2-}\, c_D \quad (61)$$

where c_D concentration of RO 96 (mmol/L)

c_{H2S} concentration of H_2S (mmol/L)

c_{HS-} concentration of HS^{-} (mmol/L)

c_{S2-} concentration of S^{2-} (mmol/L)

k_0 rate constant of H_2S decolorizing RO 96 (L $mmol^{-1}$ min^{-1})

k_1 rate constant of HS^{-} decolorizing RO 96 (L $mmol^{-1}$ min^{-1})

k_2 rate constant of S^{2-} decolorizing RO 96 (L $mmol^{-1}$ min^{-1})

Eq.(61) is transformed to:

$$-\frac{dc_D}{dt} = (k_0\, c_{H2S} + k_1\, c_{HS-} + k_2\, c_{S2-})\, c_D \quad (62)$$

Since $c_{S(II)} = c_{H2S} + c_{HS-} + c_{S2-}$ [Eq.(56)], the fraction constants for the respective sulfide species α_0, α_1, and α_2 can theoretically be determined using the dissociation constants of the sulfide species K_1 and K_2 (Table 15) as following (Riedel 1994):

$$c_{H2S} = \alpha_0\, c_{S(II)} \quad (63)$$

$$c_{HS-} = \alpha_1\, c_{S(II)} \quad (64)$$

$$c_{S2-} = \alpha_2\, c_{S(II)} \quad (65)$$

where: $\alpha_0 = (1 + K_1\, c_{H^+}^{-1} + K_1\, K_2\, c_{H^+}^{-2})^{-1}$ (66)

$\alpha_1 = (c_{H^+}\, K_1^{-1} + 1 + K_2\, c_{H^+}^{-1})^{-1}$ (67)

$\alpha_2 = (c_{H^+}^{2}\, K_1^{-1}\, K_2^{-1} + c_{H^+}\, K_2^{-1} + 1)^{-1}$ (68)

K_1 and K_2 dissociation constants (mol/L) (s. Table 15)

c_{H^+} concentration of proton (mol/L)

$\alpha_0 + \alpha_1 + \alpha_2 = 1$ (69)

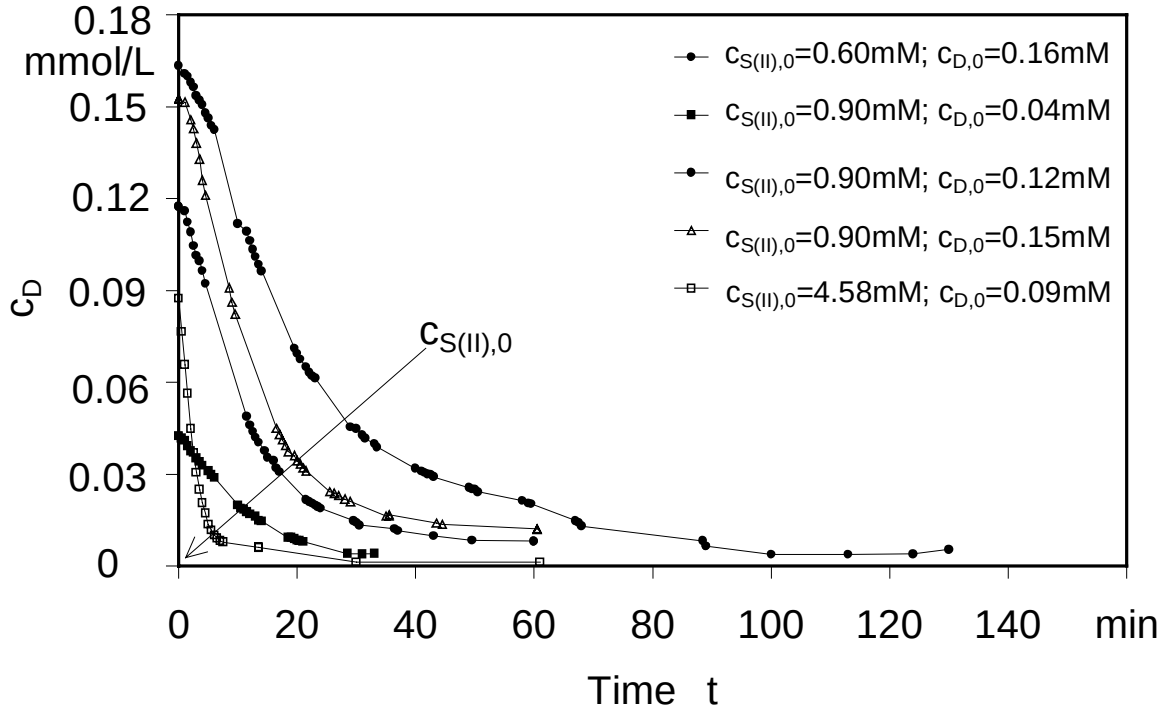


Fig. 34. Chemical decolorization of RO 96 in the variation of both levels of sulfide and RO 96: T=37 °C, pH=7.1

Table 15. Dissociation constants in equilibrium for sulfides at 35 °C (Gupta et al. 1994b)

Reactions	K-Value	pK-Value
$H_2S \longleftrightarrow HS^- + H^+$	$K_1 = 10^{-6.82}$	$pK_1 = 6.82$
$HS^- \longleftrightarrow S^{2-} + H^+$	$K_2 = 10^{-11.37}$	$pK_2 = 11.37$

K_1 and K_2 : Dissociation constants in equilibrium (mol/L)

$$K_1 = \frac{c_{H^+}\, c_{HS^-}}{c_{H2S}} \quad ; \quad K_2 = \frac{c_{H^+}\, c_{S2-}}{c_{HS^-}}$$

$pK_1 = -\log K_1$; $pK_2 = -\log K_2$

The fraction constants for the respective sulfide species α_0, α_1, and α_2 depend on pH. Putting Eq.(63)-(65) in Eq.(62), it follows:

$$-\frac{dc_D}{dt} = (k_0\alpha_0\, c_{S(II)} + k_1\,\alpha_1\, c_{S(II)} + k_2\,\alpha_2\, c_{S(II)})\, c_D \qquad (70)$$

It is simplified to:

$$-\frac{dc_D}{dt} = k\, c_{S(II)}\, c_D \qquad (71)$$

where $k = k_0\,\alpha_0 + k_1\,\alpha_1 + k_2\,\alpha_2$;
rate constant (L $mmol^{-1}$ min^{-1}) (72)

Eq.(72) shows that at a given pH and temperature, k is constant, since α_0, α_1, and α_2 are constant.

For all evaluations of the decolorization kinetics with sulfide, the concentration of sulfide at any time t excluding the initial concentration was not measured but estimated using the following stoichiometry between dye reduction and sulfide oxidation according to Eq.(58)-(60):

$$\frac{dc_D}{dc_{S(II)}} = Y_{D/S(II)} = 0.5 \qquad (73)$$

where $Y_{D/S(II)}$ = yield coefficient of dye (reduction) on sulfide (mmol/mmol)

Eq.(73) was transformed via integration to:

$$c_{S(II)} = c_{S(II),0} - 2\,(c_{D,0} - c_D) \qquad (74)$$

The model of Eq.(71) was examined for the case of great excess in sulfide compared with the dye concentration (Fig. 35). Owing to the great excess in sulfide, Eq.(71) was converted to Eq.(75), assuming that $k\, c_{S(II)}$ remained almost constant (i.e. negligible change of the concentration of sulfide during the decolorization):

$$\frac{dc_D}{dt} = -k'\, c_D \qquad (75)$$

where $k' = k\, c_{S(II)}$;
rate constant (min^{-1})

Eq.(75) was transformed into the following after separation of variables and integration under the start condition $c_D = c_{D,0}$ at t=0:

$$\ln(c_D / c_{D,0}) = -k'\, t \qquad (76)$$

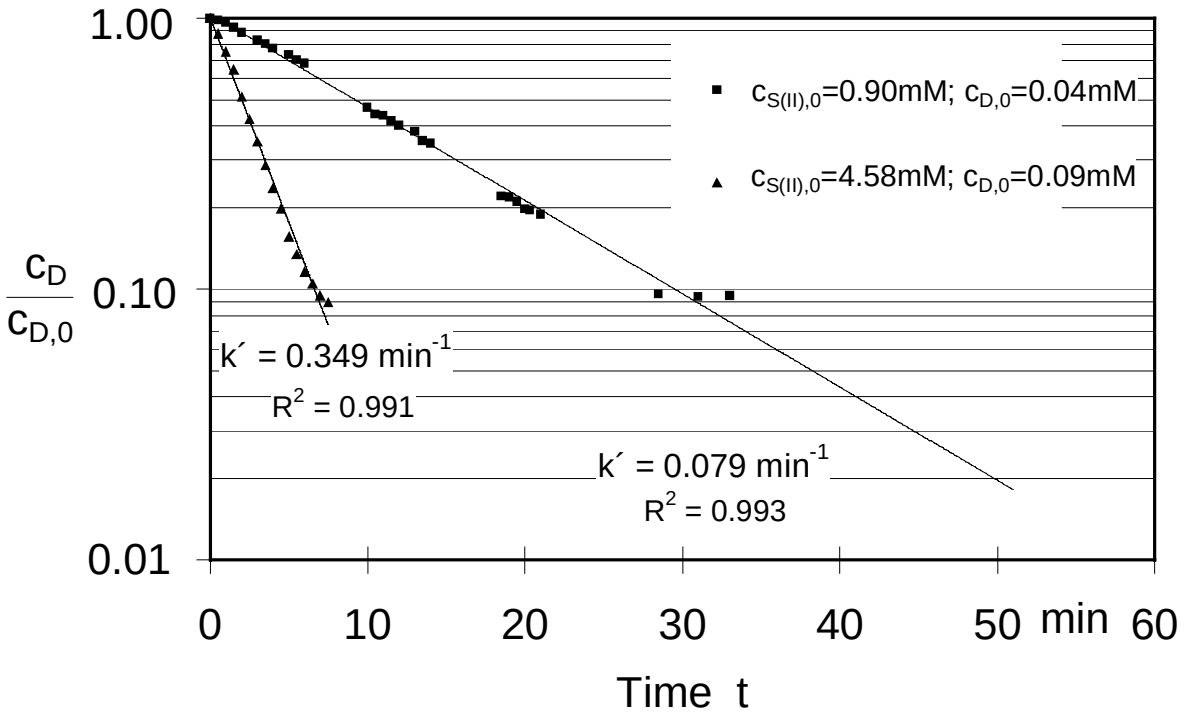


Fig. 35. Determination of decolorization kinetics of RO 96 assuming nearly constant concentration of sulfide: T=37 °C, pH= 7.1

Fig. 35 shows that the data points of $\ln(c_D/c_{D,0})$ vs. time t approached a straight line (e.g. at $c_{S(II),0}$ = 4.58 mmol/L; $c_{D,0}$ = 0.09 mmol/L). Thus the first order with respect to the dye concentration held good for describing the azo dye reduction with sulfide. It is logical that the rate constant k´ for the initial sulfide concentration of 4.58 mmol/L was much higher than that for 0.90 mmol/L. The rate constant k´ comprised the term of sulfide concentration.

Analogous to the case of excess in sulfide level, the reaction order with respect to the concentration of sulfide for the model of the Eq.(71) was inspected in the case of excess in the dye concentration (Fig. 36). A slight decolorization of RO 96 (0.17 mmol/L as initial concentration) occurred. This was clear in view of the excess of RO 96 compared with the sulfide content. The change of the dye concentration (0.01 mmol/L decolorizatiion) was only ≤ 5 % during the decolorization. Stoichiometrically 0.34 mmol/L of sulfide should be necessary for a complete decolorizaiton of 0.17 mmol/L RO 96. The starting concentration of sulfide (0.02 mmol/L) was not measured because of the detection limit, but was recovered by the decolorization of 0.01 mmol/L RO 96. At the excess in dye concentration compared with sulfide concentration, Eq.(71) was arranged to Eq.(77), since k c_D could be regarded as nearly constant.

$$\frac{dc_D}{dt} = - k'' c_{S(II)} \qquad (77)$$

where k´´ = k c_D; rate constant (min^{-1})

Using the stoichiometry between the dye and sulfide of Eq.(73), Eq.(77) was transformed into the following after separation of variable and integration, due to the start condition $c_{S(II)} = c_{S(II),0}$ at t=0:

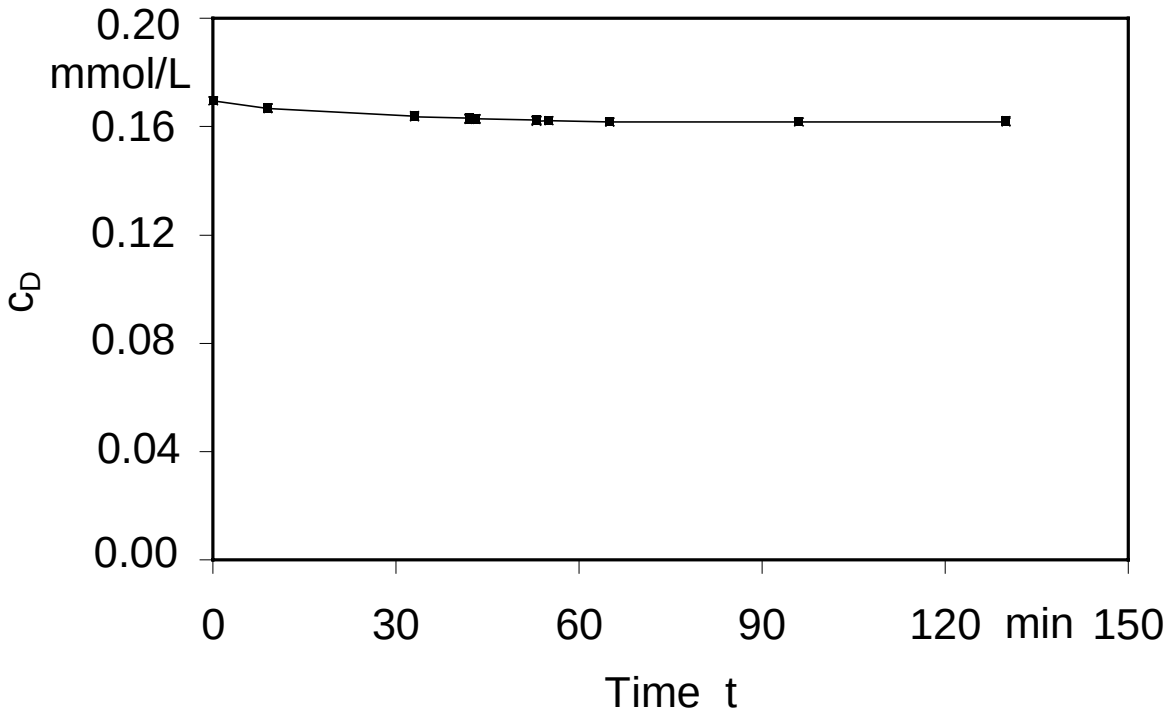
$$\ln(c_{S(II)}/ c_{S(II),0}) = - 2k'' t \qquad (78)$$

The data points of $\ln(c_{S(II)}/c_{S(II),0})$ vs. time t, based on the decolorization course of Fig. 36(a), were matched to a straight line, to give the rate constant k´´ [Fig. 36(b)]. Thus the first order with respect to sulfide concentration was valid for the decoloriaztion kinetics of RO 96 with sulfide.

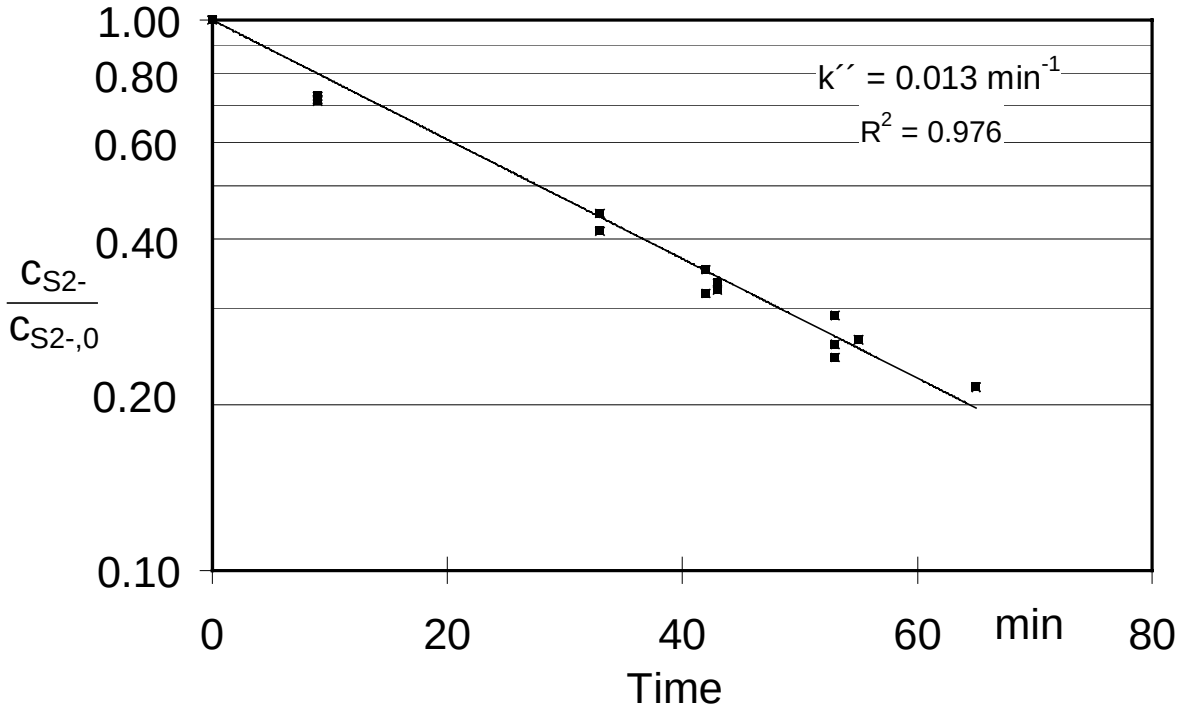
In order to test the model of Eq.(71) under the influence of both concentrations of sulfide and the dye RO 96, the following Eq. was developed by assuming sulfide as a reduction equivalent and using the yield coefficient of dye reduction on sulfide of Eq.(73), analogous to the derivation processes from Eq. (20) to (25):

$$\beta = \frac{1}{(c_{S(II),0} - 2\, c_{D,0})} \left[\ln \frac{c_{D,0} \{c_{S(II),0} - 2\,(c_{D,0} - c_D)\}}{c_D\, c_{S(II),0}} \right] = k\, t \qquad (79)$$

Using Eq.(79) the decolorization courses in the variation of sulfide and dye concentration were evaluated (Fig. 37). The chemical decolorization of RO 96 by sulfide corresponded to the kinetics of the first order with respect to both dye and sulfide concentration. The rate constant k was obtained as 0.09 L $mmol^{-1}$ min^{-1} at pH=7.1 and 37 °C.



(a)



(b)

Fig. 36. Incomplete decolorization of RO 96 at limited level of sulfide (a) and determination of decolorization kinetics (b): $c_{S(II),0}$=0.02 mmol/L, $c_{D,0}$ = 0.17 mmol/L, T= 37 °C, pH=7.1

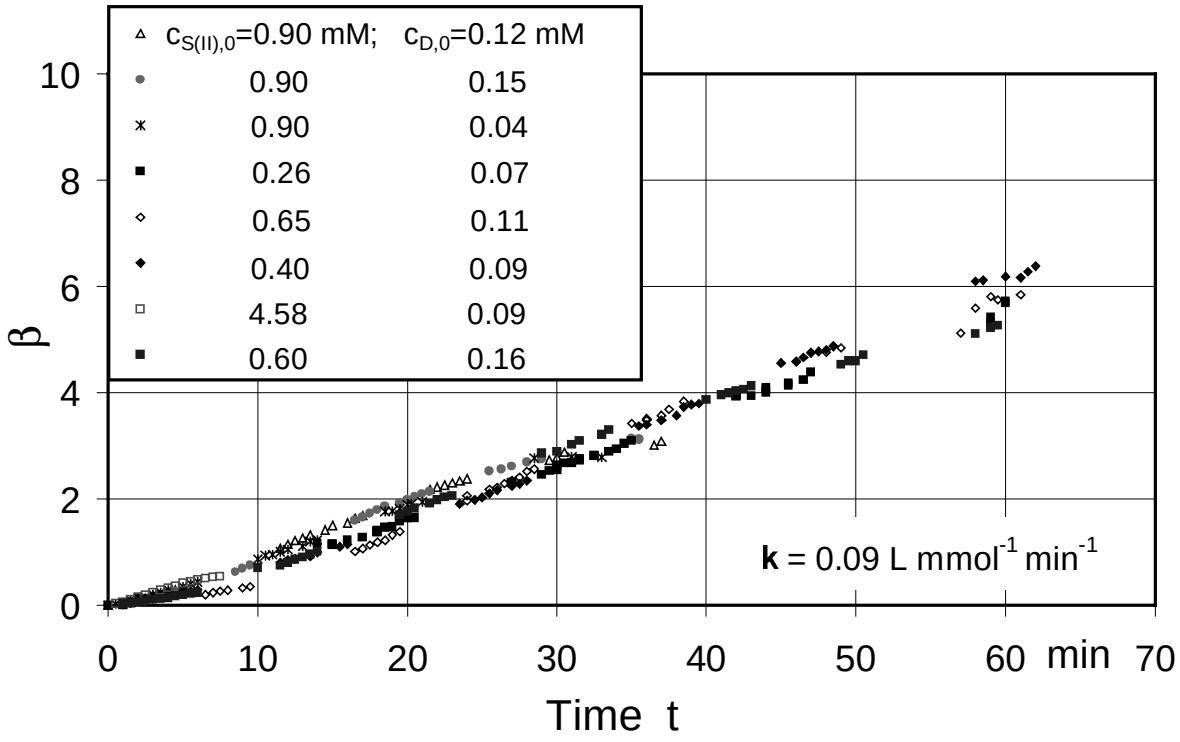


Fig. 37. Determination of decolorization kinetics of RO 96 with sulfide in variation of both dye and sulfide level: at 37 °C, pH = 7.1:

$$\beta = \frac{1}{(c_{S(II),0} - 2\, c_{D,0})} \left[\ln \frac{c_{D,0}\,\{c_{S(II),0} - 2\,(c_{D,0} - c_D)\}}{c_D\, c_{S(II),0}} \right] \qquad (79)$$

Detailed information about the respective rate constants for sulfide in various concentrations of dye and sulfide is summarized in Table 16. The rate constants at the tried initial concentrations of sulfide (0.26-0.90 mmol/L) were focused near 0.09 L $mmol^{-1}$ min^{-1}, although the reaction constant k (0.08 L $mmol^{-1}$ min^{-1}) at 4.58 mmol/L of sulfide was 15 % distant from the average (0.091 L $mmol^{-1}$ min^{-1}) (Table 16). It is not yet confirmable whether an influence of the initial concentrations of sulfide on the decolorization rate constant could possibly exist. Chen and Moriss (1972) showed that the specific oxygenation rate constant of sulfide depended on the initial sulfide concentrations. However, a simple comparison of the reaction with O_2 (Fig. 32) and with an azo dye should be cautious because of the different reaction mechanisms.

Table 16. Rate constant k in variation of initial concentrations of sulfide and dye at pH 7.1 and 37 °C

$c_{S(II),0}$ (mmol/L)	$c_{D,0}$ (mmol/L)	k (L mmol^{-1} min^{-1})	R^2	$\frac{c_{S(II),0}}{c_{D,0}}$
0.90	0.04	0.091	0.992	21.2
0.90	0.12	0.093	0.981	7.7
0.90	0.15	0.094	0.986	5.9
0.26	0.07	0.090	0.991	3.6
0.65	0.11	0.094	0.969	6.2
0.40	0.09	0.095	0.978	4.4
0.60	0.16	0.092	0.989	3.8
4.58	0.09	0.080	0.993	52.6
average		0.091	0.985	
standard deviation		0.005		

6.2.3. Influence of pH on decolorization kinetics with sulfide

In order to inspect the pH influence on the decolorization kinetics with sulfide, pH was varied from 7.1 to 6.3. Fig. 38 shows the time course of the concentration of RO 96 at pH 7.1 and 6.3. In spite of the lower initial concentration of sulfide ($c_{S(II),0}$=0.90 mmol/L) at pH 7.1 the decolorization of RO 96 occurred faster than that at pH 6.3 ($c_{S(II),0}$ =1.10 mmol/L) (70 % vs. 42 % within 15 min). This gave a clue that the rate constant for decolorization with sulfide at neutral pH could be higher than that at acid pH. To confirm this idea, further decolorization courses were tracked to compare the rate constants k for the decolorization in the variation of pH.

Fig. 39 illustrates the results of the comparison of rate constants at pH 6.3, 6.5, and 7.1 in various initial concentrations of RO 96 and sulfide. The rate constants were attained using Eq.(79). The rate constant k at pH 6.3 and 6.5 amounted to 0.06 and 0.08 L mmol^{-1} min^{-1} respectively, whereas that at pH 7.1 was 0.09 L mmol^{-1} min^{-1}. This implies the favorable decolorization at the neutral pH over that at the acid pH. Detailed information about the respective rate constants at pH 6.3 and 6.5 is summarized in Table 17.

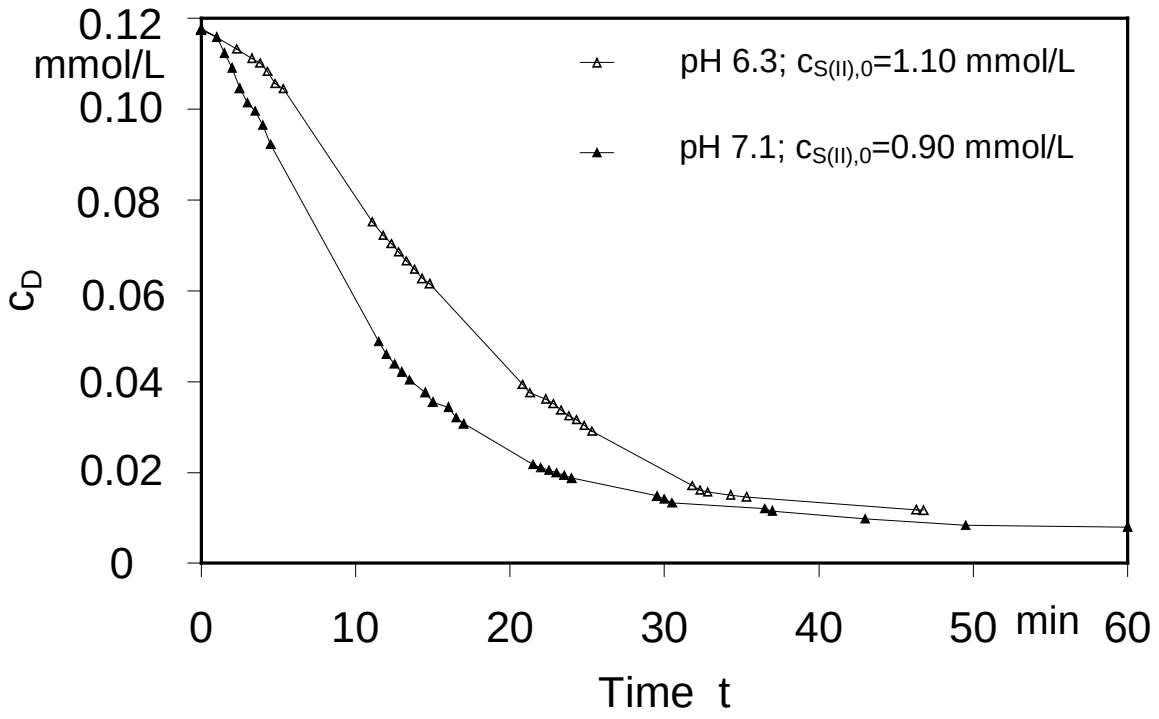


Fig. 38. Decolorization of RO 96 with sulfide at pH 6.3 and 7.1: $c_{D,0}$=0.12 mmol/L, T=37 °C

It is logical that pH determining the distribution of sulfide species (S^{2-}, HS^-, H_2S) affected the decolorization kinetics. In aqueous solution at pH<6, H_2S is predominant, whereas HS^- is dominant everywhere from pH 8 to 11.5 (s. Fig. 40; calculated for 35 °C from Kroiss and Wabnegg 1983). The decrease of rate constant for the decolorization in relation to the pH-decrease from 7.1 to 6.3 (Fig. 39) could be coupled to the protonation of HS^- to H_2S, which is thought to be less oxidizable. The fraction of H_2S of sulfide species (S^{2-}, HS^-, H_2S) in aqueous solution at 35 °C is 35 % at pH 7.1, 68 % at pH 6.5, 77 % at pH 6.3 (s. Fig. 40; Kroiss and Wabnegg 1983).

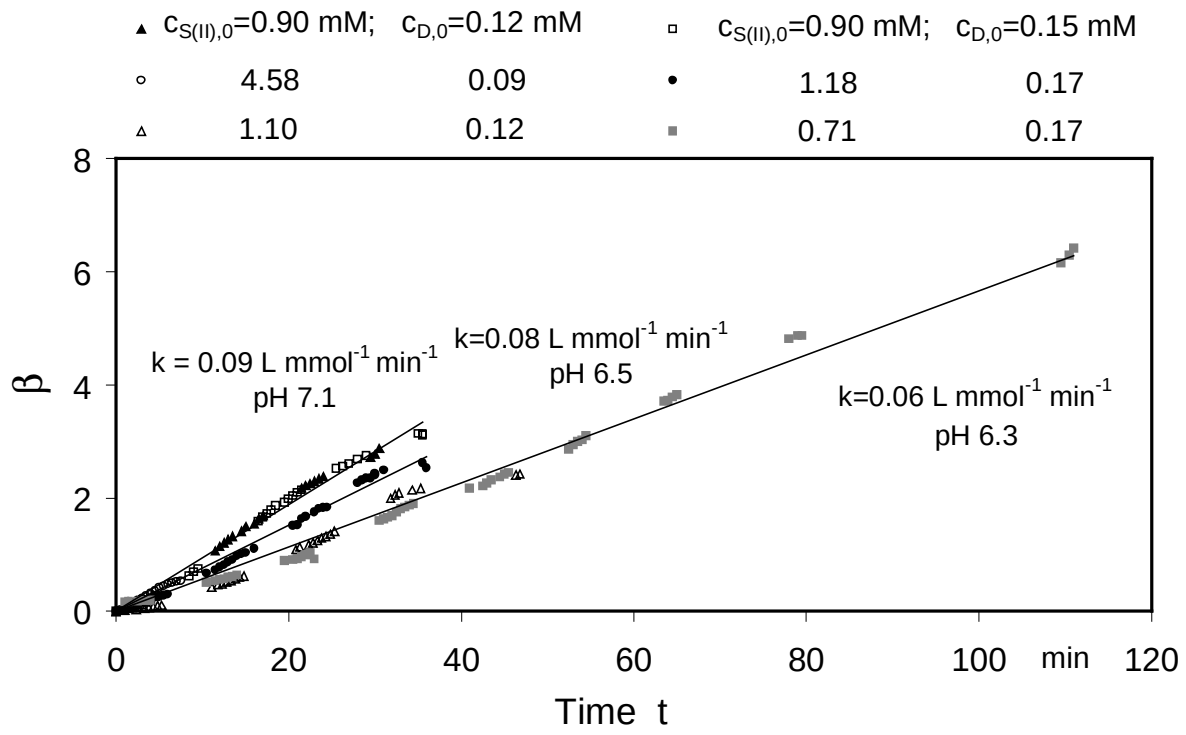


Fig. 39. Comparison of rate constants k for decolorization of RO 96 with sulfide at pH 6.3/ 6.5/ 7.1 and 37 °C in various initial concentrations of dye and sulfide:

$$\beta = \frac{1}{(c_{S(II),0} - 2\, c_{D,0})} \left[\ln \frac{c_{D,0} \{c_{S(II),0} - 2\,(c_{D,0} - c_D)\}}{c_D\, c_{S(II),0}} \right] \qquad (79)$$

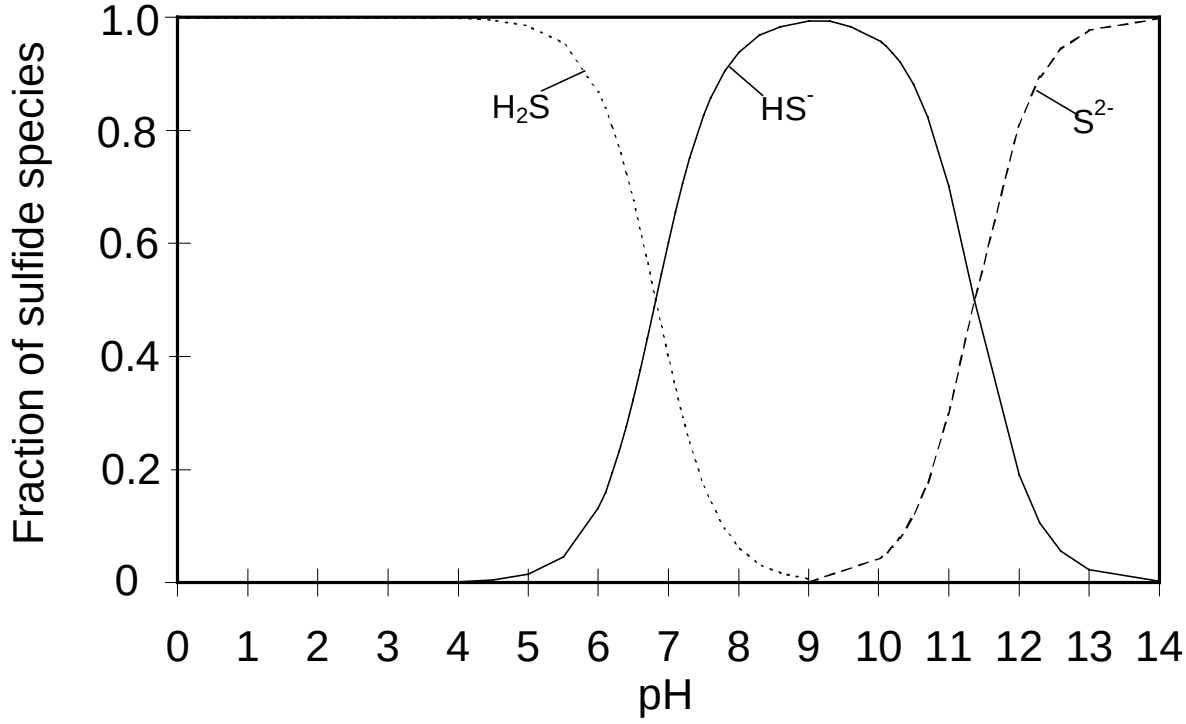


Fig. 40. Fraction of sulfide species (H_2S, HS^-, S^{2-}) at 35 °C depending on pH: calculated based on the dissociation constants of Gupta et al. (1994b)

To confirm an advantageous decolorization of RO 96 with sulfide at neutral pH compared with that at acid pH, the span of pH was enlarged between 4 and 9 in the decolorization (Fig. 41). At the same initial concentrations of RO 96 and sulfide the decolorization rate at pH 9.0 was much higher than that at pH 4.1 (~95 % decolorization within 25 min vs. ~88 % within 230 min).

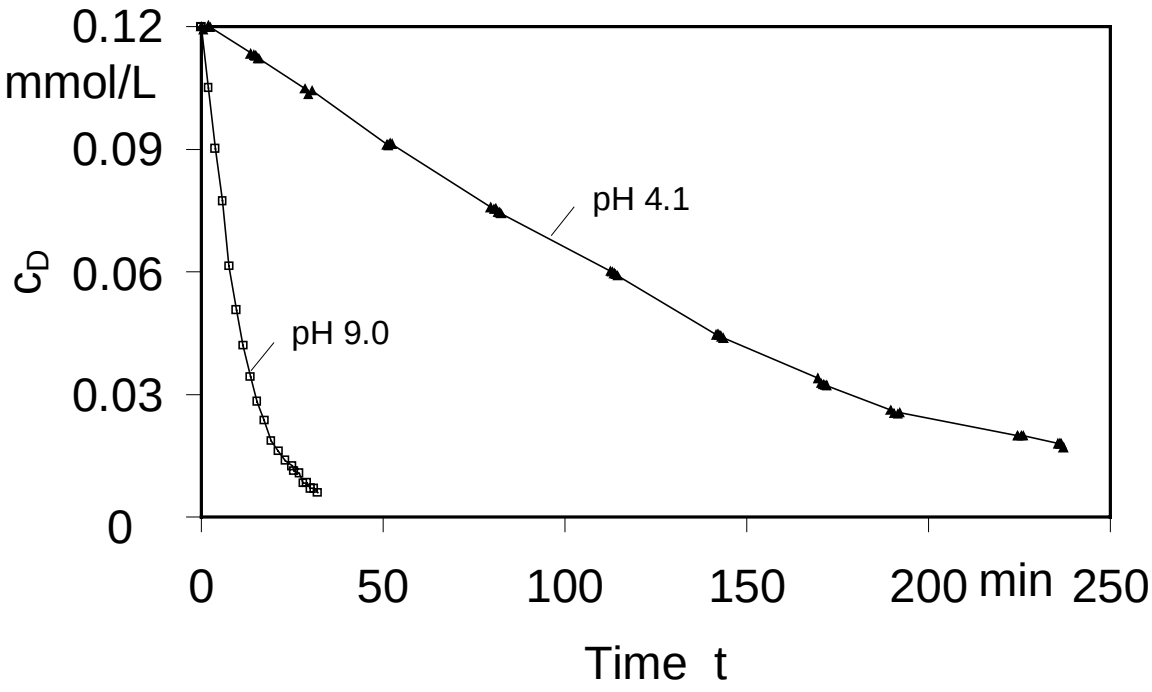


Fig. 41. Comparison of decolorization course of RO 96 with sulfide at pH 4.1/ 9.0: $c_{D,0}$=0.12 mmol/L, $c_{S(II),0}$=0.90 mmol/L, T=37 °C

Table 17. Rate constant k in variation of pH for various initial concentrations of sulfide and RO 96 at 37 °C

$c_{S(II),0}$ (mmol/L)	$c_{D,0}$ (mmol/L)	k (L mmol^{-1} min^{-1})	R^2	pH
1.18	0.17	0.076	0.984	6.5
0.71	0.17	0.057	0.990	6.3
1.10	0.12	0.055	0.957	6.3
1.36	0.17	0.009	0.922	4.1
0.90	0.12	0.009	0.956	4.1
0.90	0.12	0.124	0.994	9.0

This was more obvious in the comparison of rate constants at pH 4.1-9.0 in various initial concentrations of the dye and sulfide (Fig. 42). The rate constants were achieved using Eq.(79). The rate constant k at pH 9.0 amounted to 0.12 L $mmol^{-1}$ min^{-1}, whereas that at pH 4.1 was 0.01 L $mmol^{-1}$ min^{-1}. Detailed information on the respective rate constants at pH 4.1 and 9.0 is summarized in Table 17.

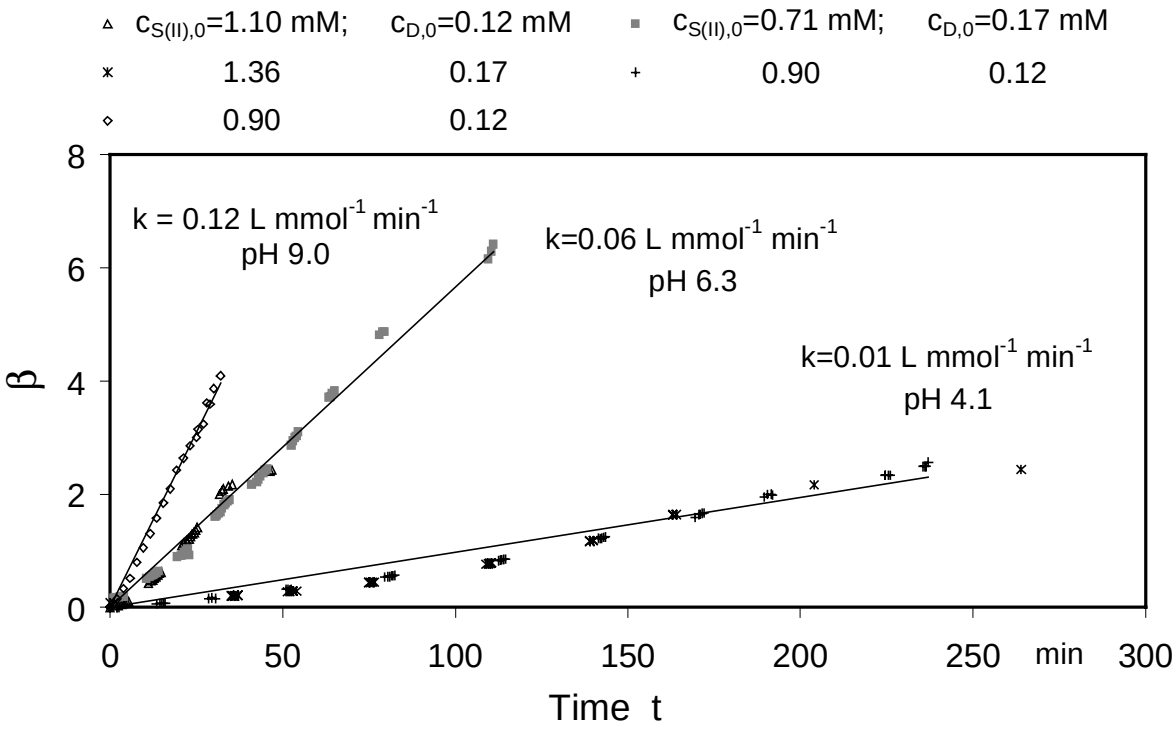


Fig. 42. Comparison of rate constant k for decolorization of RO 96 with sulfide at pH 4.1/ 6.3/ 9.0 and 37 °C in various initial concentrations of RO 96 and sulfide:

$$\beta = \frac{1}{(c_{S(II),0} - 2\,c_{D,0})} \left[\ln \frac{c_{D,0}\,\{c_{S(II),0} - 2\,(c_{D,0} - c_D)\}}{c_D\,c_{S(II),0}} \right] \qquad (79)$$

The influence of pH on the decolorization of RO 96 with sulfide can be quantified using the following Eq.(62), (70), and (71):

$$-\frac{dc_D}{dt} = (k_0\,c_{H2S} + k_1\,c_{HS^-} + k_2\,c_{S2-})\,c_D \qquad (62)$$

$$= (k_0\alpha_0\,c_{S(II)} + k_1\alpha_1\,c_{S(II)} + k_2\,\alpha_2\,c_{S(II)})\,c_D \qquad (70)$$

$$= k\,c_{S(II)}\,c_D \qquad (71)$$

where $k = k_0\,\alpha_0 + k_1\,\alpha_1 + k_2\,\alpha_2$;

rate constant (L $mmol^{-1}$ min^{-1}) (72)

Based on the results of Table 17, H_2S and HS^- were of interest, considering the almost non-availability of S^{2-} in range of pH 4-9 (s. Fig. 40). Thus $\alpha_2=0$, and then Eq.(70) is simplified to:

$$-\frac{dc_D}{dt} = k\, c_{S(II)} c_D = (k_0\, \alpha_0 + k_1\, \alpha_1)\, c_{S(II)} c_D \qquad (80)$$

where $k = k_0\, \alpha_0 + k_1\, \alpha_1$;

rate constant (L $mmol^{-1}$ min^{-1}) at pH 4-9 (81)

At pH=4.1 $\alpha_0=1$and $\alpha_1=0$ (s. Fig. 40), indicating that $c_{S(II)} = c_{H2S}$. Thus Eq.(80) is transformed to:

$$-\frac{dc_D}{dt} = k\, c_{S(II)} c_D = k_0\, c_{S(II)} c_D = k_0\, c_{H2S}\, c_D \qquad (82)$$

From the results of Table 17, k = 0.01 (L $mmol^{-1}$ min^{-1}) at pH 4.1. The decolorization kinetics at pH 4.1 was determined by H_2S alone, to give $k= k_0 = 0.01$ (L $mmol^{-1}$ min^{-1}).

At pH=9 $\alpha_0=0$ and $\alpha_1=1$(s. Fig. 40), indicating that $c_{S(II)}=c_{HS-}$. Thus Eq.(80) is transformed to:

$$-\frac{dc_D}{dt} = k\, c_{S(II)} c_D = k_1\, c_{S(II)} c_D = k_1\, c_{HS-}\, c_D \qquad (83)$$

From the results of Table 17, k = 0.12 (L $mmol^{-1}$ min^{-1}) at pH 9. The decolorization kinetics at pH 9 was determined by HS^- alone, to give $k= k_1 = 0.12$ (L $mmol^{-1}$ min^{-1}).

Because k_0 and k_1 were obtained, and $\alpha_0$and α_1 were determinable according to the dissociation constants and pH 4-9, the correlation of k vs. pH could be obtained using Eq.(81) (Fig. 43). The calculated and measured k corresponded to each other. In light of the chemical decolorization kinetics with sulfide, the basic pH is preferential over the acid or neutral pH. Taking a biological system with SRB into the consideration of a technical decolorization process, a pH-optimization for the decolorization rate with sulfide and the maximum specific growth rate of SRB (Konish et al. 1996) has to be made at neutral or weak basic pH (e.g. 7.0-7.3). The weak basic pH may contribute to a decrease in the toxicity of H_2S to SRB.

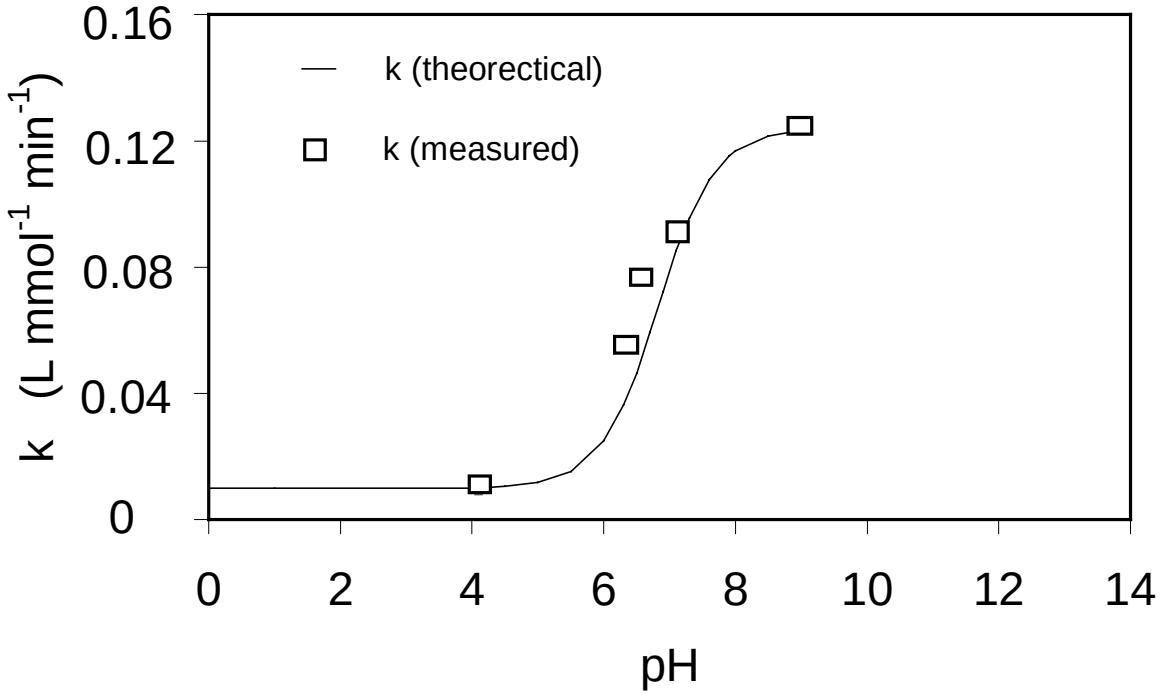


Fig. 43. Theoretical and measured rate constants k in decolorization of RO 96 by sulfide at pH 4-9: T=37 °C.

6.2.4. Comparison of kinetic models

A comparison of decolorization kinetic models should be careful by considering a complete reflection of the related complex conditions (e.g. characteristics of reduction equivalent, delay phase, redox state, competition of electron acceptors, etc.). These experiments for the decolorization of the azo dye RO 96 with sulfide have shown the kinetic model of the first order with respect to both dye and sulfide concentration in the form of the Eq.(71).

Both Wuhrmann et al. (1980) and Carliell et al. (1995), who hypothesized the decolorization with coenzymes ($FADH_2$; $FADH_2$), found first order kinetics of decolorization controlled only by the dye concentration. One possible explanation for the results can possibly be that reduction equivalents (e.g. $FADH_2$) had great excess in comparison with the dye concentration, or the rate of production of such reduction equivalents remained nearly constant. In both situations only the dye concentration might affect the decolorization kinetics. However it cannot be excluded from Carliell et al. (1995) that a sufficient amount of sulfide produced via the reduction of sulfate (originating probably from the dye additives) by SRB possibly brought about a chemical reduction of the azo dye (C. I. Reactive Red 141), thereby showing the first order kinetics. The fact that their rate constants were different for the initial dye concentrations (0.25-0.44 h^{-1} for 50-250 mg/L) makes this remark likely.

The decolorization rate of some azo food dyes was zero order with respect to dye concentration (Dubin and Wright 1975). The authors interpreted the decolorization kinetics in terms of the redox potential difference between the dye and an extracellular electron carrier. However it should be noted that the reduction rates reported referred to data obtained during the first 24 h of anaerobic incubation, thereafter the reduction rates changed. Meyer et al. (1979) reported that no general correlation between the redox potentials of azo dyes and the reduction rates existed.

It can be explained by the high rate constants of decolorization with sulfide that the decolorization through SRB was active in the bacterial consortium with sulfate (Chap. 4) and the decolorization in sulfate respiration of *D. desulfuricans* occurred very rapidly (Chap. 5.2). The reduced coenzymes (e.g. $FAMH_2$) were also reported to show a high rate of decolorization in the cell-free extracts (complete decolorization of 0.05 mmol/L Red 2G by ~17 mmol/L FMN within ~5 min; Gingel and Walker 1971). However, in biological systems with intact cells some factors such as the dye transport at the cell-membrane or in the cell, or the rate of degradation of electron donor can limit the rate of decolorization with coenzymes.

6.3. Summary

1. RO 96 was chemically decolorized by cysteine or ascorbate under the exclusion of O_2. The decolorization kinetics showed the first order with respect to both the concentration of the dye and the respective reducing agent, to give the rate constant for cysteine k_{cys} of 0.0002 L $mmol^{-1}$ h^{-1} and that for ascorbate k_{asc} of 0.0010 L $mmol^{-1}$ h^{-1} at pH=7.1 and 33 °C.
2. The chemical decolorization of RO 96 by sulfide under the exclusion of O_2 corresponded to the kinetics of the first order with respect to both the dye and sulfide concentration. The advantageous decolorization of RO 96 with sulfide at neutral or basic pH (7.1; 9.0) occurred compared with that at acid pH (4.1; 6.3; 6.5). This is attributed to an increase in the fraction of HS^-, which was even more oxidizable than H_2S, of total sulfide species (predominantly HS^- and H_2S at pH 4-9) in aqueous solution at the neutral or basic pH. The rate constants k for the decolorization at 37 °C were obtained as 0.01 L $mmol^{-1}$ min^{-1} for pH=4.1, 0.09 L $mmol^{-1}$ min^{-1} for pH=7.1, and 0.12 L $mmol^{-1}$ min^{-1} for pH=9.0.

7. SUMMARY

Azo dyes are widely used in textile finishing, and have become of concern in wastewater treatments because of their color, bio-recalcitrance, and potential toxicity to animals and humans (Levine 1991). Thus the wastewater with azo dyes must be decolorized and furthermore mineralized in appropriate systems combining biological and chemical processes. In this research the decolorization mechanisms of azo dyes were studied. Real waste water from textile finishing often contains high sulfate concentrations. Sulfide (H_2S, HS^-, or S^{2-} depending on pH) is produced by SRB in the anaerobic process. The decolorization of azo dyes takes place as a chemical reaction with sulfide. In the depletion of sulfate the decolorization occurs also via the fermentation of organic complexes (e.g. pyruvate, lactate) by SRB.

Following circumstantial influences on these decolorization processes through SRB and other possibilities of decolorization were studied:

1. The mono-azo dye RO 96 was decolorized in the anaerobic mixed culture in the presence of acetate and sulfate as the energy source. Inhibition of SRB by molybdate (8 mmol/L) caused a decrease in the decolorization rate of RO 96. Increasing concentrations of the inhibitor (20-80 mmol/L) caused a corresponding decrease in the decolorization rate, verifying the significant contribution of SRB to the decolorization process. The kinetics of decolorization inhibition by molybdate could be interpreted as non-competitive.
2. In the presence of acetate and sulfate, inhibition of MPB with BES (10, 20 mmol/L) did not significantly affect the decolorization of RO 96, implying that MPB are ineffective in decolorization. With the BES, the decolorization of RO 96 occurred even slightly faster than without inhibitor. This may be attributed to the competition between SRB and MPB for acetate, with the inhibition of MPB leading to a more rapid degradation of acetate by SRB and thereby bringing prompter sulfide development for the decolorization.
3. In the presence of lactate and sulfate as the energy source, SRB gave a significant contribution to the decolorization of RO 96.
4. The mechanism of the decolorization of azo dyes in the anaerobic mixed culture based on the extracelluar chemical reduction with sulfide was postulated. Sulfide produced via sulfate respiration by SRB chemically decolorizes azo dyes.
5. Under sulfate-rich conditions, sulfide produced via sulfate respiration by *D. desulfuricans* using pyruvate or lactate as energy and carbon source chemically decolorized the azo dyes RO 96 and RR 120 (di-azo dye). This chemical decolorization is suggested not to be linked to ATP-gain in the sulfate respiration.
6. In depletion of sulfate (≤ 0.1 mmol/L), the decolorization of the azo dyes RO 96 and RR 120 occurred in correlation with the fermentation of pyruvate by *D. desulfuricans*. It is

suggested that the electrons liberated from the pyruvate oxidation are transferred via (co-)enzymes (e.g. Fd) to the dyes as terminal electron acceptors, resulting in decolorization, instead of to the H^+, leading to the production of H_2. This decolorization is suggested to be connected to ATP gain via acetate production in the fermentation.

7. The decolorization of RO 96 with cysteine and ascorbate under the exclusion of O_2 showed the kinetics with the first order with respect to both the concentration of the dye and the respective reducing agent, to give the rate constant for cysteine k_{cys} of 0.0002 L $mmol^{-1}$ h^{-1} and that for ascorbate k_{asc} of 0.0010 L $mmol^{-1}$ h^{-1} at pH=7.1 and 33 °C.
8. The chemical decolorization of RO 96 by sulfide under the exclusion of O_2 corresponded to the kinetics of the first order with respect to both the dye and sulfide concentration. This was confirmed in the case of a great excess in sulfide or dye respectively, and under influence of both. The advantageous decolorization of RO 96 with sulfide at neutral or basic pH (7.1; 9.0) occurred compared with that at acid pH (4.1; 6.3; 6.5). This is attributed to an increase in the fraction of HS^-, which was even more oxidizable than H_2S, of total sulfide species (predominantly HS^- and H_2S at pH 4-9) in aqueous solution at the neutral or basic pH. The rate constants k for the decolorization at 37 °C were obtained as 0.01 L $mmol^{-1}$ min^{-1} for pH=4.1, 0.09 L $mmol^{-1}$ min^{-1} for pH=7.1, and 0.12 L $mmol^{-1}$ min^{-1} for pH=9.0.

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