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Metabolism of Azo Dyes by *Lactobacillus casei* TISTR 1500 and Effects of Various Factors on Decolorization

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Abstract

Lactobacillus casei TISTR 1500 was isolated from soil of a dairy wastewater treatment plant in Chiang Mai province, Thailand, as the most active azo dye degrader of 19 isolates. Growing cells and resting cells of this strain completely degraded methyl orange, thereby decolorizing the medium. The strain stoichiometrically converted methyl orange to *N,N*-dimethyl-*p*-phenylenediamine and 4-aminosulfonic acid, which were identified by HPLC, GC, and GC-MS analyses. The enzyme activity that cleaved the azo bond of methyl orange was localized to the cytoplasm of cells grown on modified MRS medium containing methyl orange. The effect of sugars, oligosaccharides, organic acids, metal ions, pH, and temperature on methyl orange decolorization by resting cells was investigated. Optimal decolorization occurred at 35°C and pH 4.0 to 8.0. The addition of sucrose or oxalic acid or galacto-oligosaccharide as an energy source led to a promotion of the degradation of methyl orange.

Key words

Lactobacillus casei; azo dyes; azo bonds; azoreductase; decolorization; factor affecting methyl orange

1. Introduction

Azo dyes have been used increasingly because of their ease and cost effectiveness in synthesis, stability, and the variety of colors available compared to natural dyes (Griffiths, 1984). They are used in the pharmaceutical, food, brewing, and cosmetic industries and are approved and certified by the U. S. Food and Drug Administration (Collier et al., 1993). However, many studies indicate that most azo dyes are toxic (Holme, 1984), carcinogenic, and mutagenic (Rafii et al., 1997).

Azo dyes are characterized by one or more azo bonds (R-N=N-R'). The dyes are reduced by bacteria from the human intestinal tract and are cleaved at the azo bonds under the low redox potential prevailing in the gut, forming the corresponding aromatic amines (Rafii et al., 1997). The reduction of azo dyes produces compounds that are more or less toxic than the parent molecule. Azoreductase activity, especially through the generation of aromatic amines, can be associated with undesirable toxicological effects; azo dyes are converted biologically to nitroaromatic compounds and *N*-hydroxyaromatic compounds, which would show carcinogenic properties (Parkinson, 1996; O'Brien et al., 1999; Haberer et al., 2003). Talarposhti et al., (2001) have reported that azo dyes are resistant to breakdown, with the potential for the persistence and accumulation of high levels of dye. Many researches have described the ability of intestinal

facultative anaerobic bacteria to degrade azo compounds (Chung et al., 1992; Seesuriyachan et al., 2002). However, little is known about the biodegradation of azo dyes by lactic acid bacteria. Lactobacilli are normal inhabitants of the digestive tract of humans and animals. Many lactic acid bacteria are able to degrade carcinogenic compounds (Haberer et al., 2003).

The goal of our study was to determine whether and how a probiotic strain of *Lactobacillus casei* degrades azo dyes. Here we report the isolation of a soil lactic acid bacterium able to degrade and decolorize methyl orange and the identification of two metabolites in the degradation pathway. In addition, effects of factors on the decolorization of methyl orange by this strain are shown.

2. Materials and Methods

2.1. Isolation and identification of azo-dye-degrading bacteria

Soil samples from a dairy wastewater treatment plant and from dairy food industries in Chiang Mai province, Thailand, were used for enrichment cultures. Approximately 1 g of soil sample was suspended in 100 ml sterilized water. One milliliter of the suspension was added to 10 ml of modified MRS liquid medium (de Man et al., 1960) containing 0.5 g methyl orange/l (1.5 mM) in a 20-ml screw-capped test tube and incubated at 35 °C for 24 h. A loop-full of each

decolorized enrichment culture was spread onto MRS agar plates containing 1.5 mM methyl

orange. Plates were incubated at 35 °C for 3 days. Colonies of lactic acid bacteria with a large

zone of decolorization were inoculated into MRS medium containing methyl orange in screw-

capped test tubes to confirm the decolorization activity. A strain showing the fastest

decolorization was selected for further studies.

The isolated strain was identified as *Lactobacillus casei* on the basis of morphological

and biochemical (API 50 CH) characterization and a 16S rRNA gene sequence analysis (1561

bp, The DDBJ/EMBL/GenBank Accession No. for the 16S rRNA sequence is AB182585)

(Seesuriyachan et al., 2002). The strain was deposited in the Thailand Institute of Scientific and

Technological Research (TISTR) culture collection under TISTR 1500.

2.2. Preparation of resting cells, cell extracts, and cell fractions

Strain TISTR 1500 was inoculated into 1 L of modified MRS medium in a 3-L flask. The

culture was incubated at 35 °C for 12 h. Cells were collected by centrifugation at 20,000×g for

10 min at 4 °C. The pelleted cells were washed twice with 0.85% (w/v) NaCl. For experiments

with resting cells, the washed cells (1 g, wet wt.) were resuspended in 10 mL of water or 0.85%

(w/v) NaCl. For cell fractionation, the washed cells (1 g, wet wt.) were resuspended in 10 mL of

20 mM Tris-HCl buffer (pH 8.0), and cells in 30-mL batches were disrupted by sonicating three

times with a Kubota 201M ultrasonic oscillator (Kubota Shoji, Tokyo, Japan) at 180 W for 5

min. Cell debris and unbroken cells were separated from extracts by centrifugation at 1,000 g

for 10 min; cytoplasm and membranes were separated by centrifugation at 20,000 g for 10 min.

2.3. Enzyme assay

Azoreductase activity in cell fractions was assayed by the modified method of

Zimmerman et al., (1982). The reaction mixture (total volume 3.0 mL) contained 2.55 mL of

118 mM sodium-potassium phosphate buffer (pH 7.5), 0.1 ml of 10.5 mM NAD(P)H, and 0.1

mL of 0.72 mM methyl orange. The reaction was started by adding 0.25 mL of the cell fraction

solution. The decrease in the absorbance of NADH or NADPH at 340 nm was monitored using

a Hitachi U-1100 spectrophotometer. Protein concentrations were measured by the method of

Lowry et al., (1951). One unit of enzyme activity was defined as the amount of enzyme that was

reduced 1 μmol of NADH per min. All experiments were carried out in triplicate.

2.4. Isolation and identification of the methyl orange (azo dyes) degradation intermediates

Pelleted cells from a 1-L culture resuspended in 0.8% (w/v) NaCl were added to a 0.23

mM methyl orange solution (150 mL) containing 20 mM sucrose to an OD_{600} of 10 ; the mixture

was incubated without shaking at 35°C . Methyl orange and accumulated products (compounds

I and II) were measured by HPLC (see Analytical methods). After incubation for 2.5 h, the

decolorized reaction mixture was concentrated to 12 mL in a rotary evaporator. A 6-mL aliquot

of the concentrated solution was adjusted to pH 11.0 and extracted three times with an equal

volume of ethyl acetate. The upper layers were pooled and evaporated to dryness (Compound I).

Compound I was incubated with 500 μL of *N,O*-bis(trimethylsilyl)-trifluoroacetamide at 90 °C

for 1.5 h. Another 6-mL aliquot of the concentrated solution (Compound II) was applied to a

Dowex 50W 100–200 mesh (Dow Chemical, Midland, USA) column (1.0 x 6.4 cm) equilibrated

with water. Compounds were eluted successively with 1 N HCl, water, and 1 N NaOH.

Fractions of 5 ml were collected at a flow rate 40 mL/h. Compound II was eluted in 1 N HCl

fractions. The fractions containing compound II were pooled and evaporated to dryness.

Compound II was mixed with 10 mL of 2.0 M trimethylsilyl diazomethane in hexane; the

mixture was incubated at room temperature for 3 h. The esterified compound II was

trimethylsilylated with 500 μL of *N,O*-bis(trimethylsilyl)-trifluoroacetamide at 90 °C for 1.5 h.

The derivatized compounds I and II were analyzed by GC–MS (see Analytical methods). The

intermediates, compound III and compound IV derived from methyl red were also isolated

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separately and drivatized to analyze them using methods described above.

2.5. Azoreductase substrate specificity

The substrate specificity of the azoreductase from strain TISTR 1500 was examined

using nine azo dyes (see Table 3). Washed cells of the strain were added to nine different 0.23

mM dye solutions (300 ml) containing 20 mM sucrose at an OD_{600} of 10 ; the mixtures were

incubated without shaking at 35 °C for 3 h. The remaining azo dyes were measured

spectrophotometrically and quantitated using the respective molar extinction coefficients of the

azo dyes in 100 mM sodium-potassium phosphate buffer (pH 7.5) (Table 3) .

2.6. Effects of various factors on methyl orange decolorization by resting cells

Washed cells of strain TISTR 1500 were added to 300 ml of 0.23 mM methyl orange to

an OD_{600} of 3.0; aliquots, each containing one of a number of additions as indicated below, were

incubated without shaking at 35 °C. All experiments were done in triplicate, and the average

values are shown.

Effects of sugars–The reaction mixture contained 20 mM D-glucose, sucrose, D-fructose, D-

lactose, maltose, D-galactose, D-mannose, D-arabinose, D-xylose, D-mannitol, D-inositol, or D-

sorbitol. A control sample was prepared without sugar.

Effects of oligosaccharides– The reaction mixture contained 10 g/L of Raftiline HP (inulin

>99.5%; glucose-fructose-lactose < 0.5%; Orafti, Tienen, Belgium), Raftilose P95 (fructo-

oligosaccharide; 95%; glucose-fructose-sucrose, 5%; Orafti), or Oligostroop (galacto-

oligosaccharide; Borculo Whey Products, Borculo, Netherlands).

Effects of metal ions– The reaction mixtures contained 20 mM sucrose and 50 μM Ca^{2+}, Co^{2+},

Cu^{2+}, Fe^{3+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, or Zn^{2+}. A control sample was prepared without metal ions.

Effects of organic acids– The reaction mixture, lacking sucrose, contained 20 mM acetic acid,

citric acid, lactic acid, oxalic acid, succinic acid, or tartaric acid. The pH of the mixture was

adjusted to pH 7.0.

Effects of pH– The reaction mixture containing 20 mM sucrose was adjusted to pH value

between 3 and 10.

Effects of temperature– The reaction mixture was incubated at 25, 35, 45, or 55°C.

2.7. *Analytical methods*

A Hitachi L-6200 HPLC system (Hitachi, Tokyo) equipped with an Inersil ODS-2

column (4.6 x 150 mm, 5 μm; GL Science, Tokyo, Japan) was used for measuring methyl

orange and its metabolites (*N,N*-dimethyl-*p*-phenylenediamine and 4-aminosulfonic acid). The

mobile phase was composed of 0.1% (w/v) H_3PO_4-acetonitrile (50:50, v/v). Samples were

eluted at a flow rate of 0.7 ml/min and room temperature. The retention times of methyl orange,

N,N-dimethyl-*p*-phenylenediamine, and 4-aminosulfonic acid were 23.9, 2.9, and 6.0 min,

respectively. The trimethylsilylated compound I and esterified and trimethylsilylated compound

II were analyzed with a Hitachi M-2500 mass spectrometer at an ionization potential of 70 eV,

coupled to a Hitachi G-3000 gas chromatograph. A TC-1 fused silica capillary column (0.25 mm

x 30 m; GL Science) was used. The column temperature was held at 100 °C for 1 min and was

then increased from 100 to 280 °C at a rate of 10 °C per min.

2.8. Chemicals

　　Tropaeolin O, crocein orange G, orange II, methyl orange, and trimethylsilyl

diazomethane were purchased from Sigma-Aldrich (St. Louise, USA). Acid red 151 and acid

orange 8 were from ICN Biomedicals (Whilst, USA). Ponceau 3R, methyl red, congo red,

NADH, NADPH, *N,N*-dimethyl-*p*-phenylenediamine, 4-aminosulfonic acid, and *N,O*-

bis(trimethylsilyl-trifluoroacetamide were from Wako Pure Chemicals (Osaka, Japan).

3. Results and Discussion

3.1. Isolation and identification of azo dye-degrading bacteria

From 46 soil samples accumulated in dairy factory and wastewater treatment facility areas, we obtained 19 isolates that grew well on MRS medium containing methyl orange and decolorized the azo dye. One isolate grew well under microaerophilic conditions at 35 °C and produced the clearest and largest decolorized zone around colonies on the modified MRS medium within 48 h. The API 50 CH tests and the analysis of 16S rRNA gene sequence showed that the isolated strain belongs to *Lactobacillus casei*.

3.2. Identification of intermediates from methyl orange

The supernatant of the reaction mixture containing resting cells and methyl orange was subjected to the analysis using HPLC. When sucrose was present in the reaction mixture, methyl orange was completely degraded within 2.5 h (Fig. 1), and the reaction mixture became colorless. Methyl orange (R_t = 23.9 min; 0.23 mM) was converted stoichiometrically to *N,N*-dimethyl-*p*-phenylenediamine (R_t = 2.9 min; 0.24 mM) and 4-aminosulfonic acid (R_t = 6.0 min; 0.25 mM). These retention times agreed with those of the authentic *N,N*-dimethyl-*p*-phenylenediamine and 4-aminosulfonic acid.

The derivatized intermediates were analyzed by GC and GC-MS (Table 1). The mass

spectra and GC retention times of the trimethylsilylated compound I and the esterified and

trimethylsilylated compound II agreed with those of the derivatized authentic *N,N*-dimethyl-*p*-

phenylenediamine (R_t = 9.8 min) and the derivatized authentic 4-aminosulfonic acid (R_t = 5.5

min), respectively. From these results, this strain probably produced an azoreductase that

cleaves the azo bond of methyl orange.

3.3. Localization of azoreductase and identification of cofactor

The azoreductase was localized in the cytoplasmic fraction (Table 2). It required NADH,

but not NADPH, as a cofactor for the decolorization activity. NADH-dependent azoreductases

from *Pseudomonas luteoda* (Chang et al., 2001), *Escherichia coli* (Rau and Stolz, 2003),

Pigmentiphaga kullae K 24 (Blümel and Stolz, 2003), *Enterobacter agglomerans*

(Moutaouakkil et al., 2003), and *Bacillus* sp. strain SF (Maier et al., 2004) have been reported.

The azoreductase from *Clostridium perfringens* strictly requires NADPH. *Streptococcus faecalis*

(Gingell and Walker, 1971) and *Sphingomonas* sp. strain BN6 (Russ et al., 2000) decolorize azo

dyes and require NADH. The precise mechanism of enzymatic reaction in *Sphingomonas* sp.

strain BN6 is unknown, but the enzyme apparently incorporates a water-soluble electron carrier,

which acts as an electron mediator, while the azo compounds are electron acceptors (Semde' et al., 1998; Russ et al., 2000).

3.4. Effect of various factors on methyl orange decolorization

Resting cells of strain TISTR 1500 required sugars, oligosaccharides, or organic acids as an energy source to promote the degradation of methyl orange (Fig. 2A–C). Sucrose was the best energy source of the sugars tested (Fig. 2A). Although D-glucose is generally known as a substrate that is taken up inside cells and metabolized easily, it took 24 h for the resting cells of strain TISTR 1500 to degrade the azo dye completely. D-Fructose, D-maltose, D-arabinose, and D-xylose did not affect the decolorization of methyl orange. The sugar alcohols D-mannitol, D-inositol, and D-sorbitol fairly promoted the decolorization. However, these findings contradicted the results of sugar fermentation tests using the API 50 CH system. The sulfonate group in a methyl orange molecule presumably obstructs the transport of these sugars into the cytoplasm of strain . Methyl orange was degraded to 39% of the normal level in the presence of galacto-oligosaccharide; fructo-oligosaccharide (12% degradation) and inulin (20% degradation) had little effect (Fig. 2B). Fructo-oligosaccharide and inulin have the similar molecular structure (Kaplan and Hutkin, 2000; Kaplan and Hutkin, 2003), but inulin contains a higher percentage of

D-glucose monomers. Since resting cells of the strain decolorized methyl orange in the presence

of inulin to a greater extent than in the presence of fructo-oligosaccharide, the cells possibly

used D-glucose in the inulin as an energy source. On the other hand, Chang *et al*. [3] reported

that the decolorization of azo dyes by *Pseudomonas luteola* is inhibited by sugars because of

their catabolite repression.

Although acetic acid and lactic acid did not activate the degradation of methyl orange

by resting cells of strain TISTR 1500 (Fig. 2C), oxalic acid, succinic acid, citric acid, and

tartaric acid led to faster and more complete decolorization of the dye within 15, 18, 21, and 30

h, respectively. The addition of all the metal ions tested, except for calcium ions, inhibited the

degradation (Fig. 2D). Possible biosorption of copper ion in the mixture was observed, because

the intact resting cells turned blue. Similar observations have been reported with *Desulfovibrio*

desulfuricans (Chen et al., 2000) and *Pseudomonas luteola* (Chen, 2002). Metal ions might

inhibit the degradation of azo dyes by directly denaturing an azoreductase or by reducing the

rate of transport of sugars and azo dyes.

Resting cells of the strain completely decolorized methyl orange between pH 4 and 8

(Fig. 2E). The degradation was inhibited at acidic and alkaline pH sides. Among the tested

temperatures, 35°C was most favorable for the decolorization, at which the azo dye disappeared

after 24 h of incubation.

3.5. Substrate specificity

Resting cells of strain TISTR 1500 decolorized various azo dyes, in adition to methyl

orange (Table 3). The highest specific decolorization rate, determined by the disappearance of

the dye, was that of methyl red at 31.0 mg^{-1} g cell^{-1} h^{-1}. In contrast, the decolorization rate of

acid red 151 by resting cells was extremely low at 0.54 mg^{-1}·g cell^{-1}·h^{-1}. The results showed that

azo dyes containing two azo bonds (acid red 151 and congo red) were more difficult to cleave,

probably because these compounds consist of polyaromatic and sulfonated groups and the

chemical structures of these dyes are more complicated. Methyl red, with a mono-azo bond and

lacking a sulfonate group, could be degraded easily. The intermediates derived from methyl red

were also analyzed by GC and GC-MS (Table 1). The mass spectra and GC retention times of

the trimethylsilylated compound III and compound IV agreed with those of the derivatized

authentic *N,N*-dimethyl-*p*-phenylenediamine (R_t = 9.8 min) and the derivatized authentic 2-

aminobenzoic acid (R_t = 5.5 min), respectively.

Azo dyes with a mono-azo bond and with a sulfonate group (methyl orange, tropaeoline

O, crocein orange G, orange II, and acid orange 8) were difficult to decolorize. Since crocein

orange G, orange II, and acid orange 8 were decolorized slowly, the methyl group in the *ortho*

position of the benzene ring probably inhibits the formation of the enzyme–substrate complex.

Blümel et al., (2002) have suggested that the sulfonate group affects the transport of these dyes

into the cells and does not affect the decolorization.

Strain TISTR 1500 converted methyl orange to *N,N*-dimethyl-*p*-phenylenediamine and

4-aminosulfonic acid, but did not mineralize this dye. The substrate specificity of this strain

indicates that probiotic bacteria can metabolize several dye compounds that are added as food

colorants, thereby decolorizing the dye solutions. Several studies indicated that aniline

derivatives including these intermediate are also toxic, carcinogenic, and mutagenic (chen et al.,

1999). Accumulated the intermediate derived from methyl orange would be eliminated from a

batch through chemical treatment process (polymerization), because these are an ampholyte ion

compound. These compounds would be converted to an inactive compound by using arylamine

N-acetyltransferase producing bacteria. The acetyltransferase have been found in various

microorganisms including Lactobacillus and several studies indicated that the enzyme can play a

role of detoxification toward aniline and its derivatives (Delomenie et al., 2001).

4. Conclusions

1. The *Lactobacillus casei* TISTR 1500 converted methyl orange to *N,N*-dimethyl-*p*-phenylenediamine and 4-aminosulfonic acid, completely azo bond broken down , but did not mineralize this dye.

2. The substrate specificity of azoreductase from strain TISTR 1500 indicates that the azo dyes containing two azo bonds and the azo dyes with sulfonate substituent is difficult to decolorize. While, the dyes with the methyl substituent at the *ortho* position show the slow rate of decolorization which is probable difficulty of the enzyme complex formation.

The aim of this work is try to isolate microorganism which has its ability to degrade azo dye normally use as food colorants in dairy factory. General, dairy wastewater is rich nutrients for bacteria growth, so we have serious problem in the use of azo dye mineralizing bacteria to treat azo dye containing wastewater. The bacteria grow well without any decolorization, solving this problem, we want to use the *Lactobacillus casei* TISTR 1500 together in application of stationary anaerobic sequencing batch reactor (SASBR) in dairy pre-treatment with lactic acid bacteria have been carried out (Seesuriyachan et al., 2001). Such the system is considered to be suitable for a coupled reaction of lactic acid fermentation and azo dye decolorization.

Acknowledgements

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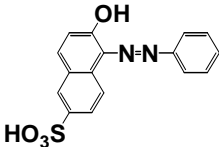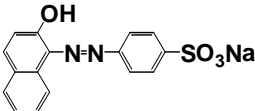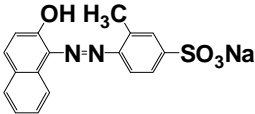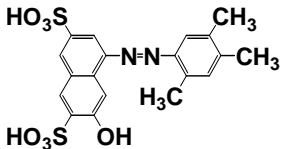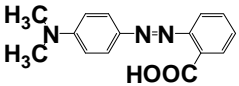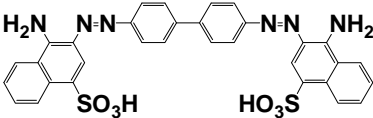TABLE 1. Mass spectra of the metabolites of methyl orange and methyl red.
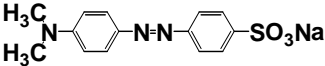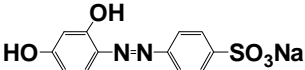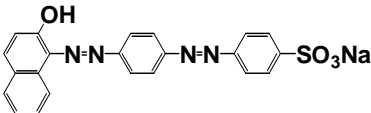
Compound	Fragment ion of trimethylsilylated compound I, III and IV and methyl esterified and trimethylsilylated compound II [m/z (assignment, relative intensity (%))]
Compound I and III (*N,N*-dimethyl-*p*-phenylenediamine)	208 (M^+, 100%), 193 (M^+-CH_3, 82.1%), 177 (6.7%), 163 (M^+-$(CH_3)_3$, 4.3%), 149 (M^+-$N(CH_3)_2$-CH_3, 4.3%), 121 (M^+-N-$Si(CH_3)_3$, 15.2%), 73 ($[Si(CH_3)_3]^+$, 25.9%)
Compound II (4-aminosulfonic acid)	258 (M^+-H, 5.6%), 243 (M^+-H-CH_3, 7.3%), 225(12.2%), 170 (M^+-H-NH-$Si(CH_3)_3$, 26.9%), 155(M^+-H-NH-$Si(CH_3)_3$-CH_3, 15.2%), 147 ($[(CH_3)_2$=O-$OSi(CH_3)_3]^+$, 100%), 135 (17.4%), 73 ($[Si(CH_3)_3]^+$, 59.1%)
Compound IV	281 (M^+, 5.2%), 266 (M^+-CH_3, 70.7%), 192 (7.9%), 147 ($[(CH_3)_2$=O-$OSi(CH_3)_3]^+$, 8.0%), 134 (M^+-CH_3-CH_3-$COOSi(CH_3)_3$, 16.5%), 73 ($[Si(CH_3)_3]^+$, 100%)

TABLE 2   Localization of azoreductase from *Lactobacillus casei* TISTR 1500.

Cell fraction	Co-factor	Specific activity (mU·mg^{-1})
Cytoplasm + membrane	NADH	0.021
	NADPH	–[a]
Cytoplasm	NADH	0.025
	NADPH	–[a]
Membrane	NADH	–[a]
	NADPH	–[a]

[a] less than 0.001

TABLE 3. The decolorization rate of azo dyes by *Lactobacillus casei* TISTR 1500.

Substrate molar extinction coefficient [a]	Chemical structure	Decolorization rate $(mg^{-1}.g\ cell^{-1}.h^{-1})$
Methyl orange $\varepsilon_{444}=9.9\times10^3$		6.1
Tropaeoline O $\varepsilon_{408}=1.2\times10^4$		5.0
Crocein orange G $\varepsilon_{463}=7.8\times10^3$		6.1
Orange II $\varepsilon_{463}=1.1\times10^4$		8.7
Acid red 151 $\varepsilon_{471}=1.5\times10^3$		0.54
Acid orange 8 $\varepsilon_{388}=3.0\times10^3$		1.1
Ponceau 3R $\varepsilon_{482}=8.6\times10^3$		8.8
Methyl red $\varepsilon_{411}=3.7\times10^3$		31.0
Congo red $\varepsilon_{465}=7.3\times10^3$		2.4

[a] The molar extinction coefficients were measured in 100 mM sodium-potassium phosphate buffer (pH 7.5).

FIGURE LEGENDS

FIG. 1. Degradation of methyl orange by resting cells of *Lactobacillus casei* TISTR 1500.

Disappearance of methyl orange (○) and appearance of *N,N*-dimethyl-*p*-phenylenediamine (□)

and 4-aminosulfonic acid (▲) in the supernatants of the reaction mixtures were measured using

HPLC.

FIG. 2. Effect of various factors on methyl orange decolorization by resting cells of

Lactobacillus casei TISTR 1500. (A) Sugars: (●) no sugar, (◆) glucose, (○) sucrose, (✳)

fructose, (◇) lactose, (✚) maltose, (□) galactose, (-) mannose, (□, dotted line) arabinose, (✕)

xylose, (△) mannitol, (▲) inositol, and (■) sorbitol. (B) Oligosaccharides: (●) no

oligosaccharide, (○) galacto-oligosaccharide, (△) fructo-oligosaccharide, and (■) inulin. (C)

Organic acids: (✕) acetic acid, (◆) citric acid, (✳) lactic acid, (●) oxalic acid, (△) succinic

acid, and (□) tartaric acid. (D) Metal ions: (●) no metal ions, (◇) calcium ions, (○) cobalt ions, (✳) copper ions, (△) ferric ions, (◆) ferrous ions, (■) magnesium ions, (✕) manganese ions, and (□) zinc ions. (E) pH: (◆) pH 3, (◇) pH 4, (●) pH 5, (○) pH 6, (▲) pH 7, (△) pH 8, (■) pH 9, and (□) pH 10. (F) Temperature: (◆) 25 °C, (○) 35 °C, (◇) 45 °C, and (●) 55 °C.
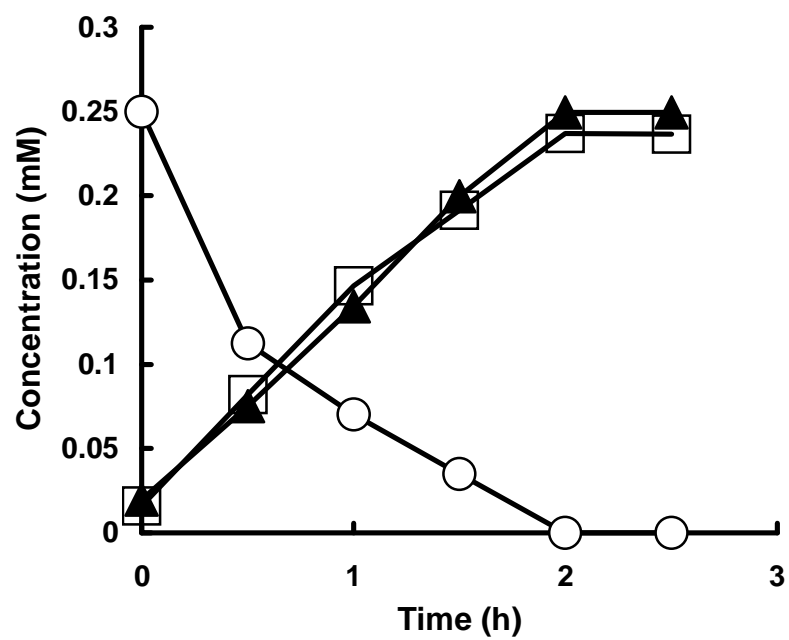
Fig. 1., Phisit *et al*.

Fig. 2., Phisit *et al*.