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Contributed Paper

Optimal Designed Formulations of A Lactic Acid Bacteria Consortium for Azo Dyes Decolorization with Sucrose As A Single Carbon Source

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ABSTRACT

This research studied the decolorization of azo dyes, methyl orange and remazol black b, by the lactic acid bacterial consortium. A process of the decolorization was separated into two stages; firstly, lactic acid bacteria (LAB) was cultivated with sucrose as a sole carbon source and then subsequently added to azo dyes decolorization under a static condition. The pure culture of each LAB strains showed high decolorized efficiency on both methyl orange and remazol black b. Then, the experimental design mixture was used to optimize the optimal proportion of mixed culture of the LAB and found the best proportion of LAB mixed culture that could decolorize azo dyes at the concentration of 100 mg/L. The azoreductase activity of the mixed culture significantly increased up to 45% which was higher than the activity by the individual strains. FTIR spectra confirmed that the lactic acid bacterial consortia had the ability on the azo dye decolorization by removing the azo group from the azo dye molecule, which was showed the absence of -N=N- stretching vibrations on methyl orange and remazol black b structures.

Keywords: decolorization, methyl orange, remazol black b, lactic acid bacterial co-culture, azo reductase

1. INTRODUCTION

Azo dyes are synthetic compounds that widely used in textile dyeing, printing, and other processes. They are characterized by an azo group consisting of two nitrogen atoms with an azo bond (-N=N-) substituted with the stabilizing, strong electron withdrawing azo group. Azo dyes can cause environmental contamination, including pollution of ground and underground water sources. The textile industry is one of the major sources of azo dye wastewater due to the large quantities of water used in dyeing processes. The processing stages and types of dyes directly affect wastewater characteristics, such as pH, dissolved oxygen, and organic and inorganic chemical composition [1]. Wastewater containing azo dyes is a serious environmental pollutant when discharged directly into the

ground and underground waters [2]. Azo dyes are toxic to human health because of the nature of the substitution groups in azo dye molecules. The effluents are strongly colored, which not only create environmental and aesthetic problems but also pose a serious potential toxic threat to ecological and human health [3, 4].

Azo dyes in wastewater can be degraded by physical or chemical methods, such as coagulation-flocculation, oxidation, absorption, and electrochemical methods. Biological methods can decolorize azo dyes, representing an environmentally-friendly and relatively low-cost treatment. A wide variety of microorganisms is capable of azo dye decolorization, including yeasts, fungi, and bacteria, particularly lactic acid bacteria (Lactobacillales), such as *Lactobacillus casei* TISTR 1500 [5], *Lactobacillus casei* LA1133, *Lactobacillus paracasei* LA0471 [6], and *Lactobacillus plantarum* No. PV71-1861 [7]. Many bacteria reduce the highly electrophilic azo bond in the dye molecule by the activity of a low-specificity cytoplasmic azoreductase, to produce colorless aromatic amines [8]. The reduction reaction in wastewater containing azo dyes requires long reaction times under aerobic and anaerobic conditions [3].

A diverse microbial population is important in wastewater treatment systems due to the combined metabolic activities of the microbial community and provide considerable advantages over the use of pure cultures in the degradation of synthetic dyes [9]. The mechanisms of degradation/decolorization and physicochemical parameters affecting azo dye removal efficiency have been reviewed by Saratale et al. [10]. The microbial community was able to decolorize and partially mineralize six azo dyes at 50 mg/L initial dye content, when an external carbon source was absented [11]. Some researchers have studied the azo dye decolorization ability using fermentation strategies, including batch, fed-batch, continuous and a sequencing batch reactor [12]. Particularly, Mozumder et al. [13] used a switched feeding system, comprising microbial fermentation in a fed-batch system, to enhance enzymatic activity and/or metabolite production. Similar feeding systems have also been used successfully in other studies. For example, the synthesis of S-adenosyl-L-methionine (SAM) by *Pichia pastoris* in a novel feeding scheme enhanced the final product concentration and specific yield of SAM. The main reason was that total ATP generation was maximized when the carbon source feeding was switched from inducible methanol to non-inducible glycerol [14]. Furthermore, a bacterial consortium was studied for azo dye decolorization using response surface methodology (RSM) based on a statistical experimental design (design of experiments, DOEs) widely used in several studies, including decolorization of methyl orange [12], reactive red 22 and reactive black 5 [15], and water-soluble azo dye congo red [16].

As aforementioned, the present work aimed to manipulate an optimal formulation of a lactic acid bacterial consortium for azo dye decolorization of mono-azo (methyl orange) and di-azo (remazol black b) bonds using a mixed design experiment under two stages of fermentation; LAB cultivation and azo dye decolorization. This process is able to separate lactic acid and its intermediates in each step. Therefore, the decolorized solution can be easily degraded using aerobic/anaerobic degradation in the medium broth and complete separation of the intermediates [5]. Furthermore, the interactions between azo dye decolorization, sucrose utilization using intracellular azoreductase activity by lactic acid bacteria were also investigated.

2. MATERIAL AND METHODS

2.1 Microorganisms and Cultivation

Five strains of the LAB with high azo dye decolorization efficiency were chosen to investigate the optimal formulation of the LAB for azo dye decolorization. Four strains were isolated from soil samples, fermented foods, and dairy food products, and subsequently identified by 16S rRNA gene sequencing and have been deposited in the Thailand Institute of Scientific and Technological Research (TISTR) culture collection as *Enterococcus faecalis* TISTR 2283, *Lactobacillus paracasei* subsp. *paracasei* TISTR 2284, *Lactobacillus casei* TISTR 2285, and *Lactobacillus paracasei* subsp. *paracasei* TISTR 2286, respectively. In addition, *L. casei* TISTR 1500 was also selected due to high efficiency on azo dye decolorization under several conditions [5, 12]. All strains were grown in modified de Man Rogosa and Sharpe (MRS) liquid medium according to Tantiwa et al. [12]. The cultures were incubated at 35°C for 12 h and then used as an inoculum seed for further experiments.

2.2 Azo Dyes Decolorization

The scheme of azo dyes decolorization with single carbon source showed in Figure 7, which explained the principle of the system using repeated-batch fermentation. In this instance, NADH was generated by two steps in the decolorization process to achieve efficient decolorization of azo dyes; first, NADH accumulated in the cell cytoplasm of LAB after incubation with 10 g/L sucrose for 6 h; second, lactic acid was reduced to acetic acid to regenerate the cofactor in the process of azo dyes decolorization.

2.2.1 Single strains of LAB decolorization

In this study, the process of bacterial azo dyes decolorization consists of two stages; LAB cultivation using sucrose as a sole carbon source and azo dye decolorization. Firstly, the inoculum seed of each strain (10% v/v) was added to 500 mL of modified MRS liquid medium and incubated at 35°C for 12 h. Then, the culture broth was centrifuged at 6,000 rpm at 25°C for 10 min. The cells pellet was washed twice with 0.85% (w/v) NaCl and resuspended with 10 g/L sucrose to give an initial OD 600 of 10 (10.85 g/L wet weight), and then incubated at 35°C for 6 h. The culture broth was then centrifuged to separate the cells and supernatant, and the cells were washed twice with 0.85% (w/v) NaCl. In the second stage, the cell pellets were added to 100 mg/L of azo dyes, and incubated with methyl orange for 12 h and remazol black b for 24 h, respectively. The culture broths were sampled every 1 h for methyl orange and 2 h for remazol black b during the incubation.

2.2.2 Formulation design and optimization

A simplex centroid design was used to determine an experimental design using Minitab Statistical 16.0 software (Minitab Inc., Pennsylvania, USA). The mixture designs consisted of 36 experiments with an experimental matrix in an initial cell proportion of 1.00, which consisted of the 5 strains LAB (Table 1). All components ranged from 0 up to 100% with different proportion conditions. The percentage (%) and rates (mg/L/h) of azo dye decolorization were used as response variables to determine optimal formulations for the lactic acid bacterial consortium. The relationships between component proportions of the different variables and response variables were described by a mixture design. A polynomial equation was employed in response surface methodology, whereas the standard forms contributed model linear, quadratic, full cubic, and special cubic equations following Equations 1-4 [17].

Linear: $Y = \sum_{i=1}^{q} \beta_i x_i$ (1)

Quadratic: $Y = \sum_{i=1}^{q} \beta_i x_i + \sum\sum_{i<j}^{q} \beta_{ij} x_i x_j$ (2)

Full cubic: $Y = \sum_{i=1}^{q} \beta_i x_i + \sum\sum_{i<j}^{q} \beta_{ij} x_i x_j + \sum\sum_{i<j}^{q} \delta_{ij} x_i x_j (x_i - x_j) + \sum\sum_{i<j<k}^{q} \beta_{ijk} x_i x_j x_k$ (3)

Special cubic: $Y = \sum_{i=1}^{q} \beta_i x_i + \sum\sum_{i<j}^{q} \beta_{ij} x_i x_j + \sum\sum_{i<j<k}^{q} \beta_{ijk} x_i x_j x_k$ (4)

The azo dye decolorization rates were used to analyze the optimal formulation of the lactic acid bacterial consortium, and the different component formulations were employed from the model.

Table 1. Experimental mixture design coded in terms of component proportions and response variables of methyl orange and remazol black b decolorization rates.

Experiments	Strains of TISTR					methyl orange	remazol black b
	2283	1500	2284	2285	2286	(mg/L/h)	(mg/L/h)
1	1.00	0.00	0.00	0.00	0.00	5.63±0.02	3.91±0.30
2	0.00	1.00	0.00	0.00	0.00	4.34±0.02	1.18±0.46
3	0.00	0.00	1.00	0.00	0.00	4.78±0.04	1.78±0.15
4	0.00	0.00	0.00	1.00	0.00	6.15±0.60	4.66±0.00
5	0.00	0.00	0.00	0.00	1.00	3.92±0.06	0.85±0.76
6	0.50	0.50	0.00	0.00	0.00	11.55±0.61	5.16±0.55
7	0.50	0.00	0.50	0.00	0.00	4.54±0.49	5.25±0.15
8	0.50	0.00	0.00	0.50	0.00	18.52±0.28	3.45±0.46
9	0.50	0.00	0.00	0.00	0.50	3.85±0.01	1.39±0.30
10	0.00	0.50	0.50	0.00	0.00	5.56±0.05	3.16±0.11
11	0.00	0.50	0.00	0.50	0.00	12.24±0.00	3.86±0.22
12	0.00	0.50	0.00	0.00	0.50	16.86±0.05	2.17±0.65
13	0.00	0.00	0.50	0.50	0.00	6.92±0.05	3.70±0.32
14	0.00	0.00	0.50	0.00	0.50	5.12±0.05	2.77±0.00
15	0.00	0.00	0.00	0.50	0.50	8.12±0.02	4.00±0.25
16	0.33	0.33	0.33	0.00	0.00	8.10±0.17	3.61±0.30
17	0.33	0.33	0.00	0.33	0.00	8.10±0.06	3.36±0.11
18	0.33	0.33	0.00	0.00	0.33	7.26±0.06	2.87±0.23
19	0.33	0.00	0.33	0.33	0.00	8.13±0.12	3.49±0.22
20	0.33	0.00	0.33	0.00	0.33	4.11±0.08	3.39±0.15
21	0.33	0.00	0.00	0.33	0.33	6.33±0.05	2.49±0.32
22	0.00	0.33	0.33	0.33	0.00	8.09±0.02	3.33±0.23
23	0.00	0.33	0.33	0.00	0.33	8.83±0.17	1.80±0.14
24	0.00	0.33	0.00	0.33	0.33	8.15±0.07	2.70±0.27
25	0.00	0.00	0.33	0.33	0.33	9.51±0.04	3.13±0.33
26	0.25	0.25	0.25	0.25	0.00	6.05±003	5.28±0.02
27	0.25	0.25	0.25	0.00	0.25	6.94±0.03	3.20±0.00
28	0.25	0.25	0.00	0.25	0.25	5.91±0.31	3.21±0.43
29	0.25	0.00	0.25	0.25	0.25	7.50±0.10	2.77±0.66

Table 1. Continued.

Experiments	Strains of TISTR					methyl orange	remazol black b
	2283	1500	2284	2285	2286	(mg/L/h)	(mg/L/h)
30	0.00	0.25	0.25	0.25	0.25	5.08±0.10	3.93±0.11
31	0.20	0.20	0.20	0.20	0.20	6.56±0.11	2.77±0.00
32	1.00	0.00	0.00	0.00	0.00	6.41±0.12	3.03±0.46
33	0.00	1.00	0.00	0.00	0.00	4.91±0.03	1.18±0.61
34	0.00	0.00	1.00	0.00	0.00	3.49±0.05	1.79±0.31
35	0.00	0.00	0.00	1.00	0.00	7.38±0.01	3.58±0.76
36	0.00	0.00	0.00	0.00	1.00	4.25±0.03	0.85±0.22

2.2.3 Decolorization of azo dyes by lactic acid co-culture using a repeated-batch system

The azo dye decolorization using a repeated-batch system with whole cell recycling was performed based on the decolorization system as aforementioned. The interval time for the repeated-batch cell cycle started with the free cell suspension, cultivated in 10 g/L sucrose for 6 h and then incubated with 100 mg/L azo dyes for 18 h. The methyl orange, remazol black b, lactic acid, acetic acid and sucrose concentrations were measured at the end of fermentation in each step. Samples were taken until the decolorization efficiency and decolorization rate remained constant.

3. ANALYTICAL METHOD

3.1 Decolorization Percentage and Rate

The UV-vis spectra of dyes were recorded from 200-800 nm using a UV-vis spectrophotometer [4]. The methyl orange concentrations were measured by a spectrophotometer (Genesys 10UV, Thermo Spectronic, Krackeler Scientific, USA) at 464 nm using the respective molar extinction coefficients of the azo dyes and all data were measured in triplicate [5]. Decolorization rate and percentage of decolorization were calculated following Equation 5 and 6, respectively.

$$\text{Decolorization rate} = \frac{C_{dye,\,t} - C_{dye,\,O}}{t_0 - t_t} \quad (5)$$

$$\text{Decolorization (\%)} = \left(1 - \frac{C_{dye,\,t}}{C_{dye,\,O}}\right) \times 100 \quad (6)$$

where $C_{dye,t}$ and $C_{dye,0}$ are the concentrations of methyl orange or remazol black b at reaction time t and 0, respectively.

3.2 Azoreductase Activity

The azoreductase activity of the LAB was investigated according to Seesuriyachan et al. [5]. The culture broth obtained after first stage of cultivation was centrifuged at 6,000 rpm, 25°C for 10 min and the cells pellet was washed twice with 0.85% (w/v) NaCl. Then, it was resuspended in 10 mL of 20 mM Tris-HCl buffer (pH 8) and sonicated using an ultrasonic bath (Sonics & Materials, USA), 3 times for 5 min each. The cells debris and supernatant were separated by centrifugation at 10,000 rpm, 4°C for 10 min and the supernatant was used to determine the intracellular azoreductase activity.

3.3 HPLC Analysis

Lactic acid, acetic acid, and sucrose concentrations were analyzed by high-performance liquid chromatography (HPLC) using a Shimadzu LC-10ATvp (Shimadzu Co. Ltd., Japan) with an RI detector and an Aminex HPX-87H column (7.8 × 1 mm, 300 mm and 9 µm particle size) from

Bio-Rad (California, USA). The samples were used directly for HPLC analysis. The mobile phase and flow rate were 5 mM H_2SO_4 in deionized water and 0.75 mL/min, respectively. Decolorized intermediates, 4-aminobenzenesulphonic acid (I) and *N,N*-dimethyl-*p*-phenylenediamine (II) were analyzed using HPLC analysis with an Inertsil ODS-3 column (4.6 × 250 mm, 5 mm; GL Science, Tokyo, Japan). The mobile phase comprised 0.1% (w/v) H_3PO_4-acetonitrile (50:50, v/v). The flow rate of the eluted sample was 0.7 mL/min at room temperature over 25 min [5].

3.4 FTIR Spectroscopy

After 10 min of decolorization process, the samples were taken and then centrifuged at 6,000 rpm at 25°C for 10 min. Then, the cell debris was discarded, but the solutions were then evaporated using a vacuum rotary evaporator at 50°C. The aromatic amine intermediates were characterized by Fourier transform infrared spectroscopy (FTIR) using a Thermo Nicolet model 6700 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The decolorized solutions were compared with control azo dyes with respect to their percent (%) transmission at different wavelengths in the mid-IR 400-4000 1/cm.

3. RESULTS

3.1 Azo Dyes Decolorization using Single Strain of Lactic Acid Bacteria

Prior to investigation of the optimal proportion of LAB co-culture for azo dyes decolorization, the free cell suspension of each strain of LAB was used to investigate the decolorization efficiency of both methyl orange and remazol black b firstly. Therefore, the percentages of azo dyes decolorization with the individual strains are shown in Figure 1. The results showed that all LAB strains can decolorized both methyl orange and remazol black b, and high efficiency of methyl orange decolorization was promoted when incubated with *L. casei* TISTR 2285 (Fig. 1A), whereas, *L. casei* TISTR 2285 and *L. casei* TISTR 1500 showed the highest efficiency of remazol black b decolorization (Figure 1B). However, the best rates of decolorization of methyl orange and remazol black b were achieved when *L. casei* TISTR 2285 was applied into the process, and the rates of decolorization were 6.15 and 4.66 mg/L/h, respectively.

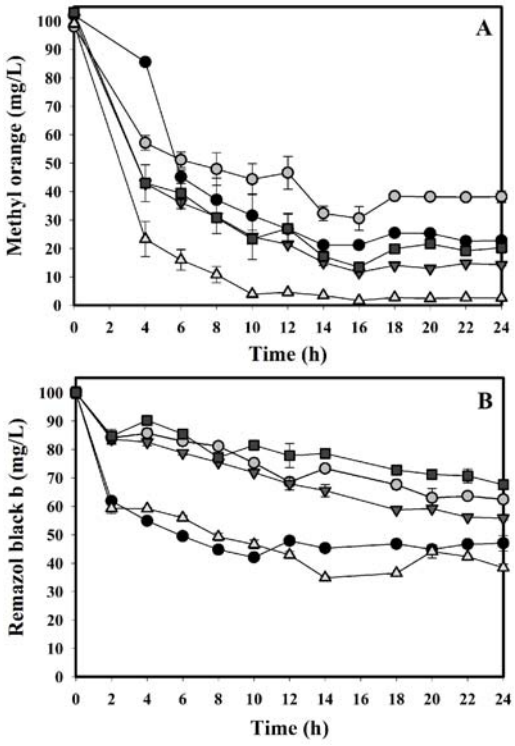

Figure 1. Methyl orange and remazol black b decolorization efficiencies by pure cultures of lactic acid bacteria operated under a switched feeding system; TISTR 2283 (●), TISTR 1500 (●), TISTR 2284 (▼), TISTR 2285 (▲) and TISTR 2286 (■).

3.2 Azo Dyes Decolorization by Lactic Acid Bacteria Consortium using Mixture Design Methodology

1. Model analysis

Mixture design coded in proportions component experimental and response variable of methyl orange decolorization rates are shown in Table 1. The decolorization rates of methyl orange were used to estimate the regression equation for ANOVA as the *p*-value, lack of fit, F-test, and multiple correlation coefficients R^2. The predicted values of the methyl orange decolorization rate matched the experimental values reasonably well, with $R^2 = 0.9497$ and *adj* $R^2 = 0.8399$, indicating that 95% and 84% of the response variability, respectively, were explained by the model. The predicted values of the remazol black b decolorization rate indicated R^2 and *adj* R^2 corresponding to 83 % and 72%, respectively, could be explained by the model. Both models suggested special cubic and quadratic models for methyl orange and remazol black b in which the regression models were significant at the 95% confidence level.

Model summary statistical data with the sequential sum of squares, sum of adjusted squares, adjusted mean of squares, *F* statistic, and *p*-value are shown in Table 2. The normal probability plots of methyl orange and remazol black b decolorization from special cubic and quadratic models are presented in Figure 2. The normal probability plots of methyl orange and remazol black b decolorization rates were linear with a 95% confidence interval. The results of the mixture surface plots also described the interaction effect of each culture formulation. Regression models for methyl orange and remazol black b decolorization rates were established. The estimated regression coefficients for methyl orange and remazol black b (mg/L/h) in terms of actual factors are shown in the special quadratic and quadratic models, respectively (Equations 7 and 8, respectively).

Table 2. ANOVA analysis of methyl orange and remazol black b decolorization rates.

Methyl Orange Decolorization Rate (mg/L/h)							
Source	DF	Seq SS	Adj SS	Ajd MS	F-ratio	P-value	
Regression	24	355.190	355.190	14.800	8.650	0.000	*Significance*
Linear	4	69.449	12.275	3.069	1.790	0.200	*Non- Significance*
Quadratic	10	155.769	263.204	26.320	15.390	0.000	*Significance*
Special quadratic	10	129.972	129.972	12.997	7.600	0.001	*Significance*
Residual Error	11	18.814	18.814	1.710			
Lack of Fit	6	10.489	10.489	1.748	1.050	0.489	*Non- Significance*
Pure Error	5	8.325	8.325	1.665			
Total	35	374.004					
S = 1.30781	PRESS = 628.021, R-Sq = 94.97, R-Sq (adj) = 83.99%						
Remazol Black B Decolorization Rate (mg/L/h)							
Source	DF	Seq SS	Adj SS	Ajd MS	F-ratio	P-value	
Regression	14	39.316	39.316	2.808	7.400	0.000	*Significance*
Linear	4	21.080	16.639	4.160	10.960	0.000	*Significance*
Quadratic	10	18.236	18.236	1.824	4.800	0.001	*Significance*
Residual Error	21	7.970	7.970	0.380			
Lack of Fit	16	6.998	6.998	0.437	2.250	0.189	*Non- Significance*
Pure Error	5	0.972	0.972	0.195			
Total	35	47.286					
S = 1.30781	PRESS = 628.021, R-Sq = 94.97, R-Sq (adj) = 83.99%						

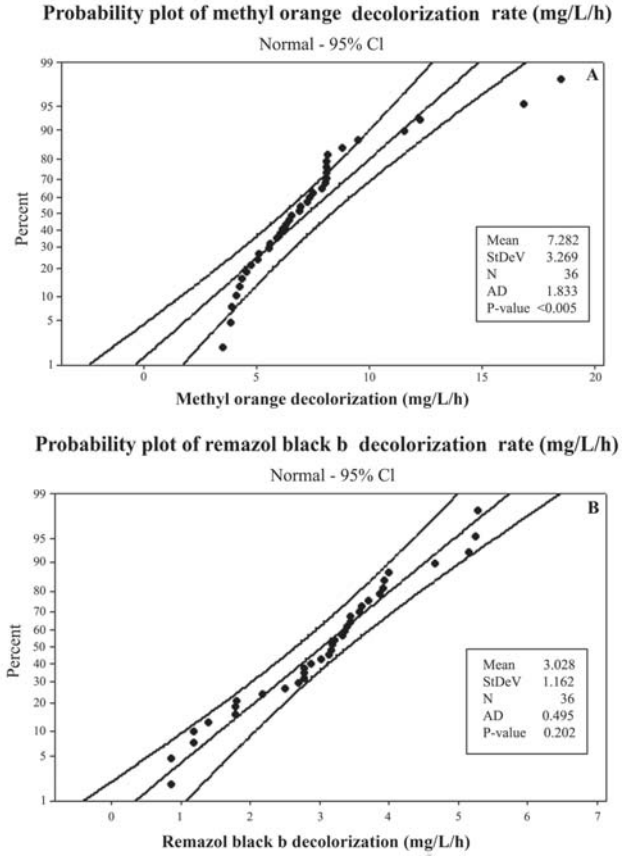

Figure 2. Normal probability plots of azo dye decolorization rate: methyl orange (a) and remazol black b (b).

$$Y_{\text{MO decolorization rate}} = 6.1X_1 + 6.1X_2 + 4.1X_3 + 6.8X_4 + 4.1X_5 + 21.0X_1X_2 - 2.9X_1X_3 + 47.3X_1X_4 - 6.3X_1X_5 + 2.4X_2X_3 + 23.5X_2X_4 + 46.9X_2X_5 + 6.4X_3X_4 + 4.1X_3X_5 + 10.5X_4X_5 + 12.4X_1X_2X_3 - 216.1X_1X_2X_4 - 115.5X_1X_2X_5 - 79.0X_1X_3X_4 + 12.5X_1X_3X_5 - 112.0X_1X_4X_5 - 55.5X_2X_3X_4 - 66.4X_2X_3X_5 - 181.7X_2X_4X_5 \quad (7)$$

$$Y_{\text{RBB Decolorization rate}} = 3.6X_1 + 1.3X_2 + 1.9X_3 + 4.2X_4 + 1.0X_5 + 8.8X_1X_2 + 8.1X_1X_3 - 3.6X_1X_4 - 3.8X_1X_5 + 4.2X_2X_3 + 3.5X_2X_4 + 2.2X_2X_5 + 1.8X_3X_4 + 3.8X_3X_5 + 3.9X_4X_5 \quad (8)$$

Equations 7 and 8 show that decolorizing LAB in terms X_1X_2 were able to decolorize methyl orange and remazol black b with coefficients of 21.0 and 8.8, respectively. The terms X_1X_4 and X_2X_4 were significant in the response variables of methyl orange decolorization. Conversely, a bacterial consortium consisting of three strains of LAB in terms $X_1X_2\ X_4$, $X_1X_2\ X_5$, $X_1X_4\ X_5$ and $X_2X_4\ X_5$ significantly decreased the decolorization efficiency of methyl orange, which was explained by Equation 7. Co-culture of defined LAB to decolorize methyl orange and remazol black b was presented in terms X_1X_4 and X_2X_4. The results show that the decolorization of azo dyes using a lactic acid bacterial consortium was faster than using pure strains of LAB.

2. Formulation design and optimization

The rates of methyl orange (Equation 7) and remazol black 5 decolorization (Equation 8) were used to analyze the optimal formulation of the lactic acid bacterial consortium. The response optimizations promoted three solutions with different component formulations as shown in Table 3. To confirm the applicability of the models, methyl orange, and remazol black b decolorization efficiencies were tested by the repeated-batch process with whole cell recycling under the optimal formulations suggested by the models. The results for the methyl orange and remazol black b decolorization rates under formulation 1 were similar to formulation 2. *L. casei* TISTR 2285 was present in both model formulations. Formulation 1 exhibited high rates of methyl orange and remazol black b decolorization, at 14.368 and 3.233 mg/L/h, respectively. Figure 3 and Figure 4 show that the profiles of decolorization efficiency, lactic acid and acetic acid production and sucrose consumption using a co-culture of LAB according to the proportions in formulations 1 and 2 were similar. Subsequently, formulations 1 and 2 were selected for use in further studies.

Table 3. Response optimizations: methyl orange and remazol black b decolorization.

Formulation	Strains of TISTR					Predicted values		Actual values	
	2283	1500	2284	2285	2286	methyl orange	remazol black b	methyl orange	remazol black b
1	0.38	-		0.62	-	15.76	3.26	14.37±0.18	3.23±0.16
2	-	0.42		0.58	-	12.23	3.80	12.46±0.85	3.82±0.62
3	-	0.38		0.30	0.32	10.06	3.07	8.80±0.64	3.48±0.22

3.3 Decolorization of Azo Dyes with the Optimal Proportion of Lactic Acid Bacterial Consortium using Repeated-batch System

The optimal formulations (formulations 1 and 2) using whole cells in co-culture were recycled to study their ability to decolorize azo dyes over the long term. The azo dye decolorization efficiency was decreased when the number of repeated-batch processes was increased. Methyl orange was completely decolorized in the first to third cycles of formulations 1 and 2 (Figure 3a and 3b), while the remazol black b decolorization efficiency was approximately below 85% after the first 4 cycles (Figure 4a and 4b). The efficiency of azo dye decolorization using formulation 1 was similar to that with formulation 2 but only in the first period. Conversely, the methyl orange decolorization decreased to 43% and 22% over 20 cycles using the proportions in formulations 1 and 2, respectively. The azo dyes decolorization efficiency profile showed that the similar trend of percentages of azo dye decolorization and lactic acid production also decreased. Conversely, acetic acid productions in the sucrose fermentation step with both formulations were stable until the end of the experiment, as shown in Figure 3e, 3f, 4e and 4f.

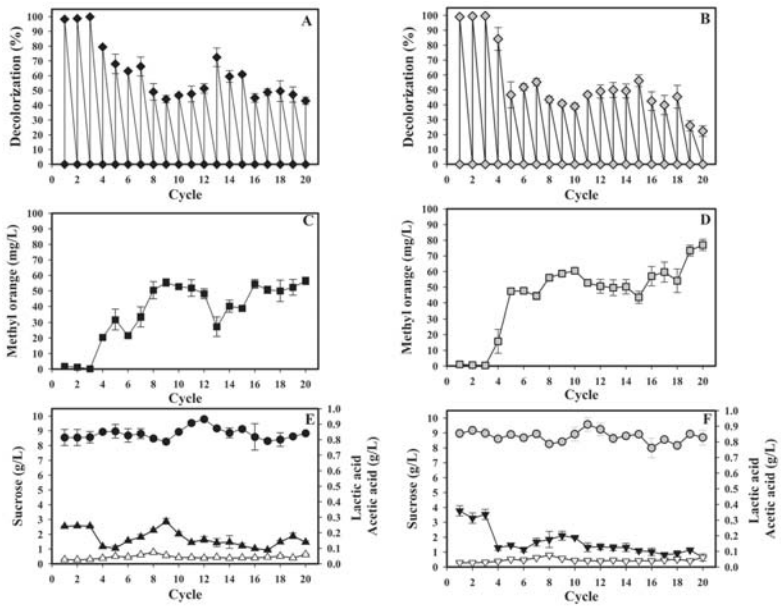

Figure 3. Methyl orange decolorization on repeated batches using the switched feeding system; Formulation 1 (a, c and e), % decolorization (◆), methyl orange (■), sucrose (●), lactic acid (▲) and acetic acid (△): Formulation 2 (b, d and f), % decolorization (◈), methyl orange (▣), sucrose (◉), lactic acid (▼) and acetic acid (▽); Figure 3e and 3f show the metabolic profile of sucrose fermentation within 6 h.

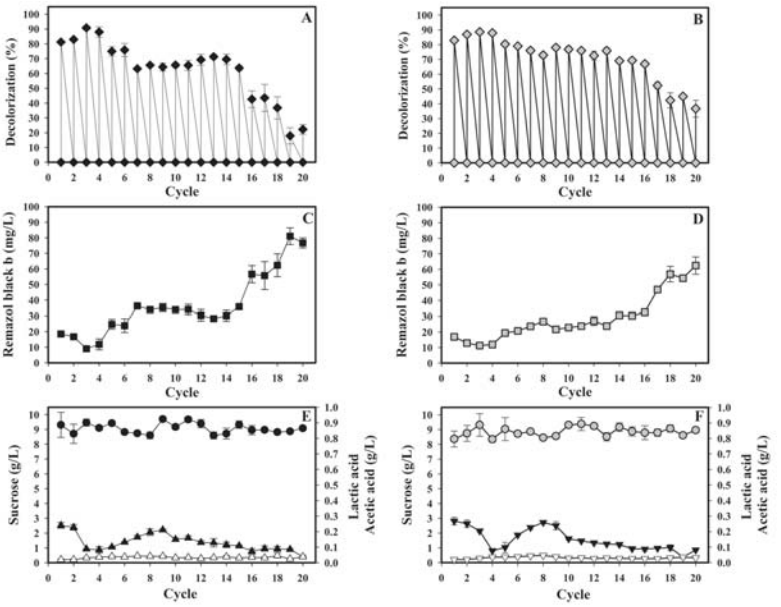

Figure 4. Remazol black b decolorization on repeated batches using the switched feeding system; Formulation 1 (a, c and e), % decolorization (◆), remazol black b (■), sucrose (●), lactic acid (▲) and acetic acid (△): Formulation 2 (b, d and f), % decolorization (◈), remazol black b (▣), sucrose (◉), lactic acid (▼) and acetic acid (▽); Figure 4e and 4f show the metabolic profile of sucrose fermentation within 6 h.

The supernatants obtained from the fermentation in 10 g/L sucrose (0-6 h) and from the methyl orange decolorization (6-18 h) were analyzed with HPLC and the results are as shown in Figure 5. At this concentration of sucrose, 0.56 g/L of lactic acid and 0.06 of g/L acetic acid were produced and secreted into cultivation broth. Whilst, in the decolorization of methyl orange during 7-18 h, the lactic acid was not present but acetic acid was increased during the incubation period.

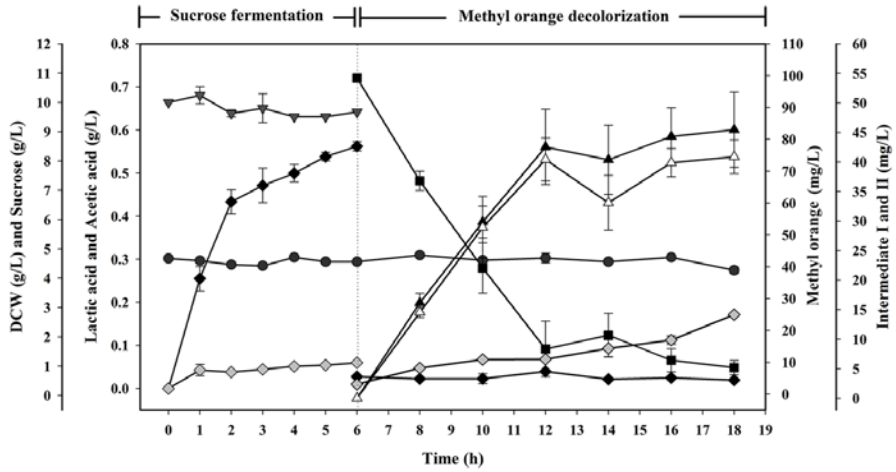

Figure 5. Metabolic and decolorization profiles of lactic acid bacteria using the switched feeding system for methyl orange decolorization; dry cell weight (●), sucrose (▼), lactic acid (◆), acetic acid (◈), methyl orange (■), intermediate I (4-aminobenzenesulphonic acid, ▲) and intermediate II (*N,N*-dimethyl-*p*-phenylenediamine, △).

3.4 FTIR Analysis

The successful azo dyes decolorization was proved through FTIR. The azo dyes, methyl orange and remazol black b, were compared via their FTIR spectra (Figure 6) with respect to their aromatic amine metabolites. The methyl orange absorption bands showed spectrum peaks (Figure 6a) that represented the stretching vibration of S=O at 1227 cm^{-1}, the stretching vibrations of asymmetric -CH_3 at 2904 and 2815 cm^{-1}, and the stretching vibration of O-H at 3453 cm^{-1}. The peak at 1598 cm^{-1}m represented the -N=N- stretching of the azo group, confirming the presence of the natural azo group in the methyl orange molecule. The remazol black b absorption bands showed spectrum peaks as illustrated in Figure 6b. The stretching vibration of the O-H band showed absorption at 3415 cm^{-1}. The stretching vibration of C-H showed an absorption at 2924 cm^{-1} while 1217 cm^{-1} represented the S=O stretching vibration. The -N=N- stretching of the azo group peaked at 1505 cm^{-1}. Peaks at 1128, 1045 and 993 cm^{-1} for asymmetric -SO_3Na stretching vibrations confirmed the presence of the functional group in the remazol black b molecule. The results of the FTIR spectra, showed the methyl orange profile was similar to remazol black b after the decolorization process; the -N=N- stretching peak disappeared, in which the -N stretching vibration and -H deformation vibrations of the primary amines were represented at 1031 and 1596 cm^{-1} for methyl orange, respectively and at 1046 and 1594 cm^{-1} for remazol black b, respectively. This demonstrated the formation of sulfonated and unsulfonated aromatic amine intermediates.

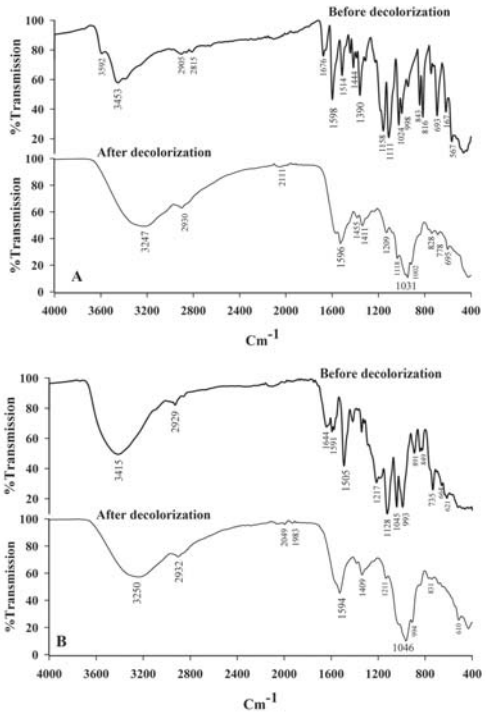

Figure 6. FTIR spectra of methyl orange (a), remazol black b (b) and solutions decolorized by lactic acid bacteria using a switched feeding system.

3.5 Enzyme Activity

The azoreductase activities were investigated after incubation with 10 g/L sucrose at 35°C for 6 h. *E. faecalis* TISTR 2283 and *L. casei* TISTR 2285 showed the highest enzyme activity and specific enzyme activity when they were compared to the individual strains (Table 4). Conversely, a co-culture of the *E. faecalis* TISTR 2283 and *L. casei* TISTR 2285 was able to enhance the specific azoreductase activity (236.41±16.81 U/g DCW).

Table 4. Azoreductase activity of lactic acid bacteria.

Strains	Enzyme activity (U/ml)	Specific enzyme activity U/g DCW
TISTR 2283	8.47 ± 0.46^{B}	180.12 ± 9.75^{B}
TISTR 1500	5.82 ± 0.66^{CD}	123.83 ± 14.06^{CD}
TISTR 2284	4.34 ± 0.40^{D}	92.31 ± 8.50^{D}
TISTR 2285	7.38 ± 0.48^{BC}	157.04 ± 10.27^{BC}
TISTR 2286	5.56 ± 0.24^{D}	118.20 ± 5.07^{D}
TISTR 2283 + TISTR 2285	11.11 ± 0.71^{A}	236.41 ± 16.81^{A}
TISTR 1500 + TISTR 2285	8.73 ± 0.79^{B}	185.75 ± 16.89^{B}

4. DISCUSSION

The selected LAB used in this study was tested for their azo dye decolorization efficiency at an initial azo dye concentration of 100 mg/L. The result showed that the individual strains presented low decolorization efficiencies and required long decolorization time under the switched feeding system. Therefore, azo dye decolorization by a lactic acid bacterial consortium was manipulated using RSM, in which the decolorization efficiencies were significantly increased by the

activity of co-cultured LAB. The co-culture of LAB in formulation 1, comprised *E. faecalis* TISTR 2283 and *L. casei* TISTR 2285 and the efficiencies of mono- and di-azo dye decolorization was higher than those of the individual strains. Thus, the co-culture community was able to enhance intracellular azoreductase activity to cleave the azo bond in the azo dye molecule. Similarly, consortium PMB11 significantly increased the enzymatic activities of lignin peroxidase, tyrosinase, 2,6-dichlorophenol-indophenol (DCIP) reductase and aminopyrine *N*-demethylase in the process of decolorizing remazol blue 59 (50 mg/L) [18].

The LAB co-culture showed higher decolorization efficiency resulting from the synergistic effect of the metabolic interactions of the individual strain [19]. For example, the consortium of three bacteria including *Paenibacillus polymyxa*, *Micrococcus luteus*, and *Micrococcus* sp. showed the metabolic activity of reactive violet 5R decolorization whereas individual strain could not show decolorization even on extended incubation [20]. This phenomenon was similar to that occurred when the extracellular metabolites of *Escherichia coli* DH5 enhanced the dye decolorization by *Pseudomonas luteola*. It was reported that *E. coli* DH5α produced extracellular metabolites as a precursor or act as a redox mediator or reducing agent to stimulate decolorization activity of *P. luteola* [21]. Therefore, the co-culture or mixed bacterial culture accelerated the decolorization efficiency of structurally different azo dyes such as methyl orange in this study, acid red 88, direct red 81, disperse orange 3 [22], and acid orange 7 [23].

Based on the previous study of *L. casei* TISTR 1500, in the form of freely suspended cells, LAB needs sucrose as an energy source to promote azoreductase activity [5, 12]. The intracellular cytoplasmic azoreductase activity requires NADH as a cofactor to cleave the azo bond in the methyl orange molecule [5]. Moreover, supplementation with a nitrogen source supplied as a yeast extract also enhanced azo dye decolorization because it worked as a co-substrate to regenerate the electron donor (NADH) in the decolorization system [24]. The decolorization metabolism of the co-cultured LAB was evident due to NADH accumulation in the lactic acid bacteria cells. Then, in the presence of the azo dye compound in the decolorization step, the accumulated NADH was able to cleave the double bond between the two nitrogen atoms and generate decolorized intermediates. However, azo dye molecule transportation into the cytoplasm of the bacterial cells is not evident because the high molecular weight of these molecules make it difficult for them to pass through the cell membrane [25].

The scheme azo dyes decolorization with single carbon source showed in Figure 7, which explained the principle of the system using repeated-batch fermentation. In this instance, NADH was generated by two steps in the decolorization process to achieve efficient decolorization of azo dyes; first, NADH accumulated in the cell cytoplasm of LAB after incubation with 10 g/L sucrose for 6 h; second, lactic acid was reduced to acetic acid to regenerate the cofactor in the process of azo dyes decolorization. The concentration of acetic acid increased during the decolorization process. The increase in acetic acid observed during azo dyes decolorization was similar to the metabolism of lactic acid in fermented cucumber by *L. buchneri* [26], whereby lactic acid was oxidized to acetic acid and 1.2-propanediol with concomitant formation of ATP and/or a cofactor. Similarly, the low lactic acid content of azo dye decolorization correlated with the acetic acid increase.

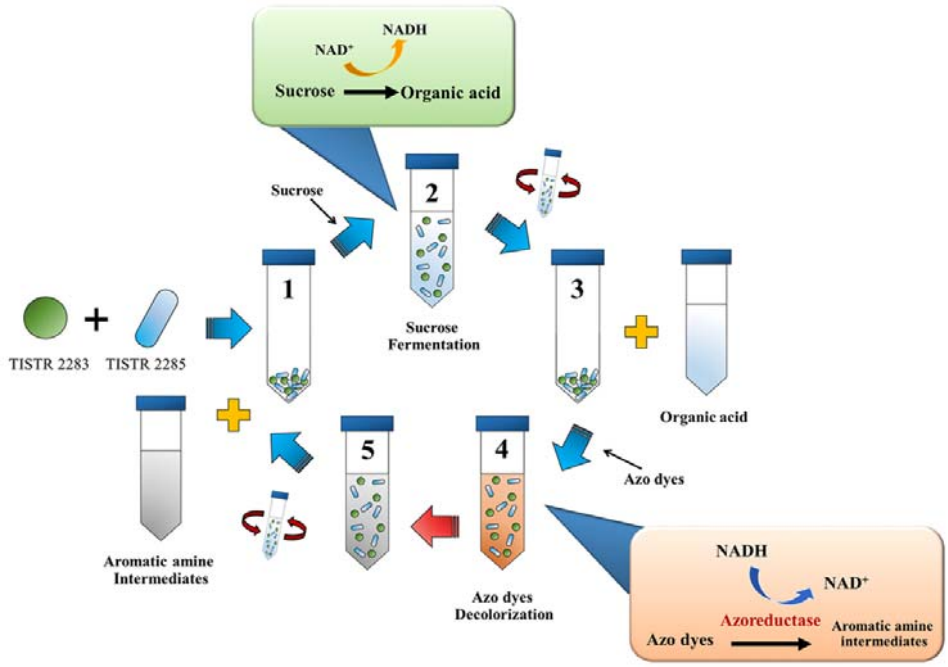

Figure 7. The scheme of azo dyes decolorization process.

To increase the azo dye decolorization efficiency, the azo dye was directly added to the culture medium consisting of a co-substrate containing carbon and nitrogen sources, which was necessary for growth and decolorization activity [27]. However, in this study azo dye decolorization process was achieved without any carbon and nitrogen sources in the decolorization step. The results showed that this process could separate the decolorization products of each step into aromatic amines and cultivation products (lactic acid and acetic acid). This decolorization may involve different mechanisms, such as enzymes, low molecular weight redox mediators and chemical reduction by biogenic reductants [3]. The azo dye decolorization by biological methods results in sulfonated and unsulfonated aromatic amines. These compounds are toxic to the aquatic environment because of their carcinogenic/mutagenic activities. Aromatic amines require treatment prior to discharge into the environment. Moreover, lactic acid has potential as a raw material for biodegradable polymers and biomedical uses [28].

5. CONCLUSION

The present work indicates a novel azo dye decolorization process by co-culture of lactic acid bacteria. The co-cultures of formulation 1 (strains TISTR 2283 and TISTR 2285) and formulation 2 (strains TISTR 1500 and TISTR 2285) completely decolorized mono- and di-azo dyes. RSM applied to a mixture design provided the best proportion of co-culture that was appropriated to promote azo dye decolorization efficiency. Thus, the lactic acid bacteria co-culture could increase sucrose utilization resulting in maximum NADH production and azoreductase activity. The decolorization process in this study will be able to completely separate a cultivation broth consisting of organic acids (lactic acid and acetic acid)

and decolorized intermediates in the form of aromatic amines, in each step.

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