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Full Paper

Nutritional requirements for methyl orange decolourisation by freely suspended cells and growing cells of *Lactobacillus casei* TISTR 1500

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Abstract: *Lactobacillus casei* TISTR 1500 possesses cytoplasmic azoreductase and can breakdown azo bonds under microaerophilic condition. It was found previously that a growing culture is more tolerant to a high initial dye concentration than freely suspended cells supplied only with sucrose. The present study is aimed at investigating the nutritive requirements for decolourisation by the growing cells and the freely suspended cells using Plackett-Burmann experimental design. In this study, the composition of the medium was found to play an important role in methyl orange decolourisation and biomass production. Sucrose, meat extract and peptone increased methyl orange decolourisation by freely suspended cells, whereas sodium acetate exerted a negative effect on decolourisation. In addition, it was observed that the yeast and meat extracts enhanced the degradation of the dye by the growing cells. Sucrose was an important factor in biomass production by freely suspended cells and growing cells. On the other hand, dipotassium hydrogen phosphate and sodium acetate decreased the biomass production. These findings promote the understanding and knowledge about the requirements of azo dye decolourisation by *Lactobacillus casei*.

Keywords: *Lactobacillus casei*, microbial decolourisation, azo dyes, methyl orange, nutritional requirements

Maejo Int. J. Sci. Technol. **2011**, *5*(01), 32-46

INTRODUCTION

Azo dyes are the most widely used synthetic colorants in comparison to natural dyes because of their many advantages, namely the ease and cost-effectiveness of synthesis, stability and availability in a variety of colours [1-2]. They are used in various industries such as pharmaceutical, food, brewing and cosmetic. However, several studies indicate that most of the azo dyes are toxic, carcinogenic and mutagenic [3-4]. Azo bonds are broken down by azoreductase to nitro-aromatic compounds [5]. Depending on the microorganisms, the biodegradation process can occur under aerobic or anaerobic conditions, or even a combination of the two. Aerobic microorganisms need to be acclimatised to produce a specific azoreductase to the dye whilst a universal azoreductase can be produced anaerobically without the process of acclimatisation [6]. In anaerobic condition, the mechanism starts with the reductive cleavage of the azo linkage and the reaction can occur in both intracellular or extracellular environments. The intracellular decolourisation requires a step of azo dye translocation from the environment into the bacterial cytoplasm [7]. However, the process of azo dye translocation across bacterial cell membrane is still under investigation. In a previous study, it was revealed that *Lactobacillus casei* TISTR 1500 requires sucrose or other types of sugars or organic acids as an energy source. The strain showed high potential of azo decolourisation by converting the dye to N, N-dimethyl-p-phenylenediamine and 4-aminobenzenesulphonic acid [5]. A few recent reports described the ability of lactic acid bacteria to degrade azo compounds. The microaerophile *Lactobacillus casei* LA 1133 degraded 35% of initial tartazine in 17 days with a growth rate at 0.052/h, and *L. paracasei* LA 0471 degraded 80% of tartazine in less than 13 days with a growth rate 0.023/h [8]. *L. acidophilus* ATCC 4356 completely reduced methyl red, orange G, Sudan III and Sudan IV while *L. fermentum* ATCC 23271 completely degraded only Sudan III and Sudan IV [9]. Also, *Oenococcus oeni* ML34 decolourised fast red up to 93% when the strain was supplied with 5 g/l of glucose [10].

Several factors such as dye structure, biomass concentration, alternative electron acceptor and redox mediator, dye concentration, and dye toxicity are known to influence the efficiency of decolourisation [11-12]. In another previous study, decolourisation by the strain TISTR 1500 of *L. casei* with a high cell density of freely suspended cells was examined to evaluate the performance of decolourisation as well as investigate the possibility of the process in azo dye treatment and factors affecting decolourisation (viz. sugars, organic acids, pH, temperature, oligosaccharides and metal ions) [7]. The nutritional requirements of the microorganisms are a key factor in biodegradation. The supplementation of wastewater contaminated with methyl red with phosphate, for example, improves decolourisation, whereas the addition of nitrate adversely affects organic reduction and decolourisation [13]. Also, yeast extract and peptone enhance decolourisation [14-15].

The strain TISTR 1500 had a high specific decolourisation rate of 14.2 mg/gCell/h and it was found that a growing culture is more tolerant to a high initial dye concentration than the freely suspended cells supplied only with sucrose [16]. Thus, in order to enhance the bacterial capacity for decolourisation, it is important to have some information related to the nutritive factors affecting the growth of the strain. The objective of the present study is to find the nutritional requirements of *Lactobacillus casei* TISTR 1500 for methyl orange decolourisation in a complex medium, particularly the MRS medium, and to create suitable culture media for growth and decolourisation.

Maejo Int. J. Sci. Technol. **2011**, *5*(01), 32-46

The Plackett-Burman experimental design was applied to screening the main components of the MRS medium to establish a supportive condition for the growing cells and the freely suspended cells during methyl orange degradation. The information thus obtained should be important for an application of the process to the treatment and biodegradation of dyestuff wastewater.

MATERIALS AND METHODS

Chemicals and Equipment

Meat extract, peptone from casein and yeast extract were purchased from Difco, Dickinson and Co. (USA). Sucrose, dipotassium hydrogen phosphate, manganese sulphate, magnesium sulphate, sodium acetate and diammonium hydrogen citrate were purchased from Fisher Scientific. Tween 80 and methyl orange (C.I. 13025) were purchased from Sigma-Aldrich.

A Sorvall centrifuge (Super T21), a Genesys 10S UV-Vis Spectrophotometer (Thermo Scientific Inc.) and a Memmert incubator were used in the experiment.

Azo-dye-degrading Microorganism

Lactobacillus casei TISTR 1500 was obtained from a culture collection (MIRCEN part unit) of Thailand Institute of Scientific and Technological Research (TISTR). Subsequently, the strain was transferred from a lyophilised tube to 10 ml of modified MRS liquid medium [17] containing 0.5 g/l of methyl orange in a 20-ml screw-capped test tube and incubated at 35°C for 24 h. The cultivation was used as inoculum for the preparation of freely suspended cells.

Screening of Factors Affecting Microbial Growth

Plackett–Burman factorial design was employed for screening the basal medium components that support the growth of the strain TISTR1500 to decolourise methyl orange at 100 mg/l. Ten components based on the MRS medium were screened. Each factor was examined at two levels: -1 for low level and +1 for high level [18-19]. Table 1 depicts the Plackett–Burman experimental design with the ten factors under investigation and the levels of each factor used in the experimental design, which are based on the following first-order polynomial model (A):

$$Y = \beta_0 \sum \beta_i \chi_i \qquad \text{(A)}$$

where Y is the response (growth of microorganisms), β_0 is the model intercept, β_i is the linear coefficient, and x_i is the level of the independent variable.

This model was used for screening and evaluating important factors that influenced the response, although it did not describe the interaction among the factors. The positive or negative magnitude of the coefficient indicates the corresponding impact on titre. A coefficient value close to zero implies a small or no effect. The P-value is the probability describing the magnitude of a contrast coefficient that results from random process variability. A low P-value indicates a “real” or significant effect. The significance of each variable is determined by applying the F-ratio. In the present study, ten assigned variables (components) were screened in the course of twelve experimental runs. The experiments on the decolourisation rate and biomass were carried out in

Maejo Int. J. Sci. Technol. **2011**, *5*(01), 32-46

triplicate. Based on the regression analysis of the variables, the confidence levels of 95% ($P<0.05$) and 90% ($P<0.1$) for each factor were considered to have a significant effect on the decolourisation and biomass production respectively.

Decolourisation of Methyl Orange and Analysis

The 12 runs of experimental media were set up as shown in Tables 1 and 2 and run under sterilised condition using aseptic technique. The culture of freely suspended cells and growing cells were prepared by the method of Seeuriyachan et al. [5, 16]. In the preparation of freely suspended cells, the strain was inoculated into 1 litre of modified MRS medium with 0.5 g/l of methyl orange in a 3-litre flask. It was then incubated at 35°C for 12 h, after which the 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 and resuspended for further experiment with an initial OD_{600} of 0.3. For growing cells, the strain was inoculated into 10 ml of modified MRS medium containing 0.5 g/l of methyl orange in a 20-ml screw-capped test tube and incubated at 35°C for 24 h. It was then used as starter for further investigation and inoculated into 800 ml of the investigated medium (12 runs) in a 1-litre Erlenmeyer flask.

All treatments were incubated in a static condition at 35°C in an anaerobic jar and samples were collected every hour. All runs were performed in triplicate. The methyl orange concentration was measured spectrophotometrically from the supernatant at 444 nm and the decolourisation rate was determined using a curve plotting of dye concentration versus time. To determine the cell dry weight of the converted biomass, a standard curve was plotted between OD_{600} and cell dry weight (CDW) [16]. All determinations were done in triplicate.

Table 1. Assigned concentrations of variables at different levels in Plackett-Burman design for decolourisation of methyl orange by *Lactobacillus casei* TISTR 1500

Variable	Medium component	Lower level (-)	Higher level (+)
X1	Meat extract (g/l)	2	20
X2	Peptone from casein (g/l)	2	20
X3	Yeast extract (g/l)	1	10
X4	Sucrose (g/l)	4	40
X5	Dipotassium hydrogen phosphate (g/l)	0.4	4
X6	Manganese sulphate (g/l)	0.01	0.1
X7	Magnesium sulphate (g/l)	0.04	0.4
X8	Sodium acetate (g/l)	1	10
X9	Diammonium hydrogen citrate (g/l)	0.4	4
X10	Tween 80 (ml)	0.2	2

Maejo Int. J. Sci. Technol. **2011**, *5*(01), 32-46

Table 2. Plackett-Burment design for 10 variables with 12 runs of experiment

Run no.	Component									
	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10
1	1	-1	1	-1	-1	-1	1	1	1	-1
2	1	1	-1	1	-1	-1	-1	1	1	1
3	-1	1	1	-1	1	-1	-1	-1	1	1
4	1	-1	1	1	-1	1	-1	-1	-1	1
5	1	1	-1	1	1	-1	1	-1	-1	-1
6	1	1	1	-1	1	1	-1	1	-1	-1
7	-1	1	1	1	-1	1	1	-1	1	-1
8	-1	-1	1	1	1	-1	1	1	-1	1
9	-1	-1	-1	1	1	1	-1	1	1	-1
10	1	-1	-1	-1	1	1	1	-1	1	1
11	-1	1	-1	-1	-1	1	1	1	-1	1
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1

RESULTS AND DISCUSSION

Nutritional Requirements for Decolourisation by Freely Suspended Cells and Growing Cells

A total of ten variables that influenced methyl orange decolourisation were analysed using the Plackett-Burman experimental design. Various medium components at different concentrations were investigated in the course of the study (Tables 1 and 2). The average decolourisation rates (observed values and predicted values) are shown in Table 3. The regression equations of the fitted model of the decolourisation and biomass production are represented in Table 4 and the predicted value of each response was generated therefrom.

To examine the fitting quality of the model, the values for each fitting method were compared. It was observed that the correlation coefficient (R^2) approaching 1 indicated a better fitting of the predicted values from the equations to the experimental values. The value of R^2 was 0.9945 for the decolourisation rate obtained using freely suspended cells, which could be interpreted as 99.45% variability in the response (Table 5). The magnitude and direction of the coefficient factor in equation (1) indicated the influence of the ten medium components on the decolourisation rate: a greater magnitude illustrated a larger effect. Variables with a confidence level greater than 95% ($P<0.05$) were considered significant.

It was found that with the response Y1 (decolourisation by freely suspended cells), four variables, namely sucrose (X4), meat extract (X1), sodium acetate (X8) and peptone (X2), had a low P-value of 0.0270, 0.0284, 0.0322 and 0.0334 respectively (Table 5). These variables thus significantly influenced the azo dye decolourisation. The estimated effects in the table indicate relative contribution of the variables on the response from the regression model. A positive value indicates that a higher setting of the variable resulted in a higher response while a negative value

Maejo Int. J. Sci. Technol. **2011**, *5*(01), 32-46

Table 3. Comparison of methyl orange decolourisation rate between observed values and predicted values generated by the linear regression models

Run no.	Decolourisation rate by freely suspended cells (g/l/h)		Decolourisation rate by growing cells (g/l/h)	
	Observed rate	Predicted rate	Observed rate	Predicted rate
1	13.6	4.34	14.11	14.24
2	12.54	12.62	9.21	9.08
3	9.84	9.76	8.66	8.79
4	13.60	13.52	10.91	11.04
5	14.90	14.82	8.72	8.85
6	8.92	9.00	16.95	16.82
7	10.1	10.18	14.48	14.35
8	7.32	7.40	5.43	5.30
9	5.25	5.17	6.61	6.74
10	8.81	8.89	7.41	7.28
11	5.32	5.24	4.76	4.89
12	4.72	4.80	1.81	1.68

Table 4. Regression equations of the fitted models of decolourisation and biomass production by *Lactobacillus casei* TISTR 1500

Response	Equation
Y1 : Decolourisation by freely suspended cells	Y1 = 8.8117 + 1.7200X1+ 1.4583X2+0.2217X3+ 1.8067X4 + 0.3617X5 - 0.145X6 - 0.3333X7 - 1.5167X8 - 0.3183X9 + 0.7600X10.....(1)
Y2 : Decolourisation by growing cells	Y2 = 9.0883 + 2.1300X1 + 1.3750X2 + 2.6668X3 + 0.1383X4 - 0.1250X5 + 1.0983X6 + 0.0633X7 + 0.4233X8 + 0.9917X9 - 1.3583X10…….(2)
Y3 : Biomass production by freely suspended cells	Y3 = 0.6075 + 0.0875X1 + 0.1025X2 + 0.1508X3 + 0.3192X4 - 0.2342X5 + 0.0758X6 - 0.0425X7 - 0.1575X8 + 0.1392X9 + 0.0692X10…….(3)
Y4 : Biomass production by growing cells	Y4 = 0.6225 + 0.0575X1 + 0.0842X2 + 0.0625X3 + 0.2625X4 - 0.1742X5 - 0.0058X6 - 0.0125X7 - 0.1642X8 + 0.0692X9 + 0.0208X10…….(4)

Maejo Int. J. Sci. Technol. **2011**, *5*(01), 32-46

indicates the reverse effect (a lower setting resulting in a higher response). Sucrose had the highest estimated effect of 3.6133 on the decolourisation by the freely suspended cells of *L. casei* TISTR 1500. This implies that sucrose was an important factor in enhancing the decolourisation at a minimum concentration of 4 g/l. It thus follows that a deficiency of this component or using a lower sucrose concentration than this level could slow down the decolourisation. Sodium acetate, on the other hand, had a negative effect on the decolourisation. Addition of ammonium citrate or sodium acetate in the MRS medium is to inhibit other types of bacteria and fungal flora while favouring the growth of *Lactobacilli* [20]. For example, sodium acetate can stimulate the growth of *Lactobacillus salivarius* CRL 1328 and its bacteriocin production [21]. It also induces the production of lactic acid in *Lactobacillus sakei* NRIC 1071 and *Lactobacillus plantarum* NRIC 1067 [22].

Table 5. Estimated effect, linear regression coefficient of model, and the corresponding analysis of variance of F-ratio and P values for methyl orange decolourisation by freely suspended cells of *Lactobacillus casei* TISTR 1500 for the ten variables by Plackett-Burman experimental design at a confidence level of 95%

Component	Estimated effect	Coefficient	Standard error	Sum of squares	Df	Mean square	F-ratio	P- value
Intercept	8.8117	8.8117	0.0767					
X1	3.4400	1.7200	0.1533	35.5008	1	35.5008	503.3200	**0.0284**
X2	2.9167	1.4583	0.1533	25.5208	1	25.5208	361.8300	**0.0334**
X3	0.4433	0.2217	0.1533	0.5896	1	0.5896	8.3600	0.2120
X4	3.6133	1.8067	0.1533	39.1685	1	39.1685	555.3200	**0.0270**
X5	0.7233	0.3617	0.1533	1.5696	1	1.5696	22.2500	0.1330
X6	-0.2900	-0.1450	0.1533	0.2523	1	0.2523	3.5800	0.3096
X7	-0.6667	-0.3333	0.1533	1.3333	1	1.3333	18.9000	0.1439
X8	-3.0333	-1.5167	0.1533	27.6033	1	27.6033	391.3500	**0.0322**
X9	-0.6367	-0.3183	0.1533	1.2160	1	1.2160	17.2400	0.1505
X10	1.5200	0.7600	0.1533	6.9312	1	6.9312	98.2700	0.0640
Total error				0.0705	1	0.0705		
Total				139.7560	11			

Note: $R^2 = 0.9945$
Adjusted $R^2 = 0.9944$
Standard Error of Estimation = 0.2656
Mean absolute error = 0.0767

As indicated in Table 6, factors with P-value less than 0.05 were considered to have a significant effect on the response Y2 (decolourisation by growing cells). Only two variables [yeast extract (X3) and meat extract (X1)] with positive effects were selected as a source of nitrogen for the bacterial growth and decolourisation at 96.98% with a P-value of 0.0302, and at 96.22% with a P-value of 0.0378, with confidence levels at 1 and 2 g/l concentration respectively. It was evident

Maejo Int. J. Sci. Technol. **2011**, *5*(01), 32-46

that yeast extract exercised the highest influence upon decolourisation by the growing cells with the highest estimated effect and regression coefficient of 5.3367 and 2.6683 respectively. Other factors proved to be statistically insignificant at a confidence level of 95%.

Table 6. Estimated effect, linear regression coefficient of model, and the corresponding analysis of variance of F-ratio and P values for methyl orange decolourisation by growing cells of *Lactobacillus casei* TISTR 1500 for the ten variables by Plackett-Burman experimental design at a confidence level of 95%

Component	Estimated effect	Coefficient	Standard error	Sum of squares	Df	Mean square	F-ratio	P-value
Intercept	9.0883	9.0883	0.1267					
X1	4.2600	2.1300	0.2533	54.4428	1	54.4428	282.77	**0.0378**
X2	2.7500	1.3750	0.2533	22.6875	1	22.6875	117.84	0.0585
X3	5.3367	2.6683	0.2533	85.4400	1	85.4400	443.77	**0.0302**
X4	0.2767	0.1383	0.2533	0.2296	1	0.2296	1.19	0.4720
X5	-0.2500	-0.1250	0.2533	0.1875	1	0.1875	0.97	0.5042
X6	2.1967	1.0983	0.2533	14.4760	1	14.4760	75.19	0.0731
X7	0.1267	0.0633	0.2533	0.0481	1	0.0481	0.25	0.7048
X8	0.8467	0.4233	0.2533	2.1505	1	2.1505	11.17	0.1851
X9	1.9833	0.9917	0.2533	11.8008	1	11.8008	61.29	0.0809
X10	-2.7167	-1.3583	0.2533	22.1408	1	22.1408	115.00	0.0592
Total error				0.1925	1	0.1925		
Total				213.7960	11			

Note: $R^2 = 0.9991$
Adjusted $R^2 = 0.9901$
Standard error of estimation = 0.4388
Mean absolute error = 0.1267

The results obtained indicated the difference in nutritional requirements between the freely suspended cells and the growing cells during process of decolourisation. The freely suspended cells of the strain TISTR 1500 required considerably more medium components than the growing cells. The strain required sucrose, meat extract and peptone to increase its decolourisation capacity. The growing cells could also increase the azo-dye decolourisation if adequate yeast extract and meat extract were supplied.

Nutritional Requirements for Biomass Production by Freely Suspended Cells and Growing Cells in the Presence of Methyl Orange

The main factors affecting biomass production were investigated by means of Plackett-Burmann experimental design using two types of cells of the strain TISTR 1500, namely the freely suspended cells at $OD_{600} = 0.3$ and the growing cells. The observed and predicted responses of the

Maejo Int. J. Sci. Technol. **2011**, *5*(01), 32-46

Table 7. Comparison of biomass production during the process of decolourisation between freely suspended cells and growing cells at several media runs generated by Plackett-Burmann experimental design

Run no.	Biomass production by freely suspended cells (g/l)		Biomass production by growing cells (g/l)	
	Observed rate	Predicted rate	Observed rate	Predicted rate
1	0.42	0.45	0.43	0.45
2	1.25	1.22	1.10	1.08
3	0.52	0.55	0.53	0.55
4	1.47	1.50	1.20	1.22
5	0.53	0.56	0.84	0.86
6	0.18	0.15	0.16	0.14
7	1.62	1.59	1.36	1.34
8	0.34	0.31	0.43	0.41
9	0.35	0.38	0.38	0.40
10	0.32	0.29	0.35	0.33
11	0.16	0.19	0.25	0.27
12	0.13	0.10	0.44	0.42

12 different runs (Table 2) are given in Table 7. It can be seen that the observed biomass production by the freely suspended cells varied between 0.13-1.62 g/l, whereas the predicted values ranged between 0.1-1.59 g/l. The first-order model was generated using the experimental data. The screening of the MRS medium components was represented via the F-ratio for ANOVA. The estimated effect of the component variables on biomass production, the values of coefficients, the F-ratio and the P-value of each component from the response Y3 (biomass production by freely suspended cells) are represented in Table 8. From the design analysis of the regression coefficient, it was found that only sucrose showed a positive-effect value with an estimated effect of 0.6383, a coefficient of the regression model of 0.3192 and a P-value of 0.0646 on biomass production in the presence of methyl orange in the mixture. On the other hand, dipotassium hydrogen phosphate (X5) showed a negative effect with an estimated effect of -0.4683, a coefficient of the regression model of -0.2342, and a P-value of 0.0878 on the same. These components were screened based on their F-ratio and P-value at a confidence level of 90% ($P<0.1$).

As indicated in Table 9, three variables, namely sucrose (X4), dipotassium hydrogen phosphate (X5) and sodium acetate (X8) were the main factors influencing decolourisation by the growing cells of *L. casei* TISTR 1500. With the response Y4 (biomass production by growing cells; Table 4), sucrose enhanced the biomass production at a confidence level of 95% during the azo-dye decolourisation with an estimated effect of 0.5250, a coefficient of the regression model of 0.2625, and a P-value of 0.0424. In contrast, dipotassium hydrogen phosphate and sodium acetate showed a

Maejo Int. J. Sci. Technol. **2011**, *5*(01), 32-46

Table 8. Estimated effect, linear regression coefficient of model, and the corresponding analysis of variance of F-ratio and P value for biomass production by freely suspended cells of *Lactobacillus casei* TISTR 1500 for the ten variables by Plackett-Burman experimental design at a confidence level of 90%

Component	Estimated effect	Coefficient	Standard error	Sum of squares	Df	Mean square	F-ratio	P- value
Intercept	0.6075	0.6075	0.0325					
X1	0.1750	0.0875	0.0650	0.0919	1	0.0919	7.25	0.2264
X2	0.2050	0.1025	0.0650	0.1261	1	0.1261	9.95	0.1955
X3	0.3017	0.1508	0.0650	0.2730	1	0.2730	21.54	0.1351
X4	0.6383	0.3192	0.0650	1.2224	1	1.2224	96.44	**0.0646**
X5	-0.4683	-0.2342	0.0650	0.6580	1	0.6580	51.91	**0.0878**
X6	0.1517	0.0758	0.0650	0.0690	1	0.0690	5.44	0.2578
X7	-0.0850	-0.0425	0.0650	0.0217	1	0.0217	1.71	0.4156
X8	-0.3150	-0.1575	0.0650	0.2977	1	0.2977	23.49	0.1295
X9	0.2783	0.1392	0.0650	0.2324	1	0.2324	18.34	0.1461
X10	0.1383	0.0692	0.0650	0.0574	1	0.0574	4.53	0.2796
Total error				0.0127	1	0.0127		
Total				3.0622	11			

Note: $R^2 = 0.9959$
Adjusted $R^2 = 0.9545$
Standard error of estimation = 0.1126
Mean absolute error = 0.0325

negative effect at a confidence level of 90% in biomass production with estimated effects of -0.3483 and -0.3283, coefficients of the regression model of -0.1742 and -0.1642, and P-values of 0.0638 and 0.0676 respectively. These results suggested that high concentrations of dipotassium hydrogen phosphate and sodium acetate at 4 g/l and 10 g/l decreased the biomass production, which was reflected by the decrease in decolourisation.

Lactic acid bacteria are fastidious microorganisms that require complex nutrients as they lack the ability to synthesise amino acids and vitamins. Thus, it is necessary to add complex nitrogen sources such as meat extract, peptone and yeast extract to their medium components. An interesting fact from this study is that sucrose is mainly required in the mixture with the freely suspended cells in order to increase the azo-dye decolourisation while meat and yeast extracts are the major stimulators of the decolourisation by the growing cells. In the case of *L. amylophilus* GV6, the strain does not require a high carbon source at 20 g/l of corn steep liquor for its growth and activity [23]. However, *Lactobacillus* sp. KCP01 requires a high carbon concentration at 25 g/l of reducing sugar for increasing the bacterial activity. In addition, all organic nitrogen sources (peptone, beef extract and yeast extract), dipotassium hydrogen phosphate and sodium acetate are positive factors for the strain [24-25]. Glucose is the main source of carbon for the growth of *Lactobacillus* sp. SK007 [26]. Under an acidic condition, glucose as a metabolisable carbohydrate like sucrose possesses

Maejo Int. J. Sci. Technol. **2011**, *5*(01), 32-46

Table 9. Estimated effect, linear regression coefficient of model, and the corresponding analysis of variance of F-ratio and P values for biomass production by growing cells of *Lactobacillus casei* TISTR 1500 for the ten variables by Plackett-Burman experimental design at a confidence level of 90%

Component	Estimated effect	Coefficient	Standard error	Sum of squares	Df	Mean square	F-ratio	P- value
Intercept	0.6225	0.6225	0.0175					
X1	0.1150	0.0575	0.035	0.0397	1	0.0397	10.80	0.1881
X2	0.1683	0.0842	0.035	0.0850	1	0.0850	23.13	0.1305
X3	0.1250	0.0625	0.035	0.0469	1	0.0469	12.76	0.1738
X4	0.5250	0.2625	0.035	0.8269	1	0.8269	225.00	**0.0424**
X5	-0.3483	-0.1742	0.035	0.3640	1	0.3640	99.05	**0.0638**
X6	-0.0117	-0.0058	0.035	0.0004	1	0.0004	0.11	0.7952
X7	-0.0250	-0.0125	0.035	0.0019	1	0.0019	0.51	0.6051
X8	-0.3283	-0.1642	0.035	0.3234	1	0.3234	88.00	**0.0676**
X9	0.1383	0.0692	0.035	0.0574	1	0.0574	15.62	0.1578
X10	0.0417	0.0208	0.035	0.0052	1	0.0052	1.42	0.4448
Total error				0.0037	1	0.0037		
Total				1.7544	11			

Note: $R^2 = 0.9979$
Adjusted $R^2 = 0.9769$
Standard error of estimation = 0.0606
Mean absolute error = 0.0175

protective effects as bacteria demand high energy for maintaining pH homeostasis [27-28].

Peptone is a rich source of amino acids and partially involves pH homeostasis mechanism. Increasing the peptone concentration in the medium can enhance the buffering capacity and bacterial survival [29]. In this case of *Lactobacillus casei* TISTR 1500, the increasing rate of azo dye degradation might have caused the buffering effect when the medium contained high peptone concentration. Both peptone and yeast extract have a positive effect on the growth of *L. fermentum* [30] and *L. lactis* subsp. *lactis* [31]. As yeast extract is a rich source of amino acids and vitamins, this could account for its positive influence on the bacterial growth [25, 32]. Attempts have been made to replace yeast extract in stimulating the bacterial growth and lactic acid production by various nitrogen sources. However, none of these sources are comparable to yeast extract, nor do they yield bacterial productivity as high as yeast extract [33].

In our previous study [5, 16], pH was seen as a limiting factor of dye decolourisation for the strain TISTR 1500. Besides, meat extract and peptone were observed to play an important role in controlling the pH [34]. For the strain TISTR 15000, the mechanism of methyl orange decolourisation starts with azo dye translocation across the bacterial membrane [16]. A low pH with a high concentration of lactic acid can lead to the disruption of some metabolic pathways. Thus, a high buffering capacity of the mixture can increase the decolourisation rate as demonstrated in the

Maejo Int. J. Sci. Technol. **2011**, *5*(01), 32-46

present study, the results of which have also shown the unconventional findings on the difference in requirements between the freely suspended cells and the growing cells. These finding suggest that during decolourisation the freely suspended cells require a high buffer capacity or pH regulation in comparison to the growing cells.

The results obtained in the present study for the strain TISTR 1500, with sucrose being the only main positive factor and the inorganic phosphate and sodium acetate exerting a negative influence on biomass production in the presence of an azo dye, apparently differ from other studies. It was observed that lactose and peptone have a positive influence on biomass production [35]. Bevilacqua et al. demonstrated an increasing trend in the biomass production when the carbon source increased up to 20 g/l in a nonlinear way at pH 6, but the interaction effect between carbon source and pH on biomass production was small [36].

As mentioned in our previous study [16], the strain TISTR 1500 possesses cytoplasmic azoreductase and the azo dye has to be imported through the bacterial membrane in the first step of the dye degradation. The study of Schär-zammaretti et al. [37] suggests that the morphology and structure of the bacterial cell wall changes depending on the composition of the medium. Both peptone and yeast extract have the major influences on the physicochemical properties of the cell wall, particularly the membrane-bound proteins. They may cause a change in the hydrophobicity of the cell wall. In MRS medium, the cell wall has a low hydrophobicity in the absence of carbohydrates. However, in the absence of peptone and yeast extract in the MRS medium, the hydrophobicity of the bacterial cell wall becomes high. Similarly, the electrical charge on the bacterial cell wall surface correlates with its N/C ratio [37]. A change in the cell wall structure and its physicochemical properties can thus affect the rate of azo dye translocation across the cell membrane. The current findings of this study should be useful for improving the culture media for the strain TISTR 1500 in order to rejuvenate the microbial cells when the strain is applied in a system of wastewater treatment.

CONCLUSIONS

The composition of the fermentation medium has been observed to be a major factor affecting the methyl orange decolourisation capacity and biomass production of the strain TISTR 1500 of *Lactobacillus casei*. A difference in the nutritional requirements of the freely suspended cells in comparison to the growing cells has also been demonstrated. Sucrose, meat extract and peptone increased methyl orange decolourisation by the freely suspended cells while sodium acetate had a negative effect on the decolourisation. Both yeast extract and meat extract enhanced the degradation of the azo dye by the growing cells. Sucrose was found to be important for the biomass production by freely suspended cells and growing cells in the presence of 100 mg/l of methyl orange. On the other hand, dipotassium hydrogen phosphate and sodium acetate decreased biomass production. These findings should promote an understanding of the requirements of azo dye decolourisation by *Lactobacillus casei*.

Maejo Int. J. Sci. Technol. **2011**, *5*(01), 32-46

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