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Optimum Concentration of Glucose and Orange II for Growth and Decolorization of Orange II by *Enterococcus faecalis* ID6017 under Static Culture

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Growth and decolorization performance of bacterial grown on azodyes-containing-medium is influenced by various concentrations of carbon sources and azodyes. The optimum level of glucose and Orange II concentration for growth and Orange II decolorization by *Enterococcus faecalis* ID6017 are reported in this paper. The experiments were carried out in liquid static culture as batch experiments. Glucose and Orange II concentrations used in these experiments were 0.45, 0.90, 1.80 g l^{-1}, and 40, 80, 120 mg l^{-1}, respectively. The specific growth rate and decolorization rate of Orange II by *E. faecalis* were highest on the medium which contained at least 0.90 g l^{-1} glucose. It is necessary to note that glucose above 0.90 g l^{-1} gave no significant difference. On the medium containing 0.90 g l^{-1} glucose and 80 mg l^{-1} Orange II, *E. faecalis* grew with the highest specific growth rate (0.28 h^{-1}) and Orange II decolorization rate (0.47 h^{-1}). The maximum specific growth rate of biomass (μ_{max}) and the half-saturation coefficient (K_S) under optimal conditions were 0.25 h^{-1} and 1.5 g.l^{-1}, respectively. The kinetics of decolorization indicated that the process followed first order kinetics with respect to the initial concentration of Orange II. The inhibition constant (K_I) was found to be 750 mg l^{-1} Orange II, indicating that Orange II concentration at e" 750 mg l^{-1} would inhibit bacterial growth to decolorize Orange II.

Key words: *Enterococcus faecalis*, azodyes, decolorization, glucose, *Orange II*

Orange II is one of synthetic azodyes which has been widely used for coloring of textiles, food, and cosmetics. Orange II (Acid-Orange-7 or *p*-(2-hydroxy-1-naphthylazo) benzenesulfonic acid sodium salt) has the molec(ular structure $C_{16}H_{11}N_2NaO_4S.5H_20$ (Fig 1: Merck Index 1968). This azodye has been used as a model substrate for azodye degradation. Orange II can be degraded by *Sphingomonas* sp. 1CX and *E. faecalis* ID6017 into two main intermediate products i.e. sulphanilic acid and 1-amino-2-naphthol (Coughlin *et al.* 1999; Meitiniarti *et al*. 2007). Both of them may be mineralized further.

Azodyes are relatively resistant to microbial degradation under conditions normally found in waste-water treatment plants. However, several microorganisms are reported to be able to transform azodyes into non-colored products or even to mineralized products (Stolz 2001). Recently, a number of studies have been focusing on microbial degradation of azodyes (Kim *et al.* 1995). This is because physicochemical methods used for color removal of the effluents show disadvantages in terms of operational problems, high cost and sludge production (Kapdan *et al.* 2000; Kodam *et al.* 2005). According to Rafii *et al*. (1990), human intestinal microbiota generally found in the effluent of waste water treatment and using of these bacteria for azodyes degradation has been reported. In industrial waste-water containing various dyes, azodye degraders should exhibit decolorizing ability for a wide range of dyes and environmental conditions (Fang *et al*. 2005). In regard to this *E. faecalis* is a bacterial species which has been reported to decolorize azodyes (Handayani *et al*. 2007). Therefore the decolorization of azodyes is an important topic for study.

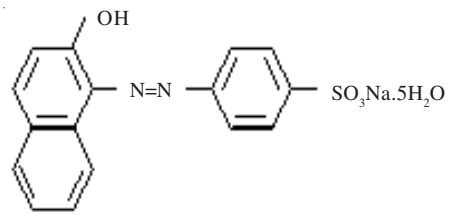

Fig 1 Chemical structrure of Orange II.

Bacterial azodyes degradation is usually initiated by cleavage of the azo bond under anaerobic conditions resulting in the formation of aromatic amine colorless products. Hence the process is called decolorization (Zimmermann *et al.* 1982; Tan 2001; van der Zee 2002).

The performance of microbial decolorization rates depends on the activity of the microorganism through a specific enzymatic process and is affected by environmental conditions, such as substrate concentration, composition of the medium, pH, carbon and nitrogen sources, and incubation temperature. *Sphingomonas* sp. strain 1CX has an ability to decolorize 20 mg l^{-1} Orange II, Acid Orange 8, Acid Orange 10, Acid Red 4, or Acid Red (Coughlin *et al*. 1997), where as *E. faecalis* ID6017 has an ability to decolorize Acid Red 27 and Reactive Red 2 up to concentration of 100 mg l^{-1} in batch system (Handayani *et al.* 2007) and *Orange II* up to concentration 120 mg l^{-1} in fedbacth system (Meitiniarti *et al.* 2006). Meitiniarti *et al.* (2005) reported that *E. faecalis* produces an Orange II reductase. Several environmental conditions, such as substrate and co-substrate concentration, could influence the enzymes activity (Tan 2001). In this paper, we discuss the optimum level of glucose as co-substrate and Orange II concentrations on the growth kinetics of *E. faecalis* and Orange II decolorization performance.

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MATERIALS AND METHODS

Microorganism and Culture Condition. *E. faecalis* ID6017 was used as a bacterial model for the decolorization of Orange II and was maintained on a slant media of Trypticase Soy Agar (TSA) at room temperature (26-28°C) as a stock culture. *E. faecalis* was grown on liquid-basal-medium consisting of (g l^{-1}): 0.25 $MgSO_4.7H_2O$, 1.98 $(NH_4)_2SO_4$, 5.55 K_2HPO_4, 2.13 KH_2PO_4, and 0.25 yeast extract. Glucose was added in concentrations of 0.45, 0.90, and 1.80 g l^{-1} Orange II (Merck, CI Acid Orange 7, CI 15510, MW= 440.41) was added in concentrations of 0, 40, 80, and 120 mg l^{-1}.

Inoculum Preparation. 48-h-old TSA slant cultures of *E. faecalis* were grown on 500 ml Erlenmeyer flask, containing basal medium without Orange II, for 24 h on a shaker (150 rpm). This culture was the pre-culture (inoculum).

Experimental Design and Culture Conditions. The experiments were carried out in two batchs using liquid medium for 9 h at room temperature. In the first experiment, three flasks each containing 80 mg l^{-1} Orange II medium were prepared and each flask was added 0.45, 0.90, and 1.80 g l^{-1} glucose. This experiment was done to determine the glucose concentration that increased bacterial growth and *Orange II* decolorization rate. The glucose concentration which resulted in increasing growth and decolorization rate, was selected and used as the limited-substrate-concentration. In the second group of experiments, the selected glucose concentration was then supplemented with 40, 80, and 120 mg l^{-1} Orange II concentrations. Experiments was carried out in triplicate.

As much as 50 ml aliquots of pre-culture were transfered to 450 ml medium in a 1 l volume growing vessels (Duran) with a rubber stopper, and incubated under static conditions at room temperature. At interval of one hour, 10 ml aliquots of each culture was removed up the late exponential growth-phase. Parameters examined were concentrations of glucose, Orange II, cell mass (biomass) and sulphanilic acid (as a decolorization product of Orange II).

Analytical Methods. Samples were centrifused at 3 326g for 30 min to separate biomass from the supernatant. The supernatant was analyzed for glucose, Orange II, and sulphanilic acid concentrations**.** After being washed twice, the biomass was re-suspended into an initial volume (10 ml) using distilled water and examined spectrophotometrically ($ë_{600nm}$). The biomass concentration in units of cell dry weight was calculated using a standard curve of optical density (OD_{600nm}; y-axis) against biomass concentration (cell dry weight) (mg l^{-1}; x-axis).

Glucose concentrations were determined spectrophotometrically ($ë_{540nm}$) using the DNS reagent method (James 1995) and Orange II concentration were determined spectrophotometrically ($ë_{482nm}$) (Zimmermann *et al.* 1982). The sulphanilic acid concentration was analyzed by injecting five to ten µl sampels of supernatant into a HPLC Shimadzu model LC-3A chromatograph, equipped with UV-detector and ODS column (150 mm x 8 mm). The mobile phase was composed of methanol and acetic acid 0.6% (v/v) = 60:40. The flow rate was 0.7 ml min^{-1}. The eluent were monitored using a UV detector at 276 nm (Chang *et al.* 2001; Supaka *et al.* 2004).

Determination of Kinetic Parameters *(k, µ, μ_{max}, K_S,* and *K_I).* The ability of bacterial strains to decolorize *Orange II* was determined by calculating the decolorization rate used equation (1) (Wuhrmann *et al*. 1980):

$$k = \frac{ln\,(C_0\text{-}C)}{(t\text{-}t_0)}$$

Note: k is decolorization rate (decrease of color intensity); C_0 and C are color concentrations (mg l^{-1}) at t_0 and t; t_0 and t are time (h).

The substrate/co-substrate consumption and decreasing of decolorization product (sulphanilic acid) was calculated using equation (2):

$$S = S_0\text{-}S_t \quad \text{or} \quad S = S_t\text{-}S_e$$

Note: S is substrate/co-substrate consumption (mg); S_0 is glucose/Orange II concentration (mg l^{-1}) at t_0; S_t is the lowest glucose/Orange II concentration or the highest sulphanilic acid concentration (mg l^{-1}); S_e is the lowest sulphanilic acid concentration (mg l^{-1}); t_0, t, and t_e are time (h).

The specific growth rate (μ) is determined from the cell mass as a first order with the following equation:

$$\frac{dX}{dt} = \mu X, \quad X = X_0 \quad \text{at t=0}$$

Integration of equation 3 yields:

$$\ln \frac{X}{X_0} = \mu t, \quad \text{or} \quad X = X_0 e^{\mu t}$$

$$\frac{1}{\mu_{max}}$$

where X and X_0 are biomass concentrations at time t and t=0.

Generally, the growth rate is restricted by the concentration of the growth-limiting substrate that can be described by the Monod Equation:

$$\mu = \frac{\mu_{max}.S}{K_S + S}$$

where μ_{max} is the maximum specific growth rate when $S>>K_S$. The constant K_S is the saturation constant or half-velocity constant and is equal to the concentration of the rate-limiting substrate when the specific rate of growth is equal to 50% of the maximum.

In this paper, the Monod equation modified by the Hanes model (called the Hanes-Monod Model) was used to analyze the values of μ_{max} and K_S (Brandt 2001):

$$\frac{S}{\mu} = \frac{S}{\mu_{max}} + \frac{K_S}{\mu_{max}}$$

A plot of against is linear with a slope of 1/u and an

intercept on the y-axis of .

When a substrate is inhibited by its own biodegradation, like Orange II, the Monod model was derivatived by incorporating the inhibition constant K_I. Among the substrate inhibition models, the Andrew's equation (7) is most widely used (Okpokwasili and Nweke 2005):

$$\mu = \mu_{max} \cdot \frac{S}{K_S + S + \frac{S^2}{K_I}}$$

where K_I is the inhibition constant (calculated by Microsoft Excel Software in this paper).

RESULTS

Optimal Level of Glucose. The addition of various concentration of glucose (0.45 to 1.80 g l^{-1}) did not give any significant difference in the specific growth rate, biomass production of *E. faecalis* or the concentration of sulphanilic acid (Table 1 and Fig 2). However, as shown in Table 1, the decolorization of Orange II was significantly influenced by the addition of glucose. By calculating glucose consumption in three initial glucose concentrations, we showed that by increasing the glucose concentration, from 0.90 to 1.80 g l^{-1}, this did not efficiently increased the specific growth rate, biomass production, and Orange II reduction by *E. faecalis*. Thus, in the second experiment, we used optimal glucose concentration i.e. 900 mg l^{-1}.

Optimal Level of Orange II. The specific growth rate of *E. faecalis* in Orange II concentration from 40 to 120 mg l^{-1} the optimal glucose concentration was not significantly different to that of the control (Fig 3). Based on the specific growth rate of *E. faecalis*, our results shown that by increasing the Orange II concentration did not increase the specific growth rate. By calculating the kinetic parameter of growth our results show that the best specific growth rate of *E. faecalis* was on the 80 mg l^{-1} Orange II-containing-medium (Table 2). In the Orange II 120 mg l^{-1} containing medium, the decolorization rate and capacity were higher than the lower Orange II concentration (40-80 mg l^{-1}) (Table 2).

The Maximum Specific Growth Rate (μ_{max}), the Half-Maximum Saturation Coefficient (K_s), and the Inhibition Constant (K_I). By calculating μ_{max} and K_s values using the Hanes-Monod model for *E. faecalis* growing on medium containing various glucose consentrations, we obtained values of μ_{max} and K_s of 0.22 h^{-1} and 0.05 g l^{-1}, respectively (Fig 4). By similar methods, the values of μ_{max} and K_s for *E. faecalis* growth on medium containing various Orange II concentrations could be determined. The values of μ_{max} and K_s were 0.25 h^{-1} and 1.5 mg l^{-1}, respectively (Fig 5). Based on the kinetic parameters obtained: m (0.26, 0.28, and 0.25 h^{-1} for 40, 80, and 120 mg l^{-1} Orange II, respectively), μ_{max} (0.25), and K_s (1.5) with various initial *S* (Orange II) concentrations, the K_I value could be calculated using microsoft excel programme by inserting the assumed K_I value as a variable. After 15 iterations, the best fit (i.e between the m values calculated by Hanes-Monod method and the m Haldane, correlation coefficient $R^2 \cong 1$) gave an Orange II K_I value of 750 mg l^{-1}.

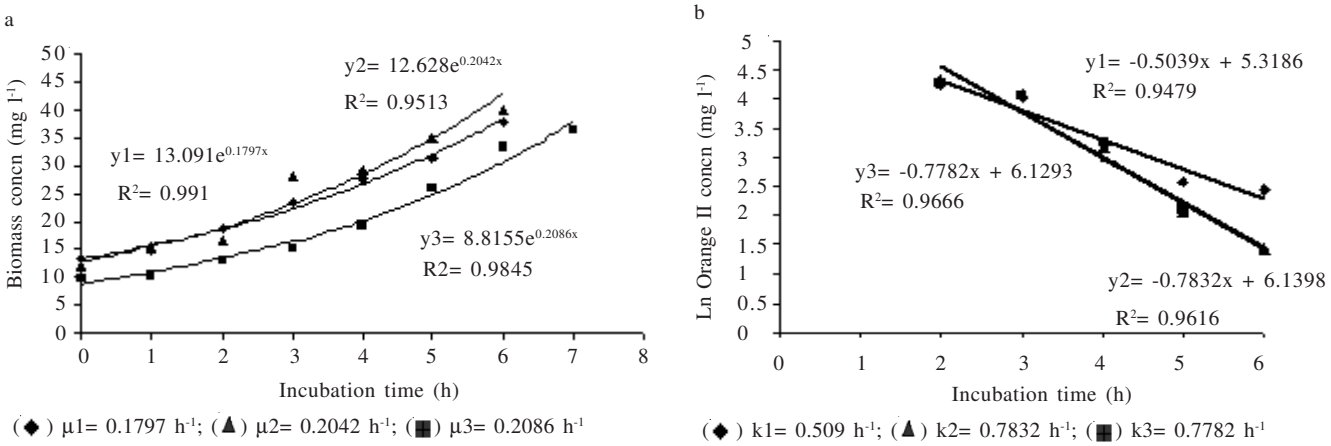
DISCUSSION

As shown by the data in Table 1 and Fig 2, increasing the glucose concentration from 0.45 to 0.90 g l^{-1}, increased specific growth rate and biomass production. However, increasing the glucose concentration from 0.90 to 1.80 g l^{-1} did not significantly increase the specific growth rate and

Table 1 The influence of glucose on *Enterococcus faecalis* ID6017 growth and its performance for Orange II decolorization in the medium containing 80 mg l^{-1} Orange II and several concentrations of glucose

Parameter of growth and decolorization	Glucose concentration (g l^{-1})		
	0.45	0.90	1.80
Specific growth rate (h^{-1})	0.18 ± 0.01	0.20 ± 0.02	0.21 ± 0.02
Biomass produced (mg)	29.3 ± 2.518.04*	30.1 ± 7.422.86*	31.3 ± 3.716.52*
Glucose consumption (g)	0.39 ± 0.005	0.67 ± 0.007	0.79 ± 0.009
Orange II reduction (mg)	65.9 ± 4.4	71.1 ± 3.2	72.5 ± 8.6
Decolorization rate (h^{-1})	0.51 ± 0,1	0.78 ± 0.2	0.77 ± 0.2
Capacity of decolorization (mg substrate/mg cell d.w.)	3.65 ± 0.3	3.11 ± 0.1	4.39 ± 0.5
Concentration of sulphanilic acid (mg l^{-1})	32.5 ± 0.3	35.5 ± 0.12	36.7 ± 0.71

*biomass produced during decolorization of Orange II.



(◆) μ1= 0.1797 h^{-1}; (▲) μ2= 0.2042 h^{-1}; (■) μ3= 0.2086 h^{-1}

(◆) k1= 0.509 h^{-1}; (▲) k2= 0.7832 h^{-1}; (■) k3= 0.7782 h^{-1}

Fig 2 Specific growth rate (a) and Orange II decolorization rate, (b) during *Enterococcus faecalis* growing on the medium containing Orange II and 0.45 g l^{-1} glucose (◆), 0.90 g l^{-1} glucose (▲), and 1.80 g l^{-1} glucos (■).

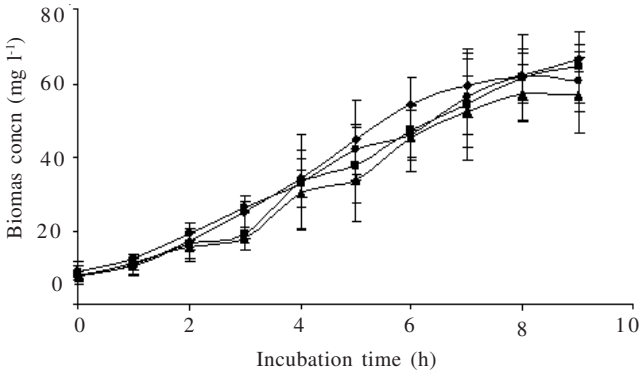


Fig 3 The growth of *Enterococcus faecalis* on the medium without Orange II (◆), with 40 mg l^{-1}; (■), 80 mg l^{-1}; (▲), and 120 mg l^{-1} (●), Orange II. Bars indicated standard error where *P*=0.05.

In contrast to the influence of glucose on bacterial growth, the decolorization of Orange II was significantly influenced by the addition of glucose. The consumption of glucose was related with the rate of decolorization and the amount of reduced Orange II. This means that additional glucose as an energy source is needed by the *E. faecalis* to decompose Orange II i.e. decolorization of Orange II is an energy consuming activity.

The addition of glucose did not give any significant difference on sulphanilic acid formation. Based on the concentration of sulphanilic acid at the end of cultivation, it showed that the concentration of sulphanilic acid was quite high. This is because this bacteria can not metabolise sulphanilic acid (Meitiniarti *et al*. 2007), although the added

Table 2 The influence of Orange II on *Enterococcus faecalis* ID6017 growth and its performance for Orange II decolourization in the medium contained 0.90 g l^{-1} glucose and several concentrations of Orange II

Parameter of growth and decolorization	Orange II concentration (mg l^{-1})			
	0	40	80	120
Specific growth rate (h^{-1})	0.28 ± 0.03	0.26 ± 0.02	0.28 ± 0.02	0.25 ± 0.03
Biomass produced (mg)	51.77 ± 8.10	45.04 ± 11.90	34.1 ± 7.1	33.72 ± 7.50
		25.10*	22.9*	23.90*
Glucose consumption (g)	695.6 ± 12.9	718.2 ± 38.6	732.7 ± 25.0	756.2 ± 22.9
Orange II reduction (mg)				
Orange II reduction (%)	n.d	33.63 ± 4.70	64.22 ± 6.38	74.45 ± 6.38
		84.00	80.27	62.00
Decolorization rate (h^{-1})	n.d	0.95 ± 0.14	0.47 ± 0.07	0.32 ± 0.05
Dye removal capacity (mg substrate/mg cell d.w)	n.d	1.55 ± 0.19	2.64 ± 0.32	2.77 ± 0.57
Concentration of sulphanilic acid (mg l^{-1})	n.d	24.2 ± 2.9	34.02 ± 3.09	37.02 ± 0,71

*biomass produced during removal of Orange II, n.d: not determined.

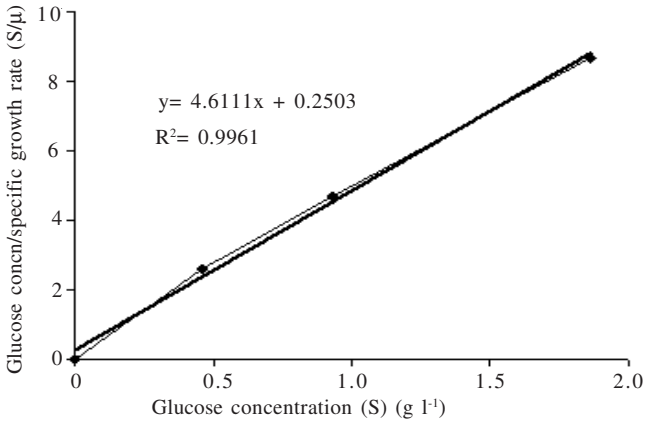


Fig 4 A plot of glucose concentration/specific growth rate (S/μ) *vs* glucose concentration (S) resulting in a straight line with slope equal to $1/\mu_{max}$= 4.61 and an intercept of K_S/μ_{max}= 0.25 for various co-substrate concentrations on growing medium of *Enterococcus faecalis*.

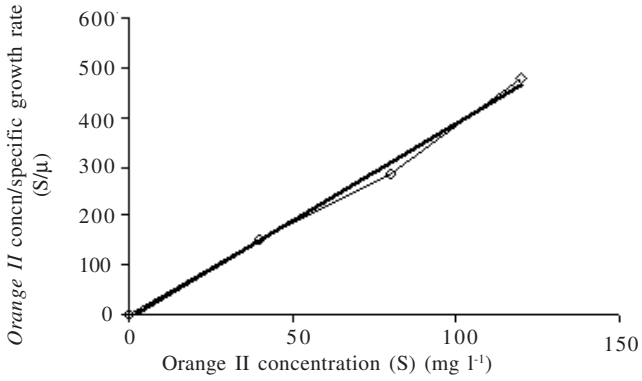


Fig 5 A plot Orange II concentration/specific growth rate (S/μ) *vs* Orange II concentration (S) resulting in a straight line with a slope equal to $1/\mu_{max}$= 3.93 and an intercept of K_S/μ_{max}= 5.89 for various substrate concentrations on growing medium of *Enterococcus faecalis.*

biomass produced by the bacteria. This might be because the glucose concentration of 0.90 g l^{-1} is adequate to support the growth of *E. faecalis*. It was also shown from glucose consumption and biomass production that these were low during bacterial growth. For their growth, bacteria require C and N sources in certain proportions (Thingstad and Lignell 1997). In the 0.90 g l^{-1} glucose containing medium, the proportions of C and N sources were suitable for bacterial growth. Thus, the bacteria grew fast and produced more biomass in this medium.

amount of glucose was related to the rate of decolorization and the amount of reduced Orange II.

The increase in Orange II concentration from 40 to 80 mg l^{-1} raised the specific growth rate of *E. faecalis*. However, when Orange II was increased up to 120 mg l^{-1}, it decreased the specific growth rate of *E. faecalis*. These results show that *E. faecalis* did not use Orange II as a growth substrate and might be toxic for bacterial growth. The highest specific growth rate of *E. faecalis* was on the medium containing 80 mg l^{-1} Orange II. This might be due to the fact that in 80 mg l^{-1} Orange II concentration, the bacteria

nutrient requirement was fullfilled and they could tolerate Orange II concentration. So, they could grow favorably.

Based on the decolorization rate (Table 2), our results show that the increase in Orange II concentration also increased the decolorization rate. This is because the reductase enzyme activity of *E. faecalis* to remove or reduce *Orange II*, was related to substrate concentration (Orange II). The kinetic analysis of decolorization indicated that the decolorization process followed first order kinetics with respect to the initial concentration of Orange II. *E. faecalis* had the highest Orange II decolorization rate on the medium containing 80 mg l^{-1} Orange II because *E. faecalis* could not use Orange II as a growing substrate. At a higher concentration, Orange II did inhibit *E. faecalis* growth.

As shown on Table 2, the increase in the decolorization rate at higher Orange II concentrations would not decrease sulphanilic acid concentration. It seems that although the sulphanilic acid concentration increased due to increasing of Orange II decolorization, *E. faecalis* could not metabolise sulphanilic acid. This would result in the accumulation of sulphanilic acid in the medium. Mendez-Paz *et al.* (2005) reported that sulphanilic acid produced by Orange II decolorization was not degrade futher and it would then be accumulated in the medium.

The μ_{max} value obtained from *E. faecalis*, grown on medium containing various glucose concentrations, was similar to the specific growth rate of *E. faecalis* which was grown on the medium contained of 0.9-1.8 g l^{-1} glucose concentration. This result showed that by increasing the glucose concentration up to 1.86 g l^{-1}, it would not efficiently increase the growth of *E. faecalis*. A low value of K_s obtained here indicated that bacteria had a very high affinity for glucose as a carbon substrate. Hence, the bacterial growth would not be affected until the glucose concentration declined to a very low level. This result also showed that using glucose at an optimal concentration is quite economic when applied in waste-water-treatment plants.

The μ_{max} values of *E. faecalis* growth with various Orange II concentrations was similar with the specific growth rate of *E. faecalis* grown on the medium containing lower than 40 mg l^{-1} Orange II concentration. This result indicated that Orange II was not a growth substrate for *E. faecalis*. Futhermore, when the K_s value of Orange II was compared with that of glucose, it showed that bacterial affinity to Orange II was lower than to glucose. This result indicated that *E. faecalis* would preferentially use glucose on the medium for rapid growth (Thingstad and Lignell 1997). Although a decrease in Orange II concentration occurred in these experiments, it is not be due to Orange II consumption as a substrate. Orange II might be used together with direct growing supporting substrate via *cometabolism*.

Based on the value (750 mg l^{-1}) of inhibition coefficient K_I obtained in this experiment, it is shown that Orange II at a concentration of ≥ 750 mg l^{-1} would inhibit growth of *E. faecalis*. The K_I value of Orange II also indicated that although μ of *E. faecalis* on the medium contained of 120 mg l^{-1} Orange II decreased, Orange II concentration used in this experiment (40, 80, and 120 mg l^{-1}) was too low to inhibit the growth of *E. faecalis*.

Based on our results, it can be concluded that by increasing the glucose (as a co-substrate) concentration up to 0.90 g l^{-1} and Orange II up to 80 mg l^{-1}, we could increase the specific growth rate and Orange II decolorizatin rate of *E. faecalis*. An increase in Orange II concentration above 80 mg l^{-1} would decrease the specific growth rate of *E. faecalis*, and when the concentration was increased to more than 750 mg l^{-1}, the growth of bacteria will be inhibited. Although the actual dye concentration in effluent is not more than 750 mg l^{-1}, we can predict that the specific growth rate of bacteria will decrease when the Orange II concentration was more than 80 mg l^{-1}. Moreover, our results show that *E. faecalis* could not consume sulfanilic acid as one of the intermediate products of azodye degradation. Accordingly, it is suggested that this bacteria should not be used alone in dye-contaminated waste-water treatment, but should be mixed with other bacterial species which could consume intermediate products of azodye degradation and had the ability to degrade other kinds of azodyes.

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