

Decolorization of Orange II by Mixed Culture of *Enterococcus faecalis* ID6017 and *Chryseobacterium indologenes* ID6016

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Previous work showed that *Enterococcus faecalis* ID6017 and *Chryseobacterium indologenes* ID6016 were able to decolorize the orange II qualitatively. In that experiment, *E. faecalis* could decolorize orange II more rapidly than *C. indologenes*. The objective of this study was to examine the decolorization of orange II by mixed culture and the growth of both bacterial species on orange II containing medium. The experiment was done in 500 mL sterilized Erlenmeyer flasks containing 285 mL growth media with 80 mg L⁻¹ orange II. Five different treatments were performed in this project, i.e. medium was inoculated with (i) 15 mL of sterile aquadest, (ii) 15 mL of *C. indologenes*, (iii) 15 mL of *E. faecalis*, (iv) 7.5 mL of *C. indologenes* and 7.5 mL of *E. faecalis*, and (v) 7.5 mL of *E. faecalis* until decolorization occurred, followed by inoculation with 7.5 mL of *C. indologenes*. Bacterial growth (total cells number), orange II, glucose, and suphanilic acid, as intermediate product of orange II decolorization, concentrations were measured every 2 h. The maximum decolorization of orange II was observed in the medium inoculated with a mixed culture of *E. faecalis* and *C. indologenes*. Decolorization of orange II occurred of growth and gave final concentration of sulphanilic acid of 7.06 mg L⁻¹. During culture both species grow in equilibrium in terms of population.

Key words: *Chryseobacterium indologenes*, decolorization, *Enterococcus faecalis*, growth, mixed culture, orange II

Penelitian sebelumnya menunjukkan bahwa *Enterococcus faecalis* ID6017 dan *Chryseobacterium indologenes* ID6016 dapat mendekolorisasi orange II secara kualitatif. Pada penelitian tersebut, *E. faecalis* dapat mendekolorisasi orange II lebih cepat daripada *C. indologenes*. Tujuan penelitian ini adalah menentukan dekolorisasi orange II oleh kultur campur dan pertumbuhan kedua spesies bakteri pada medium yang mengandung orange II. Penelitian dilakukan dalam botol Erlenmeyer 500 mL yang berisi 285 mL media dengan orange II 80 mg L⁻¹. Penelitian dilakukan dengan 5 perlakuan, yaitu medium diinokulasi dengan (i) 15 mL air suling, (ii) 15 mL kultur *C. indologenes*, (iii) 15 ml kultur *E. faecalis*, (iv) 7,5 mL kultur *C. indologenes* dan 7.5 mL kultur *E. faecalis*, dan (v) 7.5 mL kultur *E. faecalis* hingga terjadi dekolorisasi, diikuti inokulasi dengan 7.5 mL kultur *C. indologenes*. Pertumbuhan bakteri (jumlah sel total), konsentrasi orange II, glukose, dan asam sufaniilat sebagai produk antara dekolorisasi diukur setiap 2 jam. Dekolorisasi maksimum orange II teramati pada medium yang diinokulasi dengan kultur campur *E. faecalis* dan *C. indologenes*. Dekolorisasi orange II terjadi pada 6 jam pertama kultur dan memberi konsentrasi akhir asam sulfaniilat sebesar 7.06 mg L⁻¹. Pertumbuhan kedua spesies bakteri berada dalam keseimbangan selama pertumbuhan ko-kultur.

Kata kunci: *Chryseobacterium indologenes*, dekolorisasi, *Enterococcus faecalis*, kultur campur, orange II, pertumbuhan

Orange II is one of synthetic azo dyes which have been widely used for textile, food, and cosmetics. Like other xenobiotic compounds, azo dyes are relatively resistant to microbial degradation under conditions normally found in wastewater treatment plants. It was reported that several microorganisms were able to transform azodyes to non-colored products or even to mineralized products (Stolz 2001).

Bacterial azodyes degradation was usually

initiated by cleavage of azo bond carried out by azoreductase of the intestinal microflora, resulting in colorless amines (Chen *et al.* 2004; Ramalho *et al.* 2004; Kodam *et al.* 2005). This process is called decolorization (Tan 2001; van der Zee 2002). Anaerobic condition is more favorable for decolorization rather than aerobic condition. It is probably related to the electron with drawing nature of azo bond and the resistance of azo dyes to oxygenases attack, or because oxygen is a more effective electron acceptor than the dyes (Dos Santos 2004).

Many researchers have reported that degradation

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of azo dyes is often carried out by mixed cultures. In the mixed cultures, a higher degree of biodegradation and mineralization can be expected when co-metabolic activities within a microbial community complement each other (Pearce *et al.* 2003). In the anaerobic condition, orange II can be degraded by single culture of *Enterococcus faecalis* into sulfanilic acid as an intermediate product (Meitiniarti *et al.* 2007). Meitiniarti *et al.* (2005) also reported that *Chryseobacterium indologenes* could grow in the medium containing sulfanilic acid. Probably, both bacteria have synergistic relationship in orange II degradation. For this reason, our group examined the decolorization of orange II by mixed culture and the growth of both bacterial species on orange II containing medium. The aim of this study was also to determine the best mixing method of both bacterial cultures on orange II decolorization.

MATERIAL AND METHODS

Microorganism and Culture Condition. *C. indologenes* ID6016 and *E. faecalis* ID 6017 were obtained from Laboratory of Microbiology, Faculty of Biology, Satya Wacana Christian University, Salatiga, Indonesia. *C. indologenes* ID6016 and *E. faecalis* ID 6017 were maintained and cultivated on slant media of Nutrient Agar (NA) and Trypticase Soy Agar (TSA), respectively. Both cultures were incubated at room temperature and sub-cultured every two weeks.

Growth Medium. The composition of growth medium (pH 6.8-7.2) for this study was (g L⁻¹): glucose, 0.90; MgSO₄·7H₂O, 0.25; KH₂PO₄, 2.13; K₂HPO₄, 5.55; (NH₄)₂SO₄, 1.98; yeast extract, 0.25; orange II or acid orange 7, 0.08. Orange II was obtained from Merck and was used for this study without any purification.

Pre-Culture Preparation. Two inoculation needles of *C. indologenes* and *E. faecalis* from 24 h slant culture were inoculated aseptically into 200 mL growth medium without orange II. Pre-culture was incubated for 24-48 h in a shaker incubator with 150 rpm agitation or until optical density (λ 550 nm) of culture was 0.4 (cell density of *C. indologenes* and *E. faecalis* were 26×10^7 CFU mL⁻¹ and 23×10^7 CFU mL⁻¹, respectively). These cultures were then used as inocula.

Culture Condition. To determine the decolorization of orange II by both bacterial cultures, the experiment was performed with 5 different treatments in 500 mL Erlenmeyer flasks containing 285 mL growth media with 80 mg L⁻¹ orange II. These treatments are: (i) Medium was inoculated with 15 mL of sterile aquadest,

(ii) Medium was inoculated with 15 mL of *C. indologenes*, (iii) Medium was inoculated with 15 mL of *E. faecalis*, (iv) Medium was inoculated with 7.5 mL of *C. indologenes* and 7.5 mL of *E. faecalis*, and (v) Medium was inoculated with 7.5 mL of *E. faecalis* until decolorization occurred, followed by inoculating with 7.5 mL of *C. indologenes*. The final cell number each treatment at 0 h was $1.2-1.3 \times 10^7$ CFU mL⁻¹.

All cultures were incubated at room temperature, under static condition until decolorization occurred, followed by 150 rpm agitation until 120 h. Bacterial growth (total cells number), orange II and glucose concentrations, and sulfanilic acid concentration as an intermediate product of orange II decolorization were observed every 2 h.

Samples were harvested and centrifuged at 3326 g for 30 min to separate supernatant and cell mass (biomass). The supernatant was used to determine the orange II and glucose concentrations. The ability of both bacterial strains to decolorize orange II was determined by subtracting the initial orange II concentration with the lowest concentration from samplings. The total cells number was determined by making a serial dilution of cell culture with sterile aquadest and poured 0.1 mL of 10⁵-10⁷ cell suspension into TSA plate media.

Analytical Methods. Samples were centrifuged at 3326 g for 30 min to separate supernatant and cell mass. The supernatant was used for determining the orange II, glucose, and sulfanilic acid concentrations. Orange II concentration was determined at λ 482 nm (Pourreza and Zareian 2009). Glucose concentration was determined using DNS method at λ 540 nm (James 1995). The cell growth was determined using total plate count in TSA plate. In TSA plate medium, the color of *C. indologenes* colony was yellowish while *E. faecalis* was white. All absorbances were measured using Shimadzu UV-Vis 1201 Spectrophotometer. The concentration of sulfanilic acid was determined by HPLC (Coughlin *et al.* 1999; Chang *et al.* 2001; Supaka *et al.* 2004).

RESULTS

The growth of *C. indologenes* as a single culture was better than its growth in the mixed culture with *E. faecalis* (Fig 1A). On the contrary, the growth of *E. faecalis* together with *C. indologenes* inoculated as a mixed culture in the early experiment was faster than the growth of *E. faecalis* inoculated as a single culture (Fig 1B). Although, total numbers of *E. faecalis* cells in

the mixed culture was half of its single culture, in the end of culture its total number of *E. faecalis* cells was found to be the same (Fig 1B).

The decolorization of orange II occurred when *E. faecalis* was present in the culture. The rate of orange II decolorization by mixed culture occurred faster than by single culture (Fig 2 and Table 1). When inoculation of mixed culture was done early during the experiment, the rate of orange II decolorization was found to be the fastest (10.98 mg h^{-1}) (Table 1).

During cells growth and orange II decolorization, glucose and sulphanic acid concentrations significantly changed (Fig 3 and 4), except when the medium was inoculated with sterile water (control) and with *C. indologenes* as single culture. However,

sulphanilic acid was only detected in the first eight hours of culture, thereafter sulphanic acid concentration decreased (Fig 4). In the growth of *C. indologenes* as a single culture, aliquot of sulphanic acid was detected until 56 h incubation.

DISCUSSION

The growth of *E. faecalis* when co-cultured with *C. indologenes* was faster than its growth as a single culture (Fig 1B). The faster growth of *E. faecalis* cells caused by the presence of *C. indologenes* cells which could consume sulphanic acid and could decrease toxic effect of sulphanic acid in this medium. Meitiniarti *et al.* (2011) reported that *C. indologenes*

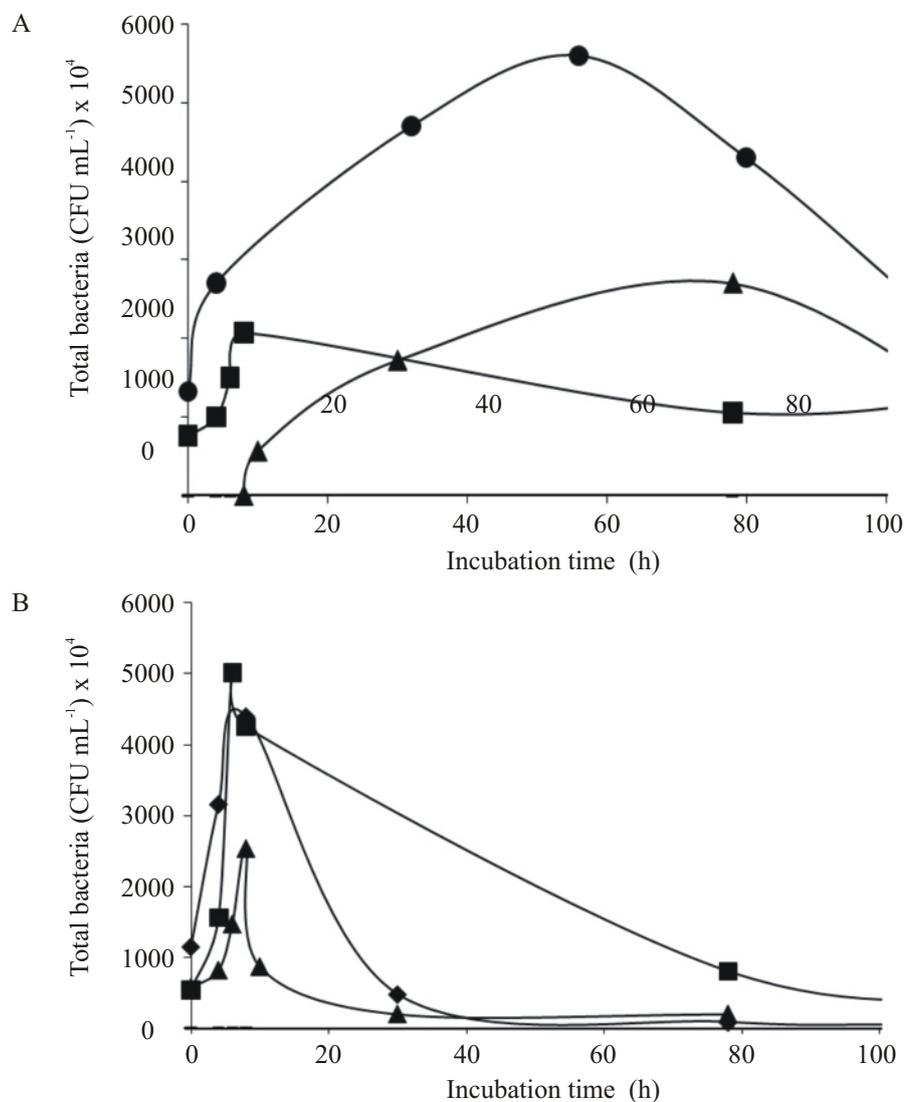


Fig 1 Total cells of *Chryseobacterium indologenes* (A) and *Enterococcus faecalis* (B) in single and mixed culture in the orange II containing medium. - : Medium was inoculated with 15 mL of sterile aquadest; ●: Medium was inoculated with 15 mL of *C. indologenes*; ◆: Medium was inoculated with 15 mL of *E. faecalis*; ■: Medium was inoculated with 7.5 mL of *C. indologenes* and 7.5 mL of *E. faecalis*; ▲: Medium was inoculated with 7.5 ml of *E. faecalis* until decolorization occurred, followed by inoculating with 7.5 mL of *C. indologenes*.

Tab 1 Values of growth and orange II decolorization parameter by single culture of *E. faecalis* and on mixed culture with *C. indologenes* after 120 h incubation

Parameter	Treatments				
	i	ii	iii	iv	v
Orange II reduction (mg) (%)	n.dt	n.dt	65.2 ± 0.2 90 ± 2	65.9 ± 0.2 89 ± 3	69.5 ± 0.3 91 ± 4
Time of decolorization (h)	n.dt	n.dt	8	6	10
Decolorization rate (mg h ⁻¹)	n.d	n.dt	8.15 ± 0.02	10.98 ± 0.02	6.95 ± 0.03
Glucose consumption during decolorization (mg)	n.d	n.dt	340 ± 7	216 ± 3	393 ± 5
Glucose consumption during 102 h (mg)	n.d	n.dt	710 ± 10	673 ± 6	693 ± 4
Sulfanilic acid concentration at decolorization (mg L ⁻¹)	n.d	n.dt	24.4 ± 0.13	23.2 ± 1.2	21.2 ± 1.2
Sulfanilic acid concentration at 128 h (mg L ⁻¹)	n.d	1.12	18.5 ± 0.5	7.06 ± 0.3	10.3 ± 0.7

n.d = not detected n.dt = not determined

i = first control

ii = early inoculation by *Chryseobacterium indologenes* (second control)

iii = early inoculation by *Enterococcus faecalis* (third control)

iv = early inoculation by both of *E. faecalis* and *C. indologenes*

v = early inoculation by *E. faecalis*, and inoculated by *C. indologenes* after decolorization

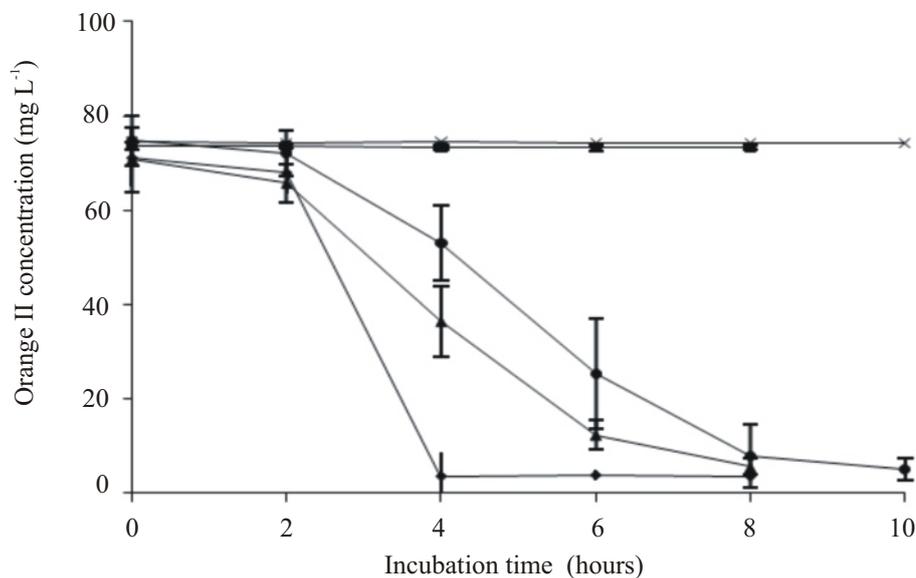


Fig 2 Decrease of orange II concentration in experiment with single culture of *Enterococcus faecalis* (control) and with mixed culture (together with *Chryseobacterium indologenes*). x: Medium was inoculated with 15 mL of sterile aquadest; ■: Medium was inoculated with 15 mL of *C. Indologenes*; ▲: Medium was inoculated with 15 mL of *E. faecalis*; ◆: Medium was inoculated with 7.5 mL of *C. indologenes* and 7.5 mL of *E. faecalis*; ●: Medium was inoculated with 7.5 mL of *E. faecalis* until decolorization occurred, followed by inoculating with 7.5 mL of *C. indologenes*. Bars represent errors bars from three replicates.

could grow on filtrate's culture of *E. faecalis* which contained sulphanic acid as intermediate product of orange II decolorization.

In the fifth treatment, where *C. indologenes* was

inoculated after orange II decolorized, *E. faecalis* grew very slowly due to the low cells density of the inoculum (5%). In low density culture, usually bacteria were less successful to colonize a natural habitat. Another factor

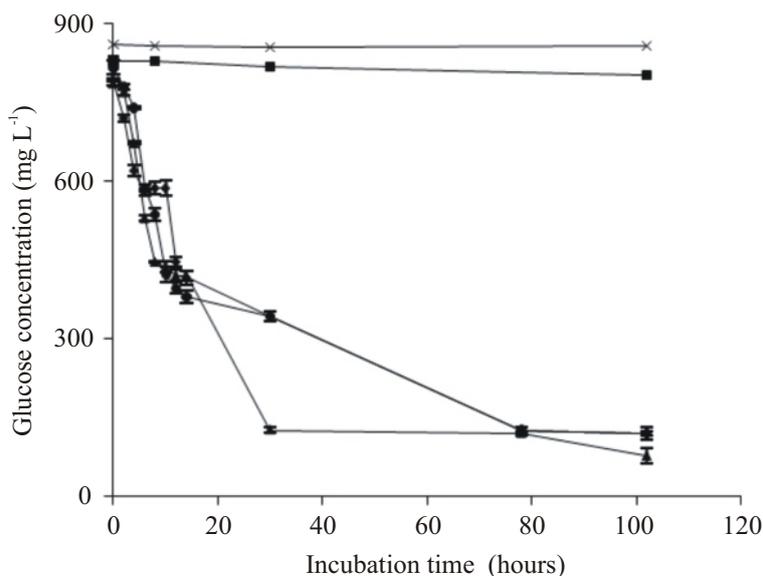


Fig 3 Change of glucose concentration in experiment with single culture of *Enterococcus faecalis* (control) and with mixed culture (together with *Chryseobacterium indologenes*). x: Medium was inoculated with 15 mL of sterile aquadest; ■: Medium was inoculated with 15 mL of *C. Indologenes*; ▲: Medium was inoculated with 15 mL of *E. faecalis*; ◆: Medium was inoculated with 7.5 mL of *C. indologenes* and 7.5 ml of *E. faecalis*; ●: Medium was inoculated with 7.5 mL of *E. faecalis* until decolorization occurred, followed by inoculating with 7.5 mL of *C. indologenes*. Bars represent errors bars from three replicates.

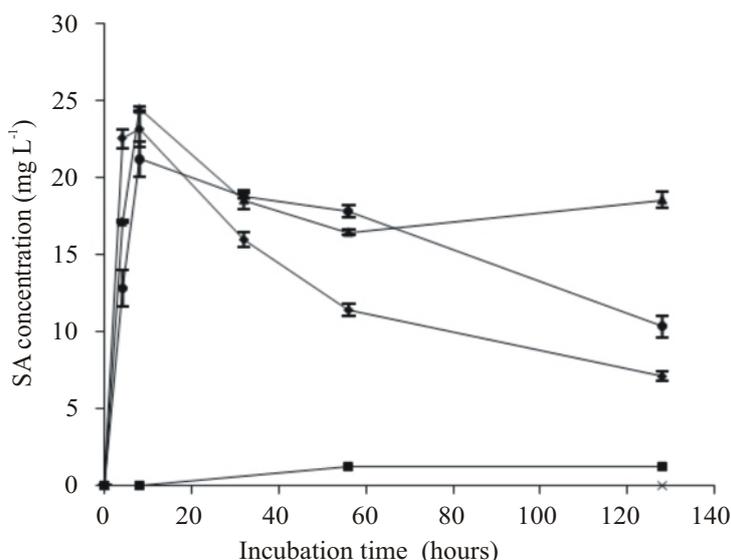


Fig 4 Change of sulphanic acid concentration in experiment with single culture of *Enterococcus faecalis* (control) and mixed culture with *Chryseobacterium indologenes*. x: Medium was inoculated with 15 mL of sterile aquadest; ■: Medium was inoculated with 15 mL of *C. Indologenes*; ▲: Medium was inoculated with 15 mL of *E. faecalis*; ◆: Medium was inoculated with 7.5 mL of *C. indologenes* and 7.5 mL of *E. faecalis*; ●: Medium was inoculated with 7.5 mL of *E. faecalis* until decolorization occurred, followed by inoculating with 7.5 mL of *C. indologenes*. Bars represent errors bars from three replicates.

that might affect the culture's growth could be the existence of intermediate product of orange II decolorization that inhibits *E. faecalis* growth. This *E. faecalis*'s low growth rate results in low decolorization rate.

The decreased of *E. faecalis* growth could be due to *E. faecalis* sensitivity to sulphanic acid. Topaç *et al.* (2009) reported that the existence of sulfanilic acid > 8 mg kg⁻¹ dry soil could decrease the number of several

bacteria. On the other hand, *C. indologenes* were more resistant to sulphanic acid and may be able use this compound as C, N, and energy sources. This ability could increase the cells number. Although there was sulphanic acid in the medium (fourth and fifth treatment, Table 1), the concentrations were not sufficient for *C. indologenes* growth.

If we compare the time needed for orange II decolorization and the rate of orange II decolorization

between single culture of *E. faecalis* and mixed culture of *E. faecalis* and *C. indologenes*, it seems that orange II decolorization performed by mixed culture was better than single culture of *E. faecalis* (Fig 2 and Table 1). Inoculation of *E. faecalis* and *C. indologenes* as a mixed culture at the early experiment resulted higher decolorization rate than inoculation of both bacteria in a successive way. In fourth treatment, time needed for orange II decolorization was the shortest and the rate of decolorization was the fastest (10.98 mg h^{-1}) (Table 1). This could be the result of a synergistic interaction between these two bacterial species. In this condition, decolorization product of orange II will be consumed by *C. indologenes*, so orange II decolorization activity by *E. faecalis* will occur fast and more effective. The capability of *C. indologenes* to consume sulphanilic acid was demonstrated by culturing of *C. indologenes* ID6016 using medium containing sulfanilic acid as C sole source also using (Meitiniarti *et al.* 2005) and the fitrate's culture of *E. faecalis* which growing on orange II containing medium (Meitiniarti *et al.* 2011).

The pattern of glucose consumption was similar with cell growth of *E. faecalis* in third and fifth treatment. Although the cell number of *E. faecalis* inoculated in the fifth treatment was half of that shown by the third treatment, in the end the cell numbers were similar. These conditions indicated that glucose was needed by *E. faecalis* for growth and orange II decolorization. Mendez-Paz *et al.* (2005) reported that the removal rate of acid orange 7 is highly favorable when glucose is added as co-substrate. Bras *et al.* (2001) also showed that the addition of glucose or acetate ions as electron donors, apparently stimulates the reduction cleavage of azo bonds caused the oxidation of organic electron donors and/or hydrogen is coupled to the colour removal process.

In the mixed culture, in addition to the presence of *C. indologenes* which could decrease toxic affect of sulphanilic acid for *E. faecalis*, probably *C. indologenes* could also produce intermediate products that were used as redox mediators for azoreductase produced by *E. faecalis*. In the degradation of 2-naphthalene sulphonate (2NS), there are several mediator redox produced by *Sphingomonas* sp. BN6 which could transfer equivalent redox to azodye (Keck *et al.* 1997). Keck *et al.* (2002) proposed that these redox mediators shuttle electrons from the cells to the azo dyes, which results in purely chemical, extremely nonspecific, extracellular reductive cleavage of the azo bond. Chang *et al.* (2004) also reported that extracellular metabolite of *Escherichia coli* strain NO₃

could stimulate decolorization of azodye by bacteria.

To conclude, inoculation of mixed culture of *C. indologenes* ID6016 and *E. faecalis* ID6017 together early during culture in orange II containing medium gave the best orange II decolorization. The rate of orange II decolorization was 10.98 mg h^{-1} . Using this treatment, the best sulphanilic acid consumption (7.06 mg L^{-1}) and equilibrium growths of both species were achieved.

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