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EMPLOYMENT OF MICROBES ISOLATED FROM RESIDENTIAL WASTEWATER TO DEGRADE CHLOROBENZENE

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Abstrak: Keupayaan konsortium mikrob daripada air sisa kediaman dalam penguraian klorobenzena (CB) telah dikaji. Konsortium ini terlebih dahulu didedahkan pada kepekatan CB yang tinggi (0.2 mg/l) selama tujuh bulan. Hasilnya, dua spesies yang paling dominan berjaya dipencilkan, iaitu 'Koloni Kuning' (YC), dan 'Koloni Putih' (WC). Sebagai perbandingan, gabungan YC and WC memberikan kadar penguraian spesifik CB, Q_s (7.12 x 10^{-6} g CB terurai/g biojisim per jam) tiga kali ganda lebih tinggi berbanding hasil gabungan bagi individu WC dan YC. Ini membuktikan wujud kesan sinergistik bagi YC dan WC dalam penguraian CB. Keputusan daripada kultur selanjar menunjukkan penambahan sumber nitrogen telah meningkatkan kadar pencairan kritikal, D_c daripada 0.08 jam^{-1} kepada 0.11 jam^{-1}. Ini membuktikan penghadan nitrogen dalam penguraian CB tidak boleh diabaikan. Keputusan juga menunjukkan penguraian CB tidak berlaku di bawah kepekatan 0.6 µg/l, di mana CB tidak dapat dikesan oleh mikrob di bawah tahap ambang ini. Hasil kajian ini boleh dijadikan panduan dalam menganggar parameter-parameter untuk kerja-kerja perskalaan pada masa akan datang atau percubaan di tapak sebenar.

Kata kunci: Konsortium Mikrob, Air Sisa Kediaman, Penguraian Klorobenzena, Mod Kultur Selanjar

Abstract: The performance of microbial consortia from residential wastewater to degrade chlorobenzene (CB) was investigated. The consortia were firstly exposed to high CB concentration (i.e. 0.2 mg/l) for seven months. As a result, two most dominant survivors, denoted as 'Yellow Colony' (YC) and 'White Colony' (WC) were isolated. In a comparison study, the mixture of WC and YC yielded three times greater maximum CB specific degradation rate, Q_s (7.12 x 10^{-6} g CB degraded/g cell per hour) than the individual WC and YC did, combined. This clarified that there was a synergistic effect of YC and WC on CB degradation. Result in a continuous culture indicated that nitrogen-enriched feed (yeast extract) has improved the critical dilution rate, D_c from 0.08 $hour^{-1}$ to 0.11 $hour^{-1}$. This proved that the nitrogen limitation could not be ignored. Our result also indicated that no degradation was witnessed below 0.6 µg/l, where CB was almost undetectable by microbes below this threshold level. Outcomes of this study have provided useful parameter estimates for future up scaling works, or on site trial.

Keywords: Microbial Consortia, Residential Wastewater, Chlorobenzene Degradation, Batch Culture Continuous Mode

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INTRODUCTION

The substantial use of CB as organic solvent, insecticide, degreaser and deodorant, and their use as intermediates in the synthesis of chemicals such as rubber processing, antioxidants, dyes and pigments, agricultural products, and pharmaceuticals, has led to a widespread release of these xenobiotic compounds into the environment (EPA 1980; Harris *et al*. 1985). These compounds have been found in a wide range of environmental media at high concentration including soils (Ding *et al*. 1992), groundwaters (Boyd *et al*. 1997), sewage sludge (Rogers *et al*. 1989a; Wang *et al*. 1992), marine and lake sediments (Masunaga *et al*. 1991; Lee & Fange 1997), and open water columns (Rogers *et al*. 1989b; Harper *et al*. 1992). They are also known as important riverine contaminants especially found in United Kingdom (Meharg *et al*. 2000). Moreover, the chlorinated benzenes (e.g. CB) that are currently being targeted by bioremediation because of its resistances (Eweis *et al*. 1998) were identified as priority pollutants by the U.S. Environmental Protection Agency (EPA 1980). Therefore, the destruction of these pollutants was emphasized in many researches and executed under safety conditions in order to protect human and environment from the hazardous effects.

Bioremediation has become increasingly important rather than chemical and physical processes. The responsibility of microorganisms for CB removal from the environment *via* enzymatically catalyzed reactions appears to be very important because of its perceived low cost, simplicity and its low adverse effect on the environment (Cookson 1995). The major mechanism of aerobic CB degradation, which *via* oxidative dechlorination usually initiated by dioxygenative hydroxylation, then leading to the formation of catechols. Finally, it undergoes the ring fission and subsequent mineralization to carbon dioxide and water. CB biodegradation under anaerobic condition has also been reported, although it occurs at a slower rate than aerobic biodegradation (Bittkau *et al*. 2004).

A wide variety of microorganisms could utilize CB as carbon and energy source in various substrates, including soil, sediment, sewage sludge and groundwater. The microbial degradation of chlorinated benzenes has been examined and the results reported that different bacterial strains such as *Pseudomonas* sp., *Alcaligenes* sp. and *Xantobacter* sp. were individually able to use CB as growth substrates (Schraa *et al*. 1986; Haigler *et al*. 1992; Spain & Nishino 1987). Besides, the indigenous microbial communities especially from the CB contaminated sites were also capable to degrade CB (Aelion *et al*. 1987; Nishino *et al*. 1994; Kao & Presser 1999; Balcke *et al*. 2004). However, the use of microbes from wastewater to degrade CB has not yet intensively investigated.

Various mathematical models have been proposed to quantitatively describe microbial growth kinetics in bioremediation systems. However, the Monod model [Eqn. (1)] is considered the basic equation (Monod 1942), then has been improved by expressions for example maintenance, diffusion or transport limitation (Pirt 1975). This Monod equation is derived from the premise that a single enzyme system with Michaelis-Menten kinetics is responsible for uptake of residual substrate concentration (S), and the amount of that enzyme or its catalytic activity is sufficiently low to be growth-rate limiting.

$$\mu = \frac{\mu_{\max} S}{K_s + S} \quad (1)$$

whereby μ = specific growth rate in hour^{-1}; μ_{max} = maximum specific growth rate when $S >> K_s$; and K_s = substrate utilization constant, numerically equal to substrate concentration when μ is half μ_{max}. In deriving a general equation for substrate dissappearance, modified Monod kinetics are assumed to be adequate for describing the growth dynamics of a bacterial culture, limited only by the concentration of the substrate (Eweis *et al*. 1998). Thus, maximum contaminant specific degradation rate, Q_s could be measured when relating the mass of cells produced per mass of substrate utilized, Y. The conversion efficiency of growth substrate into cell material has resulted in Equation (2). The higher value of Q_s signifies the better bioremediation system.

$$Q_s = -\frac{\mu}{Y} \quad (2)$$

Besides that, chemical oxygen demand or COD can also be an indirect measure for determining the level of organic matter presence on bioremediation. COD is a measure of the oxygen required to oxidize organic matter or carbon containing compound using a strong chemical oxidant. An oxidant is a compound that will readily give or donate oxygen atoms during a chemical reaction.

This study aimed at investigating the kinetic of microbial isolates from residential wastewater to degrade CB in both batch and continuous modes. Investigations would be focused on the isolation approach, comparison of the specific CB degradation rate of the identified isolates and their combinations, and the behavior or CB degradation at different CB levels.

MATERIALS AND METHODS

Chemicals and Growth Medium

The 99.9% purity CB (Fischer Scientific, Germany) has been used throughout this study. CB at concentration of 0.2 mg/l was introduced immediately into the liquid phase by a 100 µl syringe in 500 ml flask cultures. In continuous mode, CB at concentration of 3.0 mg/l was fed continuously by a peristaltic pump into the 2 l bioreactor. Sterilized liquid mineral medium consisted of 3.3 g/l dipotassium hydrogen phosphate (K_2HPO_4), 1.9 g/l sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$), 4.5 g/l ammonium sulphate [$(NH_4)_2SO_4$] and 0.2 g/l magnesium-heptahydrat ($MgSO_4.7H_2O$) was supplied for enrichment.

Isolation and Culture of Microorganisms

Ten liters residential wastewater originated from a local treatment plant were collected and filtered. In the enrichment step, 25 ml of the filtered wastewater was added into 225 ml sterilized mineral medium in 500 ml Erlenmeyer flask, and shaken at 150 rpm on the orbital shaker (Infors, Switzerland). Once the optical

density (OD) reached about 0.8, this enriched culture was immediately kept at 4ºC not more than two days.

For adaptation study, 25 ml of the enriched culture was transferred into a 500 ml Erlenmeyer flask containing 225 ml of mineral medium supplemented with 0.2 mg/l CB, and then kept in static condition for seven months at 37ºC. 0.1 ml of the culture was then transferred to the nutrient agar, and left at 37ºC for two days. The grown isolates were physiologically and biochemically characterized through the Gram staining method (Benson 1994) and biochemical tests (MacFaddin 2000) such as catalase, oxidase, urease, citrate, and indole test. The test results were compared with the classification scheme in Bergey's Manual (Goodfellow 1994) as shown in Tables 1 and 2.

Table 1: Characterization results for YC.

Type of test	Results	Observation	Inference
Gram staining	–ve	Red cocci/short rod	Short rod shape, Gram negative bacteria
Catalase	+ve	Bubbles were formed immediately	Able to produce catalase enzyme to breakdown hydrogen peroxide
Oxidase	+ve	Blue color formed	Able to produce oxidase enzyme
Urease	–ve	No color changes of the slant	Not able to produce enzyme urease
Citrate	–ve	No growth with intense blue	Could not utilize citrate as the sole of carbon source
Indole	+/–ve	Medium surface became orange	Could produce the precursor for indole formation

Note: * +ve = positive; -ve = negative

Table 2: Characterization results for WC.

Type of test	Results	Observation	Inference
Gram staining	+ve	Dark blue cocci	Cocci shape, Gram positive bacteria
Catalase	–ve	No bubbles formed	Not able to produce catalase enzyme
Oxidase	+ve	Blue color formed	Able to produce oxidase enzyme
Urease	–ve	No color changes of the slant	Not able to produce enzyme urease
Citrate	–ve	No growth with intense blue	Could not utilize citrate as the sole of carbon source
Indole	+/–ve	Medium surface became orange	Could produce the precursor for indole formation

Note: * +ve = positive; -ve = negative

The identified colonies on nutrient agar plate were propagated in 250 ml mineral medium in 500 ml Erlenmeyer flask at ambient temperature until the OD reached 0.8. In batch studies, 25 ml of this culture was then added into 225 ml fresh mineral medium in 500 ml Erlenmeyer flask, and shaken at 150 rpm for 48 h at ambient temperature. 5 ml samples were drawn periodically to determine the OD and CB concentration. In the chemostat studies, the culture was grown in 2 l bioreactor (1.5 l working volume). A multichannel peristaltic pump (Masterflex model 7520-57, USA) had been used to feed and harvest the liquid from the bioreactor continuously. Different dilution rates were obtained by altering the pump speeds ranged from 1.0 to 2.5 ml/min.

Analytical Methods

The OD was measured by Shimadzu UV-160 at 600 nm. One OD unit was assumed equivalent to 1 g/l of cell dry weight (Wang 2005). The CB was detected by the High Pressure Liquid Chromatography (HPLC) as described by Dilmeghani and Zahir (2000). Column of C18 types with 3.9 mm by 30 cm was used in HPLC and the mobile phase for isocratic detection was methanol:water with ratio of 70:30. The flow rate was fixed at 1.0 ml per minute with volume injection at 5.0 µl during 5 min run time.

RESULTS AND DISCUSSION

The potential of employing microbes originated from residential wastewater to degrade CB was investigated. Twelve types of bacteria were identified, and two of them were successfully isolated after seven months adaptation in 0.2 mg/l CB solution. Two dominant bacteria, designated as YC and WC were capable of utilizing CB as a sole carbon source. Through morphological identifications, Gram staining, and biochemical tests, YC was most likely belongs to the Gram negative bacterium from either *Alcaligenes, Pseudomonas*, *Sphingobacterium*, *Flavo-bacterium*, or *Xantobacter*. Meanwhile, WC was a Gram positive bacterium that closely related to *Aerococcus*, *Enterococcus*, *Trichococcus*, *Pediococcus*, and *Vagococcus*. These results are in agreement with the common CB degrader species reported in the literatures (De Bont *et al*. 1986; Schraa *et al*. 1986; Van der Meer *et al*. 1987; Spiess *et al*. 1995; Sommer & Gorisch 1997; Carvalho *et al*. 2002; Gobel *et al*. 2004).

A comparison study on CB degradation was performed by inoculating 10% (v/v) of YC, WC, mixture of YC and WC, and fresh wastewater into 0.0553 mg/l CB solution. The result in Figure 1 indicates that the Q_s (g CB/g cell per hour) achieved in the mixture of YC and WC was three times greater than in the individual culture of YC and WC, combined. This concludes that there was a synergistic effect of YC and WC in degrading CB. A superior performance of the mixture between Gram positive and Gram negative to degrade CB was also being witnessed by Nishino *et al.* (1992). They found that a consortium of these bacteria isolated from groundwater and soil contamination with CB was able to mineralize 54% of a 2.23 µmol/l solution *via* the modified ortho pathway within seven days. However, the actual reason for this phenomenon is still not yet fully

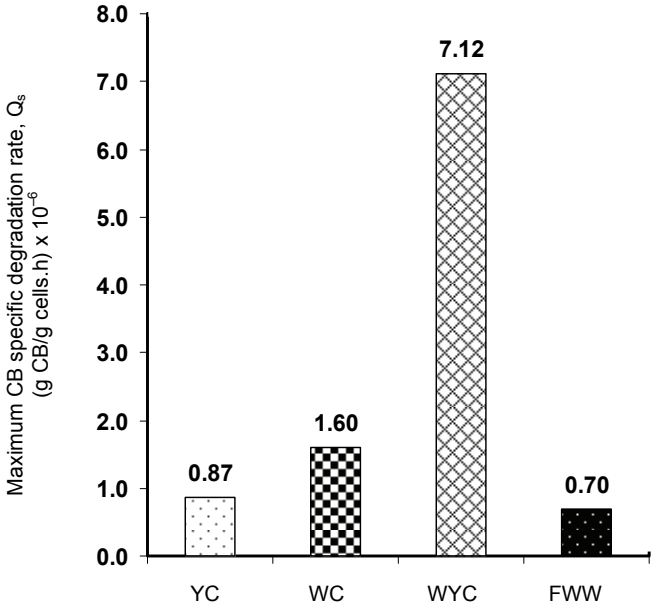

Figure 1: Comparison of the specific degradation rates of CB by YC, WC, the mixture of YC and WC (WYC), and microbes from fresh wastewater (FWW).

understood. It is also interesting to note that Q_s achieved by the mixture of WC and YC was ten times higher than the one achieved by microbes in fresh wastewater. The most possible reason is that both WC and YC may have been long adapted to high level CB in comparison to the microbes in fresh wastewater.

Stanburry and Whitaker (1984) noted that the advantages for continuous mode are able to maintain defined conditions and allowed the use of low toxic concentrations. At steady-state, the specific growth rate (μ, per hour) is balanced by the dilution rate (D, per hour). In this study, the results from continuous mode revealed that the nitrogen supply had improved the Q_s five folds (Fig. 2); and enhanced the D_c from 0.08 per hour to 0.11 per hour (Fig. 3). Thus, it was evident that nitrogen source is a crucial rate limiting substrate that can not be ignored during the CB degradation. For a balanced growth, the carbon source and the nitrogen source are co-metabolized for energy generation and growth. The nitrogen supply to organic molecules was often necessary for cellular growth and maintenance with maximum microbial activity (Alexander 1981), which tend to make them more stable biologically in degrading toxic compound. In another continuous study at dilution rate of 0.04 per hour, there was no significant difference of degradation kinetics between adapted (to CB for two days) culture and the unadapted one as shown in Figure 4. It can be implied that the microbes in residential wastewater may have been long exposed to CB in their natural habitat prior to this experiment. Hence, short-term adaptation on the culture step

as demonstrated in Mihelcic and Luthy (1988) work on polycyclic aromatic hydrocarbon degradation might not be compared with this study.

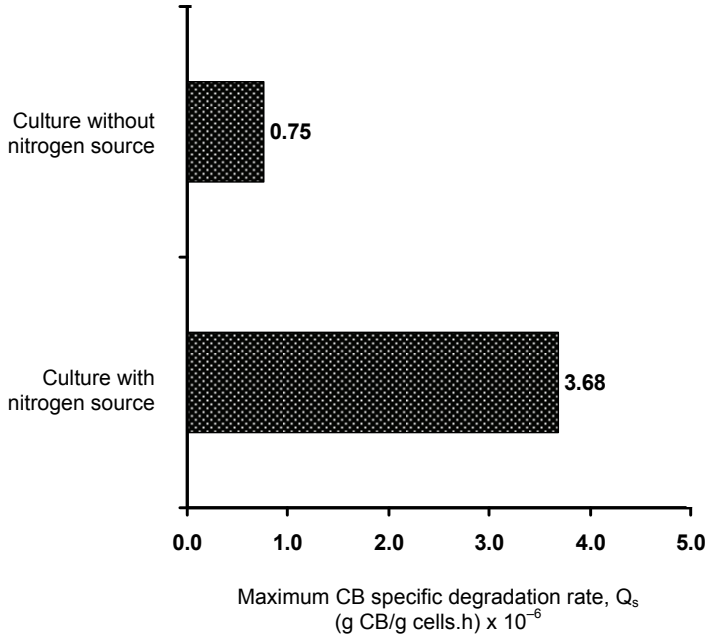

Figure 2: Specific degradation rates by culture supplied with and without nitrogen source (yeast extract).

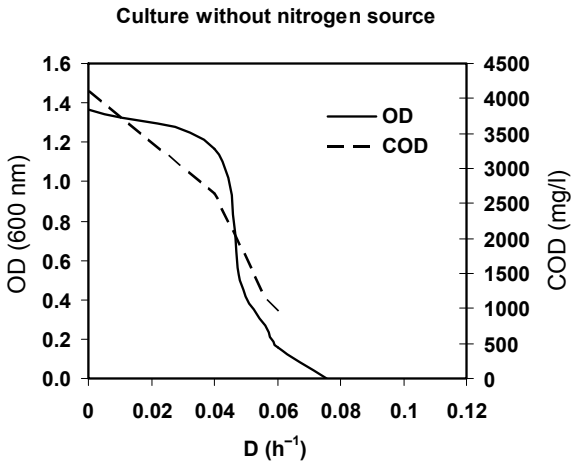

Figure 3: Growth and COD trend by culture with and without nitrogen source at different dilution rate, D *(continued on next page)*.

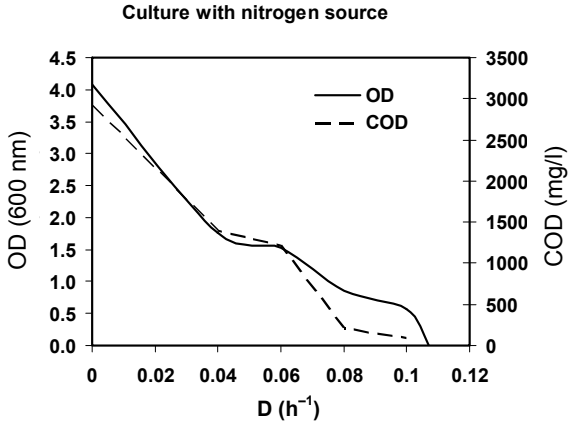

Figure 3: *(continued)*

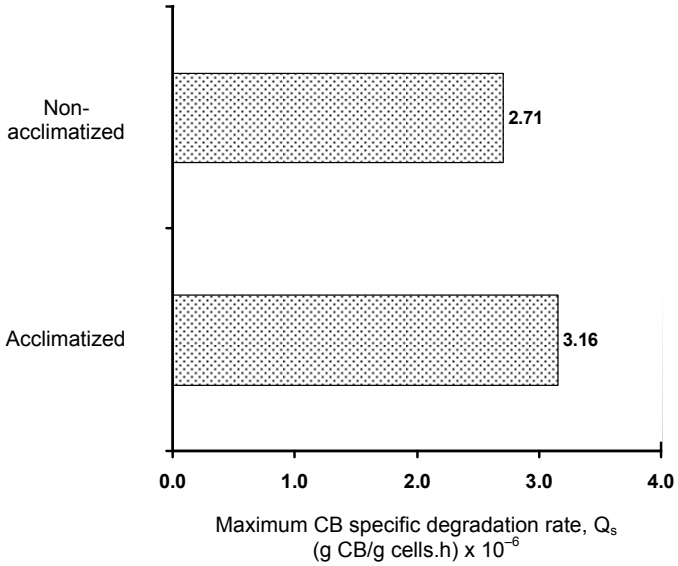

Figure 4: Specific degradation rate by culture with short-term acclimatization and without acclimatization prior to degradation test.

It is generally understood that the degradation rate obeys the first order kinetic, that is the rate of reaction strongly depends on the reactant's initial concentration. Five CB concentrations, namely 0, 0.0006, 0.0553, 0.1659 and 0.3317 mg/l were tested on fresh wastewater in batch mode. The aim of this study was to observe the characteristic of the first order kinetic degradation of CB

by our isolates, and finally to approximate the threshold level of CB degradability. Lower concentrations were chosen due to poor solubility of CB in aqueous phase. The representative in Figure 5 clearly showed that the Q_s (g CB/g cell per hour) was proportional to the initial CB concentration, which was exactly predicted earlier to follow the first order kinetic. It is also worth noting that no degradation was witnessed between 0.0006 mg/l and 0.0553 mg/l. One may conclude that this residual concentration was far below the threshold, in which the concentration of substrate below where cells cannot utilize the CB. Some other residual concentrations of variety of pollutants have also being recorded by other workers such as Rittmann and McCarty (1980), Button (1985), Schmidt *et al*. (1987), Van der Kooij and Hijnen (1988) and Van der Meer *et al*. (1987). Most importantly, the ability of our isolates from local wastewater to reduce CB level under Maximum Contaminant Level (MCL) set by Environmental Protection Agency (EPA 1999), i.e. 0.1 mg/l within two days has clearly indicated that the approach introduced, and the parameters estimated in this study has provided a useful guideline for future works.

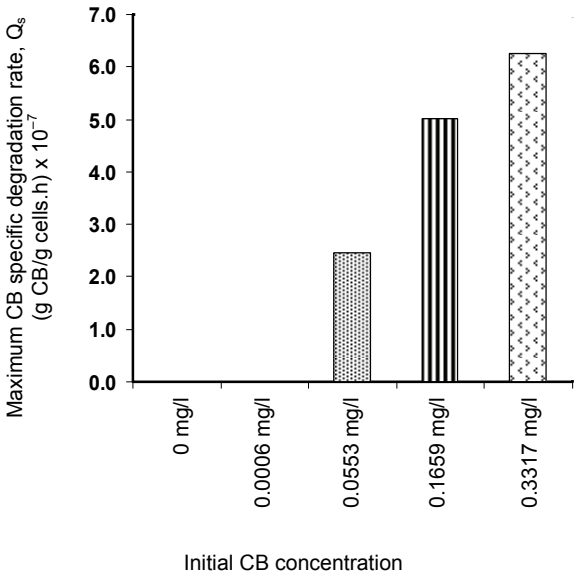

Figure 5: The specific degradation rates by cultures with different initial concentration of CB (mg/l), i.e. 0, 0.0006, 0.0553, 0.1659, and 0.3317 mg/l.

CONCLUSION

The potential of exploiting microbes from wastewater to solve chlorinated aromatic compound contamination problem has been successfully investigated in this study. The combination of two isolates, Gram positive and Gram negative bacteria have had synergistic effect, which successfully degraded CB in greater degree of degradation compared to pure individual one. In a chemostat study, the nitrogen source was found to be a critically limiting substrate. This study also found that the short-term adaptation step, as proposed in other work, has not produced a significant effect in degrading CB. Study in batch cultures revealed that the CB degradation rate perfectly obeyed the first order kinetic, and the residual CB concentration was between 0.0006 mg/l and 0.0553 mg/l.

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