

Aerobic degradation and metabolite identification of the *N*-heterocyclic indole by the *Pseudomonas putida* strain mpky-1 isolated from subtropical mangrove sediment

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Abstract: Aerobic biodegradation and metabolite identification of indole by the *Pseudomonas putida* strain mpky-1 isolated from coastal sediment of the Inner Deep Bay of Hong Kong was investigated in this study. This strain had 99.1% similarity with *P. putida* known. The biochemical degradation pathway of indole involved an initial hydroxylation reaction at the C-2 position to form oxindole followed by a second hydroxylation at the C-3 position to isatin prior to the cleavage of the 5-member carbon ring. This bacterium grew better at 22°C though it was capable of growth at low temperature (15°C in this study) with a longer lag phase. Both the bacterial specific growth rate and the biodegradation rate increased from 0.0035/hr to 0.0249/hr and from 15°C to 30°C, respectively. *P. putida* mpky-1 grew quicker at pH 6.4 (specific growth rate, 0.0115/hr) than pH 7.4 (specific growth rate, 0.0066/hr) and pH 8.4 (specific growth rate, 0.036/hr) although the lag time of bacterial growth at pH 7.4 and pH 8.4 (15.01 hr and 15.00 hr, respectively) was very similar. The decrease in bacterial growth rate was observed when salinity increased from 5‰ to 30‰. *P. putida* mpky-1 may adapt to the Mai Po and Inner Deep Bay and degrade indole due to the polluted condition.

Keywords: aerobic degradation, indole, heterocyclic aromatics, metabolic pathway, *Pseudomonas putida*, environmental factors

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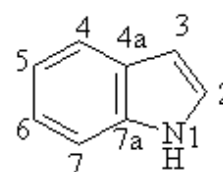
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1. Introduction

Organic aromatic compounds contain either homocyclic or heterocyclic nucleus and two-thirds of known organic chemicals are heterocyclics containing heteroatom (Kaiser et al., 1996). Organic heterocyclic compounds have cyclic structure(s) with more than one type of dissimilar atoms besides carbon atoms. Indole is a typical example of the aromatic *N*-heterocyclic compounds, in that there is a nitrogen atom in the pyrrole ring (Figure 1). Indole has a benzene ring fused with a pyrrole nucleus in the 2, 3-positions of the pyrrole ring (Sumpter and Miller, 1954). Heterocyclic aromatic compounds are more toxic than homocyclic ones and some of them including indole, 1-methylindole, 2-methylindole and 3-methylindole are also mutagenic and carcinogenic posing a serious threat to organisms including human (Dai-ley, 1981; Wilkes, 1981; Yip and Gu, 2016).

The Mai Po and Inner Deep Bay wetland, including the Mai Po Marshes, the extensive intertidal mudflats and freshwater fishponds, is the largest remaining wetland in Hong Kong. It was listed as a wetland of international importance



Indole

Figure 1. The chemical structure of indole with numerical labeling of the carbon and nitrogen position

under the Ramsar Conservation in September 1995. This wetland serves as breeding, feeding, resting and refueling station for between 50,000 to 80,000 birds in winter (Kueh and Chui, 1996). The mudflat, drained by the tide from the Inner Deep Bay, is one of the most ecologically important habitats in the Ramsar site. It is an important feeding ground for birds that migrate along the east Asian/Australian flyway. For instance, it supported over 67,000 wintering waterfowl in 1996 (Zheng et al., 2000). Of the 359 species

recorded in Mai Po, there are 106 bird species recorded on the mudflat, with 19 of them found only in this habitat (Zheng et al., 2000). However, this mudflat is subjected to intense pollution pressure because of the increasing discharge, including agricultural, domestic and industrial waste from Pearl River, other streams and, in particular, Shenzhen River, Yuen Long Creek and Kam Tin River. The extent of pollution in terms of metals and organics has also impacted the infaunal community (Shen et al., 2010; 2012; 2015; Zhao et al., 2012a, b). Yuen Long Creek and Kam Tin River are 2 of the 12 Priority Rivers in Hong Kong and were severely polluted (Chan, 1997).

The objectives of this study were to isolate and characterize aerobic indole-degrading bacteria from the sediment of mangrove; elucidate the biochemical indole-degradation pathway carried out by the isolated aerobic bacteria; and investigate the effect of environmental parameters including temperature, pH and salinity on the growth of the isolated indole-degrading bacterium.

2. Materials and Methods

2.1. Sources and Isolation of Indole-degrading Bacterium

Sediment from the mudflat of Mai Po Inner Deep Bay of Hong Kong SAR was used as an inoculum to enrich microorganisms capable of using indole as the sole source of carbon and energy. The microorganism was isolated with the enrichment culture technique. The mineral salts medium (MSM) used for the primary culture and several successive transfers consisted of: 0.8 g/L K_2HPO_4 ; 0.2 g/L KH_2PO_4 ; 0.05 g/L $CaCl_2$; 0.5 g/L $MgCl_2 \cdot 6H_2O$; 0.01 g/L $FeCl_3 \cdot 6H_2O$; 1 g/L $(NH_4)_2SO_4$; 18 g/L NaCl. The medium was adjusted to pH 7.5 with dilute NaOH or HCl and autoclaved at 120°C, 2 atm for 15 minutes. For the primary culture, about 2 g of sediments (wet weight) were added into each sterilized 250 ml conical flask containing 100 ml pre-sterilized MSM. 0.1 ml indole (0.035 g indole in 1 ml methanol) (purity, 99%) (Aldrich) was added to each flask after passing through a 0.2- μ m-pore-size-membrane syringe filter (Gelman, Ann Arbor, Michigan). The cultures were incubated on shaker at 120 rpm/min and 22°C. The experimental set up included triplicate for each treatment and one control (without sediments).

During the experiment, 1.0 ml of aliquot sample was withdrawn from each of the conical flasks and filtered through 0.2- μ m-pore-size-membrane syringe filter for analyzing the concentrations of indole in samples taken at daily intervals. The samples were identified and quantified by external standards of indole and possible degradation intermediates on a HPLC (Agilent 1100 series, Agilent Technologies) as described below (in Analysis of substrate compounds and identification of intermediates).

The microorganism was isolated using the enrichment culture technique. The new microsomes were made by inoculating 1 ml of previously established microsomes into flask containing freshly made MSM with indole when all

indole in the previously estimated microsomes had been exhausted mostly. Composition of the culture medium and the concentration of indole remained the same in all subsequent enrichment transfers. Four successive transfers were carried out in order to achieve a stable consortium and isolation of bacteria was initiated afterwards. All treatments were triplicates in each of the transfers.

2.2. Analysis of Substrate Compounds and Identification of Intermediates

The filtrates of culture samples were quantified by using HPLC (Agilent 1100 series, Agilent Technologies) consisting of a quaternary low-pressure degasser, a quaternary high-pressure pump, a model 7725i manual sample injector with a 20 μ l sample loop, and diode array and multiple wavelength detectors as described earlier (Gu et al., 2002; Yip and Gu, 2016). External standards were used to generate the calibration curve to derive the concentrations of parent compound or degradation intermediates from indole.

2.3. Identification of the Microorganisms

2.3.1. Purification of Culture

In order to purify the indole-degrading bacteria from enrichment cultures for identification, both spreading plate and streaking plate were used. Spreading plates were prepared using the fourth enrichment transfer culture by transferring 0.1 ml of the diluted culture (10^{-4} and 10^{-6}) to the center of each plate and spreading with pre-sterilized glass beads evenly before incubation. The selective agar plates were consisted of MSM as described above, 0.28 mM indole plus 1.5% agar (Lab M, United Kingdom). Spreading plates were carried out in duplicate for each dilution. All materials used above were autoclaved at 120°C, 2 atm for 15 minutes and procedures were conducted aseptically. After 4 days of incubation at 22°C, a number of tiny, separated, individual colonies could be seen on agar plates.

Individual colonies with different morphological appearances were streaked onto freshly made Nutrient Agar plates (Lab M, United Kingdom) and incubated for 2 days at 22°C. The streaked plates were assessed for purity based on colonial morphology and microscopic observation.

2.3.2. Confirmation of Pure Culture

Two of the well-separated, individual colonies on each streaked plate were chosen, then inoculated into pre-sterilized Nutrient Broth (Lab M, United Kingdom) serving as the subcultures. After incubation with shaking (200 rpm) at 22°C for 24 hours, fixed mounts were prepared for each subculture with incubation loops as described above. The slides were assessed under light microscope (Olympus CH-2, Olympus Optical Co. Ltd., Japan) to confirm the uniformity of cellular morphology and purity of the culture.

2.3.3. Preservation of Pure Cultures

One ml of each pure culture grown in Nutrient Broth medium was pipetted aseptically into sterile cryovials, amended with 0.3 ml pre-sterilized dimethyl sulphoxide (DMSO) and was stored frozen at 70°C as the Bacterial Culture Collection deposited in the Laboratory of Ecotoxicology. Pure cul-

tures were also streaked onto Nutrient Agar slants (Lab M, United Kingdom) and served as stock during experiments.

2.3.4. Smear Preparation and Gram Staining

Pure culture was routinely examined for purity. In addition, Gram staining was also conducted on the pure cultures. Heat-fixed culture mounts on glass slide were stained with crystal violet (primary stain) for 20 s. After the stain was washed off with distilled water gently, the smears were covered with Gram's iodine solution for 1 min. Afterwards, the smears were washed with 95% ethyl alcohol for 10 to 20 s to decolorize the Gram-negative bacteria. A counter-stain, Safranin was added to the smears for 20 s and then excess stain was washed off gently with distilled water (Benson, 1998). The Gram stained fixed mounts were observed under light microscope to determine the Gram stain reaction assess the cellular morphological features.

2.3.5. API 20NE Multitest System

The biochemical utilization profile of the isolated bacteria were investigated using the API 20NE Multitest Kit (BioMérieux, France), which is a convenient test system consisting of 20 microtubes containing different chemical substrates, and was used to further identify the isolated bacteria. A number of colonies were chosen from a fresh culture on Nutrient Agar plates to make a bacterial suspension. Tests were carried out following the instructions as described in the manufacturer's menu (BioMérieux 07615F, 2001). The biochemical results obtained were used for possible identification with similarity to those in the database by using Analytical Profile Index (BioMérieux, 1997).

2.3.6. 16S rDNA Sequencing

One pure colony of each pure culture was chosen from Nutrient Agar plates and was inoculated into pre-sterilized Nutrient Broths (Lab M, United Kingdom) for incubation on a shaker (200 rpm) for 24 hours at 22°C prior to the DNA extraction. The DNA extraction was carried out following the methods as described by Chachaty and Saulnier (2000). 1.5 ml of the bacterial culture was concentrated in a microcentrifuge to a compact pellet. The supernatant was discarded prior to in the addition of 567 µl Tris-EDTA buffer. It was mixed thoroughly and incubated with 30 µl sodium dodecyl sulfate and 3 µl of proteinase K for 1 hr at 37°C for lysis of bacterial cells' walls and digesting proteins. The content was then mixed thoroughly with 100 µl of 5 M NaCl for precipitation of cell wall debris, denatured protein and polysaccharides to CTAB. Then it was subsequently mixed thoroughly and incubated with 80 µl Hexadecyltrimethyl ammonium bromide (CTAB) and sodium chloride solution for 10 min at 65°C and 750 µl chloroform and isoamyl alcohol at room temperature. The viscous aqueous supernatant was collected carefully and the complete elimination of proteins and CTAB precipitates was completed by adding 500 µl phenol/chloroform and isoamyl alcohol (in 25:24:1) and centrifugation. Afterwards, DNA was recovered from the resulting supernatant by isopropanol precipitation (Chachaty and Saulnier, 2000).

DNA extracted stored in centrifuge tube at -4°C was sent to MIDI Labs, USA for 16S rDNA sequencing after ampli-

fication with PCR. Primers used for the amplification correspond to *E. coli* positions 005 and 531.

The obtained 16S rDNA sequences were analysed with BioEdit Sequence Alignment Editor (BioEdit version 5.09, Tom Hall 2001, North Carolina State University) by comparing with the public sequence database of National Center for Biotechnology Information (NCBI). The phylogenetic tree was produced by analyzing the alignment product with Molecular Evolutionary Genetics Analysis (MEGA version 2.1, Kumar et al., 2001).

2.4. Confirmation of Indole-degrading Ability of Isolated Bacterium

One individual colony on the streaked plate of pure culture was selected and inoculated into pre-sterilized MSM with 0.25 mM filter sterilized indole as described previously. During the experiment, 1ml of aliquot sample was withdrawn from each of the triplicate conical flasks and filtered through 0.2-µm-pore-size-membrane syringe filter for analysis of indole concentrations of over time of incubation (once a day). The concentration of indole was monitored by HPLC as described above. At the same time, the optical density of culture media at 600 nm (UV-1201V Spectrophotometer, Shimadzu) was measured for the bacterial growth.

2.5. Degradation under Different Environmental Conditions

Similar preparation as described above, treatments with different salinity, different pH and different incubation temperature were initiated to examine the environmental conditions on growth of *P. putida* mpky-1. The initial indole concentration for all treatments was set at 0.58 mM, which was also confirmed by HPLC analysis. Sodium chloride was used in preparing different salinity levels (0.5%, 1.8% and 3.0%) with a constant pH 7.4 for testing the salinity effects on bacterial growth. For testing the pH effects on the bacterial growth, pH values (6.4, 7.4 and 8.4) were adjusted with dilute HCl or NaOH. The cultures of 1.8% salinity and pH 7.4 were incubated at three different temperatures (15°C, 22°C and 30°C) in order to investigate the temperature effect. The cultures for salinity and pH effects were all incubated at 22°C. All treatments were incubated in shakers (200 rpm) and were carried out in triplicate.

The bacterial growth representing their ability in utilizing indole was monitored as optical density at wavelength 600 nm (UV-1201V Spectrophotometer, Shimadzu) at a regular time intervals (eight times per day) and the sampling times were progressively decreased as the bacterial growth curve became stable or dropped. Their specific growth rate (μ_m), maximum biomass (A) and lag time (λ) under different test conditions were calculated with the Gompertz model (Equation 1) which was found to be the most suitable model to fit the bacterial growth data and easy to use (Zwietering, 1990) among many developed models for describing the bacterial growth curve (Gu, 2016b; Richards, 1959; Schepers et al., 2000).

$$X = A \exp \left\{ -\exp \left[\frac{\mu_m e}{A} (\lambda - t) + 1 \right] \right\}$$

where X = Optical density; t = Time; μ_m = Specific growth rate; A = Maximum biomass; λ = Lag time.

2.6. Scanning Electron Microscopy (SEM)

One ml of the pure culture of bacterial isolate was filtered onto a 0.2 μm polycarbonate membrane filter (Gelman Science), the membrane filter was subsequently fixed with 3% glutaraldehyde in 0.2 M sodium cacodylate buffer overnight. The specimens were washed with 0.1 M sodium cacodylate three times for 2 minutes each rinse. They were fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate for 8 hours and rinsed with sodium cacodylate buffer and distilled water. The fixed samples were dehydrated with ethanol series of 40% to 80% ethanol in 10% increments and then 80% ethanol to 100% ethanol in 5% increments. They were further treated at SEM Unit of The University of Hong Kong for further preparation of critical-point drying and coating and SEM observation.

3. Results and Discussion

3.1. Degradability of Indole through Enrichment Cultures

In order to isolate the indole-degrading bacterium from sediments of Inner Deep Bay, the microorganisms in sediments were enriched with indole serving as the sole source of carbon and energy. Microorganisms were obtained using enrichment technique and subsequently characterized and identified using a combination of morphological, biochemical and molecular DNA techniques. The initial concentration (0.14 mM - 0.44 mM) of indole was confirmed by High-Performance Liquid Chromatography (HPLC). Indole concentration in culture flasks was monitored throughout the experiments. Indole in the initial (primary) culture (Figure 2) and all subsequent enrichment transfers (the first to the fourth) disappeared within 5 days (Figures 3, 4). At the same time, our sterile controls showed a negligible loss of chemical throughout the incubation period (Figures 2-4). Rate of complete indole metabolism was progressively increasing as evidenced by the shortening of time for complete degradation of indole. In the first enrichment transfer (Figure 3), very little amount of isatin was also detected when the initial concentration of indole was almost doubled compared with other cultures. In fact, the apparent lag phase before indole degradation disappeared in the third and fourth enrichment transfers (Figure 4) indicating the enrichment of specific bacteria and its ability in utilizing indole. In the initial culture and first 3 enrichment transfers, the production of oxindole was observed as indole disappeared (Figure 4). The appearance of oxindole and isatin indicated that they are transformation intermediates in indole degradation by microorganisms from the sediment.

The isolated bacterium was observed to be straight-rod shaped with the estimated size of $0.6 \times 2.4 \mu\text{m}$ (Figure 5) and stained Gram-negative. It was identified as *Pseudomonas putida* (% identity, 93.5) based on biochemical utilization profile using the API 20NE Multitest Kit (BioMérieux,

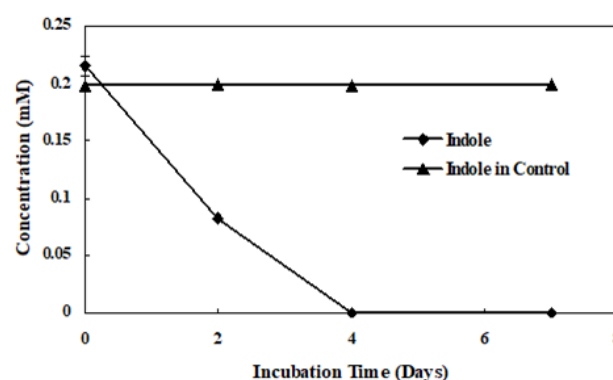


Figure 2. Degradation of indole in the initial enrichment cultures amended with sediment under incubation contribution. Bars represent standard deviations of triplicates

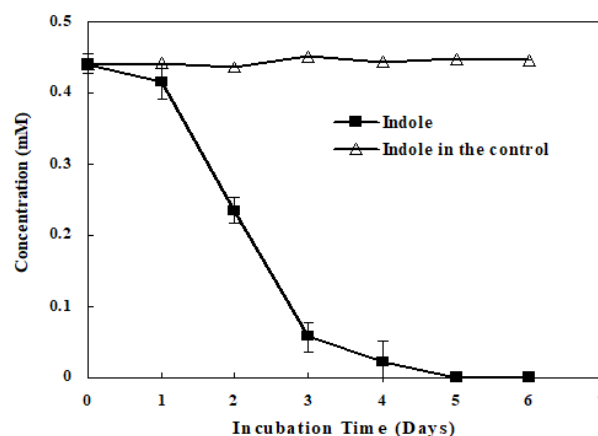


Figure 3. Degradation of indole in the first enrichment transfer cultures. Bars represent standard deviations of triplicates.

France) (Table 1). It was further confirmed to be *Pseudomonas putida* using data from 16S rDNA sequence. The neighbor joining tree (not shown) obtained showing that sample A, sample B, and *Pseudomonas putida* ATCC 17514 were grouped closely together and the difference among them was very little, less than 2.7%. We assigned our bacterial isolate *P. putida* strain mpky-1.

3.2. Environmental Effects on Bacterial Growth

Growth of *P. putida* strain mpky-1 on 0.58 mmol/L indole as the sole source of carbon and energy under different environmental conditions were investigated (not shown). Based on the biomass data obtained as O.D. at different times during incubation, Gompertz model was used to calculate parameters important growth related (Table 2) including specific growth rate (μ_m), lag time (λ) and maximum biomass (A). The model described temperature data best (r , 0.991-0.998), followed by pH data (r , 0.854-0.992) and salinity data (r , 0.792-0.978). *P. putida* strain mpky-1 grew quickest at temperature 30°C (μ_m , 0.0249/hr) compared with other conditions under the same treatment. *P. putida* also grew

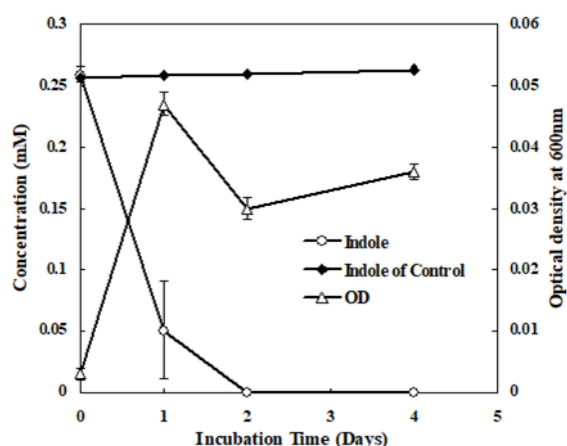


Figure 4. Degradation of indole in the forth enrichment transfer cultures. Bars represent standard deviations of triplicates.

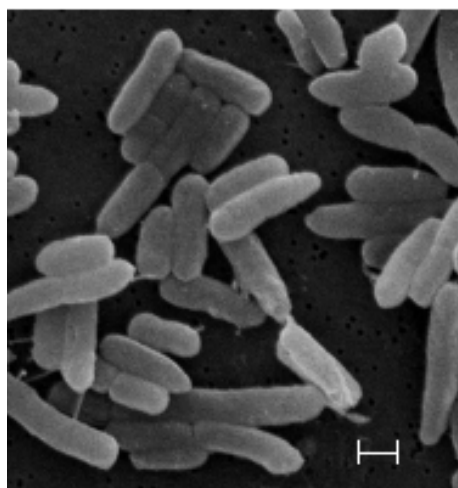


Figure 5. A scanning electron micro-graphs of the indole-degrading bacterium on surface of membrane filter after 12 hours of incubation. (scale bar, 1μm).

quickest at pH 6.4 (μ_m , 0.0115/hr) and salinity 5‰ (μ_m , 1.8211/hr) compared with other conditions. The lag phase (λ) was longer at 15°C (23 hr), lower pH (pH 6.4) (19 hr) and higher salinity (30‰) (25 hr). The total biomass was highest at 15°C, low pH (pH 6.4 and 7.4) and all salinity (A , 0.0976-0.1098).

3.3. Isolation and Characterization of Bacteria

Indole-degrading bacterium *Pseudomonas putida* strain mpky-1 was successfully isolated from the sediments of Inner Deep Bay of Hong Kong using enrichment and molecular techniques. This study emphasized the microbial degradation of indole by *P. putida* mpky-1. The degradation biochemical pathway was studied in details and the environmental factors including temperature, pH and salinity for the bacterial growth on indole as a sole source of carbon and energy were also monitored. The specific growth rate, lag time and maximum biomass were calculated using the bacterial growth stimulation equation for a better understanding of environmental factors on the degradative bacterium.

Table1. Biochemical utilization profile by *Pseudomonas putida* mpky-1

Substrates	Tested Results
Nitrate	+
Tryptophane	—
<u>Glucose</u>	—
<u>Arginine</u>	+
<u>Urea</u>	—/+ (24hr/48hr)
Esculin	—
Gelatine	—
p-nitrophenyl-β-D-galactopyranoside	—
Glucose	+
Arabinose	—
Mannose	+
Mannitol	—
N-acetyl-glucosamine	—
Maltose	—
Gluconate	+
Caprate	+
Adipate	—
Malate	+
Citrate	+
Phenyl-acetate	+
Oxidase	+

Note: Results indicated 93.5% identity (+) Positive result, i.e. *Pseudomonas putida* was capable of metabolizing the tested chemicals. (—) Negative result, i.e. *Pseudomonas putida* could not utilize the tested chemicals. Underlined chemicals were tested under anaerobic condition.

The isolated indole-degrading bacterium was identified as *Pseudomonas putida* based on phenotypic characteristics, biochemical utilization profile, and 16S ribosomal DNA sequence. High level of confidence in both API test (% identity, 93.5) and 16S rDNA sequencing (97.5% similarity) strongly suggested that the isolated bacterium was *Pseudomonas putida*. We assigned it as strain mpky-1. Its morphology has been documented by scanning electron microscopy. No apparent lag phase in the degradation curves (Figures 3, 4) indicated that the acclimation has successfully resulted in an effective indole-degrading microorganism.

Table 2. Cells growth of *Pseudomonas putida* strain mpky-1 under different condition. Its specific growth rate (μ_m), lag time (λ) and maximum biomass (A) under different environmental factors were calculated with the Gompertz model.

Experimental conditions	μ_m (/hr)	λ (hr)	A	r
Temperature 15°C	0.0035	23.0088	0.1221	0.9908
Temperature 22°C	0.0067	15.0166	0.0976	0.9787
Temperature 30°C	0.0249	16.6753	0.0911	0.9977
pH 6.4	0.0115	19.3757	0.0983	0.9925
pH 7.4	0.0066	15.0194	0.0985	0.9798
pH 8.4	0.0036	15.008	0.0521	0.854
Salinity 5‰	1.8211	4.1843	0.1014	0.7924
Salinity 18‰	0.0067	15.0166	0.0976	0.9787
Salinity 30‰	0.0017	25.2438	0.1098	0.9311

3.4. Degradation of indole and Mechanism Involved

Indole was metabolized in the initial culture, enrichment cultures and *P. putida* strain mpky-1. During the degradation of indole, two intermediates were observed on HPLC chromatogram. They were identified as oxindole (1,3-dihydro-2H-indole-2-one) and isatin (indole-2,3-dione) by comparing the metabolites with the authentic standards by using HPLC retention time and UV-visible spectrum. The identical retention times between the isolated metabolites and authentic standards indicated that the metabolites from indole degradation had identical properties to the authentic standards. During the degradation of indole, *P. putida* mpky-1 transformed indole by hydroxylation at the C-2 position and dehydrogenation to oxindole which was then further metabolized.

The little peak representing isatin with retention time 1.932 min appeared on the HPLC chromatogram but it could not be quantified because its concentration was too low. No significant accumulation of isatin in this investigation suggested the rapid transformation rate of isatin by the *P. putida* strain mpky-1. However, it may be detectable by increasing the initial concentration of indole and decreasing cell density of the inoculum because these two combined can decrease the microbial degradation rate of indole and hence its metabolites including isatin. Therefore, I propose that transformation steps of indole by *P. putida* mpky-1 were hydroxylation and then dehydrogenation at the C-2 position to oxindole (tautomer) followed by another hydroxylation and then dehydrogenation at the C-3 position to isatin (indole-2,3-dione) (Figure 6). Although no other metabolites were detectable and identified, as predicted by previous studies (Gu and Berry, 1991; Gu et al., 2002; Madsen and Bollag, 1989), the formation of isatin would be followed by the ring cleavage between C-2 and C-3 on the pyrrole ring of indole. Again, the failure of detecting other degradation products in this study may be the results of too rapid utilization of indole and its metabolites by *P. putida* mpky-1.

The mechanism of hydroxylation at C-2 position of indole was initially reported in microbial degradation of indole under various anoxic conditions except for aerobic one (Bak and Widdel, 1986; Fetzner, 1998; Gu and Berry, 1991; Gu et al., 2002; Johansen et al., 1997; Kamath and Vaidyanathan, 1990; Li et al., 2001; Licht et al., 1997; Shanker and Bollag, 1990; Wang et al., 1984). Under aerobic conditions, the first step of indole biotransformation was usually the hydroxylation at the position of C-3 instead of C-2 to form indoxyl which is an unstable compound and is quickly oxidized to indigo (blue pigment) in the presence of oxygen (Allen et al., 1997; Claus and Kutzner, 1983; Fujioka and Wada, 1968; Oshima et al., 1965; Sakamoto et al., 1953; Sebek and Jäger, 1962). Due to the instability of indoxyl, it is difficult to detect it directly on HPLC. However, the presence of blue pigment, indigo was proposed to be an indirect indication for the suggestive appearance of indoxyl in most previous studies (Allen et al., 1997; Claus and Kutzner, 1983; Fujioka and Wada, 1968; Oshima et al., 1965; Sakamoto et al.,

1953; Sebek and Jäger, 1962). During my experiment, no blue pigment has been observed in any of the experiments. Therefore, together with the evidence that oxindole was successfully identified on HPLC chromatogram, the first step of indole biotransformation should be the hydroxylation at the position of C-2. In fact, it is not surprising that a different mechanism is carried by *P. putida* strain mpky-1.

Under aerobic reaction, the source of oxygen for the hydroxylation reaction is molecular oxygen (a nucleophile). A nucleophile tends to attack the position with less electron density. Indole is an excessive molecule. In the pyrrole ring of indole, the position of C-2 has lower π charge density than C-3 does (Figure 6) so the hydroxylation prefers the position of C-2. Similarly, *P. putida* Chin IK being reported to utilize quinoline through first hydroxylation at C-2 position which has lower π charge density compared with other positions in the heterocyclic ring (Blaschke et al., 1991) indicated that π charge density might determine the position of hydroxylation carried out by microorganisms enzymatically. Hydroxylation at the C-2 position of pyridine in both aerobic and anaerobic quinoline degradation (Blaschke et al., 1991) suggested that aerobic and anaerobic degradation of the same compound could be preceded in the similar mechanisms. Different microorganisms might degrade the same compound in number of ways. During the experiment, growth of *P. putida* mpky-1 occurred utilizing both indole and oxindole while sulfur-reducing bacteria *D. indolicum* DSM 3383 could grow only by the transformation of oxindole (Licht et al., 1997).

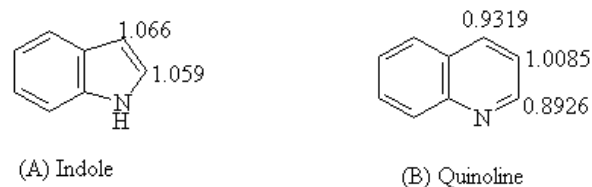


Figure 6. π charge distribution in the heterocyclic ring of indole and quinoline (modified from Li et al., 2001)

Degradability of a chemical is dependent highly on its chemical structure such as the presence of substituted groups. 0.04 mM of 1-methylindole and 0.1 mM of 2-methylindole could not be metabolized throughout the experimental incubation period (30 days) in the initial cultures using the same sediment from Mai Po (data not shown). Due to the inconclusive results of these enrichment cultures, no further attempts were made to isolate the possible bacteria involved because the transformation of the substrate was not substantiated. However, in the initial culture with indole, 0.22 mM of indole was metabolized within 4 days. Obviously, it demonstrated that the degradation rate of indole was much quicker than that of 1-methylindole and 2-methylindole. The similar results have also been reported under methanogenic and sulphate-reducing conditions (Gu and Berry, 1991; Gu et al., 2002; Johansen et al., 1997). Moreover, it has been reported that 3-methylindole (Gu and Berry, 1991; Johansen et al., 1997) and 7-methylindole (Jo-

hansen et al., 1997) could be hydroxylated to 3-oxindole and 7-oxindole respectively, but they could not be further degraded under some conditions. Mineralization of 3-methylindole was only reported under methanogenic conditions in an enrichment consortium obtained from pig manure lagoon (Gu and Berry, 1992). It is probably due to the presence of methyl groups preventing the attack by hydroxylation enzymes in microorganisms.

3.5. Effect of Environmental Factors

The metabolic activities of microorganisms often influence the fate of organic pollutants in the environments. However, the biodegradability of a chemical may be misleading if based on results from only one site or one type of environment. Therefore, the study of bacterial response to environmental factors is very important. To my knowledge, environmental factors affecting indole metabolism was only investigated under anaerobic conditions for the concentration of sewage sludge, temperature and pH were reported in that study (Madsen et al., 1988). The present study investigated the effect of temperature, pH and salinity on growth of *P. putida* mpky-1 with indole as a sole source of carbon and energy. According to the degradation curve obtained, the bacterial growth can provide further information relating to indole-degradation.

The effect of temperature on the growth of the bacterium *P. putida* mpky-1 was measured at incubation temperature of 15°C, 22°C and 30°C. Based on the experimental results, *P. putida* mpky-1 showed optimal growth at 30°C because the specific growth rate (μ_m) was 3-8 times higher than that of 15 and 22°C. The bacterial specific growth rate was higher at higher temperature (Table 2). This further indicated that non-pathogenic *P. putida* might have great potential in bioremediation. The similar maximum biomass was achieved under all three temperatures indicated the indole could be metabolized at all three temperatures. Some bacteria such as psychrotrophs may have nonzero growth and biodegradation rates at temperature as low as 0°C (Onysko et al., 2000). Temperature is one of the critical factors in designing a biological treatment process and hence studying temperature effects on microbial kinetics. Furthermore, lag phase was short at higher temperature suggesting a quick entry to active metabolism.

The pH value representing the concentration of hydrogen and hydroxyl ions affects the growth of microorganisms either directly or indirectly via its influence on the ionic state and the availability of many inorganic ions and metabolites to the cells. *Pseudomonas* sp. is one of the heterotrophic bacteria capable of withstanding acidic environment (Schlegel and Jannasch, 1992). *P. putida* mpky-1 grew quicker at pH 6.4 (μ_m , 0.0115/hr) than in pH 7.4 (μ_m , 0.0066/hr) and pH 8.4 (μ_m , 0.036/hr) although the lag time of bacterial growth at pH 7.4 and pH 8.4 (15.0194 hr and 15.0080 hr, respectively) was similar. However, the maximum biomass (*A*) achieved at pH 8.4 was much lower than that at pH 6.4 and pH 7.4, indicating that *P. putida* mpky-1 could not metabolize the indole or oxindole efficiently for energy source and

growth. Therefore, the pH regime should be considered for the application of this bacterium.

The effect of salinity on the growth of *P. putida* mpky-1 was investigated for salinity of 4‰, 18‰ and 30‰. Although the *r*-value for the bacterial growth at salinity of 5‰ was too low to be reliable, it is already ecologically significant. The low *r*-value was a result of missing data points in the lag phase period. However, the decrease in bacterial growth rate was observed from salinity of 5‰ to 30‰ (data not shown). In other word, the bacterium is sensitive to higher salinity.

In conclusion, *P. putida* strain mpky-1 was isolated for its ability to completely degrade indole as the sole source of carbon and energy. The bacterium was confirmed based on morphological, biochemical and molecular techniques. Degradation of indole was carried out through two intermediates, oxindole and isatin which has been only observed in anaerobic enrichments. The best environmental combination for indole degradation by *P. putida* strain mpky-1 is at temperature 22°C, pH 6.4 and salinity of 5‰. This bacterium may be an important species degrading *N*-heterocyclic aromatic compounds in the environment.

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Conflict of Interest Declaration

Authors have no conflict of interest in the research reported here.

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