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Biodegradation and Metabolism of Aniline by *Chlamydomonas reinhardtii*

Chi Zhu[1,2]*, Bing Li[1], Shui Wang[1], Wenjie Chang[1] and Weixiang Wang[1]

[1]Jiangsu Provincial Academy of Environmental Science,
Jiangsu provincial key laboratory of environmental engineering, Nanjing 210036, China.
[2]State Key Laboratory of Lake Science and Environment, Nanjing Institute of Geography and Limnology, Chinese Academy of Sciences, Nanjing 210008, China.

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The biodegradation and responses of detoxification enzymes in the freshwater green algae, *Chlamydomonas reinhardtii,* upon exposure to 10 mg/L concentration of aniline were investigated. Results showed that aniline was metabolized, and bioaccumulation and adsorption were 1.2%. The removal of aniline over an 8-day period in the control medium and with the group of ABT was 32% and 35%, respectively. With algae culture the removal of aniline was 56%. About up to 24% of total aniline was biodegraded or biotransformed by *C. reinhardtii* in 8 days. *C. reinhardtii* biotransformed aniline into acetylaminobenzene and the cytochrome P-450 was the key enzyme in the metabolic pathway.

Key words: Aniline; Degradation; Chlamydomonas reinhardtii; Detoxification; Enzyme; N-acetylation.

Aniline is a natural product and an important industrial chemical. It is used in a number of manufacturing processes, particularly in the dye industry (Aoki and Nakanishi, 1990). Aniline is highly toxic and it is readily absorbed through the skin in dangerous amounts and is fatal. Aniline is recognized as a serious environmental contamination because it is increasingly released into soil and water environments each year owing to its use in the production of dyes, plastics, herbicides, and pesticides. It is marked as one of the priority pollutants.

There were reports of soil and activated sludge bacteria which were able to use aniline as a sole source of carbon and in some cases biochemical studies of aniline metabolism have been initiated (T. Kayashima *et al*., 2013; Asakura and Okazaki, 1995; Cousins and Freestonc, 1995; Bollage and Russel, 1976). Previous studies on the aerobic microbial degradation of aniline have demonstrated that it can be metabolized to catechol as the first intermediate, liberating ammonia, and subsequently undergoing metabolic transformations through *or*- *tho*- or *meta*-ring cleavage pathways (Surovtseva, E.G.. *et al*., 1980; Zeyer, J. *et al*., 1985;) in order to enter central metabolism. Under anaerobic conditions, the first report of a pure culture capable of aniline dissimilation was made by Schnell *et al*. (Schnell, S. and Schink, B. 1991) who demonstrated that *Desulfobacterium* was capable of metabolizing aniline via reductive deamination of 4-aminobenzoyl-CoA, in a process that was linked to sulfate reduction. A novel microorganism, strain HY99, was found to aerobically metabolize aniline via catechol and 2-hydroxymuconic semialdehyde intermediates, and to transform aniline via *p*-aminobenzoate in anaerobic environments. Strain HY99 was able to metabolize aniline under anaerobic conditions linked with nitrate reduction

* To whom all correspondence should be addressed.
Tel : +86–25–86619653; Fax : +86–25–86619260
E-mail: zhuchi126@126.com

(Kahng, H.Y., and Kukor, J.J. *et al*., 2000). A highly efficient aniline degrading Strain H62 could degrade 100% of aniline at the concentrate of 800mg/L after 15h (Sheng duohong *et al*., 2002).

In addition, fungi were known to transform anilines to the N-formyl-N-malonyl, N-acetyl, N-succinyl, N-hydroxyglutaryl and N-glycosyl conjugates (Hallinger, *et al*., 1988; Still and Herett, 1976; Winkler and Sandermann, 1992) as well as to unusual cyclic succinimides (Arjmand and Sandermann, 1987). It was found that the fungus, *Fusarium* sp. and *Rhizopus* sp. utilized aniline as a sole nitrogen, carbon and energy source, with production of acetanilide and catechol. *Fusarium* sp. utilized 70% of 10 mmol aniline and produced 3.55mM ammonia during 30 days. *Rhizopus* sp. utilized 65% of 10 mmol aniline during 30 days (Emtiazi G and Satarii M, 2001).

More recently, higher plant had the ability to absorb and detoxify aniline. Transformation of aniline in axenic seedling of maize (Zea mays L.), kidney bean (phaseolus vulgaris L.), pea (Pisum sativum L.) and pumpkin (Cucurbita pepo L.) were studied.

The metabolism of aniline in the zebrafish (Brachydanio rerio) was studied. And the biotransformation product of aniline in the zebrafish was analyzed by HPLC. Aniline was transformed into acetanilide (Zok S and Gorge G *et al*., 1991). The metabolism of aniline in medaka (Oryzias latipes) was also investigated, the evidence suggested that polar conjugates were the dominant in via aniline metabolites (Bradbury SP et.al., 1993). The activities of two enzymes from phase I of biotransformation, aniline hydroxylase and ethylmorphine N-demethylase were investigated in turkey embryos and poults (Darko S *et al*., 2003). The biotransformation of aniline in rainbow trout (Oncorhynchus mykiss) isolated liver cells was investigated. It was showed that aniline was mainly metabolized to acetanilide and to a lesser extent to 2-aminophenol by isolated hepatocytes (Lafuente, A. *et al*., 1999). In 1980, perfusion of isolated rat liver with [^{14}C] aniline revealed apparent enzyme kinetics for 4-aminophenol formation (Eyer P and Kampffmeyer H *et al*., 1980).

Algae are a group of microorganisms that can play a role in determining the fate of toxic compounds but this has not been widely studied. Algae have a potential for removal of organic compounds that includes accumulation and degradation (Stock S.L. and Rittman B.E., 1986). A number of compounds are accumulated by algae including pesticides DDT, aldrin, dieldrin, endrin, lindane, mirexmethoxychlor, toxaphene, parathion, carbaryl, and chlordane (Mouchet P., 1986). Degradation of low phenol and catechol to CO_2 by some freshwater algae has been described (Ellis B.E., 1977). Phenolic was found to be degradaed easily by *Stains of Chlorella sp., Scenedsmus obliquus* and *Spirulina maxima* (V. Klekner and N. Kosaric 1992). In 2002 Gabriele Pinto and Antonino Pollio found that the two green microalgae, *Ankistrodesmus braunii* and *Scenedesmus quadricauda* could degrade phenols (400mg ml^{-1}) with a removal greater than 70% (Gabriele Pinto and Antonino Pollio, 2002). Procaryotic and eukaryotic photoautotrophic marine algae, including cyanobacteria (blue-green), green algae and diatoms (red and brown), were shown to metabolize a two ring PHA, naphthalene, to a series of metabilites (C. E. Cerniglia, *et al*., 1979; 1980; 1980; 1982) with the capability of conjugating the metabolites (C. E.Cerniglia, *et al*. 1980; 1982). Cyanobacteria have been shown to degrade both naturally occurring aromatic hydrocarbons (Cerniglia, C. E. *et al*., 1979; 1980; 1980; EillB. E. 1977; Narro, M. L, *et al*., 1992), and xenobiotics (Megharaj, M., *et al*., 1987). Rontani *et al*. (1997) reported the rate and mechanism of light-dependent degradation of sterols in senescent cells of *Skeletonema costatum*, a diatom widespread in coastal waters. Wang *et al*. (2007) investigated the photodegradation of aniline in the presence of the freshwater algae *Nitzschia hantzschiana*, *Chlorella vulgaris*, *Chlamydomonas sajao* and *Anabaena cylindrica*. Liu *et al*. (2007) investaged the methods for the determination 4-Bromoaniline (4-BA) in green algae *Chlamydomonas reinhardtii*, they found the continuous-flow microextraction- high-performance liquid chromatography (CEME-HPLC) technique was a satisfactorily method.

In animals and higher plants, selected xenobiotics are degraded or biotransformed through phase I (cytochrome P-450) and phase II (conjugation) enzymes. The cytochrome P-450 system is believed to be involved in the biotransformation of herbicides in unicellar green algae *Chlorella sorokiniana* and *Chorella fusca*

(Thies *et al*., 1996), but degradation of the PAH benzo(a)pyrene (B[a]P) in freshwater green alga, *S. capricornutum*, was mediated through dioxygenase and the metabolites were then conjugated to sulfate and glucose (Warshawsky *et al*., 1988, 1990). For plants, glutathione conjugation is a major pathway to detoxify organic xenobiotic (Wolf AE, 1996). It was found that the wheat (Tritieum aestivum) and soybean (Glycine max) shared a common metabolism for 2,4-DCP and 2,4-DCA since the malonylated glucoside conjugates were found as the final major metabolites (Pascal-Lorber, 2003). DCA was metabolized to N-malonyl-DCA in soybean and N-glucosyl-DCA in Arabidopsis (Lao SH, *et al*., 2003). From the above, we could see that the algae metabolize the xenobiotics in two ways.

Algae are found in the environment, are capable of producing their own food through photosynthesis and are present in a wide range of sizes from unicellular to structures comparable to terrestrial plants. The metabolism of xenobiotics has mainly been investigated in higher plant species. However, the mechanisms of the degradation of toxic compounds by algae, and the reason why algae perform these reaction and what is the extent of the degradation, are not understood. Thus, we selected common widely used green algae, *Chlamydomonas reinhardtii,* and tested them as to whether or not they have the capability to degrade aniline.

MATERIALS AND METHODS

Chemicals

Aniline (99% purity), 1-aminobenzotriazole (ABT) were purchased from Sigma Chemical Co. All other chemicals used were of analytical grade.

Culture conditions

An axenic culture of *Chlamydomonas reinhardtii* was obtained from FACHB-Collection of Institute of Hydrobiology, Chinese Academy of Sciences. The *Chlamydomonas reinhardtii* was culture in CHU-11 medium in 250 ml Erlenmeyer flasks at 25°C under illumination of 45mEm^{-2}s^{-1} with a 12:12 h light : dark cycle in an environmental chamber. pH of the medium ranged from 7 to 8, and was not adjusted throughout the experiment.

Removal of aniline

Exponential phase *Chlamydomonas reinhardtii* were inoculated into CHU-11 medium (100ml) to a cell density of concentration of 1×10^5 cell ml $^{-1}$. Aniline (EC50=70 mg l^{-1}) was added to a final concentration of 10 mg l^{-1}. In order to determine whether cytochrome P-450 was involved in the biotransformation of aniline, the effect of aniline on the growth of the algae in the presence of specific cytochrome P-450 inhibitors (ABT) was investigated. The EC50 of ABT on the *C. reinhardtii* was 48 μM. The following treatments were set up: (a) aniline added to control medium without algae cells, (b) aniline added to medium with algae cells, and (c) aniline plus 30u mol/l ABT in medium with algae cells. Three replicates of each were set up for the control and treatment samples. Each treatment contained an initial density of 1×10^5 cell ml $^{-1}$. All replicates were incubated for 8 days under the same condition as described above. During the incubation period, 4 ml samples were collected from each replicated at days 0,2,4,6 and 8 for analysis.

The concentration of aniline was measured using the following protocol. Culture media samples (4 ml) were centrifuged at 12000rpm for 2min to remove the algae cells and aniline was measured using spectrophotometer method with N-(1-naphthyl) ethylenediamine in the supernatant fluid. The density of algae cells in the culture was counted using a hematocytometer, and growth rates were determined over time.

Enzyme assays

The results of the prerious experiment showed that the growth rate of the green algae were not affected by 10mg l^{-1}aniline, the activities of the antioxidant enzymes (i.e. superoxide dismutase (SOD), peroxidase (Pox), and conjugation enzyme (GST) were, therefore, determined in the algae cells after 96h exposure to aniline.

Enzyme-Extract Preparation

Cells from the control and treatment samples (96h old) were harvested by centrifugation at 10000rpm for 10 min at 4°C. The cell pellet was resuspended with 10ml 0.1 M sodium phosphate (pH 7.6) containing 0.1%Triton X-100 and transferred onto the glass bead surface of a bead-beater. Additional buffer (0.8mL) was added to top

off the beads to ensure that the suspension was distributed evently. The beadbeater was placed in an ice bath and allowed to cool for 5 min .The sample was homogenized for three 20-s bursts with 30-s intervals to allow cooling. The homogenate with beads was then transferred to the centrifugation tubes with 4 ml sodium phosphate buffer (pH7.6). The homogenate was centrifuged at 10000 rpm for 10min at 4°C and the resulting pellet discarded. The supernatant was used as a source of cell-free extract for use in enzyme activity assays. Spectrophotometric measurements were performed with ELISA. SOD (EC1.15.1.1) was assayed using the method of beyer and Fridovich (1987). Reduction of NBT was determined at 560 nm, and 1 unit of SOD activity was defined, as the amount of enzyme required to inhibit NBT reduction by 50%. POD (EC1.11.1.7) activity was determined using the method of Putter (1975). The rate of increase in absorbance at 470 nm was measured at 25°C. Enzyme activity was calculated with an extinction coefficient of 26.6 $mM^{-1}cm^{-1}$ for tetragaiacol. GST (EC2.5.1.18) activity was determined using CDNB as the substrate (Habig and Jakoby, 1981). The increase in absorbance at 340 nm was measured at 25°C and the enzyme activity was calculated with an extinction coefficient of 9.6$mM^{-1}cm^{-1}$. Protein content of the homogenates was determined according to Bradford (1976), using crystalline bovine serum albumin as the standard.

The analysis of the medium extracts with GC-MS

The analysis of derivatives of hydrolytic products of aniline was performed on a FINNIGAN Trace 2000GC directly connected to Single quadrupole Mass spectrometer (THERMO Electron GC-MS). The mass spectrometer operated in the EI auto mode with 70-ev ionisation energy was used for full scan at a m/z range from 35to 350 Da. Standard injection port, 250°C, splitless injection, 0.2ul injection volume; capillary column,hp-5(30m,0.25mmi.d and 0.32µm film thickness; column temperature program, 50°C(1min)—-10°C/min—-180°C(1min)—-20°C/min—-240°C; carrier gas flow rate, helium 1.0ml/min.Instrument control and mass spectrometry data were managed by a Saturn GC/MS workstation

The samples at different time intervals were taken in a separatory funnel. The residues and derivatives of aniline were extracted three times, each with 15ml of trichloromethane and vigorous shaking for 3 min. The organic phase was collected and combined together. The extract was filtered with a glass funnel filled with anhydrous sodium sulfate, then was concentrated to near dryness on a rotary vacuum flash evaporator by rotary evaporator, and the final volume was made in tirchloromethane for GC/MS analysis.

RESULTS

Kinetics of the biodegradation of aniline by Chlamydomonas reinhardtii

In the present study, the growth rate of the *C. reinhardtii* was not affected compared to the control. The removal of aniline over an 8-day in the control medium and *C. reinhardtii* culture was 32% and 56%, respectively (Fig.2). The removal of aniline in the control over time may be attributed to photodecomposition and evaporation. The additional removal of aniline in the *C. reinhardtii* culture (24%) may be due to factors such as the adsorption of the chemical to the cell surface, aniline uptake, or biodegrdadation or biotransformation of chemical by the *C. reinhardtii.* To differentiate the possibilities of aniline being: (a) adsorbed and stored; and (b) degraded/biotransformed by the *C. reinhardtii* cells, residual concentrations of aniline were measured in *C. reinhardtii* cells. These results showed that the amount of aniline adsorbed or

Table 1. Induction of enzyme activities (unites per mg protein) upon exposure to aniline

Enzymes	Concentration of aniline				
	0 mg l^{-1} (control)	10 mg l^{-1}	30 mg l^{-1}	50 mg l^{-1}	100 mg l^{-1}
GST	16.5±0.2	17.5±0.9	20.4±0.2	64.9±3.4	23.2±1.3
SOD	43±1.3	49.2±0.9	62.2±17.6	90.5±12.9	196.3±11.3
POD	2.8±0.3	3.2±0.1	3.6±0.5	11.6±3.9	4.2±0.9

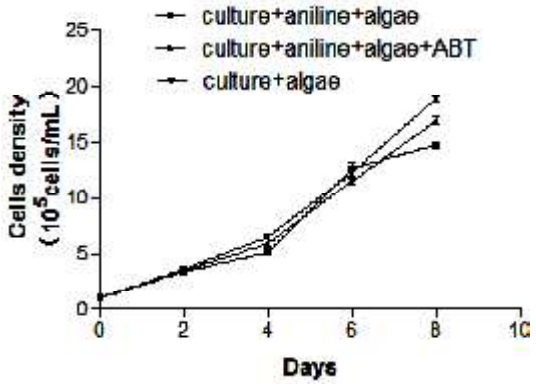

Fig. 1. Growth of *Chlamydomonas reinhardtii*

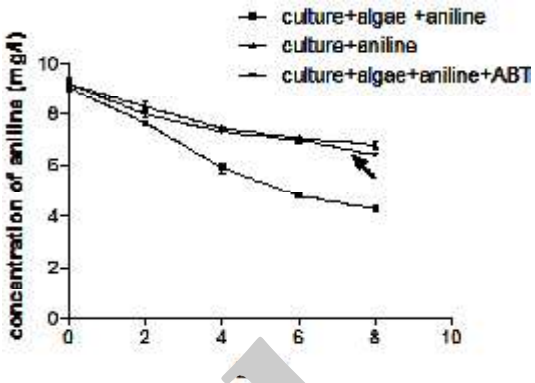

Fig. 2. Removal of aniline by algae. Arrows indicate addition of ABT

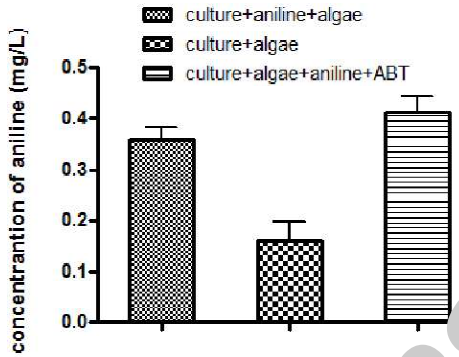

Fig. 3. Bioaccumulations and Adsorption of aniline by algae on the 8th day

stored by *C. reinhardtii* cells (0.12µg/mL), and only accounted for 1.2% of total loss of aniline. Thus, it could be concluded that up to 24% of aniline in the *C. reinhardtii* culture was due to biotransformation or biodegradation by the *C. reinhardtii*. In this study, the loss of aniline in the *C. reinhardtii* culture was also positively correlated with the exponential growth of the *C. reinhardtii* (Fig.2). The biodegradation processes of aniline fit a one-order biodegradation kinetic equation ($\tilde{N}3(S_t/S_0) = 0.1007t-0.0095$, $r^2=0.9540$, $p=0.0233$), and therefore, further supports the hypothesis that

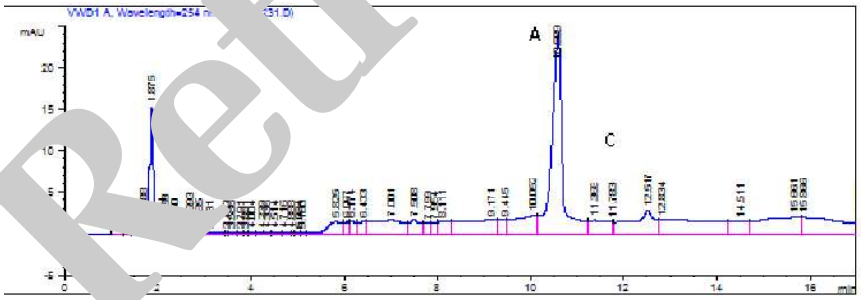

Fig. 4. The profile of HPLC on the 8th day without algae

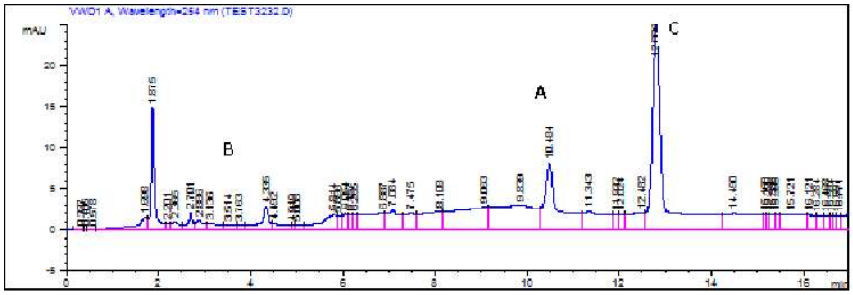

Fig. 5. The profile of HPLC on the 8th day with algae

adsorption, uptake and biodegradation of aniline resulted in a loss of the chemical from the test solution.

The addition of 30 µM ABT inhibited the removal of aniline. Up to 35% of the total aniline in 8 days (Fig. 3) was eliminated, compared to the removal of aniline (56%) in the *C. reinhardtii*

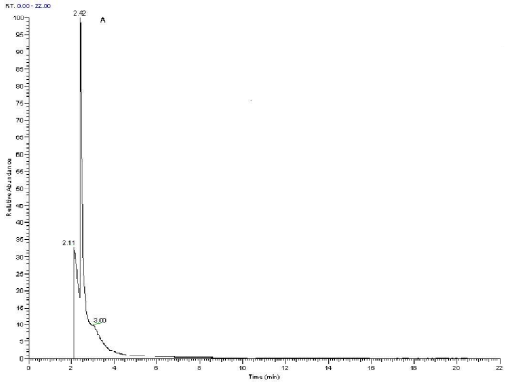

Fig 6. The control of medium without algae on 4th day by GC

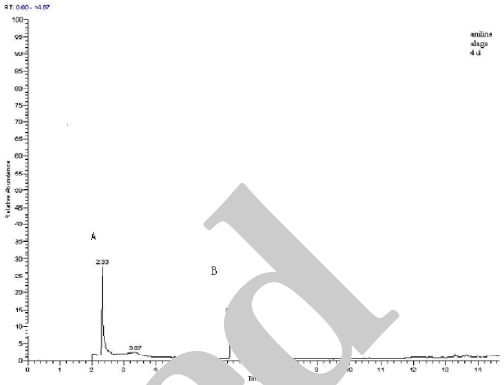

Fig 7. The profile of GC on the 4th day with algae

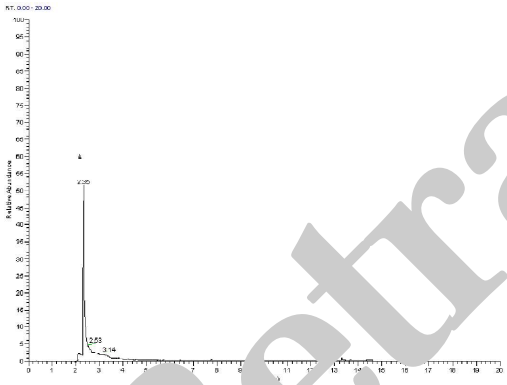

Fig 8. The control of medium without algae on 8th day by GC

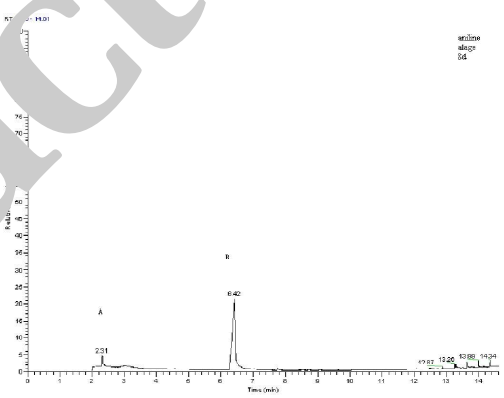

Fig 9. The profile of GC on the 8th day with algae

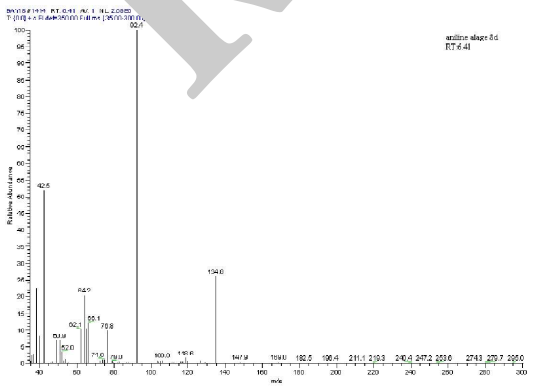

Fig 10. Mass Spectrum of metabolite in the medium with algae and aniline on the 8th day (Fig. 7. peak B)

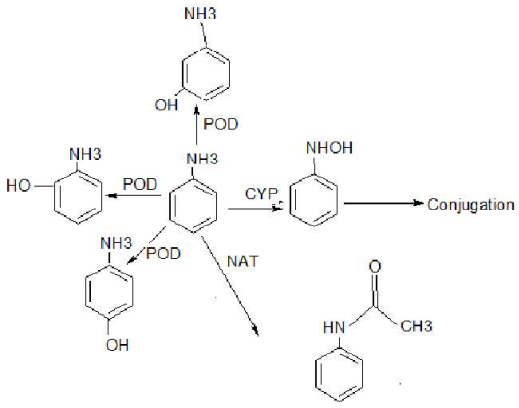

Fig 11. Proposed scheme of aniline metabolism in *C. reinhardtii*

culture. It was concluded *C. reinhardtii* contained active species of Cyt P450 and could act an important role in the metabolism of aniline.

Responses of conjugation and antioxidant enzymes

A short-term (96 h) experiment was conducted to investigate responses of conjugation and antioxidant enzyme when *C. reinhardtii* was exposed to aniline. Activity of GST increased (4 times) when was exposed to 50 mg l^{-1} aniline. Significant increases in the activities of SOD (4 times) when exposed to 100 mg l^{-1} aniline and POD (4 times) when exposed to 50 mg l^{-1} aniline. The result was in agreement with the report that the transformation of Bap in marine and freshwater algae depended on the presence and activity of enzymes locatized in the plant cells and the most important enzyme systems for detoxification of Bap are O-diphenol oxidase, Cyt P450, and peroxidase (Kirso U and Irha N, 1998).

Degradation product

By analysis of the medium extracts with GC-MS, the main product of the biodegradation of aniline was acetylaminobenzene.

DISCUSSTION

Corbett and Chipko (1978) found that the green alga *Chlorella* could convert nitrosobenzene and phenylhydroxylamine to N-phenylacetohydroxamic acid and N-phenylglycolhydroxamic acid. But the alga could not metabolize aniline (Corbett MD, 1978). In the present study, the removal of aniline was high in an axenic culture of *C. reinhardtii,* as compared to the control medium, and up to 24% of aniline disappeard in *C. reinhardtii* culture within 8 days. Furthermore, the removal of anline increased with an increase in *C. reinhardtii* biomass, while accumulation and adsorption of aniline within *C. reinhardtii* cells was negligible. The experimental evidence presented in this study, therefore, demonstrated that *C. reinhardtii* was able to metabolize aniline. The low bioaccumulation in *C. reinhardtii* cells may be due to the low log K_{ow} (0.98 in freshwater) of aniline. pH of the medium (7-8) would not effect the undisssociate forms of aniline and hence bioaccumulation potential.

In the higher plants, xenobiotics are generally metabolized through sequential phases of degradation, conjugation and compartmentation (Komo²a *et al*., 1995). Although the cytochrome P-450 system is considered to be the principal enzyme complex involved in phase I transformation, POD has also been reported to play a significant role in phase I reaction in higher plants, such as Lemna minor (Roy *et al*., 1995). Recently, cytochrome P-450 activities have been demonstrated in the marine *chlorophytes*, *chrmophytes* and *rhodophyted* (Pflugmacher and Sandermann, 1998). Thies *et al*., (1996) reported that addition of 20 µM PBO and 20 µM ABT reduced the P450-catalysed N-demethylation of herbicide merflurazon by 46% and 43% in green algae (C. Sorokiniana). In this experiment, however, 10 mg L^{-1} aniline (EC50=50 mg L^{-1}) and 30µM ABT showed no effect on *C. reinhardtii* growth rate. The removal of aniline over an 8-day period in the control medium and with the group of ABT was 32% and 35%, respectively. With algae culture the loss of aniline was 56%. It was found that the biodegradation could be totally inhibited by cytochrome P-450 inhibitor ABT (1-aminobenzotriazole). The results therefore implied that the cytochrome P-450 system was important in the detoxification of aniline in *C. renhardii*. In the study, Cyt P450 was very important enzyme in the metabolism of aniline.

POD is considered to be an important enzyme in metabolizing benzo-[a]-pyrene in many marine *chlorophytes* (*Enteromorpha intestnalis, Cladophora glomerata*) and *chara* (*Chara aspera*) (Kirso and Irha, 1998). Plant cell peroxidases were found to be involved in the activation of the promutagenic aromatic amines by tobacco cells (Wagner *et al*., 1989, 1990). In this study, POD activity was induced by aniline in *C.renhardii*. The result indicates POD was involved in the metabolism of aniline in the *C.renhardii*. In this study, a 4 fold increase in POD activity was found when *C.renhardii*.was exposed to aniline for 96h.

Sulfate and glucuronide conjugates were metabolic products of chlorophenols in the fish (Ahlbory and Thunberg, 1980), while methylation of chlorophenols usually occurred in bacteria (Allard *et al*., 1987). In higher plants, glucose conjugation is the major form of chlorophenol metabolites. In this study, the activity of the conjugation enzyme aniline-GST was detected, and high levels of glucosyltransferase and GST activitied have been reported in the marine macroalgae (Pflugmacher and Sandermann, 1998).

Likewise, GST activity has also been detected in freshwater algae, and was suggested to play a role in atrazine metabolism in the algal species (Tang *et al*., 1998). In this study, a 4 fold increase in GST activity was found when *C.renhardii*.was exposed to aniline for 96h.

Significant increases in the activities of antioxidant enzyme (SOD) were evident,after *C.renhardii*. was exposed to aniline for 96h. The results suggested that there was an increase in the production of oxyradicals by the *C.renhardi.* Roy and Hanninen (1994) reported the induction of SOD in the aquatic plant, *E.crassipes*, after exposure to PCP. This was related to the generation of oxyradicals by hydroxyquinones and catechols produced via biotransformation of PCP.

From above, we could conclude that not only Cyt P450 ,but also POD, SOD play an important role in the biodegradation of aniline.We detected the metabolite by HPLC. Peak B (Fig. 6) observed was accounted for the product of biodegradation. And other peaks detected in the control and the medium with algae and aniline were the same. Peak A represents aniline, Peak C represents the product of hydrolyzation and photolysis (Fig. 5. and Fig. 6).

The re-analysis by GC of cell-free supernatants of algae incubated with 10mg/L aniline indicated the formation of metabolite with retention time 6.35 min and 6.41 min (Fig. 7. peak B; Fig. 9. peak B). Other peaks observed were the same in the medium with algae and without algae. We could see that the retention time of metabolite was the same on the 4th day and the 8th day. The areas of the peaks were increased as the time. So it was apparent that peak B was the main product and peak A was aniline. The product of hydrolyzation and photolysis.were not detected by GC. And the metabolite of hydroxylation-aniline was not detected neither. Mass Spectrum of metabolite retrieved from the chemical library of spectra was identified acetylaminobenzene. The structure of compound was N-acetylation of aniline.

The ability of algae to metabolize aromatic compounds has been reported previously. Thus, Ellis (1997) showed that $^{14}CO_2$ was formed [^{14}C] catechol by six algal strains, and some of these organisms also metabolized phenol to CO_2. The demonstration that the ability to oxidize aromatic hydrocarbons is widespread in the algal kingdom. In the fish medaka N-acetylation was the dominant route of in vivo metabolism for 4-chloroaniline, with no indication of ring hydroxylation, while the evidence suggested that polar conjugates were the dominant in vivo aniline metabolites (Bradury SP, 1993). In 1981, the cyanobacteria *Agmenellum quadruplicatum* strain PR-6 and *Oscillatoria sp.* strain JCM grown photoautotrophically in the presence of aniline metabolized the aromatic amine to formanilide, acetanilide, and p-aminophenol. (Carl E. Cerniglia, J. P. Freeman *et al*., 1981). In humans the N-acetylation of amines was common to allow amines conserved to be excreted (See Weber, 1987). In 1998, Miadokova E *et al*., found that algae *C. reinhardtii* could matobolism 2-AF and the metabolites from 2-AF were substrates for O-acetyltransferase. A comparison of alga 2-AF activation with mammalian activation system (S9 mix) proved they shared the same pathway (Miadokova E, *et al*., 1998).

Human arylamine N-acetyltransferase is a polymorphic phase II xenobiotic-metabolizing enzyme which catalyzes the biotransformation of primary aromatic amines, hydrazine and carcinogens. The N-acetylation is a widely pathway of detoxification for aromatic amines in mammals such as humans and rats. N-acetylation of aromatic amines, intermolecular N, O-transacetylation of N-hydroxylamines, and intrmolecular N, O-transacetylation of arylhydroxamic acids in both mammlas and bacteria are catalyzed by the same enzyme, N-acetyltransferase (NAT) (Hein *et al*., 1994; Mattano *et al*., 1980). In 1976, Lloydia found that the plant tissue (Catharanthus roseus, Apocynum cannabinum and Conium maculatum) has the capability of transfer aniline to acetanilide (Carew DP, Bainbridge T, 1976). In mammalian: the arylamines were substrates for mammalian activation by cytochrome P-450-mediated pathway, as well as by cellular peroxidases (Eling *et al*., 1988). The first step in the mammalian hepatic activation of aromatic amines is N-hydroxylation. Cellular acetytransferases were essential in further metabolizing N-hydroxyarylamines to their ultimate mutagenic products (Saito *et al*., 1985). It was found that metabolites from the polycyclic aromatic amine 2-aminofluorene were substrates for O-acetyltransferase in C. reinhardtii (Miadokova E, *et al*., 1998).

In this paper the initial enzyme in the alga's catabolic pathway was tentatively identified as an aniline hydroxylase, and another pathway was the conjugation of N-acetylation. But the hydroxylase of aniline was the major way of biodegradation of aniline. And then the aniline was transferred to acetylaminobenzene. The pathway was similar to the higher plants and animals, the algae detoxified through enhancing the organic hydrophile. But how was the toxicity of the acetylaminobenzene was unknown. It was concluded that the evolutionary 'green liver' concept derived for xenobiotic metabolism in higher plant species was also valid for constitutive soluble acetyl-transferases of lower plant species.

From the above, we could assume that the metabolic pathway of aniline in *C. reinhardtii* was shown in Fig. 11.

Cytochrome P450 enzymes catalyze a rang of oxidative transformations, including carbon hydroxylation, heteroatom oxidation, and hydrocarbon desaturation.

N-acetyltransferases (NATs) are polymorphic xenobiotic metabolizeing enzymes. NATs catalyze the transfer of an acetyl group from acetylcoenzyme A to arylamine, hydrazine and arylhydroxylamine substrates. NAT has also been identified in the bacterium Salmonella typhimurium.

The first step was hydroxylation of aniline. Bioactivation of aniline could proceed by peroxidase mechanism and via P-450 mediated reactions. Following hydroxylation, the N-hydroxy-aniline was further conjugated. But the intermediates were not detected. Aniline could be a substrate for NAT forming acetylaminobenzene. N-acetylation of aniline prior to N-hydroxylation was sometimes viewed as a detoxification step since aniline was a better substrate for CYP than the acetylated product.

In summary, *C. reinhardtii* had the capability of biotransforming aniline into acetylaminobenzene and the cytochrome P-450 is the key enzyme in the metabolic pathway. To a lesser extent aniline was transferred to acetylaminobenzene. The results indicated that *C. reinhardtii* played significant role in the fate of aniline in aquatic environment.

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