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ORIGINAL PAPER

Biodegradation of pyridine by an isolated bacterial consortium/strain and bio-augmentation of strain into activated sludge to enhance pyridine biodegradation

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Abstract Pyridine and pyridine based products are of major concern as environmental pollutants due to their recalcitrant, persistent, toxic and teratogenic nature. In this study, we describe biodegradation of pyridine by an isolated consortium/strain under aerobic condition. Batch experiment results reveal that at lower initial pyridine concentrations (1–20 mg l^{-1}), almost complete degradation was observed whereas at higher concentration (30–50 mg l^{-1}), the degradation efficiency was dropped significantly. This may be due to inhibitory effect of pyridine at higher concentrations. The value of decay and yield coefficient was also determined. Furthermore, the bio-augmentation of isolated consortium/strain into the activated sludge consortium in different quantity has been also done and the effect of bio-augmentation on degradation has been studied. The results reveal that as the quantity of bio-augmentation increases, the degradation of pyridine increases. At 25% bio-augmentation, complete degradation of 20 mg l^{-1} of pyridine can be achieved within 96 h of incubation. Thus, the study concluded that the bio-augmentation of the isolated consortium/strain into the sludge enhances the pyridine degradation efficiency of the biomass.

Keywords Bio-augmentation · Biokinetic · Activated sludge · N-heterocyclic compound · Pyridine · Growth kinetics

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Introduction

The rapid expansion and increasing sophistication of the chemical industries in the past century and particularly over the last 30 years has meant that there has been increasing amount of complexity of toxic waste effluents. Xenobiotic compounds are generally man made synthetic aromatic compounds having a persistent toxic nature and are characteristically difficult to degrade in the environment. Biological degradation of xenobiotic compounds is considered to be one of the challenging tasks. Biological degradation unlike physicochemical methods is a natural process in which microorganisms make use of organic pollutants present for its growth and other cellular processes.

Aromatic heterocyclic compounds are of major concern as environmental pollutants due to their recalcitrant, persistent, toxic, and teratogenic nature. Heterocyclic compounds are generated by many

industries. Most of these chemicals are toxic to human health (Sims and O'Loughlin 1989; Liu et al. 1998; Padoley et al. 2006; Mudliar et al. 2008). Pyridine, one of the important N-heterocyclic compounds, occurs in the environment as a result of oil shale retorting, coal gasification, and pesticide use (Sims and Sommers 1985; Stuermer et al. 1982; Liu et al. 1998) and has potential application in manufacturing of dyes, explosives, pesticides, and pharmaceuticals (Kaiser et al. 1996; Liu et al. 1998).

The presence of pyridine in the environment creates severe health hazards because pyridine is toxic, teratogenic, and at higher concentration results in weakness and ataxia (Browning 1965; Padoley et al. 2006; Mudliar et al. 2008). The amounts of pyridine bases produced worldwide were estimated as: pyridine-26,000 t/year, 2-methylpyridine-8,000 t/year, 3-methyl pyridine- 9,000 t/year, 4-methylpyridine^{-1}500 t/year), 5-ethyl-2-methylpyridine- 8,000 t/year (Shimizu et al. 1993). Thus the production of the pyridine based compound in large quantity is severe concern for the environmentalist. Hence, there is an urgent need to develop a bioremediation system for the remediation of pyridine based compounds from environment.

Various physico-chemical methods such as adsorption, chemical oxidation or incineration are available for the treatment of waste emissions containing pyridine and its derivatives (Devinny et al. 1999). However, these processes are energy intensive, require high capital and operating costs and also generate secondary waste streams (Mudliar et al. 2008). Alternatively, biological treatment methods can provide a better option in view of their low capital and operating cost and the treatment results in formation of innocuous products.

Various studies showed that microbes under aerobic and anaerobic conditions are able to utilize pyridine and its derivatives (Gupta and Shukla 1975; Sims and O'Loughlin 1989; Kaiser et al. 1996; Fetzner et al. 1998; Leenheer and Stuber 1981; Mohan et al. 2003; Mudliar et al. 2008).

But for designing a system for bioremediation of pyridine using an isolated strain, the studies on applicability of the isolated consortium/strain into the activated sludge should be tested but unfortunately, there is not much study related with the bio-agumentation of isolated consortium/strain into the activated sludge. Hence, in this study, we describe the biodegradation of pyridine by the isolated consortium/strain and its applicability in the activated sludge for the treatment of pyridine containing wastewater. The bio-augmentation of isolated consortium/strain into the activated sludge consortium in different quantity has been done and the effect of bio-augmentation on degradation has also been studied.

Materials and methods

Microorganism/consortium

The pyridine degrading microorganism/consortium has been isolated from pesticide contaminated dumpsite and was maintained on the plates containing pyridine as sole carbon source by periodic subculturing. The culture was stored at 4°C.

Media and conditions

The minimal mineral media used for the study was composed of (mg per liters of deionized water) KH_2PO_4-175, K_2HPO_4-570, Na_2HPO_4-668, $NaHCO_3$-20, NH_4NO_3-185.5, and $MgSO_4$-225 in the media. Media was sterilized by autoclaving at 121°C and 15 lb/in^2 for 20 min. All components were autoclaved separately to avoid formation of precipitate. After cooling, the solutions were mixed under sterile conditions. The study was conducted at 30°C in an incubator with shaking at 50 rpm.

Batch studies on biodegradation of pyridine using isolated consortium/strain

In this study, the whole cells were used as enzyme system for pyridine degradation. The cell biomass was obtained by growing the bacterial culture in the incubator at 30°C. The culture was grown for fifteen days in minimal mineral media supplemented with addition of 10 mg l^{-1} of pyridine everyday. The cells were harvested at 4°C by centrifugation, suspended with minimal mineral media and pyridine of concentrations 1, 2, 5, 10, 20, 30, 40, and 50 mg l^{-1} in different flasks for biokinetic study. The samples at particular time interval were withdrawn and analyzed for the bacterial growth via optical density (O.D) and

mixed liquor volatile suspended solids (MLVSS) and pyridine degradation via colorimetric method (Mohan et al. 2003). The control experiments was carried out using autoclaved biomass at all initial concentrations to determine the loss in the pyridine concentration due to bio-sorption and also the control experiment without the biomass was performed to determine any type of abiotic degradation.

Batch studies on effect of bio-augmentation of isolated consortium/strain into the activated sludge biomass on pyridine degradation efficiency

In the study, the isolated consortium/strain is bioaugumented with the biomass of activated sludge (procured from an activated sludge plant treating wastewater from pharmaceutical industry) in different quantity and the effect of bio-augmentation on degradation was studied. The studies were done with addition of different quantity of isolated consortium/strain to the biomass, ranging from 0 to 25% (by wt.). The study was conducted such that the overall biomass quantity remains same in the system. The study was carried out at an initial pyridine concentration of 20 mg l^{-1}. To determine the effect of bio-augmentation on pyridine degradation, the pyridine concentration was analyzed at different time intervals.

Analytical methods

Liquid sample for chemical analysis were taken with sterile pipette for analysis of substrate, the samples were centrifuged and the supernatant was stored at 4°C until analyzed. Cell growth was monitored by measuring the OD of the culture broth samples at 620 nm and MLVSS as per APHA (1998). The pyridine present in the aqueous phase was monitored colorimetrically at 450 nm as mentioned by Mohan et al. (2003).

Results and discussions

Enrichment and isolation

During the acclimatization process certain enzymes in the bacteria are induced so that they are available for taking part in metabolism reaction. This is much more important when dealing with toxic compounds such as pyridine, and these too at high concentrations. In this study, it was envisaged to degrade pyridine using an isolated bacterial consortium/strain at the initial pyridine concentration up to 50 mg l^{-1}. To initiate the acclimatization procedure 5 mg l^{-1} of pyridine as carbon source along with the media described earlier was used for growth.

After 48 h significant growth was observed; the media turned milky. Then, 10 mg l^{-1} of pyridine from stock solution was added. Thereafter, 10 mg l^{-1} of pyridine was added periodically after 24 h along with media. After continuous addition of pyridine for about 2 weeks, more than 99% degradation was observed. Before starting the kinetics studies, a last enrichment was done by growing this acclimatized culture on 25 mg l^{-1} concentration of pyridine.

Morphological and bio-chemical characteristics of isolated consortium/strain

The isolate showed fluorescent pigmented colonies and was found out to be gram negative. The detailed rapid biochemical test (Otto and Pickett 1976) were performed and it was found that the isolate showed positive tests for Citrate, Lactate, Malonate, Fructose, D-Glucose, Acetamide, Cellobiose, Butyrate, Propionate, Glutamine, Galactose, Glycerol, Betaine, D-Ribose, and D-Mannose. The isolate showed negative test for the Nicotinate, Tartrate, Nicotinamide, m-Inositol, Sorbitol, Phenylalanine, and Sucrose.

Effect of initial concentration of pyridine on the degradation efficiency of isolated consortium/strain

Various researchers reported that batch tests need to be design with appropriate biomass and substrate concentrations for analyzing the abilities of the bacterial strains for the biodegradation (Bielefeldt and Stensel 1999). Hence batch flask experiments were conducted to examine the effect of various initial concentration of pyridine on pyridine degradation efficiency by isolated consortia/strain. The measurements of substrate concentration and bacterial growth were followed till the substrate concentration became steady. The initial concentration of pyridine was

varied in the range between 1 and 50 mg l^{-1}. The control experiments was carried out using autoclaved biomass at all initial concentrations to determine the loss in the pyridine concentration due to biosorption and also the control experiment without the biomass was performed to determine, if any other type of abiotic degradation is occurring or not.

Figure 1 shows the bacterial growth and substrate degradation pattern with respect to time at initial pyridine concentration of 10 mg l^{-1}. It can be seen from Fig. 1 that the cell density increases with decrease in the substrate concentration. Almost similar pattern was also observed at other initial concentrations.

The degradation of pyridine was observed more than 99% at initial pyridine concentrations between 1 and 20 mg l^{-1} and about 52% at initial pyridine concentration of 50 mg l^{-1}, after 96 h of incubation. However, no significant removal in pyridine was observed in the control flask containing autoclaved biomass. The removal due to biosorption was about 1–3% at different initial pyridine concentrations. About 1–3% pyridine removal was observed due to abiotic activities in the other control flask without biomass. Hence from both control experiment data it can be concluded that about 1–3% pyridine was lost due to volatilization and biosorption was negligible as the loss in pyridine concentration in the control flask containing autoclaved biomass is same as volatilization.

Figure 2 shows the degradation pattern of the pyridine with time for different initial pyridine concentrations. Results of these studies show that the higher the concentration of the pyridine above a critical initial pyridine concentration (20 mg l^{-1}), the lesser the degradation, may be due to inhibitory effect at higher

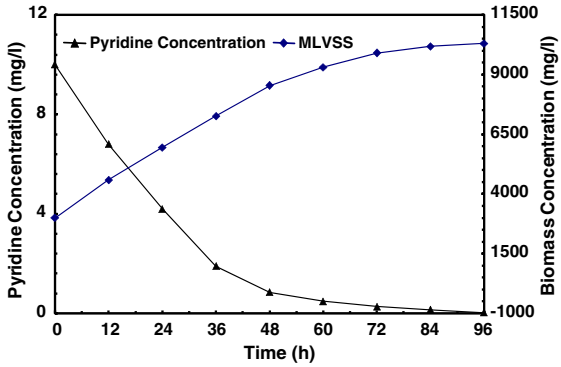

Fig. 1 Bacterial growth and substrate degradation pattern with respect to time at initial pyridine concentration of 10 mg l^{-1}

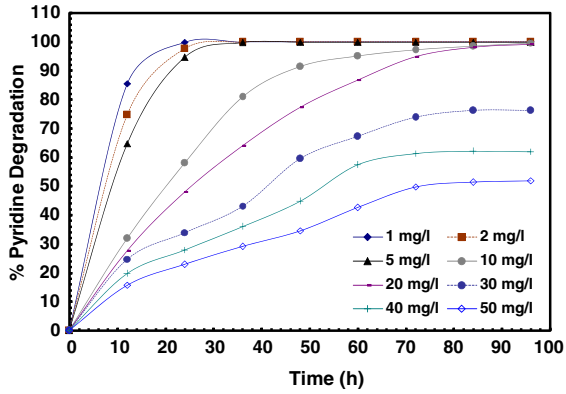

Fig. 2 Effect of initial pyridine concentration on the pyridine degradation by isolated pyridine degrading consortium/strain

concentrations (Rhee et al. 1997). At each of initial concentration there was a period of exponential growth when the substrate was being consumed at faster rate. It was observed that toward the end of the substrate consumption curve, there is a region of relatively lower rate of substrate removal. Two possible reasons may be (a) drop in pH and (b) deficit in availability of oxygen. This phenomenon is also reported by various researchers during batch degradation studies for toxic organics (Kumar et al. 2005; Arutchelvan et al. 2006; Lodha et. al. 2007).

During the experiments the pH in each flask dropped from 7 to about 6.2. The decrease in pH during the experiment may be due to acid formation because of the biotransformation of pyridine to acids. It is assumed that pyridine is degraded after removal of N atom from the cleavage ring. The N atom is removed by the enzymatic reaction and remaining ring subsequently converts to acid and thereafter to gases like CO_2, etc. (Rhee et al. 1997). Subsequently the dissolved oxygen concentration was also monitored after the experiment and the DO concentration was dropped from about 3.2 to 0.69 mg/L. This drop in the DO concentration may be due to the consumption of the DO by the microbes in the batch reactor and limitation of air transfer through the cotton plugs used to cover the flask in the shaker.

Evaluation of growth kinetics of isolated consortium/strain

In order to evaluate growth kinetics, the biomass growth data from the batch experiments were plotted

on a semi logarithmic graph as mentioned by Park et al. (2002), Kumar et al. (2005), and Lodha et al.(2007). The lag phase was observed in the initial stage of the growth curve, thereafter linear increment in the biomass concentration was observed (exponential phase) at all initial pyridine concentrations, which indicated that the pyridine was the limiting substrate in this region and the culture growth was exponentially. In the present research work also these plots have been used to calculate specific growth rate (μ) for that particular initial pyridine concentration using eq. (1).

$$\ln\left(\frac{X}{X_0}\right) = \mu_g t. \tag{1}$$

The specific growth rates were obtained at several different concentrations of pyridine ranging from 1 to 50 mg l^{-1} and it was found that the specific growth rate increases with the increase in pyridine concentration up to a certain concentration level (20 mg l^{-1}), then it starts decreasing with increase in the concentration as shown in Fig. 3. The same trend has been reported for other toxicants like phenol, catechol, technical HCH, TNT, etc. (Kumar et al. 2005; Lodha et al. 2007; Park et al. 2002). The specific growth rates were 0.0015 h^{-1} at 1 mg l^{-1}, and 0.01233 h^{-1} at 50 mg l^{-1}. The maximum specific growth rate was determined to be 0.0212 h^{-1} at 20 mg l^{-1}. This suggests that pyridine is inhibitory type of substrate.

Endogenous data or decay coefficient

The decay coefficient for the isolated culture was determined as mentioned by Kumar et al. (2005). The microbial growth curve shows a decline in the number of viable cells after the complete consumption of substrate. During this declining phase, the endogenous decay in the bacterial population persists. This part of the growth curve in a batch reactor has been modeled by following equation (2).

$$\frac{dX}{dt} = -K_d X. \tag{2}$$

In order to determine the value of K_d, the growth batch run was continued at initial pyridine concentration of 10 mg l^{-1}, rather the measurement of cell concentration was continued further for another 72 h. even after the complete consumption of pyridine. The selection of particular growth run was arbitrary, assuming the K_d is not dependent on initial substrate concentration. The data of this region were plotted as log (Biomass) versus time (Fig. 4). The negative slope gives decay rate coefficient. The value of the decay rate coefficient obtained is 0.0055 h^{-1} for pyridine.

Yield coefficient

The Yield coefficient for the isolated pyridine degrading strain/consortia was determined as mentioned by Kumar et al. (2005). The batch studies were continued till the pyridine concentration reaches steady state condition. The amount of biomass present just at the end of exponential phase was used in calculating the biomass produced as a result of consumption of substrate. The yield coefficient was determined using the following equation (3).

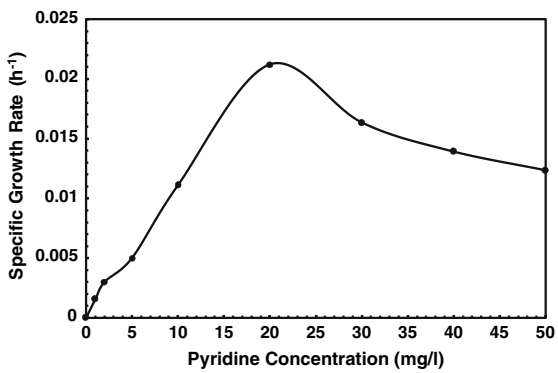

Fig. 3 Effect of initial Pyridine concentration on the specific growth rate of the isolated pyridine degrading consortium/strain

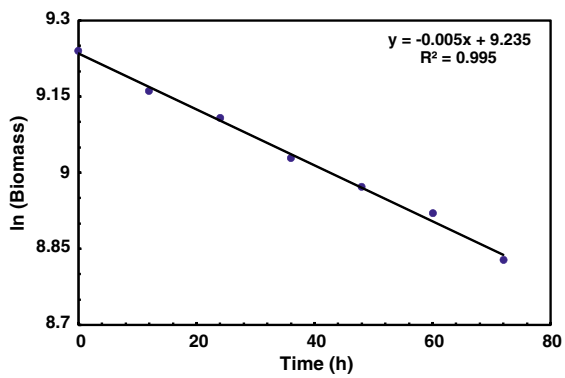

Fig. 4 Estimation of decay coefficient (K_d) for isolated pyridine degrading consortium/strain

$$X - X_0 = Y_{X/S}(S_0 - S). \quad (3)$$

Figure 5 shows the plot used to determined yield coefficients for pyridine. The value of the yield coefficient for pyridine was determined to be 0.155 g of biomass/g of pyridine. Padoley et al. (2006) reported pyridine degradation ability of the *P. pseudoalcaligenes–KPN* in the concentration range of 25–200 mg l^{-1} of pyridine and indicated a yield coefficient (Y) value of 0.26, a half-saturation rate constant (K_S) value of 37.29 mg l^{-1}, and a maximum specific growth rate constant (μ_{max}) value of 0.2 day^{-1} but the studies did not determined the value of inhibition constant.

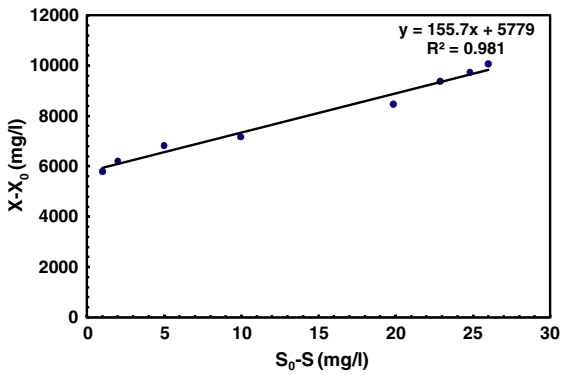

Fig. 5 Plot to calculate yield coefficient (*Y*) for growth of isolated consortium/strain on Pyridine

Effect of bio-augmentation of isolated consortium/strain on biodegradation of pyridine

During the studies, the total quantity of biomass was taken similar as taken for the isolated consortium/strain in earlier experiments and all other conditions were maintained same. The study was conducted for the initial pyridine concentration of 20 mg l^{-1}. The results of the studies are shown in the Fig. 6. The result reveals that, as the quantity of the isolated consortium/strain in the biomass of activated sludge increases, there is an increase in the pyridine degradation efficiency of the biomass. The biodegradation of pyridine at 0% bio-augmentation was about 21% after 96 h of incubation, whereas at 10% bio-augmentation more than 50% pyridine degradation was observed after 96 h. of incubation. However, at 25% bio-augmentation, almost complete removal of

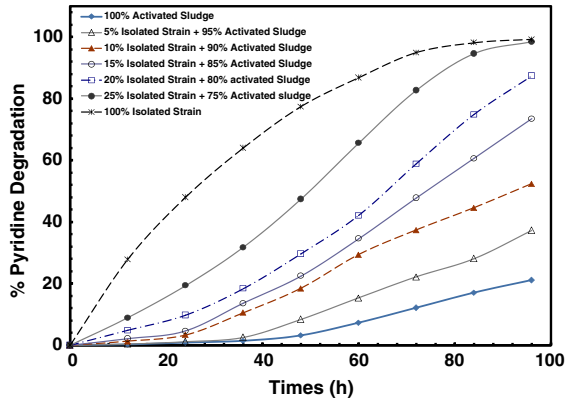

Fig. 6 Effect of bio-augmentation of isolated pyridine degrading consortium/strain on the Pyridine degradation efficiency of activated sludge biomass

pyridine (more than 98%) was observed after 96 h of incubation. Thus, it can be concluded that the isolated consortium/strain can successfully enhance the capacity of pyridine degradation of activated sludge after bio-augmentation.

Conclusion

The isolated bacterial consortium/strain was found to be capable of degrading pyridine under aerobic conditions. The result reveals that the degradation at low initial pyridine concentrations (1–20 mg l^{-1}) is higher than that at the high concentrations (30–50 mg l^{-1}). This may be due to toxicity and inhibitory effect of pyridine at higher concentrations. Based on μ versus S curve, maximum specific growth rate was found out to be 0.0212 h^{-1}. The yield coefficient and the decay coefficient were found to be 0.155 g of biomass/g of pyridine and 0.0055 h^{-1}, respectively. Furthermore, the bio-augmentation of the isolated bacterial consortium/strain showed a significant improvement in the pyridine degradation efficiency of the activated sludge. The 25% bio-augmentation of isolated consortium/strain in activated sludge results in almost complete degradation of pyridine (20 mg l^{-1}) within 96 h of incubation.

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