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Microbial degradation of pyridine by *Paracoccus* sp. isolated from contaminated soil

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ABSTRACT

A pyridine-degrading strain was isolated from the contaminated soil near the pesticide plant, identified as *Paracoccus* sp., and designated as strain KT-5, on the basis of its partial 16S rRNA gene sequence analysis. The effect of different co-substrates including glucose, ammonium chloride and trace elements on biodegradation of pyridine by *Paracoccus* sp. KT-5 was investigated. The results showed that when the initial concentration of pyridine was about 900 mg L^{-1}, 100 mg L^{-1} of glucose increased the growth of strain KT-5 and the removal of pyridine, but did not affect the release of nitrogen in the pyridine ring as ammonia. In addition, strain KT-5 was able to utilize 100 mg L^{-1} of glucose and 900 mg L^{-1} of pyridine simultaneously as the carbon source. 100 mg L^{-1} of ammonium chloride inhibited the growth of strain KT-5 in 900 mg L^{-1} of pyridine, and also slightly decreased the removal of pyridine, but did not affect the release of nitrogen in the pyridine ring as ammonia. However, lacking of trace elements not only inhibited the growth of strain KT-5 in 900 mg L^{-1} of pyridine, but also decreased the removal of pyridine, while it did not affect the release of nitrogen in the pyridine ring as ammonia.

1. Introduction

Pyridine and its derivatives are an important class of aromatic N-heterocycles. The pyridine ring occurs in nature in the form of pyridine coenzymes (nicotinamide and pyridoxal derivatives), plant alkaloids and natural products (e.g. dipicolinic acid in Bacillus spores). On the death and decay of the host species, these pyridine compounds are returned to the soil in the end. Nevertheless, when trace levels of these compounds were released into soils, they were rapidly degraded and even mineralized without contaminating the environment [1,2]. Coal gasification, retorting of oil shale and pesticide use have become anthropogenic sources of pyridine and its derivatives. As an industrial solvent and raw material of herbicide synthesis, pyridine and its derivatives are widely used in chemical, pharmaceutical and oil industries. Thus these pyridine compounds inevitably find their way into effluents (coke plant wastewater and pesticide wastewater), and then are released into the environment, in the end, reaching the biosphere [3–5]. The process is going to constitute a danger for human and other living organisms because of carcinogenic toxicity of pyridine and its derivatives [6,7]. Therefore, pyridine resulting from industrial activities should be eliminated before it enters the environment [8].

An attractive method for eliminating pyridine is biodegradation to convert it to essentially harmless compounds such as CO_2, H_2O and NH_4^+. Since 1910s, researchers have isolated some microbial organisms degrading pyridine and its derivatives including hydroxypyridine, alkylpyridine and carboxypyridine, and elucidated microbial metabolic pathway of these compounds [1–16]. There were some examples as following. A group leaded by Cain [1,2,5,9–12] from U.K. obtained some pure cultures with the ability to utilize pyridine as sole source of carbon, nitrogen and energy, and its hydroxy derivatives as principal carbon source by elective culture either from sewage of an activated-sludge plant or a few crumbs of soil. Five pure strains were selected for detailed study. *Achromobacter cycloclastes* 7N and 2L were able to convert 3-hydroxypyridine to pyridine-2,5-diol, but strain 7N had an ability to accumulate large amounts of pyridine-2,5-diol, and strain 2L transformed pyridine-2,5-diol to maleate or fumarate and NH_3 catalyzed by pyridine-2,5-diol dioxygenase, while *Achromobacter* G2 metabolized 2-hydroxypyridine by the same pathway, but not utilized 3-hydroxypyridine. Only *Agrobacterium* 35S could utilize pyridine and 4-hydroxypyridine (maximum concentration up to 0.025%, w/v), and catalyzed 4-hydroxypyridine into pyridine-3,4-diol by 4-hydroxypyridine-3-hydroxylase, further into 3-formiminopyruvate and 3-formyloyruvate by pyridine-3,4-diol dioxygenase. Another pyridine-degrading isolate *Nocardia* Zl grew best at pH 8.0 and pyridine concentrations in the range of 0.1–0.2% (v/v), and utilized slowly 3-hydroxypyridine. The following research indicated that *Nocardia* Zl mineralized pyridine ring by

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glutarate semialdehyde pathway. A *Micrococcus luteus* isolated by Sims et al. [3] from a Chalmers silt loam soil could utilize pyridine as the sole source of carbon, energy and nitrogen in concentrations up to 0.1% (v/v), and the cleavage of the pyridine ring was involved in succinate-semialdehyde pathway, and did not require initial hydroxylation of the ring. On the contrary, two pyridine-degrading strains isolated by Zefirov et al. [4] from soil taken near the pharmaceutical plant were identified as *Arthrobacter crystallopoietes* and *Rhodococcus opacus*. Their cleavage pathway of the pyridine ring was involved in initial hydroxylation of the ring. In addition, some researchers investigated potential application of pure pyridine-degrading culture in cleaning wastewater containing high-concentration pyridine. Lee et al. [8] found out the difference in pyridine degradation between freely suspended and Ca–alginate-immobilized cells of *Pimelobacter* sp. The data acquired showed that when the immobilized cells were used for pyridine degradation, a high volumetric pyridine degradation rate in the range of 0.082–0.129 g L^{-1} h^{-1} could be achieved by the immobilized cells because of the high cell concentration. Pyridine at a concentration of 2–4 g L^{-1} was kept degrading for 2 weeks.

However, there are few reports on different co-substrates' impact on the growth of pure pyridine-degrading culture and capacity of its degrading pyridine. Rhee et al. [17] found that glucose and acetic acid promoted pyridine biodegradation of a pyridine-degrading strain *Pimelobacter* sp. Xiong et al. [18] demonstrated the same result by *Paracoccus denitrificans* W12, meanwhile, suggested that phenol and quinoline inhibited elimination of pyridine.

The objective of this study was to investigate the effect of different co-substrates including glucose, ammonium chloride and trace elements on biodegradation of pyridine by *Paracoccus* sp. KT-5, isolated from contaminated soil, and the transformation of nitrogen in the pyridine ring under different conditions.

2. Materials and methods

2.1. Media

Pure culture was maintained in common bacterial medium LB agar containing 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl, 15 g L^{-1} agar and the final pH was adjusted to 7.2. Modified #1780 broth (1780-1) from ATCC used for the enrichment and isolation culture of microorganisms contains: 0.61 g L^{-1} K_2HPO_4, 0.39 g L^{-1} KH_2PO_4, 0.25 g L^{-1} KCl, 0.13 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.15 g L^{-1} yeast extract, 1.0 mL trace element solution, per 1000 mL trace element solution contains $CaCl_2{\cdot}2H_2O$ 0.0004 g, $FeSO_4{\cdot}7H_2O$ 0.04 g, $MnSO_4{\cdot}4H_2O$ 0.04 g, $ZnSO_4{\cdot}7H_2O$ 0.02 g, $CuSO_4{\cdot}5H_2O$ 0.005 g, $CoCl_2{\cdot}6H_2O$ 0.004 g, NaCl 1.0 g, $Na_2MoO_4{\cdot}2H_2O$ 0.005 g and pH was kept natural. 15 g L^{-1} agar was added to 1780-1 to become solid plant. In order to investigate pyridine biodegradation, #1780 broth from ATCC was modified again as 1780-2, in which 0.15 g L^{-1} yeast extract was replaced with 900 mg L^{-1} of pyridine as the carbon and nitrogen source. As the co-substrate medium, 100 mg L^{-1} of glucose was supplied as extra carbon source and 100 mg L^{-1} of ammonium chloride was supplied as extra nitrogen source. The medium (1780-3) without trace elements was basically the same as 1780-2 except for not containing trace element solution.

2.2. Isolation

Enrichment cultures were initiated by mixing 95 mL of 1780-1 with 5 g of excess solid collected from the polluted soil in the pesticide plant. Cultures were carried out in 500 mL Erlenmeyer flasks and incubated aerobically at 25 °C on a rotary shaker (180 rpm). Pyridine at concentrations in the range of 200–800 mg L^{-1} was used as the sole carbon and nitrogen source. Following incubation for one month, 0.1 mL of the enrichment cultures was spread to fresh solidified 1780-1 containing 200 mg L^{-1} of pyridine. Several pyridine-degrading bacteria were isolated by streak plating on solidified 1780-1 containing 200 mg L^{-1} of pyridine.

2.3. Identification of the isolate

Isolation and purification of chromosomal DNA were carried out according to TIANamp Bacteria DNA Kit (TianGEN BioTECH (Beijing) Co., Ltd.). 16S rRNA gene was amplified with the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACCTTGTTACGACTT-3′). The PCR conditions were 95 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min 30 s, with a final step at 72 °C for 10 min. The product was directly sequenced with the primer 27F (Beijing Sinogenomax Research Centre Co., Ltd.). The sequencing result was submitted to GenBank for BLAST analysis.

2.4. Pyridine degradation experiments

The isolate was inoculated in 15 mL tubes containing 5 mL LB liquid medium, and incubated in a shaker at 25 °C and 200 rpm until optical density of the cells was 2.87. Exponentially growing cells were collected on 4000 rpm for 15 min. The pellet was washed twice with 1780-2, resuspended in the same volume of 1780-2. 3% (v/v) of the cell supernatant was inoculated in 15 mL tubes containing 5 mL different mediums, and incubated in a shaker at 25 °C and 200 rpm. Since pyridine is a volatile compound, the tubes that contained the same concentration of pyridine without the bacteria inoculum were used as a negative control under the same condition, and the data acquired showed that the amounts of pyridine volatilization were all less than 5% in the experimental conditions. Three parallel tubes were tested to insure the accuracy of analysis. Subsamples were collected at regular intervals and centrifuged after the biomass was examined; the supernatant was retained for analysis of all substrates and products. For an extra carbon source experiment, 100 mg L^{-1} of glucose was selected and supplied in 1780-2 as an extra carbon, for an extra nitrogen source experiment, 100 mg L^{-1} of ammonium chloride was selected and supplied as extra nitrogen source; in order to investigate effect of trace elements on pyridine degradation, the mixed trace element solution was removed from 1780-2 as a new medium 1780-3.

2.5. Analytical methods

Cell growth was monitored by measuring optical density of culture broth samples at 600 nm (OD_{600}). Cell dry weight (mg L^{-1}) was determined gravimetrically by drying the harvest cells in an oven at 105 °C for 8 h after centrifugation. The concentration of pyridine in the samples was determined by reverse phase high-performance liquid chromatography (HPLC) using an Shimadzu LC-20A C18 column (4.6 mm × 250 mm) by 5 μm with UV detection at 254 nm. The mobile phase consisted of 70:30 (v/v) methanol:water at a flow rate of 1 mL min^{-1}. NH_4-N was determined by CM-05A multifunction water quality detection apparatus (Beijing Shuanghui Jingcheng Electric Product Co., Ltd.). Glucose was measured as described by Carroll et al. [19].

3. Results and discussion

3.1. Isolation and identification of pyridine degrader

Eighteen bacteria strains grown on plates were isolated as the active pyridine degraders from the enrichment cultures. The isolates were inoculated into 1780-2, and incubated aerobically in a shaker at 25 °C and 200 rpm for 2 days. The ability of the bacteria

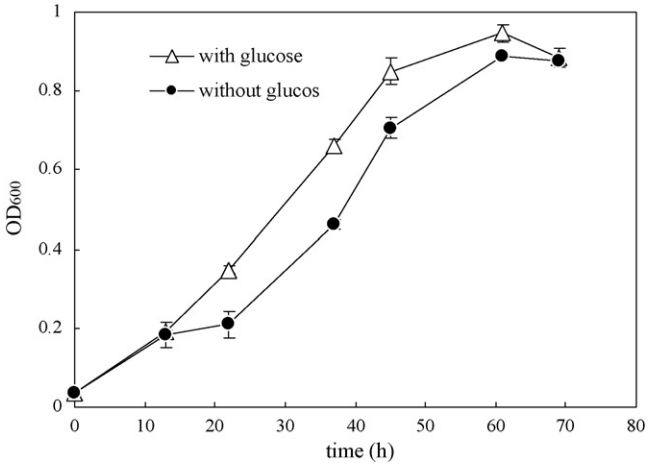

Fig. 1. Effect of extra carbon source on the growth of strain KT-5 (pyridine: about 900 mg L^{-1} and glucose: 100 mg L^{-1}).

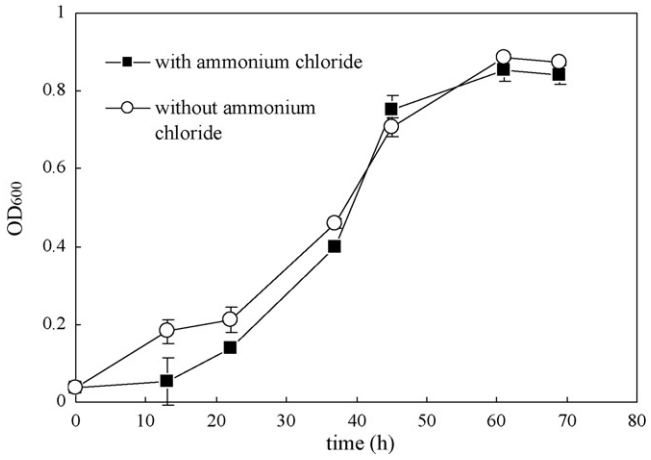

Fig. 2. Effect of extra nitrogen source on the growth of strain KT-5 (pyridine: about 900 mg L^{-1} and NH_4Cl: 100 mg L^{-1}).

to degrade pyridine was confirmed by pyridine removal rate. As a result, one of the isolates, designated as KT-5, was chosen for further study based upon its ability of highest pyridine removal rate. And then, the strain KT-5 was identified as *Paracoccus* sp. according to its partial 16S rRNA gene sequence analysis. The 16S rRNA gene sequence obtained from the strainKT-5 was deposited at the GenBank database under the accession number FJ611936.

3.2. Effect of different co-substrates on the growth of strain KT-5

Generally, for the bacteria, glucose and ammonium chloride are accessible carbon and nitrogen source, respectively. On the other hand, trace mineral elements are necessary assistant ingredients used for biocatalyst of the bacteria. Therefore, the effect of three substrates together with pyridine on the growth of strain KT-5 was firstly investigated.

3.2.1. Effect of extra carbon source

Compared with the control without glucose, glucose increased the growth of strain KT-5 since 13 h (Fig. 1). Under two different conditions, the growth of strain KT-5 experience few lag, and attained stationary at 61 h after inoculation. The cell growth rate was calculated as the following formula:

$$\mu = \frac{C_2 - C_1}{t_2 - t_1}$$

where μ is the cell growth rate (mg L^{-1} h^{-1}), C_1 is cell concentration (mg L^{-1}) at time t_1 and C_2 is cell concentration (mg L^{-1}) at time t_2. When glucose (100 mg L^{-1}) was added to 1780-2, the growing rate of cells was 7.82 mg L^{-1} h^{-1}; when no glucose was added the growing rate of cells was 5.22 mg L^{-1} h^{-1}. Therefore, glucose improved the growth of strain KT-5 in pyridine. In addition, it was validated that strain KT-5 was able to utilize pyridine as the sole carbon and nitrogen source.

3.2.2. Effect of extra nitrogen source

When ammonium chloride was added to media 1780-2, after the initial 22 h lag, strain KT-5 grew to log phase, and attained stationary at 61 h (Fig. 2). When ammonium chloride (100 mg L^{-1}) was added to 1780-2, the growing rate of cells was 4.85 mg L^{-1} h^{-1}; when no ammonium chloride was added the growing rate of cells was 5.22 mg L^{-1} h^{-1}. As a result, ammonium chloride inhibited the growth of strain KT-5 in pyridine.

3.2.3. Effect of trace elements

As described above, detected trace elements were a mixed trace element solution in the experiments, and per 1000 mL trace element solution contains $CaCl_2{\cdot}2H_2O$ 0.0004 g, $FeSO_4{\cdot}7H_2O$ 0.04 g, $MnSO_4{\cdot}4H_2O$ 0.04 g, $ZnSO_4{\cdot}7H_2O$ 0.02 g, $CuSO_4{\cdot}5H_2O$ 0.005 g, $CoCl_2{\cdot}6H_2O$ 0.004 g, NaCl 1.0 g, and $Na_2MoO_4{\cdot}2H_2O$ 0.005 g. When the mixed trace element solution existed, the growing rate of cells was 5.22 mg L^{-1} h^{-1}; when no mixed trace element solution, the growing rate of cells was 1.74 mg L^{-1} h^{-1}. It was evident by the data that lack of the mixed trace elements prevented KT-5 growing well in pyridine (Fig. 3), because many trace elements composed of essential ingredients of biocatalyst of strain KT-5 growing in pyridine.

3.3. Effect of different co-substrates on pyridine biodegradation

Many reports suggested that accessible carbon and nitrogen source stimulated bacterial ability to utilize refractory substrates. However, the reverse consequences had been also reported [18,20–23]. Consequently, effect of the three substrates on pyridine biodegradation by strain KT-5 was still investigated.

3.3.1. Effect of extra carbon source

Whether glucose was added to the media 1780-2 or not, pyridine (about 900 mg L^{-1}) was completely removed within 61 h of inoculation without lag phase (Fig. 4). This was identical with the

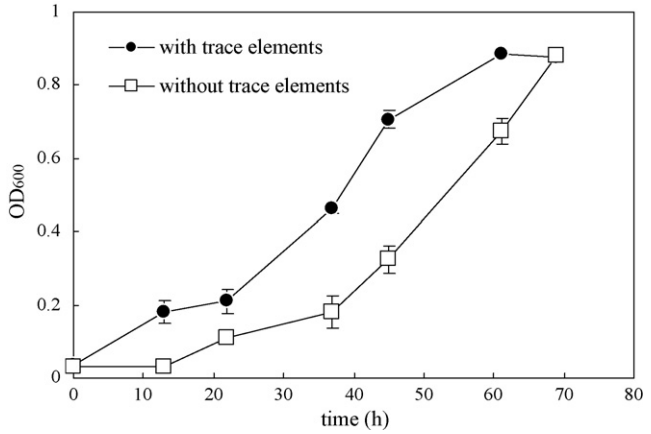

Fig. 3. Effect of trace elements on the growth of strain KT-5 (pyridine: about 900 mg L^{-1}).

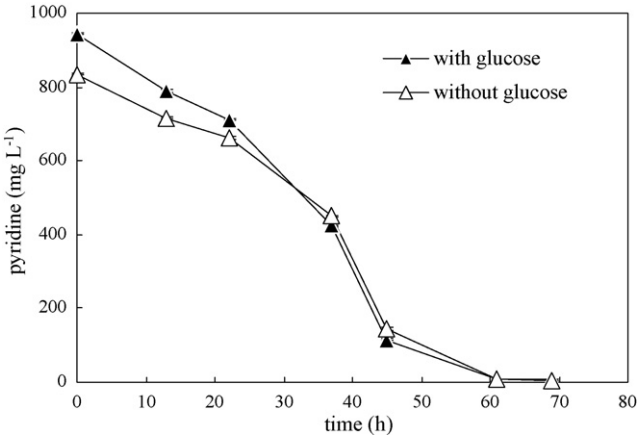

Fig. 4. Effect of extra carbon source on pyridine degradation by strain KT-5 (pyridine: about 900 mg L^{-1} and glucose: 100 mg L^{-1}).

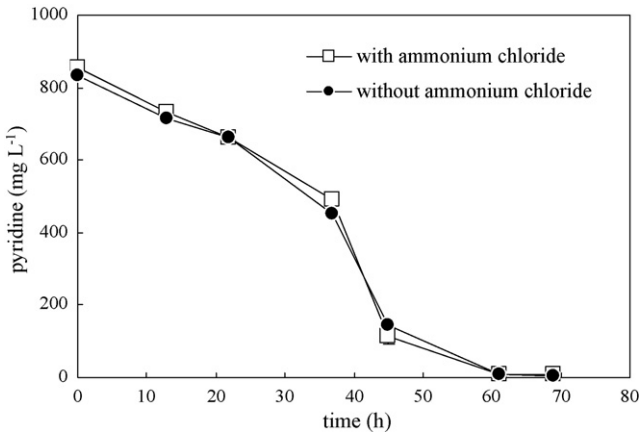

Fig. 5. Effect of extra nitrogen source on pyridine degradation by strain KT-5 (pyridine: about 900 mg L^{-1} and NH_4Cl: 100 mg L^{-1}).

growth curve of strain KT-5 (Fig. 1). The pyridine-degrading by strain KT-5 complied with first-order kinetics within the initial 37 h. The kinetics equation was as following:

$$\ln c = a + kt$$

where c is pyridine concentration, t expresses time and k is the first-order rate constant.

When containing 100 mg L^{-1} of glucose, kinetics equation was expressed as:

$$\ln c = 6.9184 - 0.0213t, \qquad R^2 = 0.9266;$$

when not containing glucose, kinetics equation was expressed as:

$$\ln c = 6.7717 - 0.0163t, \qquad R^2 = 0.9397.$$

Since the pyridine-degrading rate of the former was a little higher than the later, glucose stimulated strain KT-5's ability to degrade pyridine, which was probably attributed to increase of biomass of strain KT-5 utilizing glucose. The initial pyridine concentration of the sample without glucose was lower than the sample with glucose possibly because of preferential utilization of glucose by strain KT-5. Rhee et al. [17], Xiong et al. [18], Quan et al. [20], Yang et al. [22] and Sharma and Thakur [23] demonstrated the same results, however, Kao et al. [21] suggested extra glucose did not stimulate biodegradation of pentachlorophenol by a pentachlorophenol-degrading strain *Pseudomonas mendocina* NSYSU.

3.3.2. Effect of extra nitrogen source

Whether ammonium chloride was added to the media 1780-2 or not, pyridine (about 900 mg L^{-1}) was also completely removed within 61 h of inoculation without lag phase (Fig. 5). This was identical with the growth curve of strain KT-5 (Fig. 2). The pyridine-degrading by strain KT-5 complied with first-order kinetics within the initial 37 h. When containing 100 mg L^{-1} of ammonium chloride, kinetics equation was expressed as:

$$\ln c = 6.7766 - 0.0148t, \qquad R^2 = 0.9775;$$

when not containing ammonium chloride, kinetics equation was expressed as:

$$\ln c = 6.7717 - 0.0163t, \qquad R^2 = 0.9397.$$

It was evident by the data that ammonium chloride had slightly resistant effect on pyridine-degrading by strain KT-5. There were few reports on effect of extra ammonium chloride on pyridine biodegradation by pyridine-degrading pure cultures.

3.3.3. Effect of trace elements

Pyridine (900 mg L^{-1} or so) was completely removed within 69 h of inoculation without containing the mixed trace element solution; while the process took 61 h containing the same mixed trace element solution (Fig. 6). The pyridine-degrading by strain KT-5 complied with first-order kinetics within the initial 37 h. When containing the mixed trace element solution, kinetics equation was expressed as:

$$\ln c = 6.7717 - 0.0163t, \qquad R^2 = 0.9397;$$

when not containing the mixed trace element solution, kinetics equation was expressed as:

$$\ln c = 6.8173 - 0.0081t, \qquad R^2 = 0.9748.$$

Therefore, trace mineral elements were the essential substrates for pyridine degraded effectively by strain KT-5. There were a few reports on effect of trace metal nutriment on pyridine biodegradation by pyridine-degrading pure cultures.

3.4. Effect of different co-substrates on the transformation of nitrogen in the pyridine ring

According to some researchers' results [5,10,24–26], nitrogen in the pyridine ring was often transformed as formamide, which, furthermore, became formate and NH_4^+. Similarly, the transformation of nitrogen in the pyridine ring was determined under different co-substrates.

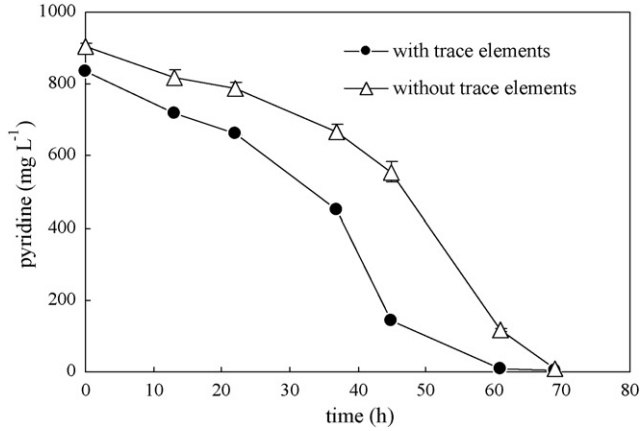

Fig. 6. Effect of trace elements on pyridine degradation by strain KT-5 (pyridine: about 900 mg L^{-1}).

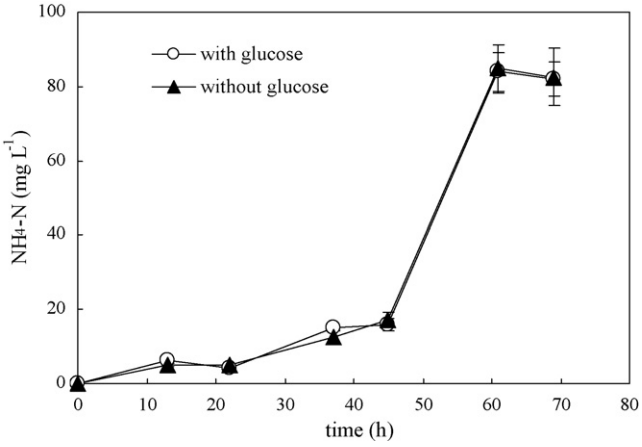

Fig. 7. Effect of extra carbon source on the transformation of nitrogen in the pyridine ring (pyridine: about 900 mg L^{-1} and glucose: 100 mg L^{-1}).

3.4.1. Effect of extra carbon source

Nitrogen in the pyridine ring was really bio-transformed into ammonium nitrogen (Fig. 7). After the initial 22 h lag, about 50% of nitrogen in the pyridine ring was converted to ammonium nitrogen within 39 h. However, the concentration of ammonium nitrogen did not decrease clearly within the following 8 h. Zhao and co-workers [25] and Sun et al. [26] found that 50–60% of nitrogen in the pyridine ring was converted to ammonium when pyridine was biodegraded by the bacteria, and that residual nitrogen in the pyridine ring was assimilated as the nitrogen source by the strains. In this study, the same results were obtained. 40–50% of nitrogen in the pyridine ring was assimilated as the nitrogen source by strain KT-5. Additionally, extra glucose did not affect biotransformation of nitrogen in the pyridine at all.

3.4.2. Effect of extra nitrogen source

Although ammonium chloride was added to degrading medium as extra nitrogen, it seemed that ammonium chloride had no impact on transformation of nitrogen in the pyridine ring by strain KT-5; moreover, extra ammonium chloride was not almost utilized within 61 h after inoculation (Fig. 8). Similarly, almost half of nitrogen in the pyridine ring was assimilated as the nitrogen source by strain KT-5; the other was converted to ammonium nitrogen.

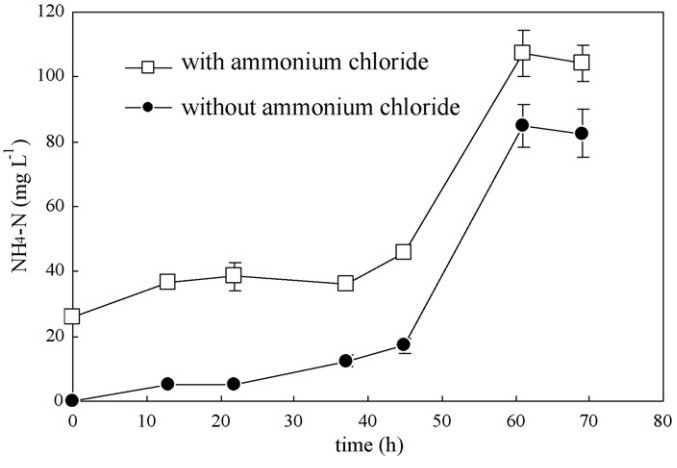

Fig. 8. Effect of extra nitrogen source on the transformation of nitrogen in the pyridine ring (pyridine: about 900 mg L^{-1} and NH_4Cl: 100 mg L^{-1}).

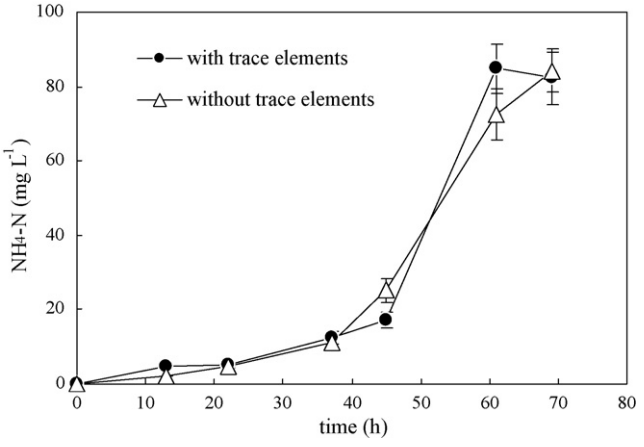

Fig. 9. Effect of trace elements on the transformation of nitrogen in the pyridine ring (pyridine: about 900 mg L^{-1}).

3.4.3. Effect of trace elements

Compared with the sample containing the mixed trace element solution as described above, almost half of nitrogen in the pyridine ring was also converted to ammonium nitrogen within 69 h without containing the mixed trace element solution (Fig. 9). Therefore, it was validated that the mixed trace element solution had no clear impact on transformation of nitrogen in the pyridine ring by strain KT-5. There were few reports on effect of trace elements on converting of nitrogen in the pyridine ring.

3.5. Utilization of carbon source by strain KT-5 under co-substrate condition

Strain KT-5 was able to utilize glucose and pyridine simultaneously as the carbon source (Fig. 10). Both pyridine-degrading and glucose utilizing by strain KT-5 conformed to the first-order kinetics.

For the removal rate of pyridine:

$$\ln c = 6.7717 - 0.0163t, \qquad R^2 = 0.9397;$$

and for the removal rate of glucose:

$$\ln c = 13 - 1.8715t, \qquad R^2 = 1$$

It can be seen that the removal rate of glucose was higher than the removal rate of pyridine by strain KT-5. Furthermore, it was still revealed from the data that strain KT-5 was able to utilize two different carbon sources independently, and that the degradation of glucose and pyridine had no obvious effect on each other.

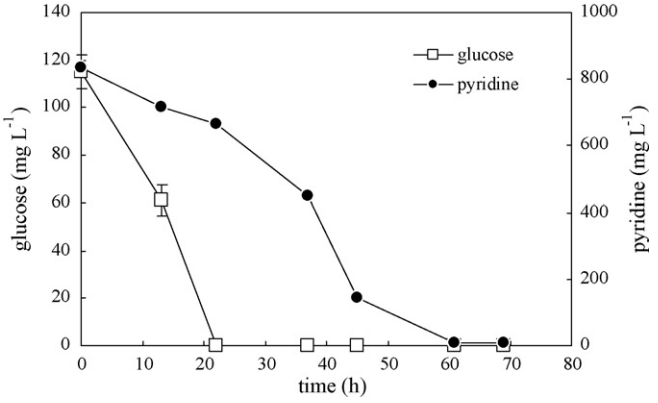

Fig. 10. Utilization of carbon source by strain KT-5 under co-substrate condition.

On the contrary, Quan et al. [20] indicated that glucose promoted biodegradation of quinoline by *Burkholderia pickettii*, but quinoline decreased utilization of glucose by *Burkholderia pickettii*.

4. Conclusions

Strain KT-5 capable of degrading pyridine was isolated from the polluted soil in the pesticide plant. According to sequence analysis of 16S rDNA, the strain was identified as *Paracoccus* sp. Different co-substrates such as glucose, ammonium chloride and trace elements had different effects on biodegradation of pyridine by *Paracoccus* sp. KT-5. Glucose (100 mg L^{-1}) stimulated the growth of strain KT-5 and increased the removal of pyridine, but did not affect the biotransformation of nitrogen in the pyridine ring as ammonia nitrogen. Furthermore, strain KT-5 was able to remove completely glucose (100 mg L^{-1}) within 22 h and pyridine (about 900 mg L^{-1}) within 61 h simultaneously as the carbon source. Ammonium chloride inhibited the growth of strain KT-5, and also slightly inhibited the removal of pyridine, but did not affect the release of nitrogen in the pyridine ring as ammonia nitrogen. However, lacking of trace elements not only inhibited the growth of strain KT-5 in 900 mg L^{-1} of pyridine, but also decreased the removal of pyridine, whereas, did not affect release of nitrogen in the pyridine ring as ammonia. In conclusion, all the data indicate that when strain KT-5 is applied to decontaminate effluent containing pyridine, accessible carbon and nitrogen source do not affect obviously removal efficiency of pyridine by strain KT-5. However, trace inorganic nutritious elements are necessary for the cell growth and removal of pyridine.

Acknowledgements

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