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Isolation and characterization of a nitrobenzene degrading yeast strain from activated sludge

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ABSTRACT

Strain Z1 was isolated from nitrobenzene-contaminated sludge. Strain Z1 was able to utilize nitrobenzene as a sole source of carbon, nitrogen, and energy under aerobic condition. Based on the morphology, physiological biochemical characteristics, and 26S rDNA D1/D2 domain sequence, strain Z1 was identified as *Rhodotorula mucilaginosa*. Strain Z1 mineralized up to 450 mg L^{-1} nitrobenzene. Kinetics of nitrobenzene degradation was described using the Andrews equation. The kinetic parameters were as follows: q_{max} = 1.50 h^{-1}, K_s = 31.31 mg L^{-1}, and K_i = 101.34 mg L^{-1}. Strain Z1 had a high-salinity tolerance. It degraded nitrobenzene effectively in 5% NaCl (quality concentration). Even in the presence of aniline or phenol, strain Z1 degraded nitrobenzene efficiently. Strain Z1 therefore could be an excellent candidate for the bio-treatment of nitrobenzene industrial wastewaters.

1. Introduction

Nitrobenzene is widely used in the manufacture of organic products, such as aniline, lubricating oils, dyes, drugs, pesticides, and synthetic rubber [1]. Its wide usage in industry has been contaminating the environment. Chronic exposure to nitrobenzene in humans can result in methemoglobinemia and liver damage [2]. Because of its toxicity, nitrobenzene is listed as a priority pollutant by the US Environmental Protection Agency [3].

Wastewater loaded with nitrobenzene can be treated with biological methods, including anaerobic method and aerobic method. Under anaerobic condition, nitrobenzene was reduced to aniline, and aniline was then completely degraded by a following aerobic process [4]. Under aerobic condition, nitrobenzene was mineralized to CO_2 and H_2O. Aerobic degradation of nitrobenzene has been studied by many researchers. Bacterial strains capable of degrading nitrobenzene were described, such as *Bacillus*, *Pseudomonas*, *Klebsiella*, *Comamonas*, *Streptomyces*, *Alcaligenes*, *Acinetobacter*, and *Flavobacterium* [2,5–8]. White rot fungi were also reported for degrading nitrobenzene [9]. However, researches to date have not described the degradation of nitrobenzene by yeast strains.

This paper reports on the isolation and characterization of a nitrobenzene degrading yeast strain. Since nitrobenzene industrial wastewater usually contains high inorganic salts (specifically NaCl) and multi-organic compounds [10], the nitrobenzene degradation by the yeast strain in high salinity or in the presence of other toxicants were tested. The purpose of this paper is to demonstrate the degradation effectiveness and application potential of the yeast strain.

2. Materials and methods

2.1. Chemicals

All of the chemicals used in this study were of Analar grade. The water used was double deionized water.

2.2. Enrichment and isolation of the yeast strain

The activated sludge used in this study was collected from a nitrobenzene-contaminated site, located in Dalian (Liaoning Province, PR China). Ten milliliters of the activated sludge was aseptically added to 90 mL of the sterilized MS medium [2] in a 250-mL flask. The pH of the MS medium was adjusted to 7.0. The 250-mL flask was supplemented with nitrobenzene to a final concentration of 200 mg L^{-1} and incubated on a rotary shaker (180 rpm) at 30 °C. When the culture became obviously turbid, 10 mL of the culture was transferred to 90 mL fresh MS medium in a new 250-mL flask with 200 mg L^{-1} nitrobenzene. This operation was repeated until the degradation of nitrobenzene came to a stable level. And then the culture was diluted and spread onto agar (1.5%) plates

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with MS medium and nitrobenzene. The agar plates were incubated at 30 °C for 4–5days. Colonies appearing on the agar plates were sub-cultured. Purity of the cultures was confirmed by microscopic examination. One of the most efficient isolates, strain Z1, was selected.

2.3. Identification of the yeast strain

The cell morphology was examined by a GEM-1200EX scanning electron microscope [2]. The 26S rDNA D1/D2 domain sequence was amplified by PCR with primers NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL4 (5′-GGT CCG TGT TTC AAG ACG G-3′). PCR mixtures contained LA Taq (0.5 μL), 2.5 mM dNTP (8 μL), 10× LA PCR buffer (5 μL), NL1 (1 μL), NL4 (1 μL), dH_2o (up to 50 μL), and genomic DNA (50 ng). The amplification conditions were a starting temperature of 94 °C for 5min; 55 °C for 1 min; 72 °C for 1 min, 30 cycles; and finally 72 °C for 5 min. The PCR instrument used was a TaKaRa Thermal Cycler Dice TP600 (TaKaRa Bio Inc.), the electrophoresis instrument used was a Mupid (ADVANCE-BIO Co., Ltd.), and the electrophoresis image-forming instrument used was an ImageMaster® VDS (Pharmacia Biotech). A partial sequence of the PCR products was determined for the isolate using an ABI PRISM™ 3730XL DNA Sequencer (Applied Biosystem). Agent using for sequencing was BigDye® Terminator V3.1 Cycle Sequencing Kit.

A maximum likelihood phylogenetic tree was generated by neighbor-joining methods. Evolutionary distance bootstrap values were determined using DNADIST program in PHYLIP.

2.4. Nitrobenzene degradation kinetics

One loop of the stock culture on the agar plates was transferred aseptically to 100 mL sterilized MS medium in a 250-mL flask with 200 mg L^{-1} nitrobenzene. Ten milliliters of the culture in the late exponential phase was aseptically inoculated into 90 mL sterilized MS medium in a 250-mL flask. The 250-mL flask was supplemented with nitrobenzene at different initial concentrations. The cultures were incubated on a rotary shaker (180 rpm) at 30 °C. All cultivations were repeated five times. A flask with same amount of the autoclaved cells (10 min, 60 °C) was used as control.

As nitrobenzene consumption rate decreased with the increase of nitrobenzene concentration in MS medium, the Andrews equation was used to describe the dependence of the specific substrate consumption rate (q) on the concentration of the substrate (nitrobenzene) [11]:

$$q = \frac{q_{\max} S}{S + K_s + (S^2/K_i)} \quad (1)$$

where $q_{\max}$ is the maximum specific substrate consumption rate, K_i is the inhibition constant, K_s is the half-rate constant, and S is the substrate concentration.

The effects of pH (3.0–12.0, 30 °C, 180 rpm) and temperature (10–50 °C, pH 7.0, 180 rpm) on nitrobenzene degradation were also investigated. Certain initial concentration of nitrobenzene (200 mg L^{-1}) was added into the MS medium. After 60 h of incubation, samples were withdrawn for the analysis of nitrobenzene concentration. All cultivations were repeated five times. A flask with same amount of the autoclaved cells (10 min, 60 °C) was used as control.

2.5. Nitrobenzene degradation

The capacity of strain Z1 to degrade nitrobenzene in high salinity was tested. Batch experiments were performed five times, and the mean values of the data are presented. Ten milliliters of the culture in the late exponential phase was aseptically inoculated into each 250-mL flask with 90 mL sterilized MS medium. Each 250-mL flask was supplement with 200 mg L^{-1} nitrobenzene and 2–7% (quality concentration) NaCl. The cultures were incubated on a rotary shaker (180 rpm) at 30 °C. Samples were withdrawn periodically for nitrobenzene concentration analysis. A flask with same amount of the autoclaved cells (10 min, 60 °C) was used as a biotic control. A flask with 200 mg L^{-1} nitrobenzene and 2–7% NaCl without cells constituted an abiotic control.

The same setup was also used for testing the capacity of strain Z1 to degrade nitrobenzene in the presence of aniline or phenol. Phenol concentrations ranged from 50 to 200 mg L^{-1} and aniline ranged from 25 to 100 mg L^{-1}. Samples were withdrawn periodically for the analyses of nitrobenzene concentration, aniline concentration, and phenol concentration.

Table 1
The physiological biochemical characteristics of strain Z1

Characteristics		Characteristics		Characteristics	
Carbohydrate fermentation		Carbohydrate assimilation		Nitrogen assimilation	
Glucose	–	Galactose	+	Nitrate	–
Galactose	–	Sucrose	+	Cadaverinc·2HCl	–
Sucrose	–	Maltose	+	L-Lysine	–
Maltose	–	Cellubiose	+		
Lactose	–	Trehalose	+		
Raffinose	–	Lactose	–		
		Raffinose	+		
		Soluble starch	–		
		D-Xylose	+		
		L-Arabinose	+		
		D-Ribose	+		
		L-Rhammose	–		
		Erythritol	–		
		Ribitol	–		
		D-Mannitol	+		
		Succinic acid	+		
		Citric acid	+		
		Inositol	–		

(+), growth; (–), no growth.

2.6. Analytical methods

The dry cell mass was determined as described by Du et al. [12]. Nitrobenzene concentrations were measured with a V-560 UV–vis spectrophotometer (JASCO, Japan) using *N*-(1-naphthyl) ethylenediamine dihydrochloride as per standard procedure [13]. Phenol concentrations were analyzed as depicted by Zhang et al. [14] and aniline concentrations by Jia et al. [15]. Prior to a total organic carbon (TOC) content analysis, samples were centrifuged (15 °C) at 20,000 × g for 20 min to remove the biomass. The TOC contents were determined using a TOC-5000 analyzer (Shimadzu, Japan) [16].

2.7. Nucleotide sequence accession number

The 26S rDNA D1/D2 domain sequence of strain Z1 was deposited at the GenBank under accession number DQ778627.

3. Results and discussion

Strain Z1 was able to utilize nitrobenzene as a sole source of carbon, nitrogen, and energy under aerobic condition. After incubation in malt juice culture at 25 °C for 3 days, the morphology of strain Z1 was ovate or elliptic with size of (2.8–5.2) μm × (2.5–6.6) μm. When strain Z1 grew on wort agar plate for 1 month at 25 °C, the colony was cheese-like, pink, glistening, and smooth with regular edge. There was no feigned hypha generated when strain Z1 grew on cornmeal agar. Table 1 shows the physiological biochemical characteristics of strain Z1.

The 26S rDNA D1/D2 domain sequence of strain Z1 was 613 bp. A maximum likelihood tree was shown in Fig. 1. Based on the

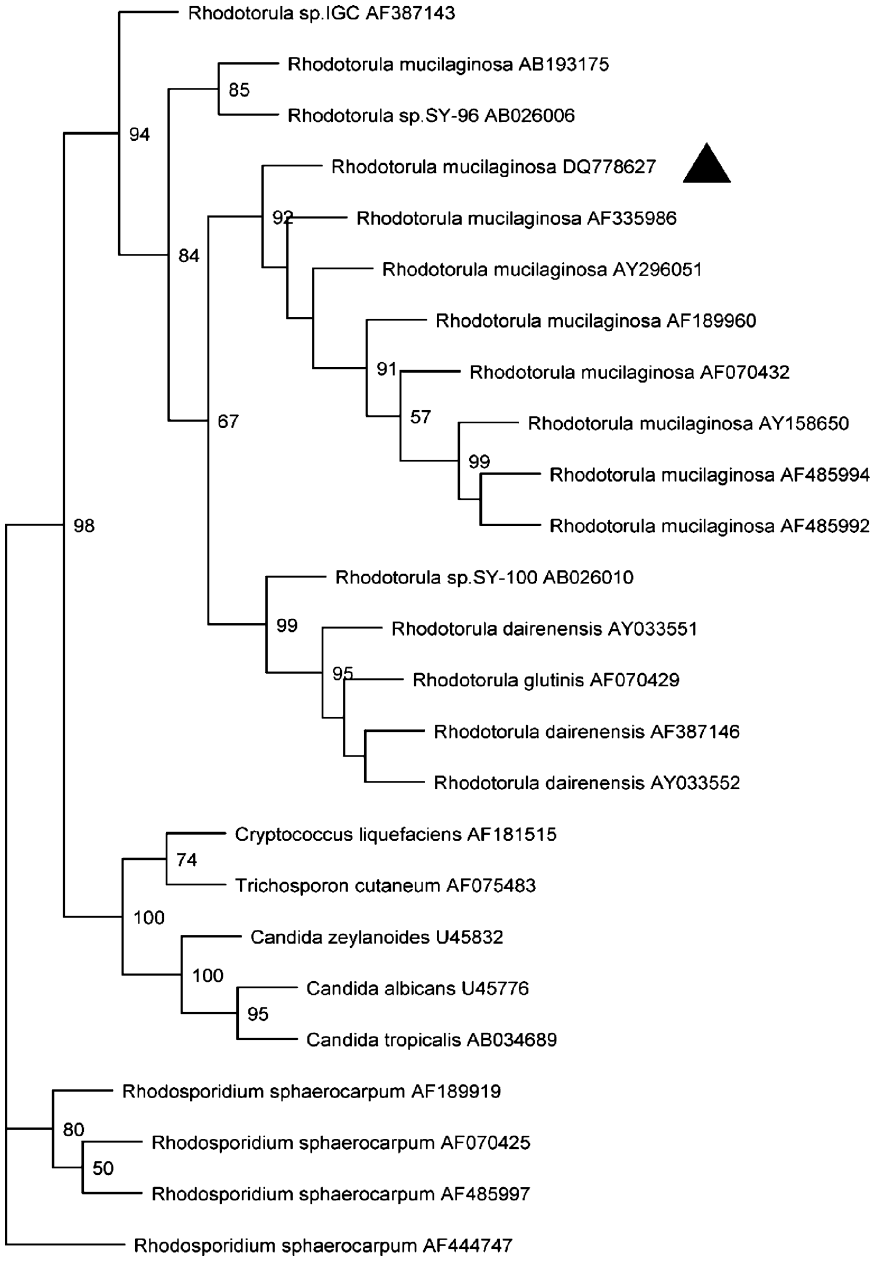

Fig. 1. Phylogenetic tree of strain Z1 based on 26S rDNA D1/D2 domain sequence comparison.

sequencing of 26S rDNA D1/D2 domain with 613-bp, the homology between strain Z1 and a *R. mucilaginosa* (GenBank accession no. AF335986) was 99%. Strain Z1 was identified as *R. mucilaginosa*. *R. mucilaginosa* spreads widely in air, soil, lakes, ocean water, and dairy products. It may colonize plants, humans, and other mammals [17]. Recent studies have reported that *R. mucilaginosa* can be used in the production of β-carotene [18]. However, reports on the degradation of nitrobenzene by *R. mucilaginosa* are very few. The finding of this paper indicates that nitrobenzene degradation can be achieved with yeast strains, not only with bacteria and white rot fungi.

Fig. 2 shows the effect of temperature on nitrobenzene degradation and cell growth. Strain Z1 grew well at 30–40 °C. The degradation rates of nitrobenzene, however, were beyond 70% at 25–40 °C. Strain Z1 was inactive when temperature was lower than 25 °C or higher than 40 °C, because considerable cell death was observed and only small amount of degradation was realized. Cultures of pH 4.0–11.0 all reached degradation extents of above 55%, and pH 7.0 was optimal (with a degradation rate of 98%, Fig. 3). In conclusion, the degradation of nitrobenzene by strain Z1 can be achieved in a wide range of pH, and a slight acidic condition was preferable. The optimal pH and temperature were pH 7.0 and 25–40 °C, respectively.

Fig. 4 shows the results of nitrobenzene consumption and cell growth profiles at initial nitrobenzene concentrations of 200 mg L^{-1}. Strain Z1 degraded 200 mg L^{-1} nitrobenzene within 60 h and over 98% of TOC was removed. These results indicated that most of the organic carbon was transformed to carbon dioxide. The TOC curve showed that TOC decreased more slowly than nitrobenzene concentration. Fig. 5 shows the degradation of nitrobenzene by strain Z1 at different initial concentrations. Strain Z1 grew on nitrobenzene up to the concentration of 450 mg L^{-1}. As shown in Fig. 4, the lag phase was extended at higher nitrobenzene concentrations. Strain Z1 completely degraded nitrobenzene with initial

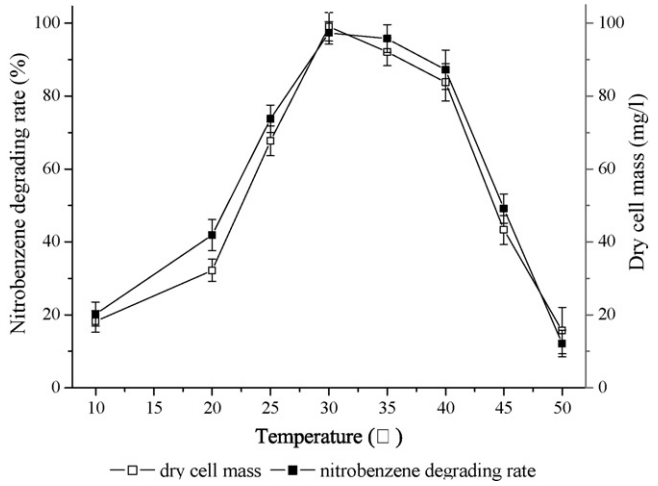

Fig. 2. Effect of temperature on cell growth and nitrobenzene degradation by strain Z1. The experiments were repeated five times. The error bars depicted the 95% confidence intervals.

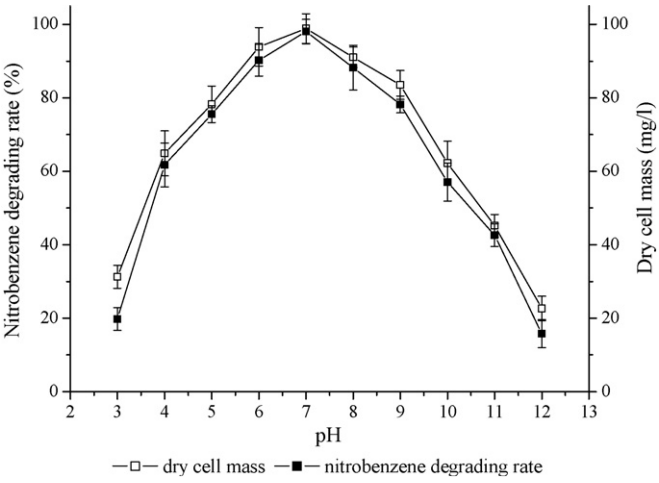

Fig. 3. Effect of pH on cell growth and nitrobenzene degradation by strain Z1. The experiments were repeated five times. The error bars depicted the 95% confidence intervals.

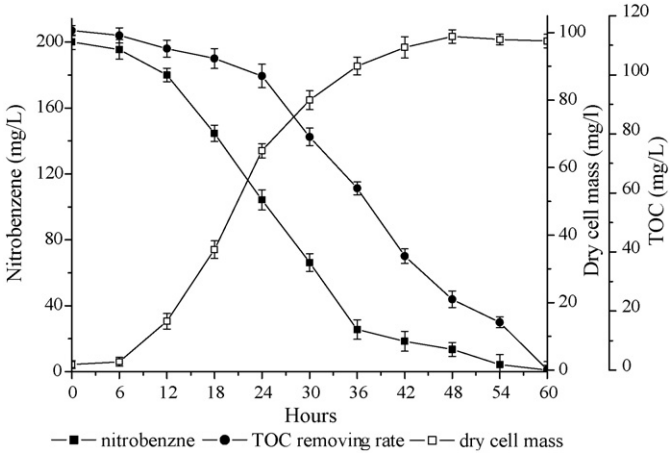

Fig. 4. Degradation of 200 mg L^{-1} nitrobenzene by strain Z1. The experiments were repeated five times. The error bars depicted the 95% confidence intervals.

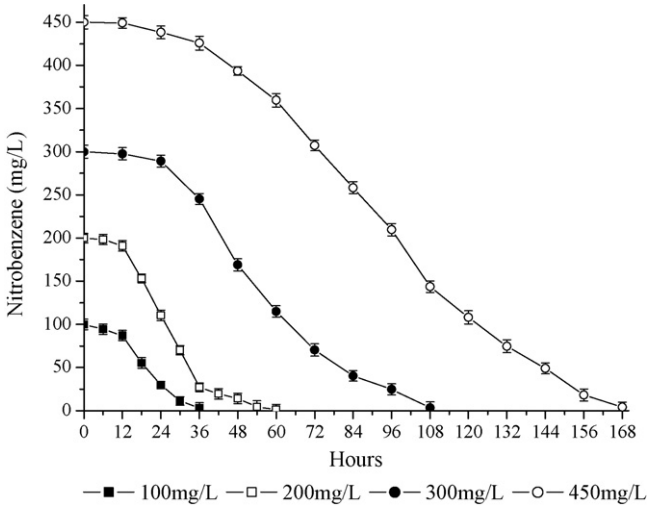

Fig. 5. Degradation of different concentrations of nitrobenzene by strain Z1. The experiments were repeated five times. The error bars depicted the 95% confidence intervals.

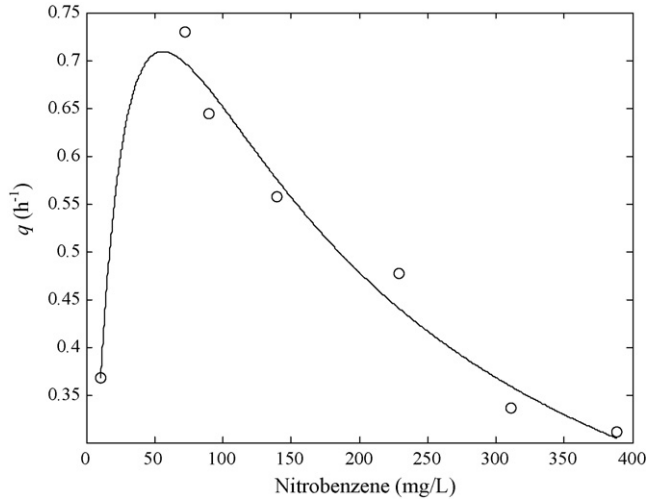

Fig. 6. Kinetic model for the nitrobenzene degradation by strain Z1.

concentration of 100, 300, and 450 mg L^{-1} within 36, 108, and 168 h, respectively. The dry cell mass increased slightly with the increase in nitrobenzene concentration (data not shown). These results demonstrated that most of the nitrobenzene was utilized by strain Z1 for the generation of energy to drive metabolic reactions, not for the production of new biomass.

The relationship between specific consumption rate (q) and initial nitrobenzene concentrations was shown in Fig. 6. When the initial concentrations of nitrobenzene were lower than 70 mg L^{-1}, the q gradually increased. At higher concentrations above 90 mg L^{-1}, the inhibition effect of nitrobenzene became prominent. It is a fact that nitrobenzene displays the inhibitory nature at high concentrations. The Andrews equation was used here to express the kinetics of nitrobenzene degradation. The value of R^2 was 0.9626, which demonstrated that the experimental data was well correlated by Andrews equation. By using a non-linear least squares regression analysis, the kinetic parameters were determined as follows: q_{max} = 1.50 h^{-1}, K_s = 31.31 mg L^{-1}, and K_i = 101.34 mg L^{-1}.

Nitrobenzene industrial wastewater usually contains inorganic salts and multi-organic compounds. The existence of these com-

Table 2
Concentrations of aniline and phenol during the degradation period by strain Z1

Multi-component system	Aniline concentration (mg L^{-1})		Phenol concentration (mg L^{-1})	
	0 h	60 h	0 h	60 h
200 mg L^{-1} nitrobenzene + aniline or phenol	25	23.12	50	47.28
	50	49.17	100	98.34
	75	73.98	150	147.01
	100	97.71	200	195.95

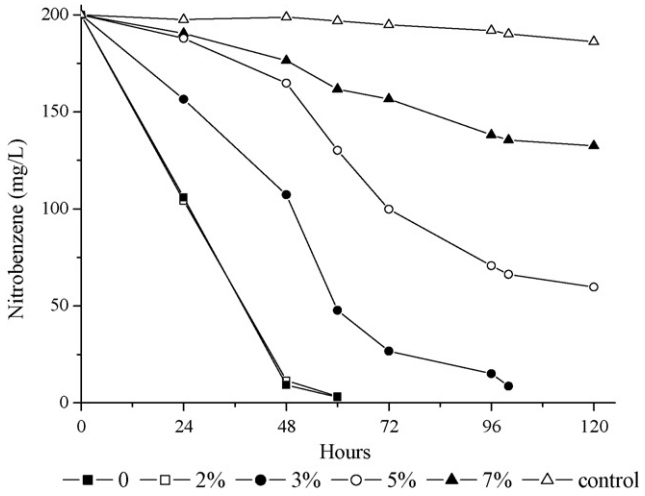

Fig. 7. Degradation of 200 mg L^{-1} nitrobenzene by strain Z1 in different quality concentrations of NaCl. The experiments were repeated five times, and the means value of data were presented.

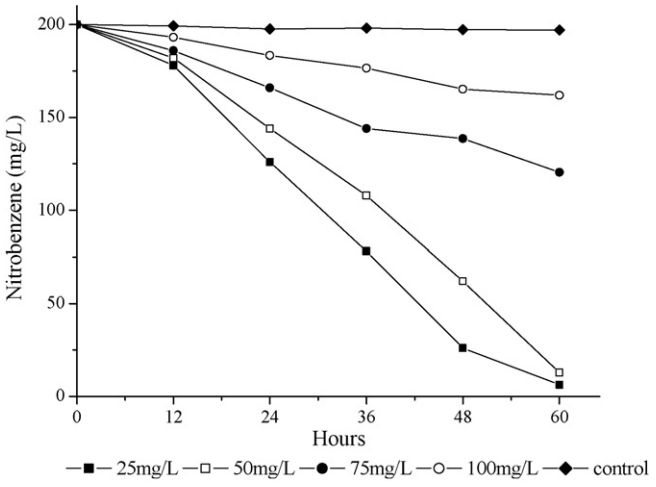

Fig. 8. Degradation of 200 mg L^{-1} nitrobenzene by strain Z1 in the presence of aniline. The experiments were repeated five times, and the mean values of data were presented.

pounds can influence the performance of biological process. It has been generally accepted that conventional microbes could not be used to treat wastewater containing salts (specifically NaCl) over 3% [19]. In this study, the nitrobenzene degradation by strain Z1 in high salinity was tested (Fig. 7). When the quality concentration of NaCl was 2%, strain Z1 completely degraded 200 mg L^{-1} nitrobenzene after 60 h. The degradation of nitrobenzene by strain Z1 in 2% salinity and 0% salinity presented no distinction. These result demonstrated that strain Z1 grew well in 2% NaCl. At a higher concentration of NaCl (3%), the nitrobenzene degradation was less effective within 60 h. However, over a longer period, 100 h, strain Z1 degraded 200 mg L^{-1} nitrobenzene efficiently. After 100 h, the degradation rate of nitrobenzene reached to 97%. Even the concentration of NaCl reached to 5%, strain Z1 degraded, up to 70%, nitrobenzene with 200 mg L^{-1} after 120 h. These results showed that strain Z1 had a high-salt tolerance. Both of the abiotic control and biotic control showed that the concentrations of nitrobenzene remained unchangeable.

The nitrobenzene degradation by strain Z1 was also tested in the presence of aniline or phenol, since these toxicants were present along with nitrobenzene in industrial wastewaters. Strain Z1 degraded 200 mg L^{-1} nitrobenzene in the presence of 50 mg L^{-1} aniline without inhibition (Fig. 8). Over 93% of nitrobenzene was removed after 60 h. At 75 mg L^{-1} of aniline, the nitrobenzene degradation rate decreased sharply. Phenol concentrations of up to 100 mg L^{-1} did not inhibit the nitrobenzene degradation by strain Z1 (Fig. 9). The degradation rate of nitrobenzene was above 95% after 60h. However, at the higher concentration of 150 mg L^{-1}, the inhibition effect of phenol on nitrobenzene degradation became predominant. These results showed that strain Z1 degraded nitrobenzene effectively even in the presence of these toxicants. The concentrations of aniline and phenol were also monitored. As can be seen from Table 2, during the degradation period, the concentrations of aniline and phenol kept invariable.

Many research works have reported the degradation of nitrobenzene by microbes under aerobic condition (Table 3). Wei et al. isolated three bacteria (*Bacillus subtilis*, *Pseudomonas mendocina*, and *Klebsiella penumoniae*), which utilized nitrobenzene as the sole source of carbon and energy. Of the three bacteria, *B. subtilis* manifested the highest nitrobenzene degradation ability [5]. *B. subtilis* degraded 200 mg L^{-1} nitrobenzene completely

Table 3
Microorganisms capable of degrading nitrobenzene under aerobic condition

Microorganism	Initial nitrobenzene concentration (mg L^{-1})	Degradation rate (%)	Degradation time (h)
Bacillus subtilis [5]	200	99	145
Pseudomonas mendocina [5]	200	70	145
Klebsiella pneumoniae [5]	200	80	145
Streptococcus [20]	100	44	72
Stapylococcus [20]	100	28	72
Corynebacterium [20]	100	21	72
Streptomyces [2]	200	99	72
A mixed culture [21][a]	100–200	–[b]	

[a] The mixed culture contained four bacterial genera (*Acinetobacter*, *Alcaligenes*, *Flavobacterium*, and *Pseudomonas*) and one yeast (*Rhodotorula*).
[b] The degradation of nitrobenzene was inhibited.

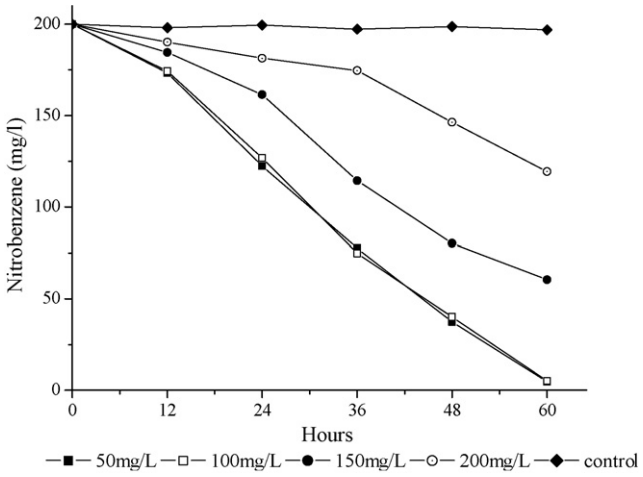

Fig. 9. Degradation of 200 mg L^{-1} nitrobenzene by strain Z1 in the presence of phenol. The experiments were repeated five times, and the mean values of data were presented.

within 145 h, while the mixture of the three bacteria did so within 120 h. Liu et al. isolated three strains, which belonged to the genera of *Streptococcus*, *Stapylococcus* and *Corynebacterium* [20]. When 100 mg L^{-1} nitrobenzene served as the sole source of carbon and energy, *Streptococcus*, *Stapylococcus*, and *Corynebacterium* degraded 44%, 28%, and 21% of nitrobenzene after 72 h, respectively. Davis et al. reported the cultures from industrial wastewater, which contained four bacterial genera (*Acinetobacter*, *Alcaligenes*, *Flavobacterium*, and *Pseudomonas*) and one yeast (*Rhodotorula*) [21]. The cultures utilized nitrobenzene as the sole source of nitrogen. When nitrobenzene concentrations were 100–200 mg L^{-1}, the biodegradation by the cultures was inhibited. Zheng et al. isolated a *Streptomyces* strain, which utilized nitrobenzene as the sole source of carbon, nitrogen and energy [2]. This *Streptomyces* strain degraded 200 mg L^{-1} nitrobenzene completely within 72 h and tolerated nitrobenzene up to 400 mg L^{-1}. The nitrobenzene degradation by laccase from white rot fungus was also tested [9]. At the concentration of 150 mg L^{-1}, over 86% of nitrobenzene was removed by 72 h. At the concentration of 200 mg L^{-1}, nitrobenzene was not degraded. Levin et al. investigated the nitrobenzene degradation by the white rot basidiomycete *Trametes trogii* [22]. At concentrations of 250–500 mg L^{-1}, more than 90% of nitrobenzene was removed within 12–24 days. Although the strain *T. trogii* tolerated nitrobenzene up to the concentration of 500 mg L^{-1}, the degradation time was long. Also, the medium used for cultivation of the strain *T. trogii* was complex. In this paper, the Z1 strain utilized nitrobenzene as a sole source of carbon, nitrogen, and energy. It degraded 200 and 450 mg L^{-1} nitrobenzene completely within 60 and 168 h, respectively. Even in 3–5% NaCl or in the presence of aniline and phenol, the Z1 strain degraded 200 mg L^{-1} nitrobenzene effectively. These results demonstrate that the Z1 strain has a higher potential for being applied to nitrobenzene industrial wastewater treatment.

4. Conclusions

A yeast strain capable of degrading nitrobenzene was isolated from nitrobenzene-contaminated sludge. This yeast strain was identified as a *R. mucilaginosa* and designated strain Z1. Strain Z1 tolerated nitrobenzene up to the concentration of 450 mg L^{-1}. It completely degraded nitrobenzene with initial concentration of 100, 200, 300, and 450 within 36, 60, 108, and 168 h, respectively. The Andrews equation was used to express the kinetics of nitrobenzene degradation. The kinetic parameters were as follows: q_{max} = 1.50 h^{-1}, K_s = 31.31 mg L^{-1}, and K_i = 101.34 mg L^{-1}. Strain Z1 had a high-salinity tolerance. It degraded 200 mg L^{-1} nitrobenzene in 3–5% NaCl efficiently. Even in the presence of phenol or aniline, strain Z1 degraded 200 mg L^{-1} nitrobenzene effectively. Phenol inhibited the nitrobenzene degradation at the concentration of 100 mg L^{-1}. Compared to phenol, aniline displayed higher inhibitory effect on nitrobenzene degradation. When the concentration of aniline reached to 75 mg L^{-1}, the degradation rate of nitrobenzene decreased sharply.

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