The Study about the Degradation of Nitrobenzene by Domesticated Activated Sludge and the Initial Validation of Toxicology before and after the Degradation

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Abstract: In this paper, the domestication of the activated sludge by which nitrobenzene can be degraded in a relatively poor nutrition is studied. The ability of activated sludge degradation of nitrobenzene is studied by degradation kinetics. The toxicology validation about the effect of activated sludge degradation of nitrobenzene is preliminarily explored. The results show that: The nitrobenzene could be the sole carbon and nitrogen source in the domestication of the activated sludge; The degradation process is in line with the feature of first order kinetics; When nitrobenzene of 130mg/L ,85mg/L and 40mg/L is degraded, the kinetic equations are : lnC = -0.0112t + 4.5767, lnC= -0.0116t + 4.5075 and lnC = -0.0098t + 4.514, the half-life t1/2 are 61.88h, 59.74h and 70.71h, the degradation rate constant K（h-1）are 0.0112,0.0116 and 0.0098; MTT assay can be used to initially study the changes of cytotoxicity before and after the biodegradation of nitrobenzene polluted water samples; The activated sludge domesticated in this experiment is preliminarily verified that it can significantly reduce the toxicity of nitrobenzene polluted water sample.

Keyword: nitrobenzene; Biodegradation; Toxicology validation; Activated sludge

1 Introduction

Nitrobenzene, also be known as the dense patch oil, is colorless or slightly yellow oil with almond-flavored liquid. Mainly used for making aniline, benzidine, azobenzene, and so on. Nitrobenzene have toxic, absorbing large quantities of steam or contamination from high concentration of it on skin can cause acute poisoning, make hemoglobin oxidated or complex, the color of blood will turn to dark brown, and cause headaches, nausea and vomiting. Chemical properties of nitrobenzene make it be more stable in the water, and be slightly soluble in water. The density of nitrobenzene is greater than water. When being discharged into water, nitrobenzene would deposit on the bottom for a long time as water pollution [1].

Traditional degradation methods of nitrobenzene are low efficiency and easy to cause secondary pollution. Full domesticated activated sludge can be efficiently and securely on the biological degradation of nitrobenzene in water, but aniline and other higher toxic compounds often be produced after degradation, and activated sludge usually be influenced by the nutrition conditions. A kind of activated sludge by which nitrobenzene can be degraded in a relatively poor nutrition is domesticated and the toxicology validation about the effect of activated sludge degradation of nitrobenzene preliminarily explored.

2 Materials

2.1 Chemicals

Fetal calf serum (FCS) (Hyclone); RPMI 1640 (Gibco); Trypsin(Gibco); Dimethyl sulfoxide (DMSO) (Beijing Soledad Lite-On Technology Co., Ltd.); MTT (Sigma); Nitrobenzene (Tianjin Guangfu Fine Institute of Chemistry); Beef extract (Beijing obo Star Bio-Technology Co., Ltd.); Peptone (Beijing obo Star Bio-Technology Co., Ltd.); Sodium chloride (Tianjin Tianxin Fine Chemicals R&D Center); Potassium dihydrogen phosphate (Tianjin Chemreagent Chemical Reagents Development Center); Dipotassium hydrogen phosphate (Tianjin Tianxin Fine Chemicals R & D Center); Sodium hydroxide (Tianjin Tianxin Fine Chemicals R & D Center); Hydrochloric acid (Tianjin Tianxin Fine Chemicals R&D Center). Benzene (HPLC pure, Tianjin Chemical Reagent Research Institute)

Trace Element Solution: $MnSO_4.H_2O$ 0.28g, $FeSO_4.7H_2O$ 0.3g, $MgSO_4.7H_2O$ 0.06g, $CaCl_2$ 1g, $CuSO_4$ 0.05g, $ZnSO_4$ 0.05g, H_3BO_3 0.05g, add them to distilled water of 1000mL.

2.2 Equipment

Agilent 6890N gas chromatograph (Agilent Technologies, Inc.); SANYO MC0175-type CO2 incubator (NAPCO); OLYMPUS inverted microscope, Olympus Corporation, Japan; GILSON pipette (France); 96-well culture plates (Orange Scientific); WELLSCAN MK3 type micro plate reader (United States Bio-Rad company); Clean Bench (Sujing Group); Constant temperature shaker (hadonglian); 752 spectrophotometer (Shanghai Spectrum Instruments Co., Ltd.); high-pressure Autoclave (SANYO); High-speed centrifuge (Shanghai Anting Scientific Instrument

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Factory).

2.3 Tumor Cell Lines

The HepG2 cells are provided by the Institute of Medical, Harbin University of Commerce.

2.4 Activated Sludge Source

Harbin City Wastewater Treatment Plant

2.5 Medium Preparation

2.5.1Domestication Medium

Take sodium chloride 5g, dipotassium hydrogen phosphate 4g, potassium dihydrogen phosphate 1.5g, 1ml by adding trace elements in 1000ml distilled water, adjust the ph value to 7.0,120 ℃ sterilization 20min, prior to use aseptic technique by adding a certain amount of nitrobenzene .

2.5.2 Enrichment Medium

Add sodium chloride 5g, beef extract 5g, peptone 10g to distilled water of 1000mL, adjusting the ph value to 7.0, sterilizes at 120℃ for 20 minutes.

3 Methods

3.1 The domestication of activated sludge

Add activated sludge for 5g to enrichment medium of 100mL, set the constant temperature shaker to 30℃, 140r/min, and culture for 24h.

Centrifuge medium at 3000r/min for 20min, remove supernatant, add nitrobenzene to domestication medium of 100ml, make the concentration of nitrobenzene to 10mg/L, and set the constant temperature shaker to 30℃, 140r/min, and culture for 3d.

Centrifuge medium at 3000r/min for 20min, remove supernatant, add the same concentration of nitrobenzene as previous step to enrichment medium of 100mL, and set the constant temperature shaker to 30℃, 140r/min, and culture for 24h. Centrifuge the culture medium at 3000r/min for 20min, remove supernatant, then add nitrobenzene to domestication medium of 100mL, make the concentration of nitrobenzene to 20mg/L, set the constant temperature shaker to 30℃, 140r/min, and culture for 3d.

Repeat the steps above in medium containing 40mg/L, 60mg /L, 80mg /L, 100mg/L, 120mg/L, 130mg/L, 140mg/L, 150mg/L of nitrobenzene, separate the final culture medium according to the conditions above after domestication, use the domestication medium without nitrobenzene to wash bacteria centrifuge for one time then continue separate by centrifuge after washing.

3.2 Degradation

3.2.1 Draw the Activated Sludge Growth Curve to Find the Slow Growth Stage of Activated Sludge

The isolated bacteria will be added as the measure as 5% of the whole volume to enrichment medium of 100mL, culture in the shaker at 30℃and 140r/min, respectively, sample 1ml at 0,4,8,12,24,28,36,48,60,72h, using spectrophotometer to measure all samples at wavelength of 600nm, make the sampling time as X-axis, OD values as the Y-axis ,draw the activated sludge growth curve.

3.2.2 Activated Sludge Degradation of Nitrobenzene Experiment

Take activated sludge in slow growth period, add it as the measure as 5% of the whole volume to domestication mediums of 100mL which contain nitrobenzene of 130mg/L, 85mg/L and 40mg/L, set the constant temperature shaker to 37℃, 130r/min, set up the control experiments without bacteria, respectively, sampling 1mL at 4, 8,12,24,36,48,72, and 96h, prepare the samples and use the gas chromatograph to measure the concentration of nitrobenzene[2].

● Analysis Method Sample treatment method: Adding 3mL of benzene in the sample bottle, oscillating for 1min, extracting for two times then combine the extract, preserve them at 4℃ for the test.

Gas chromatography conditions: Column: HP-5 column (30m×0.25mm×0.25μm); Mobile phase: high-purity nitrogen gas (N_2, 99.99%); Flow rate of carrier gas: 0.8mL/min; Sample volume: 1μL; Split ratio: 50:1; Column temperature: 120℃; Injection port temperature: 260℃; electron capture detector temperature: 240℃ [3, 4].

●MTT assay

HepG2 human hepatoma cells and human gastric cancer cell SGC-7901 was cultured with the RPMI1640 culture medium which containing 10% volume fraction of fetal bovine serum. Set incubator to 37℃, 5% of the CO_2. Using the cells in logarithmic growth phase for the experiment [5].

Take the cells in logarithmic growth phase, use complete culture medium to adjust cell concentration to 5 $\times 10^4$/L, then put the cells into 96-well plate, as 100μL each hole, set humidified incubator to 37℃, 5% of the CO_2, culture for 24 h.

Put the nitrobenzene samples that before and after be depredated to 96-well plate as 100μL each hole, make the concentrations of the respectively 1, 1/2, 1/4 times initial concentration of the samples; Blank control group: plus RPMI1640 medium for 100μL; set three flat-shaped holes for each concentration. Set incubator as 37℃ and 5% humidified CO2, then culture for 72h.

Use quickly flap method to discard the supernatant, add MTT test solution ($0.5g•L^{-1}$) 100μL to each well, continue to foster the supernatant discarded after 4h, then add DMSO to each hole for 200μL, dissolved it using the micro-oscillator, measure the absorbance values of each hole by using the micro plate reader in detective wavelength of 570nm. Calculate the cell viability, cell growth inhibition rate.

$$\text{Cell viability}(\%) = \frac{\text{OD values of cells in the experimental group}}{\text{OD value of control cells}} \times 100\%$$

$$\text{Cell growth inhibition rate}(\%) = 1 - \text{Cell viability}(\%)$$

4 Results

4.1 Growth curves of activated sludge

The growth curve of activated sludge which be got from the experiment shows as Figure 1.

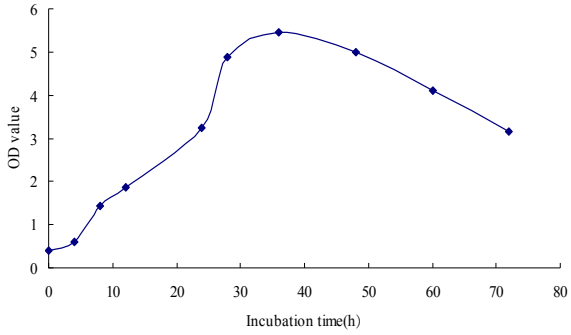

Figure 1. The grows curve of activated sludge

The activated sludge in slow growth period has both strong oxidation and adsorption capacity of organic matter, and also has a good settling characteristic. From the growth curve, we can see that the activated sludge that be cultured for 28 ~ 36h is in the slow growth period, which consistent with the requirement of experiment.

4.2 Nitrobenzene Standard Curve and Linear Range

Dissolve the nitrobenzene standard in benzene; respectively prepare six different concentrations of nitrobenzene standard solutions as 1.2, 2.16, 3.12, 4.08, 5.04 and 6.0mg/L. After testing them by gas chromatography methods, make the nitrobenzene concentration (C) as the value of abscissa, the integral area (AUS) as the longitudinal coordinates, then we got the standard curve: y = 1E+07x + 2E+06, r=0.9983. The results showed that the nitrobenzene of 1.2-6.0mg/L have good linearity.

4.3 Determination of the Recovery Rate of Nitrobenzene

Take the sterilized culture mediums with different concentrations of nitrobenzene, make them be extracted and determined according to experimental method, then we got the nitrobenzene extraction percent recovery is (86.65 ± 2.20)% (n = 5).

4.4 The Kinetics Analysis to Nitrobenzene Degradation

In order to understand the dynamics property of degradation, the test studied degradation rates of nitrobenzene which initial concentration are 130mg/L, 85mg/L and 40mg/L in a under the conditions of inoculating a certain amount of activated sludge. Measured results shown in Figure 2, Table I.

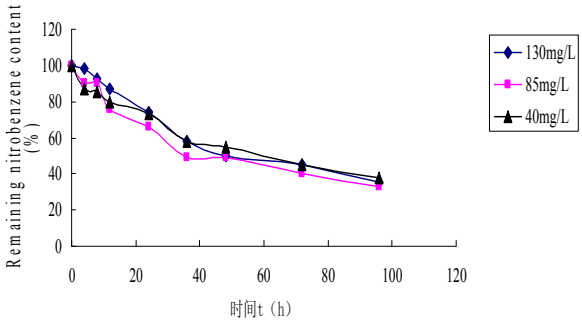

Figure 2. Growth curve of nitrobenzene

TABLE I. DEGRADATION KINETICS OF NITROBENZENE

The initial concentration of nitrobenzene /mg·L^{-1}	Kinetic equation	Half-life $t_{1/2}$/h	Degradation rate constant K/h^{-1}	Correlation r
130	lnC = -0.0112t + 4.5767	61.88	0.0112	0.9812
85	lnC = -0.0116t + 4.514	59.74	0.0116	0.9686
40	lnC = -0.0098t + 4.5075	70.71	0.0098	0.9844

Figure2 shows that the activated sludge can use nitrobenzene as the sole carbon and nitrogen sources and in that way, the nitrobenzene could be depredated. Table 1 show that the degradation process is in line with the feature of first order kinetics. When nitrobenzene of 130mg/L, 85mg/L and 40mg/L is degraded, the kinetic equations are: lnC = -0.0112t + 4.5767, lnC= -0.0116t + 4.5075 and lnC = -0.0098t + 4.514.

4.5 MTT assay results

MTT results are shown in table II.

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TABLE II. THE INHIBITORY EFFECT OF NITROBENZENE ON HEPG2 CELL（$\bar{X}\pm S$，N=3）

Content	The number of parallel samples	OD value	inhibition rate （%）
Control			
Before degradation	3	1.168±0.022	-
1	3	0.670±0.028	42.620±2.379
1/2	3	0.742±0.066	36.453±5.616
1/4	3	0.866±0.006	25.807±8.018
After degradation			
1	3	0.919±0.093	21.297±7.935*
1/2	3	1.021±0.032	12.590±2.735**
1/4	3	1.043±0.064	10.650±5.450*

**P<0.01 vs. control * P<0.05 vs. control

MTT assay detected the changes of cytotoxicity before and after the biodegradation of nitrobenzene polluted water samples, the result is shown in the table. Nitrobenzene acting on HepG-2 cells for 72h, then we can see that cell growth was inhibited to varying degrees, the growth inhibition rate reduced when the nitrobenzene concentration is decreased, it is statistically significant when be compared with the control group and the various concentrations of nitrobenzene before degradation.($P<0.05$ or $P<0.01$).

5 Conclusions

The domestication of the activated sludge by which nitrobenzene can be degraded in a relatively poor nutrition, and the degradation process is in line with the feature of first order kinetics.

MTT assay is able to initially study the changes of cytotoxicity before and after the biodegradation of nitrobenzene polluted water samples, so this method can be used to study the degradation effect of the activated sludge method, the degradation conditions and the way of domestication.

After the initial identification of MTT assay, the activated sludge domesticated in this experiment is preliminarily verified that it can significantly reduce the toxicity of nitrobenzene polluted water sample.

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