ISOLATION AND CHARACTERIZATION OF A HIGH SALT-TOLERANT AND GLYPHOSATE-DEGRADING STRAIN OF \textit{Agrobacterium tumefaciens} BZ8

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(Submitted: December 15, 2015; Revised: April 16, 2016; Accepted: April 16, 2016)

Abstract – In this study, a high salt-tolerant and glyphosate-degrading strain named BZ8 was isolated from activated sludge. According to 16S rDNA sequencing methods, morphological, physiological and biochemical analysis, strain BZ8 was identified as Agrobacterium tumefaciens. The growth and glyphosate-degrading capability of \textit{A. tumefaciens} BZ8 were investigated and the results showed that the optimum conditions for glyphosate degradation under 6% of NaCl concentration were found as follows: inoculation size of 10% (v/v), incubation temperature 37°C and initial pH of 5.0. Salt tolerance test showed that \textit{A. tumefaciens} BZ8 grew well and could thoroughly degrade 2000 mg/L glyphosate in 36 h if the concentration of NaCl was lower than 6%, while the degradation rate decreased gradually with increasing NaCl concentration. But the glyphosate degradation rate could still reach 62% when the salt concentration was 8%. In addition, the kinetic parameters for \textit{A. tumefaciens} BZ8 grown on 100-2800 mg/L glyphosate according to Haldane’s model could predict the cell growth tendency successfully. These results showed that \textit{A. tumefaciens} BZ8 could be used to control glyphosate wastewater with high salt content. Therefore, it has potential application.

Keywords: salt-tolerant; glyphosate-degrading strain; identification; growth kinetics.

INTRODUCTION

Glyphosate is the most widely used herbicide in the world because it is a cheap, high efficient, low residue, broad-spectrum and foliar applied weed-killer. It can be used on non-cropland as well as on a great variety of crops. Nowadays, China is the largest exporter of glyphosate in the world. However, during the production of glyphosate, the discharge of wastewater can cause serious water pollution problems and environmental damage (Xu et al., 2007). This is because the wastewater mainly contains glyphosate, NaCl and so on (Amoros et al., 2007). It was reported that producing one ton of glyphosate could cause production of 5-6 tons of glyphosate wastewater. Therefore, it is necessary to find safe and effective treatment methods for glyphosate wastewater.

Up to now, several technologies, which can be defined as physical methods, chemical techniques and biological treatments, have been developed for the treatment of glyphosate wastewater. Among these, physical and
The screened strain was characterized and identified based on its morphological, biochemical properties and 16S rRNA gene sequence analysis. The obtained cells of exponential growth phase in SM were harvested by centrifugation at 12000 rpm, 4℃ for 20 min and washed with sterile water. By using the method mentioned by Zhai et al. (Zhai et al., 2012), the genome of the strain was extracted.

Part of the 16S rRNA gene was amplified by PCR using the forward primer: Eu27F (5'-AGAGTTTGATCATGGCTCAG-3') and the reverse primer: 1492R (5' -TACGCTACCTTGTAGACTT-3'). DNA amplifications were performed in 50 μL reactions containing approximately 100 ng of total DNA, 4 μL of 10× buffer, 4 μL of 25 mmol/L MgCl2, 4 μL of 2.5 mmol/L dNTPs, 2 μL of each of 10 mmol/L primer and 0.4 μL of 5 U/μL Taq polymerase. The PCR conditions were as follows: 94℃ for 3 min followed by 30 cycles of denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s and primer extension at 72℃ for 2 min, followed by a final step at 72℃ for 10 min. PCR products were purified using a commercial kit (Sangon, Shanghai, China), and then the obtained purified PCR products were sequenced by Sangon Biotech Company (Shanghai, China). The sequencing result was submitted to GenBank for BLAST analysis. A phylogenetic tree was constructed by the neighbor-joining method using the software MEGA 4.0 (Tamura et al., 2007).

Biodegradation experiments

The stored culture of strain BZ8 maintained on a nutrient agar slant was inoculated into 100 mL SM supplemented with 2000 mg/L glyphosate. Then the bacterial sub-culture
at the late exponential phase was used as inoculum. The biodegradation experiments were performed in 500 mL flasks with a working volume of 100 mL at 200 rpm.

In order to investigate the influence of initial inoculation size, initial pH and temperature on glyphosate degradation, replicate flasks with SM supplemented with 2000 mg/L glyphosate and 60 g/L NaCl were conducted at initial inoculum sizes varying from 2% to 15%, initial pH ranges from 3 to 7 and temperatures from 20℃ to 42℃, respectively. A similar experimental procedure was used for testing the capacity of the strain BZ8 to degrade glyphosate under the optimized conditions.

The effect of NaCl concentrations on glyphosate degradation was investigated by applying different NaCl concentration (20, 40, 60, 80 and 100 g/L) at a constant glyphosate concentration of 2000 mg/L.

For the estimation of intrinsic kinetic parameters, a pure culture of strain BZ8 was inoculated into 100 mL SM in 500 mL flasks with 60 g/L NaCl under the optimized conditions. The SM was supplemented with glyphosate concentrations varying from 100 to 2800 mg/L. All the experiments were performed in triplicate. Culture samples were regularly taken for measurement of biomass and glyphosate concentration, and results were shown as means ± confidence intervals.

**Glyphosate degradation kinetics**

The most commonly used kinetic model to describe microbial growth is the Monod kinetic model. However, this model does not represent the growth kinetics of inhibitory substrates. Haldane’s model developed from the Monod kinetic model has been used widely to characterize the metabolic inhibition of substrate on the microbial growth (Li et al., 2010). Therefore, in this study, Haldane’s equation was used to describe strain BZ8 growth kinetics. For each flask with a certain initial glyphosate concentration, the specific growth rate (μx, 1/h) was calculated as:

$$\mu_x = \frac{r_x}{\rho_x} = \frac{d\rho_x}{dt} / \rho_x$$

where rx is the cell growth rate (mg/L/h) and ρx is the cell concentration (mg/L). Then μx was modeled using Haldane’s model described as follows:

$$\mu_x = \frac{\mu_{\text{max}} \rho_S}{K_S + \rho_S + \rho_S^2 / K_{SI}}$$

where $\mu_{\text{max}}$ is the maximum specific cell growth rate (1/h), $\rho_S$ is the glyphosate concentration (mg/L), $K_S$ is the saturation constant (mg/L), and $K_{SI}$ is the self-inhibition constant (mg/L).

The yield coefficient (mg dry cell mass/mg glyphosate) $Y_m$ was calculated using the following equation:

$$Y_m = \frac{M_{\text{max}} - M_i}{\rho_S - \rho_{Sr}}$$

where $M_{\text{max}}$ is the maximum biomass concentration (mg/L), $M_i$ is the initial cell concentration (mg/L), $\rho_S$ is the initial substrate concentration (mg/L), and $\rho_{Sr}$ is the residual glyphosate concentration when the cell concentration reached the maximum (mg/L).

**Analytical procedures**

The cell concentration was monitored spectrophotometrically at 600 nm. The uninoculated sterile medium was used as a control. The optical density (OD) value was then converted to dry cell mass (DCW) using a calibration curve (Vasiliadou et al., 2008). The relationship between DCW and OD was found to be DCW (mg/L)=287.3× OD600−3.27; R²=0.9997. The concentration of glyphosate in the culture was determined by a high performance liquid chromatography (HPLC) method on a 1100 series HPLC (Agilent, American) as described by Kawai et al. (1991).

**RESULTS AND DISCUSSION**

**Characterization and identification of glyphosate-degrading strain BZ8**

Several strains were isolated from activated sludge by the method mentioned above. The glyphosate degradation ability was confirmed by the glyphosate degradation rate. Among them, a bacterium, named BZ8, was found to exhibit the highest glyphosate degradation ability and thus was chosen for further study. Colonies of strain BZ8 were smooth, round, moderate humidity, neat edges and white opaque. This bacterium is non-spore-forming, aerobic and gram-negative rod-shaped. It was V.P experiment-negative, nitrate reductase-positive, catalase-negative, utilization of citrate, hydrolysis of starch, glutin-positive and indole production-negative. It also utilizes sucrose, maltose, glycerol, D-galactose, xylose, D-glucitol, mannitol as sole carbon sources for growth.

The 16S rRNA gene is a highly conserved gene and was used for the phylogenetic analysis of taxa at higher levels. The 16S rRNA gene sequences of strain BZ8 were obtained (comprising 1385 nucleotides) and submitted to GenBank (http://www.ncbi.nlm.nih.gov). The sequence displayed the highest similarity (99%) to that of *A. tumefaciens* (GenBank accession AB535688.1 and FR828338.1). A phylogenetic tree was constructed based on the 16S
rRNA coding gene sequences of the isolate and the nearest relatives (Fig. 1). Combined with the morphological, physiological and biochemical analysis, the strain can be identified as *A. tumefaciens*. So, this strain was named *A. tumefaciens* BZ8.

Effects of inoculation size, initial pH, temperature and salinity on glyphosate degradation

Fig. 2 illustrates the effect of inoculation size on the glyphosate degradation of *A. tumefaciens* BZ8. Experiments were carried out with the same initial glyphosate concentration of 2000 mg/L. It was obvious that the glyphosate concentration decreased with time for different inoculation sizes. Glyphosate was thoroughly degraded after 54 h, 48 h, 34 h and 36 h at inoculation sizes of 2%, 5%, 10% or 15%, respectively (Fig. 2). The effect of inoculum size on cell growth of strain BZ8 is also shown in Fig. 2. As shown in Fig. 2, with the increase of inoculum size, the lag period of the cell growth shortened gradually, while the cell growth rate and the substrate utilization rate were accelerated. Table 1 presents the values of glyphosate degradation rate with respect to inoculation size. The glyphosate degradation rate was greatly increased with inoculation size up to 10% (v/v), but it kept almost constant as the inoculation size further increased up to 15%. Taking into account the growth of strain BZ8 and glyphosate degradation, 10% (v/v) was chosen as the optimum inoculum size and used for all following experiments.

Glyphosate degradation and the cell growth were also tested at various initial pH values (Fig. 3). As expected, the pH greatly influenced glyphosate degradation as well as cell growth in the strain BZ8 culture system. Fig. 3 illustrates that the growth of strain BZ8 was positively related with the glyphosate degradation. The optimal pH for *A. tumefaciens* BZ8 growth occurred at pH 5.0. The shortest time to degrade glyphosate thoroughly occurred at pH 5.0. Apparently, a pH value of 5.0 would be the optimum for degradation of glyphosate by this bacterial strain. However, there was no distinct difference in glyphosate degradation rate as pH increased from 4.0 to 6.0 (Table 1). Although the glyphosate biodegradation efficiencies of the other two systems (pH 3.0 and pH 7.0) were significantly delayed, the strain BZ8 was still capable of entirely consuming glyphosate. This special pH adaptability indicates that the strain BZ8 could be applied to acidic or neutral conditions without altering the pH.

The influence of temperature on the glyphosate degradation and growth of strain BZ8 is illustrated in Fig. 4. The results show that strain BZ8 could effectively degrade glyphosate in the temperature range from 30°C to 42°C. Especially under the temperature of 37°C, BZ8 showed higher efficiency of glyphosate degradation as compared to the other three temperatures. The glyphosate degradation rate was seen to decrease with increasing or falling temperature (Table 1). However, at a low temperature of 20°C and a high temperature of 42°C the strain BZ8 could still degrade glyphosate thoroughly within 70 h and 40 h, respectively. Therefore, strain BZ8 is a potentially useful microorganism that could function at both comparatively low and high temperatures. This broad temperature adaptability may be important for biodegradation in glyphosate-contaminated environments that undergo daily and seasonal temperature changes.

**Figure 1.** Phylogenetic tree of strain BZ8 based on 16S rRNA gene sequence analysis. Bootstrap values obtained with 1000 repetitions are indicated as percentages at all branches. The scale bar indicates 0.002 substitutions per nucleotide position.

_Brazilian Journal of Chemical Engineering_
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Table 1. Glyphosate degradation rates of *A. tumefaciens* BZ8 under different conditions

<table>
<thead>
<tr>
<th>Initial inoculum size (%)</th>
<th>Glyphosate degradation rate (mg/L/h)</th>
<th>Initial pH</th>
<th>Glyphosate degradation rate (mg/L/h)</th>
<th>Culture temperature (℃)</th>
<th>Glyphosate degradation rate (mg/L/h)</th>
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</thead>
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<td>2</td>
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<td>6</td>
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</tr>
<tr>
<td>—</td>
<td>—</td>
<td>7</td>
<td>40.93</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*A. tumefaciens* BZ8 grew well over the temperature ranges from 30°C to 42°C, which was significantly different at 20°C (Fig. 4). It reached the stable phase after 36 h at 30-42°C. At 20°C, the stable phase was recorded after 66 h. The fastest cell growth speed of BZ8 occurred at 37°C. According to these results, the temperature of 37°C was applied in the following set of experiments.

The actual glyphosate wastewater normally contains various concentrations of NaCl, which directly influences the activity of glyphosate-degrading microorganisms. In this study, strain BZ8 was inoculated into SM medium with 2000 mg/L glyphosate at different NaCl concentrations and incubated at 37°C for 36 h. As shown in Fig. 5, the strain grew well and glyphosate could be completely degraded in 36 h when the concentration of NaCl was lower than 60 g/L. With increasing salt concentration, the cell growth was inhibited gradually and the glyphosate degradation rate decreased greatly. The possible reason is that high salinity could cause osmotic stress or inhibit the reaction pathways in the organic degradation process. This results in a significant decrease in biological treatment efficiency or biodegradation kinetics. In addition, high salt content induces cell lysis, which causes increased effluent solids. However, the glyphosate degrading rate of strain BZ8 could still achieve 62% when the salt concentration reached 80 g/L. Compared with the other salinities, strain BZ8 grew difficultly at the salinity of 100 g/L, and the total glyphosate removed was only 35%. Therefore, strain BZ8 could be applied for comparatively high salinity glyphosate wastewater treatment.

Growth kinetics of *A. tumefaciens* BZ8

The consumption of glyphosate and growth of *A. tumefaciens* BZ8 at various initial glyphosate concentrations were investigated with the same NaCl concentration of 60 g/L (Fig. 6). As shown in Fig. 6,
with the increase of glyphosate concentration, the time to degrade glyphosate thoroughly gradually shortened. It was obvious that the strain BZ8 could completely degrade 2800 mg/L glyphosate within 56 h (Fig. 6a). This maximum biodegradable glyphosate concentration of BZ8 was higher than Bacillus cereus CB4 (Fan et al. 2012) and Arthrobacter sp. N4 (Bazot and Lebeau, 2008), and it is inferred that strain BZ8 could tolerate a relatively higher concentration of glyphosate than most glyphosate-degrading microorganisms in previous reports.

The biomass and glyphosate concentrations were linearly related for most of the active growth phase, so the yield coefficient (Ym, mg/mg) was calculated by linearizing the decline of glyphosate with the increment of cell mass using Equation (3). All coefficients of correlation (R2) were found to be above 0.99. The growth yields observed in this study varied between 0.201 mg/mg and 0.353 mg/mg as the initial glyphosate concentration was varied from 100 to 2800 mg/L (Fig. 6b). The highest value (0.353 mg/mg) was found at a glyphosate concentration of 200 mg/L, where the maximum specific growth rate was also obtained (Fig. 7).

When the concentrations were between 0 and 200 mg/L, the values of specific growth rate increased gradually with increase of glyphosate. There was a slight decrease in specific growth rate as the glyphosate concentrations increased from 200 mg/L to 400 mg/L; however, beyond 400 mg/L, with an increase of glyphosate concentration, a remarkable decline in specific growth rate occurred (Fig. 7).
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The phenomenon could be explained by the fact that the sole carbon source of glyphosate was consumed mainly for assimilation into biomass and energy for cell growth and maintenance. When the inhibition effect of glyphosate becomes predominant above the glyphosate concentration of 400 mg/L, the proportion of the total glyphosate converted to energy for cell growth and maintenance increased as the specific growth rate decreased. More energy is required to overcome the effect of substrate inhibition at high glyphosate concentrations, while the proportion of the total substrate assimilated into biomass decreases as specific growth rates decrease. Therefore, substrate inhibition is known to reduce both specific growth rate and the yield coefficient.

The specific growth rate (µx) was estimated using Equation (1) by performing a linear least-squares regression on the semi-logarithmic plot of the biomass concentration over cultivation time in the exponential growth phase. Then, the µx data were employed to determine Haldane’s parameters by nonlinear least-squares regression analysis using Matlab 7.0. The Haldane parameters for strain BZ8 grown on glyphosate were obtained as µmax = 1.28 h⁻¹, Ks = 84.82 mg/L, and Ki = 227.59 mg/L (R²=0.992). It is clear that Haldane’s equation was strongly correlated with the experimental data (Fig. 7). This indicates that Haldane equation was suitable to describe the process of strain BZ8 glyphosate degradation in terms of cell growth behavior.

CONCLUSIONS

In present study, a high salt-tolerant and glyphosate-degrading strain BZ8 was isolated and identified as A. tumefaciens. The growth and glyphosate-degrading capability of the strain BZ8 were investigated and the optimum degradation conditions were obtained. Salt tolerance tests showed that the strain grew well and could thoroughly degrade 2000 mg/L glyphosate in 36 h when the concentration of NaCl was lower than 6%, and the glyphosate degradation rate could still reach 62% when the salt concentration was 8%. The kinetic parameters for strain BZ8 grown on glyphosate according to Haldane’s model were µmax = 1.28 h⁻¹, Ks = 84.82 mg/L, and Ki = 227.59 mg/L. The results demonstrate that strain BZ8 has a remarkable potential for application in the disposal of industrial glyphosate wastewater.

ACKNOWLEDGEMENT

The present research was financially supported by Anhui Provincial Natural Science Foundation (Grant No. 1508085QC56), the Key research and development project of Anhui Province (Public welfare research linkage17040704067), the National Natural Science Funds (grant no. 31501461), the foundation of Huainan science and technology planning project (grant no. 1095), the Liquor Making Biological Technology and Application of key laboratory of Sichuan Province (grant no. NJ2014-18), the Key project of Natural Science Foundation of Anhui Provincial Department of Education (Grant No. KJ2015A279), the Key projects of domestic and foreign research and training of outstanding young and middle aged backbone talents in Universities (gxfxZD2016203).

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