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Isolation and characterization of *Sporobolomyces* sp. LF1 capable of degrading chlorimuron-ethyl

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Abstract

A yeast strain which was capable of degrading sulfonylurea herbicide chlorimuron-ethyl named as LF1 was isolated from a chlorimuron-ethyl contaminated soil near the warehouse of the factory producing chlorimuron-ethyl in Shenyang City, Northeast China. The strain was identified as *Sporobolomyces* sp., based on its morphological and physiological characteristics and the phylogenetic analysis of 18S rRNA gene sequence. So far, this is the only yeast strain of *Sporobolomyces* sp. which is able to degrade chlorimuron-ethyl. Incubation tests showed that when the initial concentration of chlorimuron-ethyl in culture was 5 mg/L, LF1 could degrade more than 77% of the herbicide after incubation for 4 d at 30°C. The possible mechanism of chlorimuron-ethyl degradation by LF1 could be the acidic hydrolysis caused by the acids from the metabolism of the yeast strain. Further study should be conducted to examine the pathways of chlorimuron-ethyl degradation by LF1 and to approach the feasibility of using LF1 to degrade the chlorimuron-ethyl in soil system.

Key words: chlorimuron-ethyl; *Sporobolomyces* sp.; metabolic acid; acidic hydrolysis
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Introduction

Sulfonylurea is a family of herbicides that can control weeds via inhibiting acetolactate synthase (ALS), a key enzyme in the branched-chain amino acid biosynthetic pathways of bacteria, fungi, and higher plants (Blair and Martin, 1988; Brown, 1990; Nemat Alla *et al*., 2008). It is widely used in many parts of the world, because of its low application rate (2–100 g/ha), high herbicidal effect, broad action spectrum, good crop selectivity, and low human and animal toxicity (DL_{50} on rat is generally > 5000 mg/kg) (Brown, 1990; Zanardini *et al*., 2002). Chlorimuron-ethyl is a member of sulfonylurea, and specifically used for the control of broadleaf and gramineal weeds before or after their germination in soybean field (Reddy *et al*., 1995). This herbicide was introduced into China in 1993, and has been used popularly in the country. Only in Heilongjiang Province, a total of 400 tons of chlorimuron-ethyl is used, and covers more than 1.33×10^6 ha of soybean field each year (Zhao and He, 2007). The fate and behavior of chlorimuron-ethyl in the environment has been widely investigated (Reddy *et al*., 1995; Choudhury and Dureja, 1996, 1997; Nilanjan *et al*., 2006; Zhang *et al*., 2007). Like other members of sulfonylurea, chlorimuron-ethyl can persist in soil for a long time, giving significant damage to crops and affecting soil functional microbes and soil enzymes (Wagner *et al*., 1995; Boldt and Jacobsen, 1998; EL-Ghamry *et al*., 2001; Gigliotti and Allievi, 2001; Soltani *et al*., 2005; Wang and Zhou, 2005; Teng and Tao, 2006, 2008; Yang *et al*., 2007; Nemat Alla *et al*., 2008). Moreover, because of its low K_{ow} and high water solubility, chlorimuron-ethyl is prone to leach through soil to groundwater, easily causing groundwater pollution via precipitation and irrigation (Briggs *et al*., 1981; Afyuni *et al*., 1997). Consequently, there is a need to find a way to rapidly detoxify the residua of chlorimuron-ethyl in the environment.

Sulfonylurea can be dissipated in soil mainly through chemical hydrolysis and microbial degradation, and its breakdown is largely dependent on soil temperature, moisture, organic matter, and pH (Blair and Martin, 1988). The first process of the breakdown is mainly chemical hydrolysis, where the sulfonylurea bridge is cleaved, making the molecule biologically inactive; the second process is microbial degradation, where a wide range of microbes can metabolize the herbicide, ultimately resulting in the complete mineralization of the chemical (Berger and Menne, 1998; Braschi *et al*., 2000; Morrica *et al.*, 2001).

The degradation of sulfonylurea is faster and more

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effective in non-sterilized than in sterilized soil, which indicates that the degradation depends prevalently on soil microbes (Joshi *et al*., 1985; Miller *et al*., 1997). Therefore, it may be feasible to utilize microbes to detoxify chlorimuron-ethyl residua in soil. However, the studies on the degradation of sulfonylurea by pure microbial cultures are relatively limited. Up to now, there are some reports about the degradation of metsulfuron-methyl, chlorsulfulor, bensulfuron-methyl, ethametsulfuron-methyl, and azimsulfuron by pure cultures (Zanardini *et al*., 2002; Yu *et al*., 2005; Zhu *et al*., 2005; He *et al*., 2006; Valle *et al*., 2006; Gu *et al*., 2007), but very little information about the degradation of chlorimuron-ethyl.

The objectives of this study were to isolate and characterize the microbes for degrading chlorimuron-ethyl under laboratory conditions, and to determine the optimal conditions for the degradation of chlorimuron-ethyl in pure culture.

1 Materials and methods

1.1 Chemicals and soil

Chlorimuron-ethyl (97% purity) is purchased from Shanghai Anpel Instrument Co., Ltd., China. Dichloromethane is of analytical reagent grade and redistilled before use. Methanol and acetic acid glacial are of chromatographic pure grade and filtrated before use. Other chemicals used in this study are all of analytical grade.

The test soil is a chlorimuron-ethyl contaminated aquic brown soil near the warehouse of the factory producing chlorimuron-ethyl in Shenyang City of Northeast China.

1.2 Enrichment and isolation of chlorimuron-ethyl degrading strains

Ten grams of 0–20 cm soil samples was added to 100 mL of nitrogen-limited minimal salts medium ($MgSO_4{\cdot}7H_2O$ 0.2 g, $MnSO_4{\cdot}H_2O$ 0.02 g, $CaCl_2$ 0.1 g, $FeSO_4{\cdot}7H_2O$ 0.01 g, KH_2PO_4 0.4 g, Na_2HPO_4 0.6 g, glucose 5.0 g, distilled water 1 L, pH 7) in 250 mL flask, and 10 mg of chlorimuron-ethyl as the sole nitrogen source for microbes was added to the mixture at 7-d intervals. After three additions of chlorimuron-ethyl, 10 mL of the soil suspension was transferred to a sterile mineral salts medium containing 100 mg/L chlorimuron-ethyl. When the mineral solution turned turbid, 1 mL of the suspension was transferred to a fresh sterile mineral salt medium containing 200 mg/L chlorimuron-ethyl. The procedure was repeated three times to increase the level of chlorimuron-ethyl to 500 mg/L. The final enriched culture was spread on the culture agar plates containing 100 mg/L chlorimuron-ethyl, and the medium comprised (per 1000 mL distilled water): (1) for bacteria: beef extract 5.0 g, peptone 10.0 g, NaCl 5.0 g, agar 15.0 g, pH 7.0, (2) for fungi: glucose 10.0 g, peptone 5.0 g, KH_2PO_4 1.0 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, 1% benzolrose 3.3 mL, agar 20.0 g, and 2 mL of 80% lactic acid were added before incubation, and (3) for actinomycetes: starch 20 g, KNO_3 1 g, NaCl 0.5 g, K_2HPO_4 0.5 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, $FeSO_4{\cdot}7H_2O$ 0.01 g, agar 15.0 g, and pH 7.2. 10 mL of 1% $K_2Cr_2O_7$ was added before incubation. Different colonies were picked up, and further purified by using streak plating method. No bacteria and actinomycetes capable of degrading chlorimuron-ethyl were obtained from the enrichment culture, but a total of 5 yeast isolates were found to possess the ability of degrading chlorimuron-ethyl, among which, strain LF1 had the highest degradation efficiency, and was selected for further studies.

1.3 Identification and characterization of LF1

The identification of LF1 was based on its colony morphology and cultural and biochemical characteristics following with reference to The Yeasts: a Taxonomy Study (Yarrow, 1998), Identification Manual of Fungi (Wei, 1979), and Illustrated Genera of Imperfect Fungi (Barnett and Hunter, 1972). The 18S rRNA gene was amplified by using polymerase chain reaction (PCR) with the universal primer pair (Hang *et al*., 2003) of EF3 (5'-TCCTCTAAATGACCAAGTTTG-3') and EF4 (GGAAGGGRTGTATTTATTAG-3'). The conditions for PCR were: denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, at 48°C for 1 min, and 72°C for 3 min, and a final extension at 72°C for 10 min. PCR fragments were purified by agarose gel electrophoresis. The 1445 bp of 18S rRNA gene sequence was determined at Takara Biotechnology Co., Ltd., Dalian, China and compared with the most similar sequence in the Genebank nucleotide sequence database based on the percentage similarities. Sequences with the greatest similarity to the isolate LF1 sequence were extracted from the database and aligned. Software DNAMAN 4.0 (Lynnon BioSoft, Vaudreuil, Canada) and CLASTAL X 1.83 (Thompson *et al*., 1997) were used for sequence analysis and contrast. The tree was generated by MEGA 3.0 (Kumar *et al*., 2004) and ultimately deposited at GenBank under accession number FJ356010.

1.4 Inoculum preparation for degradation study

The strain LF1 was cultured in 250 mL flask containing 100 mL of YM medium (peptone 5.0 g, yeast extract 3.0 g, malt extract 5.0 g, distilled water 1000 mL) supplemented with 100 mg/L chlorimuron-ethyl. At its exponential phase, LF1 was collected by centrifugation. The pellets were washed two times with 0.2 mol/L KH_2PO_4-Na_2HPO_4 buffer, re-suspended in the same buffer, and used for inoculation.

1.5 Degradation of chlorimuron-ethyl by LF1

LF1 was isolated from the minimal salts medium (MSM) supplemented with chlorimuron-ethyl as the sole nitrogen source. To have a better growth of LF1, the degradation test of chlorimuron-ethyl by LF1 was performed in MSM supplemented with nitrogen sources NH_4NO_3 (MSNM) and chlorimuron-ethyl. An inoculum (5%, *V/V*) of LF1 was inoculated into MSNM, and all cultures were incubated in a rotary shaker at 150 r/min. The degradation efficiency was determined and estimated by the removal

percentage of chlorimuron-ethyl from the liquid culture.

1.6 Determination of optimum degradation conditions of chlorimuron-ethyl by LF1

Three levels of chlorimuron-ethyl (5, 10, and 50 mg/L), NH_4NO_3 (0.1, 1, and 5 g/L), and temperature (20, 30, and 40°C) were installed. Each treatment was tri-replicated, with un-inoculation as the control.

1.7 Quantification of chlorimuron-ethyl by HPLC

The culture of 20 mL was transferred to a tube, and extracted three times with 20 mL CH_2Cl_2 for each. After dehydration with anhydrous sodium sulfate, the organic phases were collected in a 100-mL flat bottom flask, and concentrated to almost dryness with rotary evaporator (EYELA Rotary Evaporator N-1000, Japan). Methanol was added to dissolve chlorimuron-ethyl, and the volume was up to 5 mL for concentration determination.

The recovery efficiency of sulfonylurea from aqueous solution is largely affected by pH (Yu *et al*., 2005; Negre *et al*., 2005; Ye *et al*., 2006), and acidic pH (< 7) was also helpful to the recovery. To detect the optimal pH for the best recovery of chlorimuron-ethyl from aqueous solution, the pH of the culture before extraction was adjusted to 2.5, 5.0, and 7.0, and each treatment was tri-replicated.

The chlorimuron-ethyl concentrations of all samples were analyzed by high performance liquid chromatography (HPLC, Agilent 1100, USA) equipped with a Zorbax SB-18 ODS Spherex column (4.6 μm × 250 mm) and DAD array detector. The operating conditions were: mobile phase methanol and 0.1% (*V/V*) acetic acid in water (70:30, *V/V*), ultraviolet detection wavelength 254 nm, flow rate 1 mL/min, injection volume 10 μL, and column temperature 25°C.

All experiments were performed in triplicates, and data were analyzed statistically by using program SPSS 13.0.

2 Results and discussion

2.1 Identification and characterization of LF1

After 5 d incubation at 30°C, the LF1 colonies on YM agar Petri dish were cream-colored, and their surfaces were glossy, with butyrous texture and entire margin (Fig. 1a). Moreover, there were some fuzzy images corresponding to LF1 colonies on the plate cover when the dish was inversed for 7 d, which proved that the strain had ballistospore. After 4 d incubation at 30°C, the vegetable cells in liquid YM medium were shown as ellipsoid, single, and gemmative under light microscope (BH-2, Olympus, Japan) (Fig. 1b), and thin ring and a little sediment began to form in test tubes. Dalmau plate culture on PDA showed that no pseudomycelium and true mycelium were presented.

Conventional biochemical tests (Table 1) showed that LF1 was negative in glucose and lactose fermentation tests. LF1 could assimilate potassium nitrate and produce acidic matter, but could not produce starch classes.

According to the above-mentioned morphological and biochemical characteristics, LF1 could be preliminary identified as *Sporobolomyces*.

The 18S rRNA gene sequence (1445 bp) of LF1 was obtained, and a phylogenetic tree based on the partial 18S rRNA gene sequence of LF1 was constructed (Fig. 2). LF1 was related to *Sporobolomyces falcatus* lineage (Hamamoto *et al*., 2000), with the sequence similarity score of 98.96%. The result of this phylogenetic analysis was consistent with that of morphological characterization and biochemical tests, and thus, the isolate LF1 was finally identified as *Sporobolomyces* sp.

2.2 Degradation of chlorimuron-ethyl by LF1

To obtain the best recovery of chlorimuron-ethyl from liquid culture, the chlorimuron-ethyl concentration was set at 5 mg/L. When the culture pH was 2.5, 5.0 and 7.0, AR (average recovery) and RSD (relative standard deviation) were 96.55% and 0.0128, 88.55% and 0.0087, and 81.95%

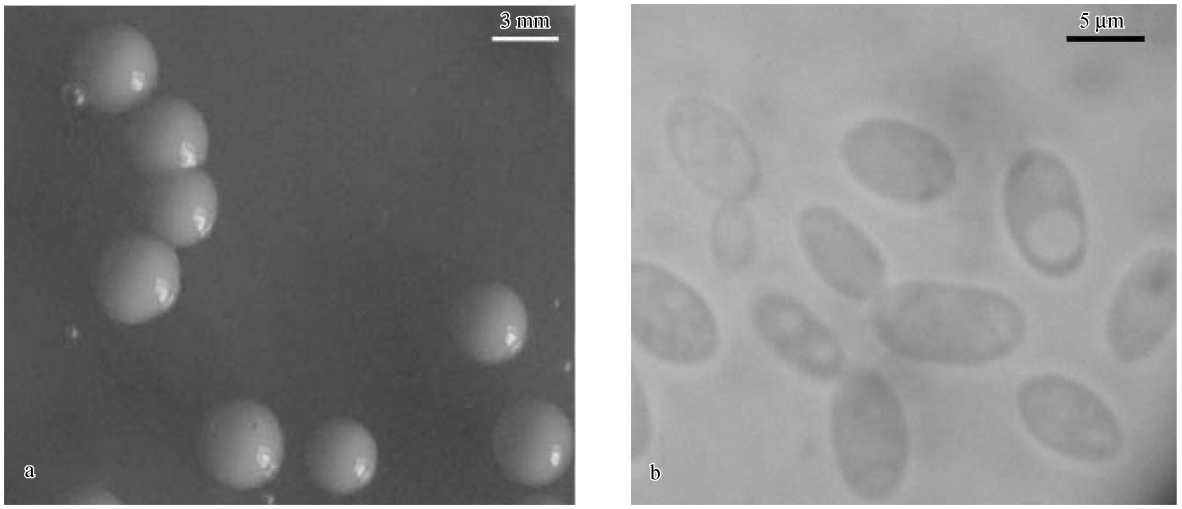

Fig. 1 Colony morphology, after 5 d incubation on YM agar Petri dish at 30°C (a), vegetative cells after 4 d incubation in liquid YM medium at 30°C (b).

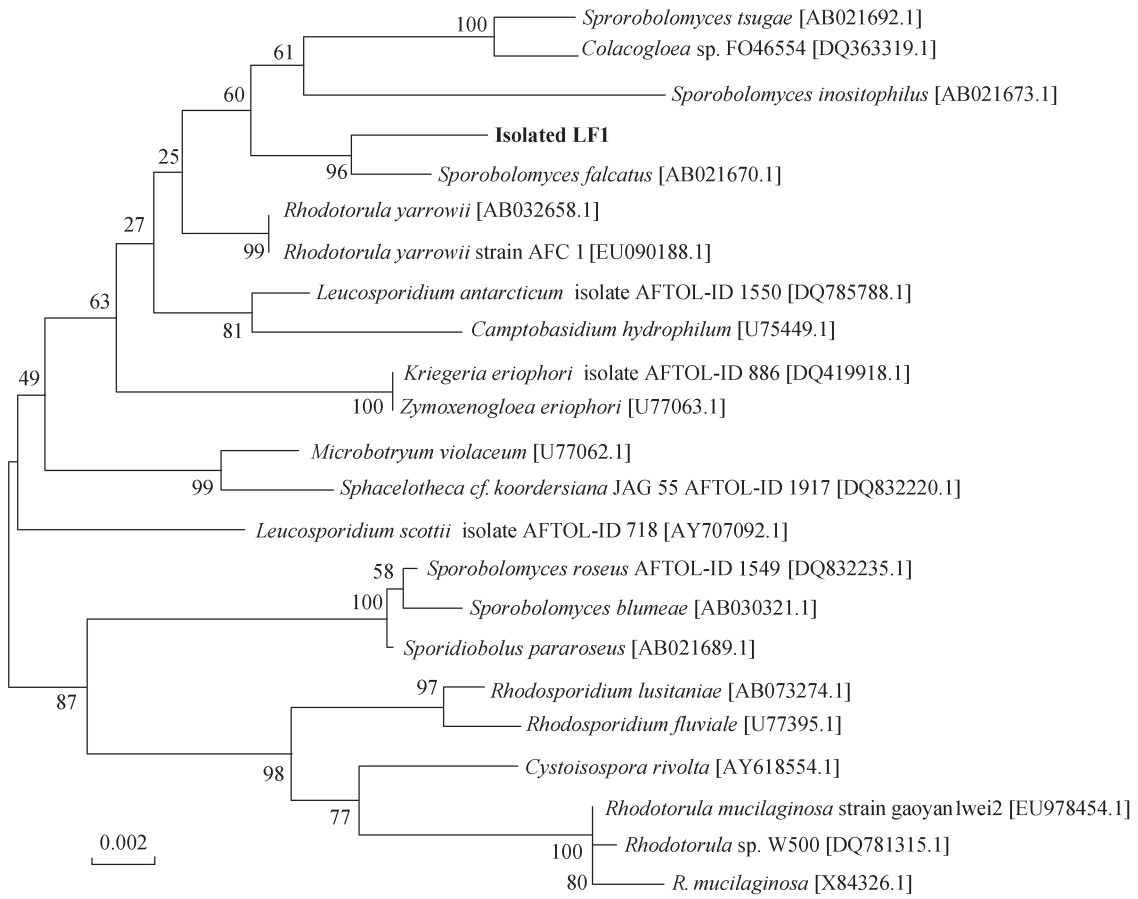

Fig. 2 Phylogenetic tree based on 18S rRNA gene sequences of LF1 and related species. The GenBank accession number for each microorganism used in the analysis is shown in square brackets after species name. The scale bar indicates 0.002 substitutions per nucleotide position. Bootstrap values obtained with 1000 resamplings were indicated as percentages at all branches.

Table 1 Biochemical tests of LF1

Fermentation of glucide		Assimilation of glucide		Assimilation of nitrate		Production of acidic matter	Production of starch classes
Glucose	–	Glucose	+	Ammonium sulfate	+	+	–
		Xylose	–				
Lactose	–	Arabinose	–	Potassium nitrate	+		
		Sucrose	+				
		Raffinose	–				
		Lactose	–				

The reactions optically resembling negative controls are scored as "–", and those similar to positive controls are scored as "+".

and 0.0126, respectively. The results indicated that the average recovery was increased with decreasing pH and low pH was favorable to the recovery of chlorimuron-ethyl. Similar results were obtained in studies on other members of sulfonylurea.

Ye *et al*. (2006) obtained better recoveries of ten sulfonylurea members by adjusting soil extracts to pH 2.5. Yu *et al*. (2005) also isolated a fungal strain capable of degrading metsulfuron-methyl under laboratory conditions, where the liquid culture was adjusted to pH 4–5 before metsulfuron-methyl was extracted to obtain the best recovery. Negre *et al*. (2005) investigated the chemical and biological degradability of cinosulfuron in flooded paddy field sediment, and the aqueous solution containing hydrolyzed cinosulfuron was adjusted to pH 4.0 to separate degradation products. The reasons are: the members of sulfonylurea are weakly acidic compounds with pK_a value 3.3–5.2 and sensitive to pH, and, to quantitatively extract these compounds from water to organic phase, the pH value of extraction solvents should be at least 2 pH units lower than the pK_a (Ye *et al*., 2006).

In order to test the effects of chlorimuron-ethyl concentration on its own degradation, the initial chlorimuron-ethyl concentration changed from 5 to 50 mg/L. As shown in Fig. 3, after 4 d incubation, the removal rate of chlorimuron-ethyl at its initial concentration of 5, 10, and 50 mg/L was, respectively, 8.97%, 6.24%, and 4.57% in the control, and 77.45%, 65.07%, and 46.44% after the inoculation of LF1.

To determine the effect of NH_4NO_3 concentration on chlorimuron-ethyl degradation, the initial concentration of NH_4NO_3 was varied from 0.1 to 5 g/L, chlorimuron-ethyl

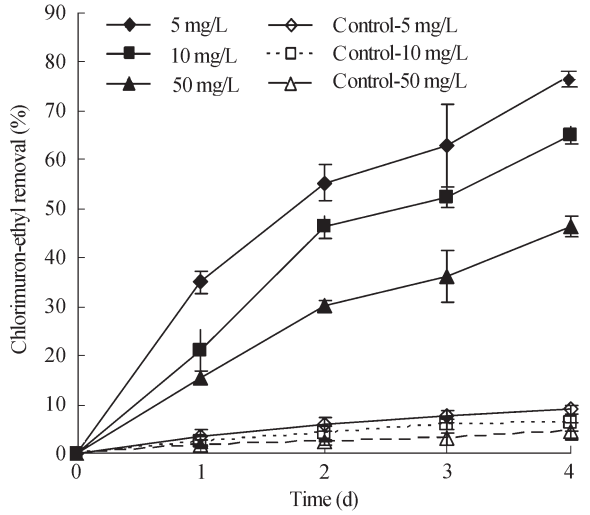

Fig. 3 Effect of initial chlorimuron-ethyl concentration on its degradation. NH_4NO_3: 1 g /L, 30°C. Error bars represent standard deviation (n = 3).

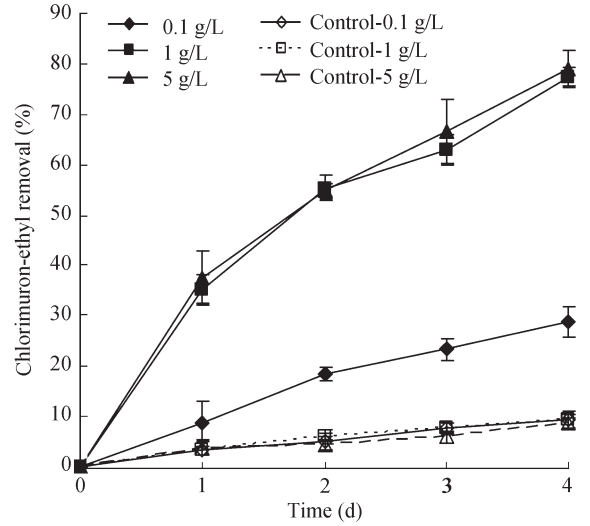

Fig. 4 Degradation of chlorimuron-ethyl at different NH_4NO_3 concentrations. Chlorimuron-ethyl: 5 mg/L, 30°C. Error bars represent standard deviation (n = 3).

concentration was 5 mg/L, and incubation temperature was 30°C. As shown in Fig. 4, the removal rate of chlorimuron-ethyl in the control was quite low and had no significant difference among the treatments of different NH_4NO_3 concentrations, but after the inoculation of LF1, the removal rate was much higher, with the treatments 1 and 5 g NH_4NO_3/L > 0.1 g NH_4NO_3/L. The result indicated that as a nitrogen source, NH_4NO_3 stimulated the growth of LF1, and thus more chlorimuron-ethyl was removed. However, when the NH_4NO_3 was excessive, its stimulation effect was somewhat decreased. In this study, the appropriate NH_4NO_3 concentration was 1 g/L.

To study the effects of temperature on chlorimuron-ethyl degradation, the incubation temperature was installed at 20, 30, and 40°C, and the initial concentration of chlorimuron-ethyl and NH_4NO_3 was 5 mg/L and 1 g/L, respectively. Figure 5 shows that in the control, higher temperature was more favorable to the breakdown of chlorimuron-ethyl, however, after the inoculation of LF1,

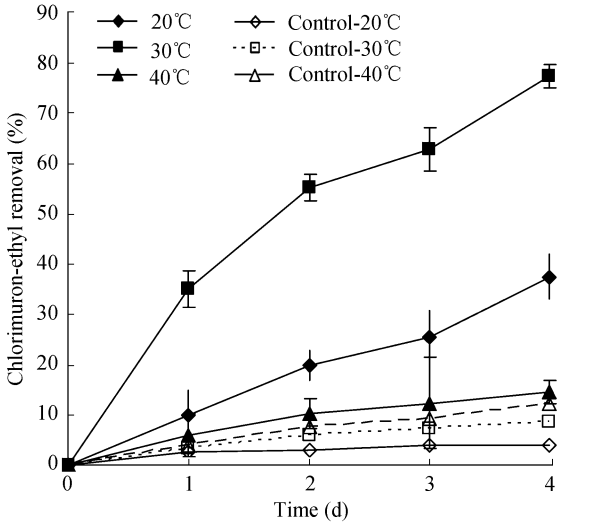

Fig. 5 Degradation of chlorimuron-ethyl at different temperatures. Chlorimuron-ethyl: 5 mg/L; NH_4NO_3 1 g/L. Error bars represent standard deviation (n = 3).

the removal percentage of chlorimuron-ethyl after 4 d incubation was 37.54%, 77.45%, and 14.50% at 20, 30, and 40°C, respectively. Therefore, 30°C was the optimal temperature for LF1 growth. In sum, the optimal degradation conditions of chlorimuron-ethyl by LF1 in this study were 5 mg/L of chlorimuron-ethyl, 1 g/L of NH_4NO_3, and 30°C.

2.3 Mechanism of chlorimuron-ethyl degradation by LF1

The dynamic changes of medium pH, LF1 growth (cell density at OD_{600}), and removal percentage of chlorimuron-ethyl during incubation in the optimal degradation conditions were tracked. Figure 6 shows that there were two phases. During 0–18 h of incubation, medium pH was decreasing while LF1 growth and chlorimuron-ethyl removal percentage was increasing; thereafter, medium pH and LF1 growth maintained at a constant level while chlorimuron-ethyl removal rate was still increasing. These phenomenon suggested that the chlorimuron-ethyl removal

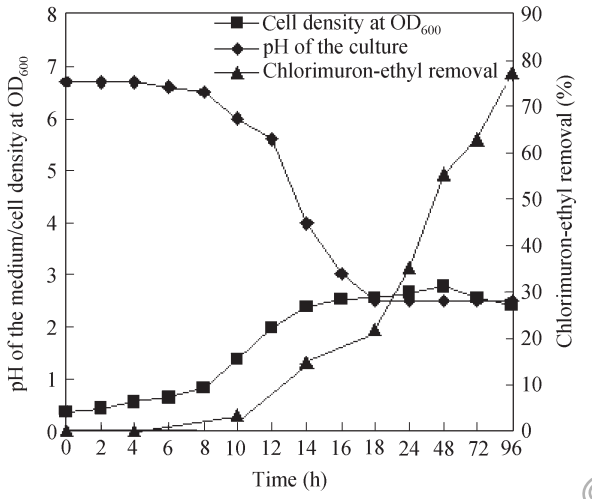

Fig. 6 Dynamic changes of medium pH, LF1 growth, and chlorimuron-ethyl degradation. Chlorimuron-ethyl: 5 mg/L, NH_4NO_3: 1 g/L, and 30°C.

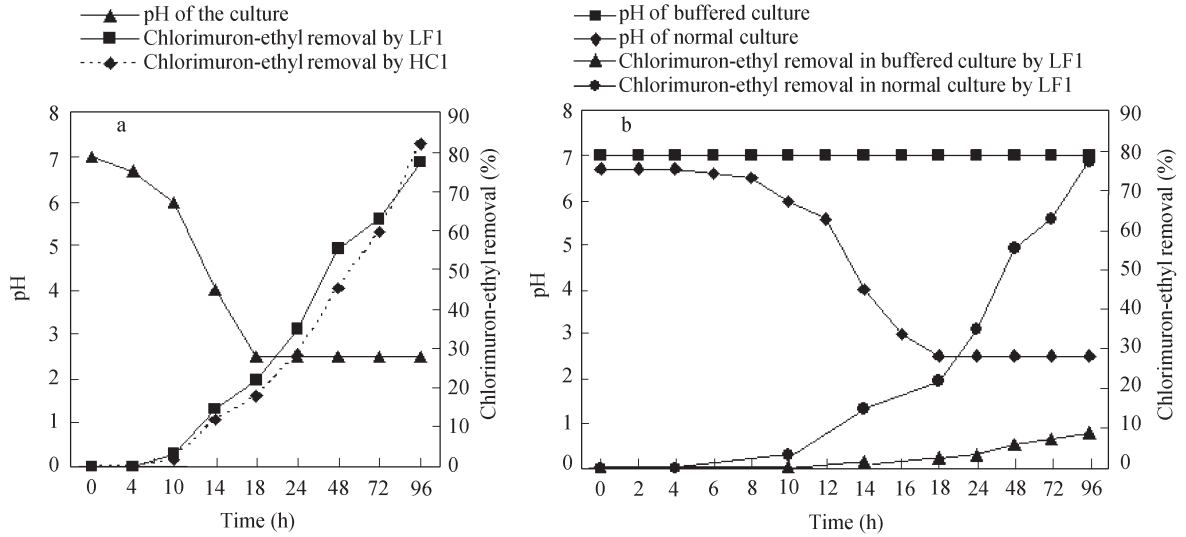

Fig. 7 Degradation process of chlorimuron-ethyl by LF1 and HCl (a); and degradation pattern of chlorimuron-ethyl in buffered and normal cultures (b).

by LF1 was pH dependent.

Some previous studies (Berger *et al*., 1998; Braschi *et al*., 2000) indicated that the degradation of sulfonylurea members was accelerated by the decrease of medium pH. Therefore, it is necessary to study whether the variation of the medium pH was the key of the degradation of chlorimuron-ethyl by LF1. Two experiments were designed to answer this question. In one experiment, LF1-inoculated culture and no LF1-inoculated medium were installed, and the medium pH was adjusted along with the culture pH by using 1 mol/L HCl at each sampling time. In other experiment, two LF1-inoculated cultures were installed, one buffered to pH 7.0 by using 0.2 mol/L KH_2PO_4-Na_2HPO_4 buffer (buffered culture) and the other not buffered (normal culture). Figure 7a shows that there was no difference in the degradation process of chlorimuron-ethyl between the culture and the medium. Figure 7b showed that in buffered culture, chlorimuron-ethyl concentration had less decrease, while in normal culture (pH decreased to about 2.5), the chlorimuron-ethyl removal rate was about 80%, which supported that the degradation of chlorimuron-ethyl by LF1 was dependent on the decrease of medium pH.

Our preliminary experiment had shown that both chlorimuron-ethyl and phosphate buffer did not affect LF1 growth, and chlorimuron-ethyl had no effect on medium pH. Consequently, the experiments mentioned above could educe a common conclusion that the degradation of chlorimuron-ethyl by LF1 was mainly due to the decrease of medium pH induced by the microbial metabolism. This conclusion is consistent with some previous studies. Berger *et al*. (1998) found that the degradation of metsulfuron-methyl by soil fungus *A. niger* only occurred when the medium pH decrease due to the citric acid from *A. niger*. Braschi *et al*. (2000) investigated the primisulfuron degradation by *Micrococcus flavus*, *Pseudomonas acidovoran*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Rhizoctonia praticola*, and *Phanerochaete chrysosporium* and found that the disappearance of primisulfuron was well correlated with the decrease of medium pH, suggesting that the degradation of primisulfuron was caused by microbial metabolic acid-induced hydrolysis. Therefore, the decrease of micro-environment pH induced by the metabolic acids from LF1 could be the main driving force of chlorimuron-ethyl degradation.

3 Conclusions

So far, there has rare report about yeast possessing the ability of degrading sulfonylurea. The strain LF1 isolated from a chlorimuron-ethyl polluted soil could be the only yeast strain of *Sporobolomyces* sp. capable of degrading chlorimuron-ethyl. Incubation tests showed that when the initial concentration of chlorimuron-ethyl in culture was 5 mg/L, LF1 could degrade more than 77% of the herbicide after incubation for 4 d. The optimal conditions of chlorimuron-ethyl degradation by LF1 were chlorimuron-ethyl 5 mg/L, NH_4NO_3 1 g/L, and 30°C. The possible mechanism of chlorimuron-ethyl degradation by LF1 could be the acidic hydrolysis caused by the acids from the metabolism of the yeast strain. Further study should be conducted to investigate the degradation pathways of chlorimuron-ethyl by LF1, and to approach the feasibility of using LF1 to degrade the chlorimuron-ethyl in soil system.

Acknowledgments

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