DOI:10.1021/jf900067f

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Rapid and Complete Degradation of the Herbicide Picloram by *Lipomyces kononenkoae*

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An enrichment culture approach was used to isolate a pure culture of the yeast *Lipomyces kononenkoae*, which had the ability to grow on the herbicide picloram. The yeast rapidly and completely degraded 50 μg mL^{-1} picloram by 48 h of growth. While *L. kononenkoae* was found to use both N atoms of picloram as a sole nitrogen source for growth, it failed to mineralize the herbicide or use it as a sole C source. Product analysis done using LC-ESI-MS indicated that biodegradation of picloram by *L. kononenkoae* proceeds via a didechlorinated, dihydroxylated, pyridinecarboxylic acid derivative. Our results are consistent with the hypothesis that the majority of picloram degradation in the soil is likely due to microbial catabolic processes.

KEYWORDS: Picloram; biodegradation; yeast; *Lipomyces kononenkoae*; dechlorination; complete degradation

INTRODUCTION

Picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid) is an acidic herbicide, within the pyridine carboxylic acid family, that is primarily used to control annual and perennial dicot weeds, shrubs, and woody vegetation (*19*, *22*). The herbicide functions as an auxin mimic, causing uncontrolled and disorganized cell growth, ultimately leading to plant death. (*6*, *12*, *19*). Picloram has been reported to stimulate RNA, DNA, and protein synthesis at low concentrations, while cell division and growth are inhibited at higher concentrations (*19*).

Since picloram has not been reported to be rapidly degraded, does not adhere well to soil particles, and is relatively water-soluble (430 μg mL^{-1}), it frequently finds its way into the surrounding environment (*5*, *10*, *19*). The persistence of picloram in the environment varies from months to years, with an average half-life of 90 days (*3*, *19*). While picloram is susceptible to rapid photodegradation, with a half-life of 2.6 days (*23*), chemically induced degradation is very slow with reported half-lives of 9 to 116 years (*4*). Although it has been postulated that microbial metabolism may account for the majority of picloram degradation in soil (*17*), up until now, no pure cultures of microorganisms have been isolated that rapidly or completely degrade picloram to support this contention.

Only a few studies have examined the microbial degradation of picloram, and many of these have used uncharacterized soil enrichment cultures (*13*), or fungi that only partially degraded the herbicide (*15*). While most degradation studies have been done under aerobic conditions, Ramanand et al. (*14*) examined the reductive dechlorination of picloram by freshwater sediments under anaerobic methanogenic conditions. These authors reported that picloram was only partially degraded to an isomer of dichloro-4-amino-2-pyridinecarboxylic acid following 50 days of acclimation and a 30 day incubation period, and then after another 90 days of incubation, another unknown metabolite accumulated. In general, microbial degradation of picloram has been found to be relatively slow and often incomplete (*8*, *9*, *13*, *15*). While the exact mechanism(s) by which microorganisms degrade picloram is unknown (*17*), Meikle et al. (*9*) proposed that degradation in soil is initiated by an oxygen-requiring ring-cleavage dioxygenase. Moreover, degradation rates have been reported to increase when conditions are favorable for an increase in overall soil microbial activity (*11*, *21*, *23*) and are reduced under anaerobic conditions (*9*, *24*). These results indicate that some pure microbial cultures or consortia have the ability to transform picloram in soils and sediments.

Here, we report the isolation and initial characterization of a pure culture of the yeast *Lipomyces kononenkoae*, which has the ability to use picloram as a sole source of nitrogen for growth. Moreover, we report here that a pure culture of *L. kononenkoae* rapidly and completely degrades picloram, likely through dechlorinated intermediates.

MATERIALS AND METHODS

Culture, Medium, and Growth Conditions. The *Lipomyces* strain used in these studies was isolated from the top 5 cm of soil obtained from a long-term weed science research site maintained by South Dakota State University, near Brooking, South Dakota. The soil had a 17 year history of routine applications of picloram (as formulated material). The strain was isolated by using an enrichment culture technique (*2*), whereby

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 Published on Web 04/24/2009

picloram was maintained at an aqueous saturation level of 430 μg mL^{-1} using a biphasic, silicone oil-based, aqueous−organic system. The aqueous phase contained nitrogen free mineral salts medium (MSM), containing (per liter) 40 mg of $CaCl_2$, 1 mg of $MnSO_4 \cdot H_2O$, 0.21 mg of Na_2MoO_4, 13 mg of $MgSO_4$, 770 mg of K_2HPO_4, 350 mg of KH_2PO_4, 350 mg of $FeSO_4 \cdot 7H_2O$, and 1000 mg of mannitol. Picloram (50 mg) was dissolved in 20 mL of sterile silicone oil by swirling the mixture overnight at 130 rpm, and the silicone oil−picloram mixture was added to 80 mL of MSM. One gram of soil was added, and the mixture was incubated at 20 °C, with shaking at 130 rpm. After 45 days, the culture appeared turbid, and 1 mL was transferred to a flask containing MSM medium supplemented with 233 μg mL^{-1} picloram (MSM-P). This process was repeated two additional times, and culture growth was subsequently enhanced by periodically transferring 2 mL cultures to fresh MSM-P medium.

The ability of the enrichment to degrade picloram was confirmed by incubating an aliquot of the mixed culture in 80 mL of MSM medium containing 79,200 dpm of 2,6-^{14}C-picloram (specific activity: 24.6 mCi mmol^{-1}, 97.5% purity) (graciously supplied by Dow AgroSciences LLC, Indianapolis, IN) in a 250 mL biometer flask. The side arm contained 15 mL of 0.5 N NaOH. The flask was inoculated with 1 mL of the mixed culture, sealed, and incubated at 25 °C with mixing. The flask was periodically flushed with fresh air, the NaOH was replaced, and 1 mL of the NaOH solutions was added to 14 mL of scintillation cocktail and counted using a scintillation counter.

A pure culture was subsequently obtained from the enrichment medium by repeatedly streaking for isolation on MSM-P medium containing agar. After 3 days of incubation, a number of white, mucoid, colonies were recovered and purified by restreaking onto the same medium. Degradation of picloram by pure cultures was determined by using high performance liquid chromatography (HPLC) analysis. Following growth in MSM-P medium, a 1 mL aliquot of culture medium was centrifuged at 13,000*g*, and the supernatant was analyzed by using a Waters HPLC (Milford, MA), fitted with a UV detector and a 25 mmx 4.6 mm Spherisorb ODS C18 column (Phenomenex, Torrence, CA), using a 60% acetonitrile with 0.1% tricholoracetic acid mobile phase at 1 mL min^{-1} and UV detection at 235 nm.

The isolates were maintained and subsequently analyzed on yeast minimal medium (YMM) containing (per liter) 0.77 g of K_2HPO_4, 0.35 g of KH_2PO_4, 0.13 g of $CaCl_2 \cdot 2H_2O$, 0.10 g of MgSO4$\cdot 7H_2O$, 0.001 g of $FeSO_4$, 120 g of mannitol, 0.01 g of histidine, 0.02 g of methionine, 0.02 g of tryptophan, 0.05 g of picloram, 1 mL of vitamin stock solution, and 1 mL of trace elements solution. The vitamin solution contained (per liter) 0.02 g of biotin, 2 g of pantothenate, 0.002 g of folic acid, 10 g of *m*-inositol, 0.4 g of niacin, 0.2 g of ρ-aminobenzoate, 0.4 g of pyroxidine HCl, 0.2 g of riboflavin, and 0.4 g of thiamine HCl. The trace element solution contained (per liter) 0.5 g of H_3BO_4, 0.0625 g of $CuSO_4$, 0.1 g of KI, 0.333 g of $FeCl_2$, 0.4 g of $MnSO_4$, 0.235 g of $NaMoO_4 \cdot 2H_2O$, and 0.712 g of $ZnSO_4 \cdot 7H_2O$ (*20*).

Pure cultures were gram stained and identified as yeast by microscopic analyses. The taxonomic and species status of the isolated strain was determined by Midi Laboratories (Newark, DE) and by sequencing the gene region encoding for 18S rRNA. The 18S rDNA was PCR amplified, using primers corresponding to positions 3334 and 3630 in the *Schizosaccharomyces japonicus* LSU rRNA gene, and sequenced. BLASTN analysis (*1*) (http://blast.ncbi.nlm. nih.gov/Blast.cgi) indicated that the 296 bp sequence had 100% identity to the corresponding gene region in *Lipomyces kononenkoae*.

Growth and Degradation Experiments. Cultures of *Lipomyces kononenkoae* were grown in YMM containing 50 μg mL^{-1} picloram for 1 week at 30 °C in side arm flasks. Microbial growth was monitored by using a Klett−Summerson photoelectric colorimeter (Klett Mfg. Co, New York, NY) fitted with a number 55 filter. Subsamples for analysis of picloram concentration (see below) were aseptically removed from the growing cultures and stored at −20 °C until the end of the growth experiment.

For resting cell studies, replicate cultures of *L. kononenkoae*, were grown as described above, centrifuged at 10,000*g* for 10 min, and resuspended in YMM to 10-fold the initial concentration. Growth of *L. kononenkoae* was initially monitored using a Beckman DU 7400 spectrophotometer at 600 nm, and degradation of picloram was determined by using a Waters HPLC as described below.

The concentration of picloram in filtered (0.2 μm, Millipore, Bedford, MA) samples, along with a series of picloram standards ranging in concentration from 0 to 50 μg mL^{-1}, were measured using a Waters HPLC fitted with a UV detector as described above. The instrument was run for 22 min in the isocratic mode at a flow rate of 0.5 mL min^{-1} using acetonitrile and 4% acetic acid (80:20) as the mobile phase. The injection volume was 250 μL, and UV detection was done at 230 and 254 nm. Standards were made using analytical grade picloram (99.8% pure) obtained from Chem Services Corp. (West Chester, PA).

Mineralization of ^{14}C-Picloram by Pure Cultures. 2,6-^{14}C-Picloram (specific activity: 24.6 mCi mmol^{-1}; 97.5% purity) (graciously supplied by Dow AgroSciences LLC, Indianapolis, IN) was used to determine if *L. kononenkoae* had the ability to mineralize the herbicide. Triplicate cultures of *L. kononenkoae* were grown in YMM containing 50 μg mL^{-1} unlabeled picloram as described above and 1.2 nM ^{14}C-picloram (0.03 μCi radioactivity). Cultures were grown in biometer flasks at 30 °C with constant shaking. Scintillation vials (7 mL), containing 1 mL of 1 M NaOH, were suspended over each culture to trap $^{14}CO_2$ produced from the mineralization of the herbicide. Vials were changed daily throughout the course of the experiment, and subsamples of culture medium were removed daily for HPLC analysis to determine the concentration of picloram remaining in the culture. Mineralization levels were calculated as the percent of added picloram metabolized over time.

Growth Yield Studies. Triplicate cultures of *L. kononenkoae* were grown at 30 °C in YMM containing 0.2 mM or 0.4 mM NH_4Cl, representing 0.5 and 1.0 mol equiv levels of the nitrogen found in picloram. A triplicate set of inoculated cultures containing 50 μg mL^{-1} picloram as the sole source of nitrogen for growth was incubated in parallel. Growth was monitored for 7 days using a Beckman DU 7400 spectrophotometer at 600 nm.

Identification of Metabolite(S). Cultures of *L. kononenkoae* were grown for 0, 5, 10, 15, 25, 35, 48, 52, and 56 h at 30 °C in YMM containing 50 μg mL^{-1} picloram. At the indicated times, cultures were centrifuged at 10,000*g*, and the supernatants were filtered through 0.2 μm filters (Millipore, Bedford, MA). Uninoculated medium containing picloram served as the negative control. The initial identification of the picloram was performed by direct infusion using a Waters MicroMass ZMD mass spectrometer (Milford, MA), equipped with an electrospray interface (ESI). Full scan spectra (100−300 amu) were acquired in positive mode at 2 scans s^{-1}. Tuning parameters were optimized for direct infusion. The capillary exit and entrance voltages were selected to optimize the formation of fragment ions, while keeping $[MH]^+$ at 100% relative abundance. The following instrument parameters were used: capillary voltage, +3.5 kV; cone voltage, +90 V; extractor voltage, +5 V; RF lens, +0.1 V; source Block Temp, 90 °C; desolvation temperature, 200 °C; and desolvation N_2 flow rate, 325 L h^{-1}. Using these run conditions, picloram exhibited base molecular ions at *m*/*z* 241, 243, and 245 ($M + H^+$), which represents the three ions that are characteristic of the isotopes for the 3 Cl atoms. There was very little fragmentation of picloram under these conditions

More detailed metabolite analyses were done by injecting 50 μL aliquots of the supernatant samples described above into a Waters Alliance high performance liquid chromatograph, equipped with a photodiode array detector, coupled to a Waters MicroMass ZMD mass spectrometer using an electrospray interface. A Zorbax, RX-C8 column (2.1 mm ID × 150 mm × 5 μm film thickness) was used for analysis at 40 °C. The mobile phase consisted of a gradient starting with 95% water (0.1% formic acid) (A): 5% acetonitrile (B); 95% A at 0 min; 95% A at 5 min; 50% A at 10 min; 3% A at 15 min; 3% A at 20 min; 95% A at 25 min; and 95% A at 30 min. The mobile-phase flow rate was 0.2 mL min^{-1}, and samples in the autosampler were maintained at 8 °C to minimize decomposition. Full scan spectra (100−300 amu) were acquired in both positive and negative mode at 2 scans s^{-1}. Using these conditions, the retention time for picloram was 10.2 min for UV detection and 10.4 for MS detection, with base molecular ions at *m*/*z* 241, 243, and 245 ($M + H^+$), the same as previously seen in direct infusion analyses. Although somewhat less sensitive than positive ion mode, full scan spectra (100−300 amu) acquired in negative mode exhibited base molecular ions at *m*/*z* 239, 241, and 243 ($M - H^-$), and major fragment ions at *m*/*z* 195, 197, and 199, which would represent loss of the carboxyl group. For UV detection, wavelengths of 210 to 400 nm were monitored, with a resolution of 1.2 nm and a sampling rate of 1 spectrum s^{-1}.

RESULTS AND DISCUSSION

Picloram is a substituted pyridine carboxylic acid herbicide containing six carbon and two nitrogen atoms per molecule (**Figure 1**). A single microorganism capable of rapidly degrading picloram was isolated following enrichment and subsequent multiple streakings for purity on agar medium. Microscopic analyses showed that the isolated organism was a yeast, and this was initially confirmed by experiments that demonstrated that efforts to kill fungi present in the cultures with antibiotics had a detrimental effect on picloram degradation ability (data not shown). Also, the requirement of vitamins for growth and the ability of the cultures to grow and degrade picloram under acid conditions (pH 3.5) were consistent with the presence of a yeast.

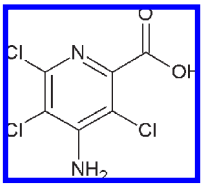

Figure 1. Structure of picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid).

DNA primers for amplification of 18S rDNA were used to amplify genomic DNA extracted from the pure culture. Strong amplification of the isolates was seen using this primer set, producing a single ~300 bp fragment. Genbank searches indicated that the sequence had 100% nucleotide similarity to *Lipomyces kononenkoae* (Genbank accession U84237).

Growth of *Lipomyces kononenkoae* Correlated with Degradation of Picloram in Active and Resting Cell Cultures. Results in **Figure 2A** and **B** shows that as *L. kononenkoae* grew in YMM

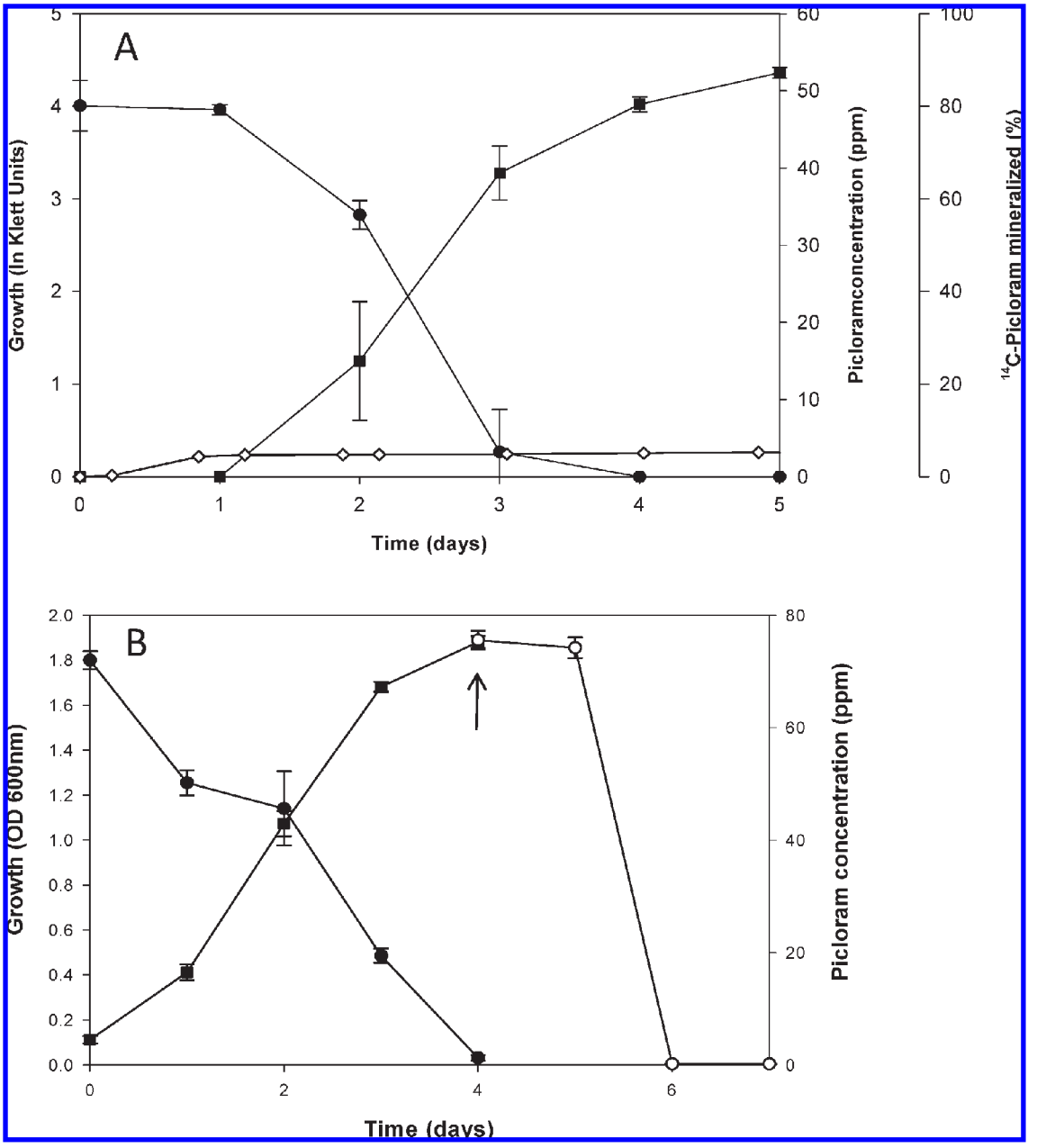

Figure 2. Degradation and mineralization of picloram by *Lipomyces kononenkoae*. (**A**) (■) Growth of *Lipomyces kononenkoae*, (●) degradation of picloram, and (◇) percent mineralization of ^{14}C-picloram over time. Error bars represent the standard deviation of duplicate cultures. (**B**) Cultures of *Lipomyces kononenkoae* growing on 50 μg mL^{-1} picloram were spiked with an additional aliquot of picloram (↑), and growth (■) and degradation (●,○) were monitored overtime. Error bars represent the standard deviation of duplicate cultures.

medium, the concentration of picloram decreased to nondetectable levels after 4 d of incubation. A small amount of cell biomass and a slow growth rate corresponded to a greater level of picloram remaining in the culture medium (see **Figure 2A**, days 0 and 1). As the yeast strain began to grow by day 2, however, the picloram within the culture medium also began to degrade at a comparable rate. Growth of the culture began to level-off around days 4 and 5, and this corresponded to the complete degradation of the picloram within the culture medium. While other microorganisms have been reported to degrade picloram, degradation has been found to be slow and incomplete (*8*, *9*, *13*, *15*). Moreover, while the yeast *Rhodotorula glutinis* has previously been reported to partially degrade picloram after extensive (1 month) incubation (*15*), to our knowledge, this is the first report of a pure yeast culture that has the ability to rapidly degrade this herbicide in a few days.

Further evidence that *L. kononenkoae* rapidly degraded picloram was seen by examining stationary phase cell cultures pregrown on 50 μg mL^{-1} picloram as substrate. Following the further addition of ~70 μg mL^{-1} picloram, these cultures demonstrated a rapid decrease in picloram concentrations after only 48 h, and at a much faster rate than that seen with the initial degradation of the compound (**Figure 2B**). This indicated that *L. kononenkoae* more actively degraded picloram following initial growth on this substrate, perhaps due to either the presence of more cells, or to the induction of an enzyme(s) required for herbicide degradation. Lag phases in the degradation of several substituted pyridines has previously been reported (*7*), although they are not likely due to issues of cell permeability or transport (*16*).

***L. kononenkoae* Did Not Mineralize or Use Picloram As the Sole Carbon Source for Growth.** Growth of *L. kononenkoae* in the presence of ^{14}C-picloram showed that only 5% of the radiolabeled material had been converted into CO_2 after 5 days of growth (**Figure 2A**), whereas HPLC analysis showed that all of the picloram had been degraded to negligible levels by this time. Moreover, growth studies indicated that *L. kononenkoae* could not use picloram aa a sole source of C for growth (data not shown).Taken together, these results indicate that while *L. kononenkoae* has the ability to use picloram as the sole N source for growth, the yeast does not mineralize this herbicide, at least from the carbon atoms labeled, or use it as a carbon source for growth.

***L. kononenkoae* Uses Both N Atoms in Picloram As a Nitrogen Source.** Since initial studies indicated that *L. kononenkoae* only used picloram as a sole N source for growth, it was of interest to determine if one or both N atoms in the herbicide served as a growth substrate. To test this, the yeast was grown in YMM medium containing NH_4Cl, at concentrations equivalent to 0.5 or 1.0 times that found in 50 μg mL^{-1} picloram. Results in **Figure 3** show that following 6 days of growth, approximately half of the overall cell density was achieved using half as much nitrogen as that found in 50 μg mL^{-1} picloram. In contrast, cultures containing picloram and NH_4Cl with the same molar equivalents of N showed nearly identical final cell densities, displaying nearly identical growth curves. These results suggest that *L. kononenkoae* used both N atoms in picloram as a source of nitrogen for growth. The use of the ring-internal N atom in nitrogen heterocyclic compounds for growth has been previously shown for many microorganisms (*7*, *18*).

Identification of the Metabolite from the Degradation of Picloram. Since the complete degradation of 50 μg mL^{-1} picloram occurred within 48 h, shorter incubation periods were used in an attempt to identify potential intermediates involved in the degradation process. LC-ESI-MS analysis of culture growth media

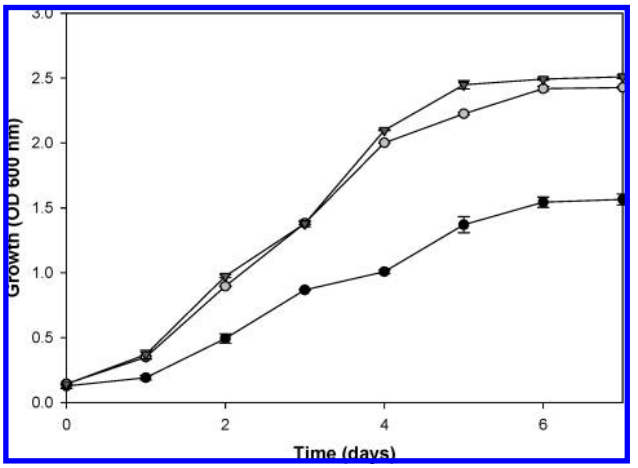

Figure 3. Growth of *Lipomyces kononenkoae* cultures on 50 μg mL^{-1} picloram (▼), 0.2 mM (0.5 mol equiv of picloram) NH_4Cl (●), or 0.4 mM (1.0 mol equiv of picloram) NH_4Cl (○) as the sole sources of nitrogen for growth. Values are the means of triplicate cultures, and error bars represent the standard deviation.

following 35 h of incubation indicated that only a picloram peak at the appropriate RT was observed by UV. Picloram was confirmed by the presence of appropriate ions by using MS in both positive and negative modes, and degradation of picloram was confirmed by a decrease in peak areas at the retention time for picloram. A decrease in the three molecular ions was observed at m/z 241, 243, and 245 ($M + H^+$) in positive mode and major fragment ions at m/z 195, 197, and 199 in negative mode. After 52 h of incubation, however, a second peak in addition to that due to picloram was observed (UV retention time = 12.2 min; MS retention time = 12.4 min). In positive mode, the unknown exhibited a molecular ion at m/z 205 ($M + H^+$) and fragment ions at m/z 187 and 160. In negative mode, only a molecular ion at m/z 203 was observed; there was no significant fragmentation. These results suggest that the unknown peak represents a didechlorinated, dihydroxylated picloram molecule. This is consistent with the postulated intermediates in the picloram degradation pathway proposed by Meikle et al. (*9*). However, given the limitations of our analysis, we could not determine which isomer of chloro-dihydroxy-4-amino-2-pyridinecarboxylic acid was produced by *L. kononenkoae* during catabolism of picloram. While it was possible to purify the unknown by liquid chromatography, the appropriate standards available for comparison of RTs are not commercially available. Nevertheless, our results suggest that picloram degradation by *Lipomyces kononenkoae* proceeds via a series of dechlorination reactions leading to the release of both N atoms, which are subsequently used to support the growth of the yeast.

While these studies demonstrated that an isomer of chloro-dihydroxy-4-amino-2-pyridinecarboxylic acid is produced in the *L. kononenkoae* picloram biodegradation pathway, our results do not indicate the mechanism by which this product is produced. Since degradation of picloram by *Lipomyces kononenkoae* was very rapid, we may not have been able to see transient initial degradation products, even with the short incubation times used in our studies. Also, the intermediates may not have accumulated in amounts easily seen with our methodology. However, like other substituted pyridines, picloram biodegradation likely involves dechlorination, deamination, and ring cleavage (*7*, *9*) A complete understanding of the complete picloram biodegradation pathway in *L. kononenkoae* will require further isotopic, mutational, gene cloning, and biochemical analyses.

ABBREVIATIONS USED

RPM, revolutions per minute; PCR, polymerase chain reaction; rRNA, ribosomal ribonucleic acid; picloram, 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid; MSM, mineral salts medium; MSM-P, MSM medium containing picloram; HPLC, high performance liquid chromatography; YMM, yeast minimal medium; LSU, large subunit; amu, atomic mass unit; RT, retention time; UV, ultraviolet; *m*/*z*, mass-to-charge ratio.

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Received for review January 7, 2009. Revised manuscript received April 10, 2009. Accepted April 13, 2009. This study was supported, in part, by a grant from the University of Minnesota Agricultural Experiment Station (to M.J.S.) and by a grant from USDA-NRCS (to R.F.T.).