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Bioconversion of alkaloids to high-value chemicals: Comparative analysis of newly isolated lupanine degrading strains

Stella Parmaki [a], Ioannis Vyrides [a], Marlen I. Vasquez [a], Viki Hartman [a], Irene Zacharia [a], Ioanna Hadjiadamou [b], Catarina B.M. Barbeitos [c], Frederico C. Ferreira [c], Carlos A.M. Afonso [d], Chrysoulla Drouza [e], Michalis Koutinas [a, *]

[a] Department of Environmental Science & Technology, Cyprus University of Technology, 30 Archbishop Kyprianou Str., 3036, Limassol, Cyprus
[b] Department of Chemistry, University of Cyprus, P.O. Box 20537, 1678, Nicosia, Cyprus
[c] Institute for Bioengineering and Biosciences, Department of Bioengineering, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001, Lisbon, Portugal
[d] Research Institute for Medicines (iMed. ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003, Lisbon, Portugal
[e] Department of Agricultural Sciences, Biotechnology and Food Science, Cyprus University of Technology, 30 Archbishop Kyprianou Str., 3036, Limassol, Cyprus

HIGHLIGHTS

- Eight aerobic and anaerobic lupanine metabolizing strains were isolated.
- Lupanine is toxic for *V. fischeri*, *D. magna* and monocotyledonous plants.
- The alkaloid acted as growth promoter for a dicotyledonous plant.
- *Rhodococcus rhodochrous* LPK211 achieved 81% removal for 1.5 g L^{-1} of lupanine.
- *P. putida* LPK411 formed 17-oxolupanine and other alkaloid derivatives as products.

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ABSTRACT

This work explores the potential for development of a lupanine valorization process evaluating different isolated microorganisms for their capacity to metabolize the alkaloid. Ecotoxicological assessment demonstrated that lupanine is toxic for *Vibrio fischeri* and *Daphnia magna* exhibiting EC_{50} values of 89 mg L^{-1} and 47 mg L^{-1} respectively, while acting both as growth inhibitor for a monocotyledonous and as promoter for a dicotyledonous plant. Among the eight aerobic and anaerobic strains isolated and identified *Rhodococcus rhodochrous* LPK211 achieved 81% removal for 1.5 g L^{-1} lupanine, while no end-products were detected by NMR constituting a promising microorganism for lupanine biodegradation. Moreover, *Rhodococcus ruber* LPK111 and *Rhodococcus* sp. LPK311 exhibited 66% and 71% of removal respectively, including potential formation of lupanine N-oxide. *Pseudomonas putida* LPK411 reached 80% of lupanine removal and generated three fermentation products potentially comprising 17-oxolupanine and lupanine derivatives with open ring structures enabling the development of alkaloid valorization processes.

1. Introduction

Lupinus albus L. constitutes species of the genus *Lupinus* in the family *Leguminosae* (or *Fabaceae*), which is cultivated along the whole Mediterranean region as well as in Central and Western Europe, America and Africa (Boschin and Resta, 2013). *L. albus* seeds can be applied as animal feed, but they also comprise an important food product for the human diet exhibiting numerous applications

Abbreviations: M9, Minimal microbial growth medium; GC, Gas chromatography; EC_{50}, Half maximal effective concentration; ITS, Internal transcribed spacer sequence; NCBI, National Center for Biotechnology Information; NMR, Nuclear magnetic resonance spectroscopy; ACN, Accession number; LD_{50}, Lethal dose; IC_{50}, Half maximal inhibitory concentration; i.p., Intraperitoneal administration; i.v., Intravenous administration; p.o., Oral administration.

* Corresponding author.
E-mail address: michail.koutinas@cut.ac.cy (M. Koutinas).

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in the manufacture of existing or novel food products as a source of supplementary protein and fiber (Erbas, 2010). The seeds typically involve 33–47% proteins and 6–13% oil (Annicchiarico et al., 2014; Erbaş et al., 2005), while the content in dietary fibers (34–39%) could be suitable for dietetic food production (Yorgancilar and Bilgiçli, 2012). However, the varieties of *L. albus* with favorable agronomic characteristics contain toxic quinolizidine alkaloids (1.9–2.7%), which are not suitable for human or animal consumption (Carmali et al., 2010).

Apart from the anti-nutritional effect alkaloids also impart a bitter taste stimulating the development of different procedures for their removal from *L. albus* seeds. Varieties with low-alkaloid content have been developed and successfully applied in areas where *Lupinus* is not endemic including Australia and Eastern Europe. Moreover, the capacity of bacterial strains to act as debittering agents has been explored demonstrating low efficiency in quinolizidine alkaloids removal from *L. albus* flour (Santana and Empis, 2001). Debittering constitutes the process that is widely used for elimination of undesirable alkaloids from *L. albus* seeds, which is performed through exhaustive boiling and leaching of seeds in water. However, this process involves consumption of large amounts of water which is eventually discarded with the effluent wastewater at the end of each leaching batch (Carmali et al., 2010; Erbas, 2010). Although the wastewater generated could be applied in agriculture as growth regulator and biocide, the specific effluents are generally discharged without prior treatment (Ciesiołka et al., 2005).

The leaching wastewaters of *L. albus* comprise a high content in quinolizidine alkaloids, which primarily consist of lupanine. The specific molecule entails a symmetric structure that includes useful functionalities for the fine chemicals and pharmaceutical industries and may serve as a starting material for semisynthesis of a range of new and known alkaloids with high added-value (Villalpando-Vargas and Medina-Ceja, 2016). Nevertheless, lupanine is not considered as an easy target for chemical modification due to the presence of stable amide and tertiary amine functionalities (Smith et al., 2002). Previous studies have reported that various strains isolated from soil (e.g. *Pseudomonas* sp.) are capable of utilizing lupanine as the only source of carbon and nitrogen (Hopper et al., 1991; Santana et al., 1996). Thus, apart from the use of biological treatment for detoxification of these effluents, bioconversion could be applied to modify this natural molecule aiming to produce new structures more prone to chemical modification reducing the cost required (Rathbone and Bruce, 2002).

In the present study, the potential for the development of a lupanine bioconversion process has been explored. The work aimed in isolating microorganisms capable of metabolizing lupanine under both aerobic and anaerobic conditions. To this end, eight microbial isolates have been identified and tested for lupanine biodegradation. The structures of the end-products formed have been assessed in lupanine bioconversion with the use of NMR, while the toxicity of lupanine on marine bacteria, crustacean, monocotyledonous and dicotyledonous plants have been also evaluated. The microorganisms isolated comprised diverse metabolic functions on lupanine enabling both the development of biological treatment and lupanine valorization processes.

2. Materials and methods

2.1. Purification of lupanine

Lupanine was purified from *L. albus* seeds following the general procedure described elsewhere (Maulide et al., 2016). 1 Kg of lupin beans (produced by Sociedad Agro Comercial Alicante LTDA, Chile) were weighted and poured into 5 L of tap water inside a 10 L glass reactor equipped with a condenser and it was gently refluxed overnight. The reactor was cooled down to near room temperature, while the water (approximately 2 L) was separated from the beans by decantation. The brown water was transferred to a plastic container that was basified with NaOH (approximately 0.5 Kg, exothermic dissolution, pH > 11) and it was cooled to room temperature by pouring the container immersed in tap water. 2 L of diethyl ether was added and the phases were vigorously shaken/stirred. Subsequently, the organic phase was separated (if necessary an additional 1 L of diethyl ether was added to facilitate organic phase separation), transferred to a glass container and dried with anhydrous magnesium sulphate. The organic solvent was removed in a rotary evaporator and collected for reuse, while the residue obtained was transferred to a smaller round bottom flask using dichloromethane that was withdrawn in the rotary evaporator and left overnight under vacuum to provide lupanine (4–5 g) as brown solid.

Lupanine was further purified from the brown solid residue by adding 20 mL of *n*-hexane per 1 g of lupanine, gently heated until full dissolution was obtained and then 0.1 g of activated charcoal was added. The mixture was heated and while still hot it was filtered under vacuum through a filter funnel containing celite. The flask that contained the mixture and the filter was washed with *n*-hexane. The solvent was evaporated in the rotatory evaporator and it was left under vacuum providing lupanine as slightly yellow solid (95% yield), pure by ^{1}H and ^{13}C NMR, elemental analysis: Found: 72.56 (C), 10.32 (H), 10.97 (N); Expected: 72.54 (C), 9.74 (H), 11.28 (N).

2.2. Acute toxicity tests

2.2.1. Acute toxicity assessment with marine bacteria

The microscale test for acute toxicity, as the bioluminescence inhibition of *Vibrio fischeri* (NRRL B11177) following 5 min and 15 min of exposure to test samples, was used as described elsewhere (Vasquez and Fatta-Kassinos, 2013). Lyophilized bacteria were reconstituted prior use. The salinity was adjusted using a concentrated NaCl (18%) solution to achieve a final salinity of 2% and the pH ranged from 7 to 8. Each experiment was conducted in triplicate, while within each experiment samples were tested in duplicate. Phenol was used as positive control and an EC_{50} ranging between 13 and 26 mg L^{-1} was considered as acceptable. Nine concentrations of lupanine that varied between 8 and 2000 mg L^{-1} were tested. The percentage of bioluminescence inhibition (I) in each sample was determined through comparison of the bioluminescence value exposed to saline control solution (I_c) to that of each test sample (I_s) using the following formula:

$$I = 100 - \left(\frac{I_c - I_s}{I_c} \times 100\right) \quad (1)$$

A linear regression of the inhibition as Γ value (Eq. (2)) was logarithmically plotted against the concentration of the compound. The EC_{50} value, as the concentration that inhibited 50% of the population, and the Toxicity Units (TU_{50}) were calculated as shown in Eq. (3). Samples were then classified as follows: non-toxic ($TU_{50} = 0$), slightly toxic ($0 < TU_{50} < 1$), toxic ($1 < TU_{50} < 10$), very toxic ($11 < TU_{50} < 100$) and extremely toxic ($TU_{50} > 100$) (Persoone et al., 1993).

$$\Gamma = \frac{\mathrm{I}}{100 - \mathrm{I}} \quad (2)$$

$$TU_{50} = 100/EC_{50} \quad (3)$$

2.2.2. Acute toxicity assessment with plants

The seeds of the dicotyledon *Sinapis alba* and the monocotyledon *Sorghum saccharatum* were exposed to lupanine for 72 h in a Petri dish. Each Petri dish contained a filter paper with 5 seeds and 1 mL of the testing solution, while five different concentrations between 6.25 and 100 mg L^{-1} were evaluated in triplicate. Each experiment was repeated at least three times. The growth inhibition, expressed as length of roots/shoots, as well as the germination index, given as the number of germinated seeds, were the endpoints evaluated. A control experiment that included only deionized water was run in parallel to the tests described above. The following equation was applied (A: average number of germinated seeds and average root and shoot length in the control, B: average number of germinated seeds and average root and shoot length in the 5 concentrations tested):

$$\%\ Inhibition = \frac{A - B}{A} \times 100 \quad (4)$$

Subsequently, growth inhibition was correlated to lupanine concentration and corresponding EC_{50} values were calculated.

2.2.3. Acute toxicity assessment with crustaceans

Invertebrates are frequently employed as test species in toxicity bioassays, among which *Daphnia magna* constitutes the most frequently applied organism for chronic and acute toxicity tests. The acute bioassay of *D. magna* incorporates a well-developed standard toxicity test where immobilization of the organism is determined following 24 h and 48 h of exposure (Verma, 2008). Each independent experiment comprised of five different concentrations of lupanine that ranged between 3.125 and 100 mg L^{-1} as well as a control. Moreover, each concentration was tested in triplicate as internal replicates and ephippia were hatched 72 h prior use. Five neonates (<24 h) were exposed to test solutions and the control for 24 h and 48 h in a multiwell plate. Immobilization was calculated in each well, while that of the control test was not significant (<5%). The percentage of immobilization was estimated via recording of the number of dead and immobilized neonates, versus the number of actively swimming test organisms in each well. Moreover, the neonates which were not capable of swimming following gentle agitation of the liquid for 15 s were additionally considered as immobilized even if they could still move their antennae. The immobilization percentage was calculated using the following equation:

$$I_C(\%) = \frac{{}_{cor}N_{imm} \times N_{tested}}{100} \quad (5)$$

I_c corresponds to the immobilization percentage at concentration C, ${}_{cor}N_{imm}$ represents the corrected number of immobilized daphnids and N_{tested} stands for the total number of daphnids tested. Moreover, in cases where immobilization in the control experiment (I_o) ranged between $0 < I_o < 5\%$, the corrected number of immobilized daphnids was calculated using Abbott's equation (Abbott, 1925). The corresponding EC_{50} value was calculated by Probit analysis, which is commonly applied to correlate a dose (concentration of lupanine) with a quantal response (% immobilization) (Bliss, 1934). For this purpose the immobilization percentage was transformed into Probit values and the concentration of lupanine was log-transformed. A linear regression of the Probit values with the log-transformed concentration was then applied and the EC_{50} values were calculated.

2.3. Mixed culture enrichment and isolation of pure strains

A minimal microbial growth medium (M9) was used for subcultures of microorganisms, which included the following composition (g L^{-1}): $Na_2HPO_4 \cdot 2H_2O$ 8.5, KH_2PO_4 3.0, NaCl 0.5, NH_4Cl 0.5, $MgSO_4$ 0.24 and lupanine 1.5. The medium was sterilized in an autoclave for 15 min at 121 °C and it was allowed to reach room temperature, while lupanine was added (as a 7.5 g L^{-1} solution) following filter sterilization (Ahlstrom, Bärenstein, Germany, 0.2 μm pore size). Solid cultures were performed using M9 medium supplemented with 1.5% (w/v) of agar.

Microbial strains capable of using lupanine as the sole carbon source have been isolated from four environmental samples that included anaerobic granular sludge obtained from an anaerobic bioreactor treating cheese whey (Charalambides Christis Ltd, Limassol, Cyprus), anaerobic digested sludge and activated sludge derived from a municipal wastewater treatment plant (Sewerage Board of Limassol – Amathus (SBLA), Moni, Cyprus) as well as wastewater from a *L. albus* snack manufacturer (Tremoceira, Charneca da Caparica, Portugal). The first three samples were passed through 0.45 μm filters (Whatman, Buckinghamshire, UK) and separated solids were used as 1% (w/v) inoculum for enrichment cultures. Furthermore, wastewater from a lupine beans processing plant was directly used as inoculum (5% v/v) in enrichment cultures. All environmental samples were cultivated under aerobic conditions, while granular and digested sludge were additionally applied under anaerobic conditions.

Aerobic and anaerobic enrichments of microorganisms were carried out using 25 mL working volume in 100 mL and 30 mL flasks respectively. All cultures consisted of M9 medium supplemented with lupanine and inoculums under sterile conditions as described above, while gas-tight flasks were applied. The flasks were incubated for a period of 7 days and the cultures were maintained at 31 °C and 100 rpm.

Following three sequential enrichment cultures 1 μL of samples was withdrawn from each flask and plated by the streak method on M9 agar medium aiming to isolate the desired strains. The inoculated solid media were sealed with parafilm and incubated for 7 days at 31 °C. Subsequently, single colonies were transferred into liquid M9 medium and cultivated cells were stored in media containing 50% glycerol, at −80 °C. Isolated strains were also maintained at 4 °C on M9 agar plates and subcultured onto fresh media every month.

2.4. Strain identification by 16S rRNA sequence analysis

Liquid cultures of the isolated pure strains were prepared using M9 medium supplemented with 1.5 g L^{-1} of lupanine and were inoculated by the strains maintained on Petri dishes. The cultures were incubated in a shaking incubator at 100 rpm which was maintained at 31 °C for 72 h. 0.5 mL of liquid culture samples were transferred into sterile eppendorf tubes (1.5 mL total volume) that contained 0.5 mL of glycerol. DNA extraction, PCR (27F/1492R primers), PCR purification (using Montage PCR Clean up kit) and sequencing alignment were performed by Macrogen (Amsterdam, the Netherlands) using the Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA) and the resulting alignment of the 16S rRNA was compared for homology in the National Center for Biotechnology Information (NCBI) database by BLASTn nucleotide tool analysis. Phylogenetic trees were constructed by the neighbor-joining method using Mega 6.0 software and the most similar sequences according to the NCBI database by BLASTn and boot strapping in 500 replicates.

2.5. Microbial cultures

The inoculum was pregrown overnight at 31 °C in M9 supplemented with 1.5 g L^{-1} of lupanine. The incubation of inoculum was performed using flasks of 100 mL (25 mL working volume) for aerobic strains and serum bottles of 35 mL (25 mL working volume) for anaerobic strains in a shaking incubator (Stuart SI600, Staffordshire, UK), which was stirred at 100 rpm. In each experiment, aerobic cultures were prepared in duplicate inoculating 3 mL of pregrown cells under sterile conditions utilizing flasks of 500 mL (150 mL working volume) and anaerobic cultures were conducted in a similar way using serum bottles of 250 mL (180 mL working volume). Anaerobic conditions were formed through flashing of serum bottles with CO_2. All experiments were performed using initial lupanine concentration of 1.5 g L^{-1}, while a control flask, prepared without the additions of cells, was utilized to evaluate that lupanine conversion was due to the microbial action. The cultures were incubated for a period of 36–42 h and they were maintained at 31 °C, pH 7 and 100 rpm. Samples were withdrawn at regular intervals and biomass concentration was determined by absorbance at 600 nm on a UV/VIS spectrophotometer (JENWAY 7315, Staffordshire, UK).

2.6. Analytical methods

2.6.1. Determination of lupanine concentration

Gas chromatography (GC) was used to determine the concentration of lupanine in microbial culture samples. The method derived from Santana et al. (1996) was used for analysis of alkaloids, which was modified as follows: culture samples were obtained at regular intervals, centrifuged at 13,000×*g* for 5 min and filtered with 0.2 μm syringe filters. 0.5 mL of the supernatant was homogenized in 5 mL of 0.5 N HCl and held at room temperature for 30 min. The homogenate was made alkaline (pH 10) with ammonia solution 25% and applied to a standard Extrelut NT 20 column (Merck, Darmstadt, Germany). The alkaloids were eluted with 60 mL of dichloromethane, while the eluates were evaporated to dryness and then taken up in 2 mL of methanol for analysis by GC. The determination of lupanine was performed using a Shimadzu GC-2014 (Shimadzu, Milton Keynes, UK) equipped with a Flame Ionisation Detector (FID), an AOC-20i auto-injector and a Zebron ZB-5 capillary column (Phenomenex, Macclesfield, UK) with dimensions 30 m × 0.25 mm × 0.25 μm. Nitrogen was used as mobile phase and the stationary phase of the column consisted of 5% phenyl and 95% dimethylpolysiloxane. 1 μL of alkaloids collected in methanol was injected and the temperature of the column was kept constant at 150 °C for 2 min followed by an increase of 15 °C min^{-1} up to 250 °C. Subsequently, the temperature was further raised to 300 °C at a rate of 30 °C min^{-1} and it was maintained at the specific temperature for an additional 15 min. The samples were analyzed in triplicate and the standard deviation was calculated. All chemicals used were obtained from Sigma-Aldrich Company Ltd (UK) and were of analytical grade.

2.6.2. Determination of final metabolic products

Nuclear magnetic resonance spectroscopy (NMR) was used to determine the final metabolic products of lupanine bioconversion in samples derived from microbial cultures. Liquid samples of 100 mL were first centrifuged for 4 min at 13,500 rpm, the supernatant solution was filtered through 0.2 μm syringe filters to remove any remaining solids and it was evaporated to dryness under reduced pressure at 40 °C in a rotary evaporator (Stuart RE300, Staffordshire, UK).

The samples were dissolved in 600 μL DMSO-d_6 (or $CDCl_3$-d) and ^{1}H NMR, ^{13}C NMR, 2D {^{1}H} grCOSY, 2D {^{1}H} TOCSY, 2D {^{1}H, ^{13}C} grHSQC and 2D {^{1}H, ^{13}C}HMBC spectra were recorded on a 500 MHz Bruker AvanceII NMR spectrometer. Standard Bruker pulse sequences were used. ^{1}H NMR experiments were performed using 12.00 μs pulse (90°), an acquisition time of 3.17 s, a spectral width of 10,330 Hz, 16 scans (+4 dummy scans) and delay time 1.00 s ^{13}C NMR experiments were performed using 9.00 μs pulse (90°), an acquisition time of 1.10 s, a spectral width of 29,762 Hz, 4096 scans (+4 dummy scans) and delay time 2.00 s 2D{^{1}H} grCOSY and TOCSY experiments were performed using 12.00 μs pulse (90°), an acquisition time of 0.97 s, a spectral width of 6667 Hz, 64 scans (+8 dummy scans) and delay time 2.00 s 2D {^{1}H, ^{13}C} grHMBC experiments were performed using 12.00 μs pulse (90°), an acquisition time of 0.25 s, a spectral width of 6329 Hz, 80 scans (+16 dummy scans) and delay time 1.41 s 2D {^{1}H, ^{13}C} grHSQC experiments were performed using 12.00 μs pulse (90°), an acquisition time of 0.17 s, a spectral width of 6009 Hz, 32 scans (+32 dummy scans) and delay time 2.00 s.

3. Results and discussion

3.1. Toxicological assessment of lupanine

Acute toxicity testing using the gram negative *V. fischeri* constitutes a microscale biomonitoring tool in environmental toxicology. Toxic substances alter the metabolic pathways of the strain that contribute to the production of light decreasing its bioluminescence capacity. Such assays comprise rapid response systems and are known to be simple and effective in the detection of toxic molecules (Parvez et al., 2006). Results can be rapidly obtained, while various sample types can be tested including single compounds, as well as synthetic and complex mixtures (Devare and Bahadir, 1994). Moreover, the rapid reproduction, short exposure duration, sensitivity to chemical compounds and important ecological role of daphnids in the aquatic food chain constitute *D. magna* as the most frequently tested organism in aquatic toxicity bioassays (Hanazato and Hirokawa, 2004). They comprise planktonic crustaceans that belong to *Phyllopoda* (*Branchiopoda*) characterized by flattened leaf-like legs utilized to produce a water current for the filtering apparatus (Ebert, 2005). The aforementioned acute toxicity tests have been globally applied for screening of potentially hazardous chemicals and monitoring of industrial effluents. Thus, the specific ecotoxicological bioassays were explored to determine the acute toxicity of lupanine on different organisms.

The toxicity of lupanine was evaluated through calculation of the half maximal effective concentration (EC_{50}) of the two aquatic organisms, the fluorescent marine bacterium *V. fischeri* and the planktonic crustacean *D. magna*, while root growth inhibition of the plants *S. alba* and *S. saccharatum* was also determined (Fig. 1). The toxicity of the alkaloid on aquatic organisms was tested in two different durations of exposure to the alkaloid. Thus, the EC_{50} values calculated for *V. fischeri* were 89 mg L^{-1} and 47 mg L^{-1} for 5 min and 15 min of exposure, while the corresponding values for *D. magna* reached 60 mg L^{-1} and 12 mg L^{-1} following 24 h and 48 h of exposure respectively. Moreover, the phytotoxicity imposed on dicotyledonous *S. alba* and monocotyledonous *S. saccharatum* was assessed during exposure to different lupanine concentrations for 96 h. Lupanine severely impacted the root growth of *S. saccharatum* demonstrating inhibition that ranged between 97 and 99.7% for the four highest alkaloid concentrations employed (100, 50, 25 and 12.5 mg L^{-1}). The alkaloid also inhibited the germination of *S. saccharatum* (40–80%) in the four highest concentrations tested. However, exposure to lupanine exhibited positive effect for the root growth and it did not affect the germination of *S. alba*, which constitutes a dicotyledonous plant similarly to *L. albus*.

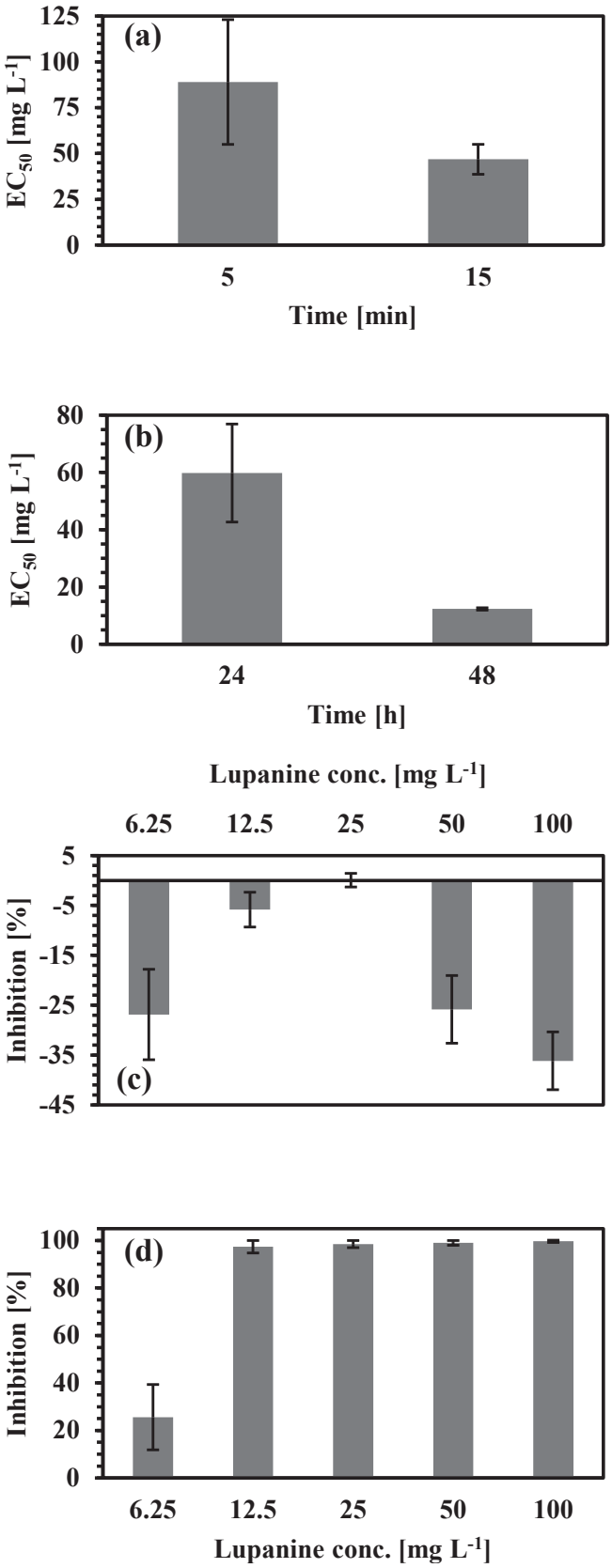

Fig. 1. Ecotoxicological assessment of lupanine on aquatic organisms and plants. EC_{50} (mg L^{-1}) values were calculated for **(a)** *V. fischeri* (5 min and 15 min of exposure to lupanine) and **(b)** *D. magna* (24 h and 48 h of exposure to lupanine). Inhibition of root growth was determined for **(c)** *S. alba* and **(d)** *S. saccharatum* (expressed as % root growth inhibition).

Ecotoxicological studies have been previously conducted to assess the toxicity of various quinolizidine alkaloids in rodents, including mice and rats, as well as guinea pigs (Table 1). Thus, the corresponding LD_{50} values for intra peritoneal administration of lupanine, sparteine, 13-hydroxylupanine and 17-oxolupanine in mice were 175, 36, 172 and 690 mg kg^{-1} respectively. These results demonstrate that lupanine and 13-hydroxylupanine express equal toxicity to mouse, while lupanine is more toxic as compared to 17-oxolupanine and less toxic than sparteine. Well-known alkaloids, such as caffeine and nicotine, have gained substantial interest relevant to their ecotoxicological assessment. The EC_{50} values calculated for caffeine using the *D. magna* assay was 161–684 mg L^{-1} and 182 mg L^{-1} for 24 h and 48 h of exposure respectively (Camacho-Muñoz et al., 2010) exhibiting lower toxicity as compared to lupanine. However, although Jiménez et al. (2017) reported that nicotine is non-toxic for *V. ficsheri* the effect of lupanine on *D. magna* was less toxic than that of nicotine, which comprised EC_{50} values that reached 4–6 mg L^{-1} and 0.79 mg L^{-1} for 24 h and 48 h of exposure respectively (Jiménez et al., 2017; Martins et al., 2007). Only a few studies have been conducted for evaluating the toxicity of alkaloids to plants. The toxic alkaloid cylindrospermopsin has been reported to inhibit the growth of *S. alba* demonstrating an IC_{50} value of 18.2 μg mL^{-1} (Vasas et al., 2002). However, quinolizidine alkaloids, such as lupanine, are important for the survival of plants acting as a defense mechanism against pathogens or predators and constitute allelopathic metabolites for competition with other plant species (Bunsupa et al., 2013). Previous studies (Cahill and Lamb, 2007) exhibit that dicotyledonous plants (e.g. *L. albus* and *S. alba*) constitute stronger competitors compared to monocotyledonous (e.g. *S. saccharatum*) suggesting that dicotyledonous develop mechanisms enabling a significant competitive advantage over other plants.

3.2. Isolation and characterization of lupanine metabolizing strains

Samples from four environmental sources (granular sludge, digested sludge, aerobic sludge and wastewater from the *L. albus* processing industry) were enriched with lupanine as the sole source of carbon under aerobic and anaerobic conditions. Following three sequential enrichment cultivations of the samples in liquid media significant growth was observed and grown cells were subsequently inoculated into solid media. Eight bacterial strains (4 aerobic and 4 anaerobic) were purified holding the capacity to grow on lupanine at a concentration level of 1.5 g L^{-1}. Based on the phylogenetic and physiological characteristics, aerobic strains were identified as *Rhodococcus ruber* LPK111 (ACN: MF455224.1, source: granular sludge), *Rhodococcus rhodochrous* LPK211 (ACN: MF455223.1, source: digested sludge), *Rhodococcus* sp. LPK311 (ACN: MF455225.1, source: aerobic sludge) and *Pseudomonas putida* LPK411 (ACN: MF455219.1, source: *L. albus* processing wastewater). Moreover, the isolated strains from anaerobic cultures of granular sludge were identified as *Pseudomonas citronellolis* LPK121 (ACN: MF455218.1) and *Rhodobacter* sp. LPK122 (ACN: MF455222.1), while the strains derived from digested sludge were identified as *Pseudomonas* sp. LPK221 (ACN: MF455221.1) and *Ochrobactrum tritici* LPK222 (ACN: MF455217.1). All strains demonstrated sequence homology higher than 99% to similar strains and their internal transcribed spacer sequence (ITS) was deposited in GenBank. Thus, the phylogenetic trees (Fig. 2) based on the ITS were constructed using the neighbor-joining method implemented in the multiple alignment software ClustalW (MEGA 6.0) to correlate the isolated strains with other microorganisms that shared similar ITS gene sequence.

Pseudomonas sp. LPK221 exhibited close similarity to

Table 1
Ecotoxicological effects of alkaloids in different organisms. Intraperitoneal administration (i.p.); intravenous administration (i.v.); oral administration (p.o.).

Alkaloid	Species	Test	Value	Reference
Lupanine	Mouse	LD_{50} (i.p.)	175 mg Kg^{-1}	(Yovo et al., 1984)
	Mouse	LD_{50} (p.o.)	410 mg Kg^{-1}	(Yovo et al., 1984)
	Rat	LD_{50} (i.p.)	177 mg Kg^{-1}	(Pothier et al., 1998)
	Rat	LD_{50} (p.o.)	1440-1664 mg Kg^{-1}	(Pothier et al., 1998)
	Guinea pig	LD_{50} (i.p.)	210 mg Kg^{-1}	(Wink, 1993)
	V. fischeri	EC_{50} (5 min)	89 mg L^{-1}	Current study
	V. fischeri	EC_{50} (15 min)	47 mg L^{-1}	Current study
	D. magna	EC_{50} (24 h)	60 mg L^{-1}	Current study
	D. magna	EC_{50} (48 h)	12 mg L^{-1}	Current study
Sparteine	Mouse	LD_{50} (i.p.)	36 mg Kg^{-1}	(Yovo et al., 1984)
	Mouse	LD_{50} (p.o.)	220 mg Kg^{-1}	(Yovo et al., 1984)
	Rat	LD_{50} (i.p.)	42-44 mg Kg^{-1}	(Wink, 1993)
	Rat	LD_{50} (i.v.)	68-75 mg Kg^{-1}	(Wink, 1993)
13-hydroxylupanine	Mouse	LD_{50} (i.p.)	172 mg Kg^{-1}	(Wink, 1993)
	Rat	LD_{50} (i.p.)	199 mg Kg^{-1}	(Wink, 1993)
17-oxolupanine	Mouse	LD_{50} (i.p.)	690 mg Kg^{-1}	(Wink, 1993)
Caffeine	Mouse	LD_{50} (i.p.)	369 mg Kg^{-1}	(Mitkov et al., 2007)
	Mouse	LD_{50} (p.o.)	527 mg Kg^{-1}	(Mitkov et al., 2007)
	D. magna	EC_{50} (24 h)	161-684 mg L^{-1}	(Martins et al., 2007)
	D. magna	EC_{50} (48 h)	182 mg L^{-1}	(Camacho-Muñoz et al., 2010)
	D. magna	LC_{50} (24 h)	684 mg L^{-1}	(Martins et al., 2007)
Nicotine	*D. magna*	EC_{50} (24 h)	4-6 mg L^{-1}	(Martins et al., 2007)
	D. magna	EC_{50} (48 h)	0.79 mg L^{-1}	(Jiménez et al., 2017)

Pseudomonas sp. IBP-A36, which holds the capacity to produce polyhydroxyalkanoates (Galia et al., 2014), while *Pseudomonas citronellolis* LPK121 and *Pseudomonas putida* LPK411 were similar to *Pseudomonas citronellolis* P3B5 and *Pseudomonas putida* OBS-2 isolated from the medicinal plants *Ocimum basilicum* and *Morinda citrifolia* respectively (Remus-Emsermann et al., 2016; unpublished results, NCBI). *Ochrobactrum tritici* LPK222 showed close similarity to *Ochrobactrum tritici* TJ3, a phenol-degrading and nitrate-reducing bacterial strain (Baek et al., 2003), and *Rhodobacter* sp. LPK122 was related to *Rhodobacter* sp. R076N. Furthermore, Fig. 2d demonstrates that *Rhodococcus rhodochrous* LPK211 was alike *Rhodococcus rhodochrous* LAZC-8, an actinomycete isolated from a clinical environment in Mexico, *Rhodococcus* sp. LPK311 was similar to *Rhodococcus* sp. K-39 (a hydrocarbon-degrading bacterial strain) and *Rhodococcus ruber* LPK111 showed close similarity to *Rhodococcus ruber* 2S12 (unpublished results, NCBI). Thus, the lupanine metabolizing strains isolated belong to genera strongly associated to the biodegradation of recalcitrant and toxic pollutants.

3.3. Biodegradation of lupanine by aerobic isolated strains

The microorganisms isolated under aerobic conditions (*Rhodococcus rhodochrous* LPK211, *Rhodococcus ruber* LPK111, *Rhodococcus* sp. LPK311 and *Pseudomonas putida* LPK411), holding the capacity to utilize lupanine as the sole carbon source, were grown in M9 medium containing 1.5 g L^{-1} of the alkaloid. The cultures were maintained at 31 °C, pH 7 and 100 rpm for 36–42 h, while the results demonstrate that lupanine was substantially biodegraded by all isolated strains (Fig. 3). *Rhodococcus rhodochrous* LPK211 achieved high removal of lupanine at 36 h, which corresponded to 81%, while under the same conditions *Rhodococcus ruber* LPK111 and *Rhodococcus* sp. LPK311 demonstrated 66% and 71% removal of lupanine within 42 h and 36 h respectively. However, although *Pseudomonas putida* LPK411 produced the lowest biomass content as compared to the three *Rhodococcus* strains, it demonstrated the shortest lag phase and significant removal of lupanine which reached 80% following 36 h of cultivation. The four anaerobic strains isolated, demonstrated very slow growth on lupanine under tested conditions (data not shown) and therefore these strains were not studied any further.

Previous studies have confirmed that *Pseudomonas* and *Rhodococcus* species hold the capacity to biodegrade a wide range of alkaloids, including among others nicotine, caffeine, codeinone and ergot alkaloids (Table 2). Wang et al. (2012) reported that *Pseudomonas* sp. S16 was capable for complete biodegradation of 3 g L^{-1} of nicotine within 10 h at 30 °C. Furthermore, *Pseudomonas* sp. GSC 1182 removed 80% of caffeine (1.2 g L^{-1}) within 48 h (Gokulakrishnan et al., 2007), while *Rhodococcus* sp. Y22 demonstrated 100% degradation of nicotine following 8 h of cultivation (Gong et al., 2009). *Rhodococcus erythropolis* MTHt3 could remove 5 mg L^{-1} of ergotamine and ergine within 24 h, which were converted to lysergic acid accumulating as final metabolic product in the media. Only a limited number of studies have led to the isolation of microorganisms capable of biodegrading lupanine. Similar performance was achieved by bacterial strains IST 20B and IST 40D demonstrating 99% removal for 1 and 2 g L^{-1} of lupanine at 27 °C respectively (Santana et al., 2002). However, the specific isolates could not be identified and any metabolites generated as end-products were not measured to assess the capacity of the strains to form added-value products from lupanine bioconversion.

3.4. Bioconversion of lupanine for the production of high-value products

Lupanine comprises a symmetric structure and may serve as a starting material for semisynthesis of a range of other alkaloids (Carmali et al., 2010). Thus, apart from current efforts focusing on the use of microorganisms for debittering of *Lupinus* flours as well as detoxification of *Lupinus* industrial wastewater, the bioconversion of lupanine into modified structures could substantially enhance the effectiveness of lupanine-based chemical conversion technologies for the production of biopharmaceuticals. In line with the above, we have performed a preliminary assessment of the final metabolic products generated during bioconversion of lupanine by the four aerobic strains isolated applying NMR analysis in samples obtained at the end of the batch experiments presented in the previous section (Fig. 4). There were no end-products detected for *Rhodococcus rhodochrous* LPK211 indicating that lupanine could be mineralized to a large extent. *Rhodococcus ruber* LPK111 and *Rhodococcus* sp. LPK311 produced the same molecule (B, Fig. 4), the

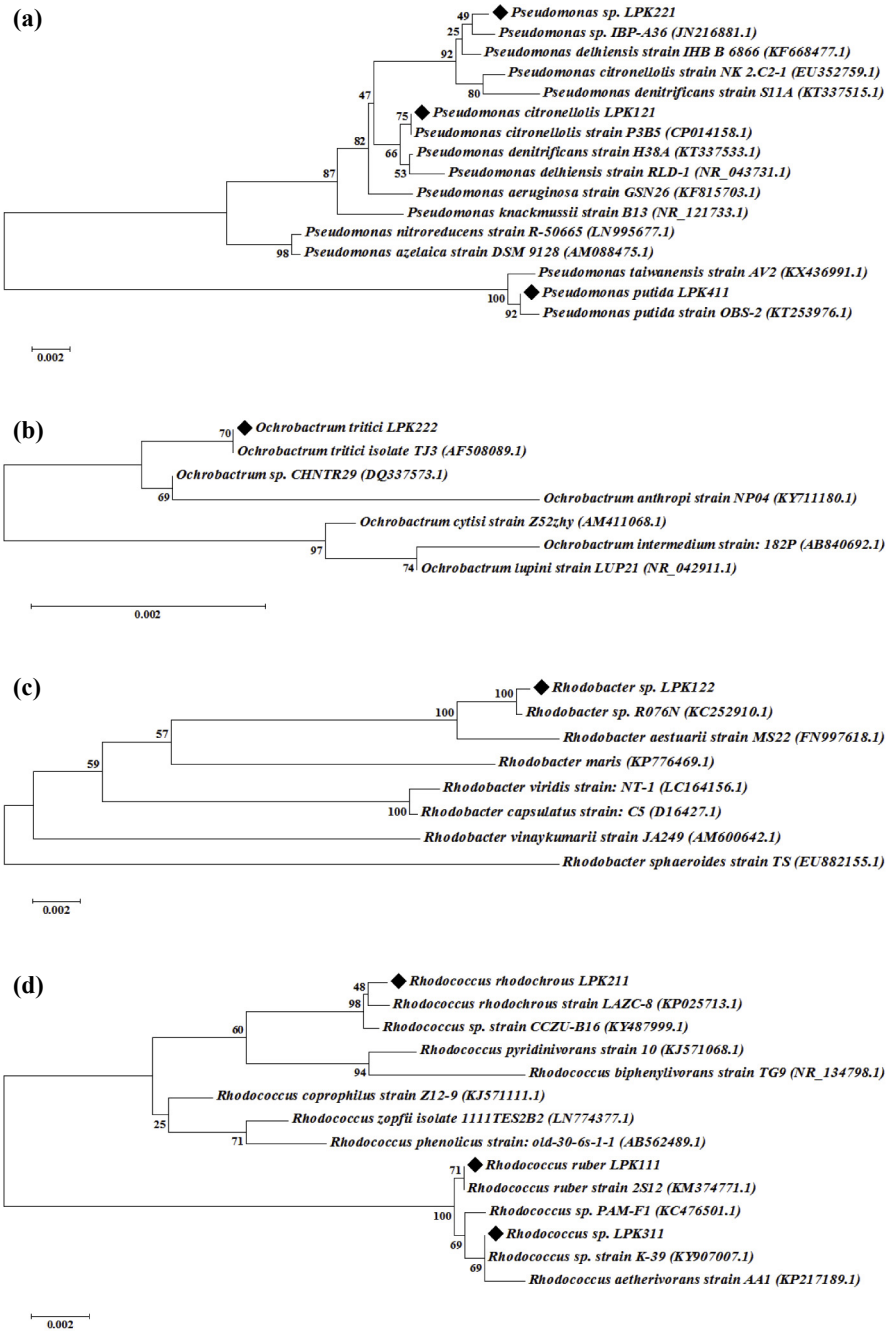

Fig. 2. Phylogenetic trees of microbial isolates obtained through neighbor-joining analysis exhibiting the position of purified microorganisms among similar strains. Bootstrap values (expressed as percentages of 500 replications) are shown at the branch points and the scale bar represents a distance of 0.002. **(a)** *Pseudomonas putida* LPK411, *Pseudomonas citronellolis* LPK121 and *Pseudomonas* sp. LPK221, **(b)** *Ochrobactrum tritici* LPK222, **(c)** *Rhodobacter* sp. LPK122 as well as **(d)** *Rhodococcus ruber* LPK111, *Rhodococcus rhodochrous* LPK211 and *Rhodococcus* sp. LPK311.

most possible structure of which constitutes 12-(l1-oxidanyl)tetradecahydro-4H-12l4-7,14-methanodipyrido[1,2-a:1′,2′-e][1,5] diazocin-4-one (lupanine N-oxide). Three compounds (molecules C, D and E in Fig. 4) have been observed as end-products of lupanine bioconversion using *Pseudomonas putida* LPK411. A ketone group at C-17 was formed at molecules C and D, while a C–N (2-1) bond was cleaved and a hydroxyl group was added to C-2 in products D and E. The potential structures of these products could be 4-(6-oxodecahydro-2H-1,5-methanopyrido[1,2-a][1,5]diazocin-4-yl) butanoic acid, 4-(decahydro-2H-1,5-methanopyrido[1,2-a][1,5] diazocin-4-yl)butanoic acid and decahydro-2H,6H-7,14-methanodipyrido[1,2-a:1′,2′-e][1,5]diazocine-6,11(7H)-dione (17-oxolupanine).

Although the fermentation metabolites presented above have not been previously identified as products formed in similar biocatalytic processes, lupanine N-oxide (molecule B, Fig. 4) as well as other epi N-oxides have been synthetically prepared from sparteine in laboratory scale (Brukwicki and Wysocka, 2003). Moreover, 17-oxolupanine (molecule C, Fig. 4) as well as lupanine, sparteine, albine, angustifoline, multiflorine and some of their derivatives

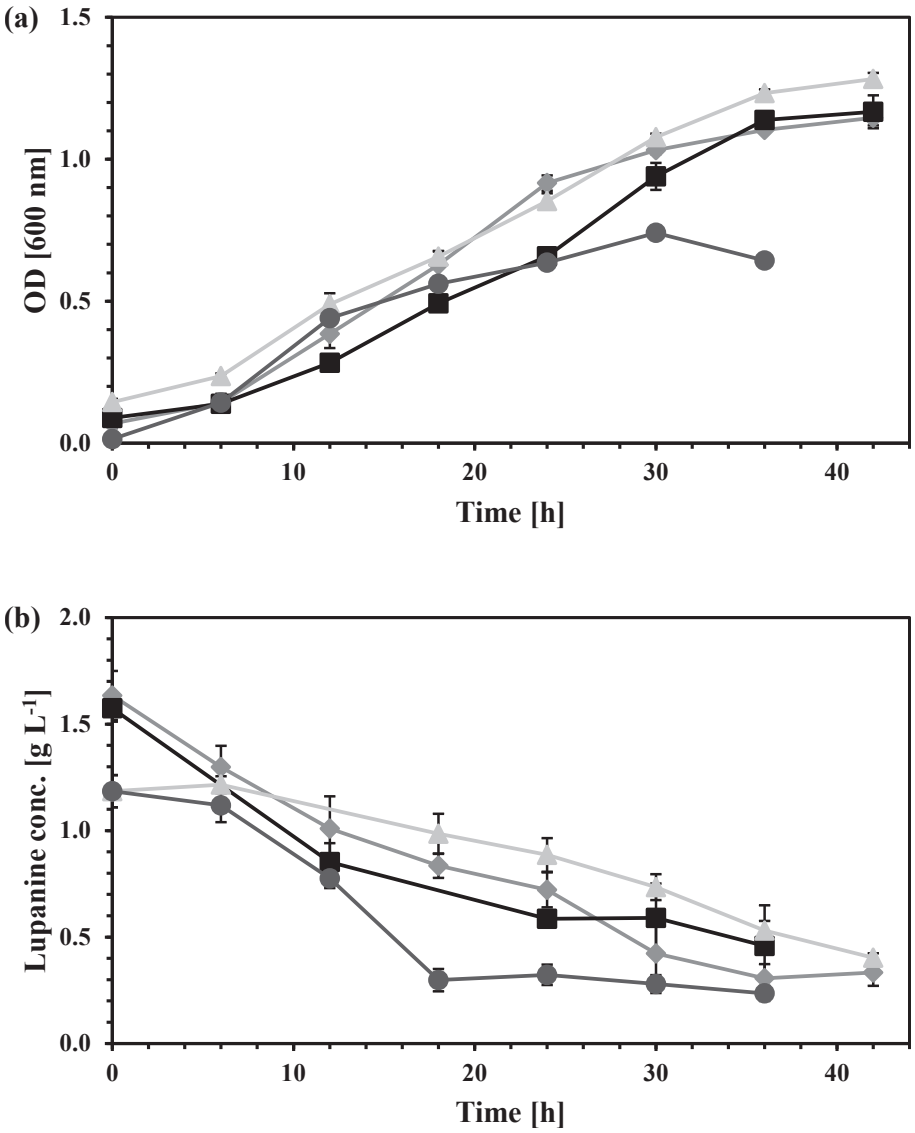

Fig. 3. Biodegradation of lupanine from aerobic microorganisms. **(a)** Microbial growth (expressed as OD) and **(b)** concentration of lupanine in fermentations performed. ■: *Rhodococcus* sp. LPK311, ●: *Pseudomonas putida* LPK411, ◆: *Rhodococcus rhodochrous* LPK211, ▲: *Rhodococcus ruber* LPK111.

Table 2
Microbial bioconversion of different alkaloids.

Microorganism	Alkaloid	Removal (%)	Time (h)	Metabolites	Conc. (g L^{-1})	Reference
IST 20B	Lupanine	99	24	3-hydroxylupanine, 13-hydroxylupanine, 17-oxosparteine	3.0	(Santana et al., 2002)
IST 40D	Lupanine	99	24	3,4- dehydrolupanine, α-isolupanine	3.0	(Santana et al., 2002)
R. rhodochrous LPK211	Lupanine	81	36	–	1.5	Current study
R. ruber LPK111	Lupanine	66	42	lupanine N-oxide	1.5	Current study
R. sp. LPK311	Lupanine	71	36	lupanine N-oxide	1.5	Current study
P. putida LPK411	Lupanine	80	36	17-oxolupanine, molecules D-E (Fig. 4)	1.5	Current study
P. alcaligenes CFR 1708	Caffeine	100	30	–	1.0	(Babu et al., 2005)
Pseudomonas sp. HF-1	Nicotine	99.6	25	–	1.3	(Ruan et al., 2005)
Pseudomonas sp. ZUTSKD	Nicotine	97	12	–	1.6	(Zhong et al., 2010)
Pseudomonas sp. S16	Nicotine	100	10	–	3.0	(Wang et al., 2012)
P. putida M10	Codeinone	100	72	14β-hydroxycodeine, 14β-hydroxycodeinone	3.0	(Lister et al., 1999)
Rhodococcus sp. Y22	Nicotine	100	8	–	1.0	(Gong et al., 2009)
R. erythropolis MTHt3	Ergotamine, ergine	100	24	Lysergic acid	0.005	(Thamhesl et al., 2015)
O. intermedium DN2	Nicotine	97.7	36	–	0.5	(Yuan et al., 2005)

have been isolated from *Lupinus* species of *L. albus*, *L. variusorientalis*, *L. hartwegii*, and *L. densiflorus* (El-Shazly et al., 2001). However, the non-rigid lupanine derivatives produced in *Pseudomonas putida* LPK411 cultures (molecules D and E, Fig. 4) constitute novel structures reported here for the first time. A few studies have been conducted to date concerning the chemical and

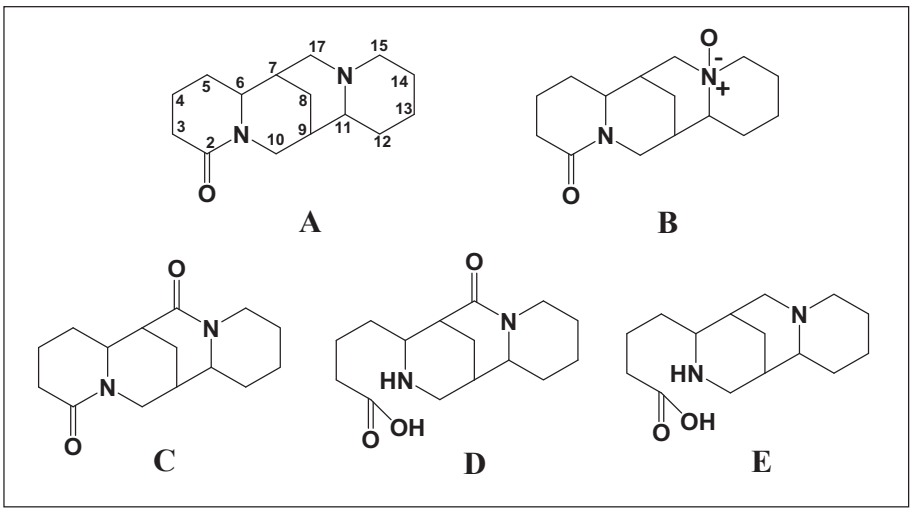

Fig. 4. Chemical structure of lupanine and final metabolic products from lupanine bioconversion identified through NMR analysis. **A:** lupanine; **B:** lupanine-N-oxide; **C:** 17-oxolupanine; **D** and **E:** novel structures.

biological conversion of lupanine. Hopper et al. (1991) reported that lupanine can be metabolized by a *Pseudomonas* sp., where the first reaction included hydroxylation of lupanine into 17-hydroxylupanine, through the action of lupanine 17-hydroxylase. The production of 3-hydroxylupanine, 13-hydroxylupanine, 17-oxosparteine, 3,4-dehydrolupanine and α-isolupanine has been confirmed employing the two bacterial strains IST 40D and IST 20B (Santana et al., 2002). Moreover, Maulide et al. (2016) achieved chemical conversion of lupanine into sparteine using $NaBH_4$ and I_2 in an organic solvent. Pertinent to the molecular structures of the final metabolic products depicted in Fig. 4, molecules C and D derived from site-specific oxidation at carbon C-17 to the corresponding amide constitute the most promising compounds for generating useful products. The formed amide bond allows the creation of new generation sparteine analogues via alkylation on the amide bond. Thus, the present study demonstrates that both known and novel lupanine-based alkaloid structures can be produced with the use of the isolated strains, which could form the basis for the development of *Lupinus* wastewater valorization strategies.

4. Conclusions

The development of a lupanine valorization process is expected to alleviate the environmental problem associated with the alkaloid content of lupine beans processing wastewater. Although lupanine was toxic to *V. fischeri*, *D. magna* and monocotyledonous plants, the alkaloid promoted root growth in dicotyledonous. The isolated microorganisms were efficient both for biological treatment and bioconversion of lupanine demonstrating potential production of a range of alkaloid-derived molecules that could serve as targets for chemical modification. These results are promising for the development of a lupanine bioconversion process based on the newly isolated *Pseudomonas putida* LPK411.

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