Valorization of alkaloid containing wastewater: Bioconversion of lupanine into added-value products by newly isolated microorganisms

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Abstract

Purpose: *Lupinus albus* constitutes an important food product for the human diet exhibiting numerous applications in the manufacture of novel food products as a source of supplementary protein and fiber. However, *L. albus* processing industries generate significant amounts of wastewater comprising a high content in toxic quinolizidine alkaloids, mainly lupanine. This work explores the potential for development of a lupanine valorisation bioprocess, removing the toxic alkaloid from industrial wastewater.

Methods: The microscale test of *Vibrio fischeri* for acute toxicity, the acute bioassay of *Daphnia magna* as well as the seeds of the dicotyledon *Sinapis alba* and the monocotyledon *Sorghum saccharatum* were used for ecotoxicological assessment of lupanine. Moreover, several strains isolated in our lab for lupanine detoxification (*Pseudomonas putida* LPK411, *Rhodococcus ruber* LPK11, *Rhodococcus rhodochrous* LPK211, *Rhodococcus* sp. LPK311) have been tested for their capacity to perform enantiomeric resolution of the racemic mixture incorporated in the wastewater.

Results: Ecotoxicological assessment demonstrated that lupanine is toxic for *V. fischeri*, *D. magna* and monocotyledonous plants, however promoted root growth in

dicotyledonous. All strains achieved high enantiomeric excess (99%-100% of L-(-)-lupanine) at 42 h. *P. putida* LPK411 constituted the most efficient microorganism exhibiting maximum enantiomeric excess at 30 h. Thus, the biodegradation of racemic lupanine was evaluated for *P. putida* LPK411 under different culture conditions and use of different immobilisation carriers.

Conclusions: These results are promising for the development of a lupanine valorisation process reducing the cost required for laborious chemical conversion into biopharmaceuticals.

Keywords

Lupanine, quinolizidine alkaloids, enantiomeric excess, biodegradation, bioconversion, toxicity assessment.

1. Introduction

Lupinus albus constitute low cost and non-genetically modified seeds [1, 2] comprising an important food product with a range of applications in food production. *L. albus* seeds typically involve 33-47% proteins and 6-13% oils [3, 4], while the content in dietary fibers (34-39%) could be suitable for dietetic food production [5]. However, the varieties with favorable agronomic characteristics contain toxic quinolizidine alkaloids (1.9-2.7%), which are not suitable for animal or human consumption [6]. Debittering constitutes the process that is widely used for elimination of undesirable alkaloids from *L. albus* seeds, which is performed through exhaustive boiling and alkaloid leaching from the seeds into water. Thus, the *L. albus* snack manufacturing process generates large amounts of lupanine containing wastewater [7].

Lupanine displays moderate acute toxicity and the symptoms show that alkaloids cause neurological effects leading to loss of motor coordination and muscular control [8]. In line with the above, the Health Authorities of Great Britain, France, Australia and New Zealand have placed a maximum limit of 200 mg kg^{-1} for the content of quinolizidine alkaloids in food products [9]. Although lupanine imposes toxic effects to the environment, the molecule entails an asymmetric 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 [10]. Nevertheless, the alkaloid is not considered as an easy target for chemical modification due to the presence of stable amide and tertiary amine functionalities [11].

Previous studies have reported that various strains isolated from soil (e.g. *Pseudomonas* sp.) are capable of utilising lupanine as the only source of carbon and nitrogen [12, 13]. Santana et al [14] reported that bacterial strains IST 20B and IST 40D achieved 99% removal for 1 and 2 g L^{-1} of lupanine at 27 ^{o}C, while the maximum lupanine removal obtained for a *L. albus* seeds extract complex medium was only 77%. Other studies showed that *Pseudomonas* (e.g. *Pseudomonas* sp. S16, *Pseudomonas* sp. GSC 1182) and *Rhodococcus* (e.g. *Rhodococcus* sp. Y22,

Rhodococcus erythropolis MTHt3) species hold the capacity to biodegrade a wide range of alkaloids, including among others nicotine, caffeine, codeinone and ergot alkaloids [15-17].

In the present work, the toxicity of lupanine has been evaluated on marine bacteria, crustaceans and plants, while the potential for the development of a lupanine valorisation process has been explored. Four microorganisms (*R. ruber* LPK11, *R. rhodochrous* LPK211, *Rhodococcus* sp. LPK311 and *P. putida* LPK411) capable of metabolising lupanine as the sole carbon source were isolated. The work aimed in evaluating the capacity of each strain to perform resolution of the racemic mixture of lupanine contained in the waste (D-(+)-lupanine, L-(-)-lupanine) through microbial decomposition of one of the optically active forms faster than the other. Thus, enantiomeric excess (e.e.) of L-(-)-lupanine was investigated during fermentation of all isolated microorganisms with the racemic mixture. Moreover, the bioconversion of lupanine was evaluated for *P. putida* LPK411 under different culture conditions (e.g. temperature, pH, lupanine concentration, immobilisation carriers).

2. Methods

2.1. Microbial cultures

Four isolated strains holding the capacity to grow under aerobic conditions on lupanine were employed in the current study. The microorganisms were maintained in glycerol stocks at -80 ^{o}C. The inoculum was pregrown overnight at 31 ^{o}C in an incubator, which was stirred at 100 rpm, while M9 media supplemented with 1.5 g L^{-1} of lupanine were used. In each experiment, cultures were prepared in duplicate inoculating 3 ml of pregrown cells under sterile conditions utilising flasks of 500 ml (150 ml working volume). The microorganisms were incubated using varying temperatures, initial lupanine concentrations and pH values according to the requirements of each experiment. 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).

2.2. Purification of lupanine

Lupanine was purified from *L. albus* seeds following the general procedure described elsewhere [18]. 1 Kg of lupin beans (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 to room temperature, while the water was separated from the beans by decantation. The brown water was basified with NaOH (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 stirred. Subsequently, the organic phase was separated and dried with anhydrous magnesium sulphate. The 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).

2.3. Analytical methods

Gas chromatography (GC): GC was used to determine the concentration of lupanine in culture samples. The method derived from Santana et al [13] was used for analysis of alkaloids, modified as follows: culture samples were centrifuged 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, an AOC-20i auto-injector and a Zebron ZB-5 capillary column (Phenomenex, Macclesfield, UK). 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 ^{o}C for 2 min followed by an increase of 15 ^{o}C min^{-1} up to 250 ^{o}C. Subsequently, the temperature was further raised to 300 ^{o}C at a rate of 30 ^{o}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.

Determination of final metabolic products: Nuclear magnetic resonance spectroscopy (NMR) was used to determine the final metabolic products of lupanine bioconversion in microbial cultures. Samples of 100 ml were centrifuged, filtered through 0.2 μm syringe filters and evaporated to dryness under reduced pressure at 40 ^{o}C in a rotary evaporator (Stuart RE300, UK). The samples were dissolved in 600 μl DMSO-d6 (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 10330 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 29762 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°), acquisition time of 0.25 s, 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°), acquisition time of 0.17 s, spectral width of 6009 Hz, 32 scans (+ 32 dummy scans) and delay time 2.00 s.

Analysis of e.e.: Samples were passed through a pipette with silica before injection in the HPLC. After evaporating to dryness, 2 mg of lupanine are dissolved in 100 μL of iPrOH, to which 900 μL of hexane are added. The enantiomeric excess of L-(-)-lupanine was determined by HPLC using a chiral column (Chiral Pak IC) and mobile phase of 53% hexane, 22% isopropanol and 25% hexane (with 0.1% diethanolamine) at a flow rate of 1 mL/min, room temperature and UV detection at 230 nm.

2.4. Acute toxicity tests

Toxicity assessment with marine bacteria: The microscale test for acute toxicity, as the bioluminescence inhibition of *V. fischeri* (NRRL B11177) following 5 min and 15 min of exposure to test samples, was used as described elsewhere [19]. Lyophilized bacteria were reconstituted prior use. The salinity was adjusted using a solution of NaCl (18%) 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 13-26 mg L^{-1} was considered as acceptable. Nine concentrations of lupanine that varied between 8-2000 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:

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

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. [20].

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

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

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 dish contained a filter paper with 5 seeds and 1 ml of the testing solution, while 5 different concentrations between 6.25-100 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. Eq. 4 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). Subsequently, growth inhibition was correlated to lupanine concentration and corresponding EC_{50} values were calculated.

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

Toxicity assessment with crustaceans: The acute bioassay of *D. magna* incorporates a standard toxicity test determining the immobilisation of the organism following 24 h and 48 h of exposure [21]. Each experiment comprised of 5 lupanine concentrations ranging between 3.125-100 mg L^{-1} as well as a control. Each concentration was tested in triplicate 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. Immobilisation was calculated in each well, while that of the control test was not significant (<5%). The percentage of immobilisation was estimated via recording of the number of dead and immobilised 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 immobilised even if they could still move their antennae. The immobilisation percentage was calculated using the following equation:

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

I_c corresponds to the immobilisation percentage at concentration C, ${}_{cor}N_{imm}$ represents the corrected number of immobilised daphnids and N_{tested} stands for the total number of daphnids tested. In cases where immobilisation in the control experiment (I_o) ranged between $0 < I_o < 5\%$, the corrected number of immobilised daphnids was calculated using Abbott's equation [22]. The corresponding EC_{50} value was calculated by Probit analysis [23].

3. Results and Discussion

3.1. Ecotoxicological assessment of lupanine

The toxicity of lupanine was evaluated through calculation of the half maximal effective concentration (EC_{50}) of *V. fischeri* and *D. magna*, while root growth inhibition of *S. alba* and *S. saccharatum* was also determined (Fig. 1). 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 97-99.7% 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*.

3.2. Lupanine bioconversion products

The bioconversion of lupanine into modified structures could substantially enhance the effectiveness of lupanine-based chemical conversion technologies for the production of biopharmaceuticals. Thus, 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 batch

experiments conducted with the use of 1.5 g L^{-1} lupanine at 31 ^{o}C and pH 7 has been performed (Fig. 2). There were no end-products detected for *R. rhodochrous* LPK211 indicating that lupanine could be mineralised to a large extent. *R. ruber* LPK111 and *Rhodococcus* sp. LPK311 produced the same molecule (a, Fig. 2), the most possible structure of which constitutes lupanine N-oxide. Three compounds (molecules b, c and d in Fig. 2) have been observed as end-products of lupanine bioconversion using *P. putida* LPK411. A ketone group at C-17 was formed at molecules b and c, while a C-N (2-1) bond was cleaved and a hydroxyl group was added to C-2 in products c and d. 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).

The non-rigid lupanine derivatives produced by *P. putida* LPK411 (molecules c and d, Fig. 2) constitute novel structures reported here for the first time. These molecules have been derived from site-specific oxidation at carbon C-17 to the corresponding amide constituting the most promising compounds for generating useful products from lupanine bioconversion. The formed amide bond allows the creation of new generation sparteine analogues via alkylation on the amide bond demonstrating that both known and novel lupanine-based alkaloid structures can be produced with the use of the isolated strains.

3.3. L-(-)-lupanine e.e. from P. putida LPK411

Lupanine exhibits numerous applications for the fine chemical and pharmaceutical industries due to asymmetric core structure of quinolizidine. However, lupanine in nature exists in a racemic mixture of two enantiomers (D-(+)-lupanine and L-(-)-lupanine), necessitating the development of separation processes recovering the enantiomers in a pure form [24]. L-(-)-lupanine may serve as a starting material for synthesis of D-(+)-sparteine, a naturally occurring compound which cannot be easily obtained from natural sources [10]. Since L-(-)-sparteine exhibits numerous pharmacological properties [11] and chemical applications as ligand or promoter for various asymmetric reactions [18], the production of L-(-)-lupanine from the wastewater through bioconversion may open a novel direction for synthesis of D-(+)-sparteine at a potentially lower cost.

L-(-)-lupanine e.e. was investigated during fermentation of *P. putida* LPK411 and three *Rhodococcus* strains (*R. ruber* LPK11, *R. rhodochrous* LPK211, *Rhodococcus* sp. LPK311). The cultures were maintained at 31 ^{o}C, pH 7 and 100 rpm. The results demonstrate that all the isolated microorganisms were capable of performing resolution degrading faster D-(+)-lupanine (Fig. 3). The e.e. of all strains ranged between 95-100% at 42 h of cultivation, while *P. putida* LPK411 reached e.e. values of 95% substantially faster (30 h) as compared to the three *Rhodococcus* strains.

3.4. Lupanine biodegradation from P. putida LPK411 at varying temperatures and pH

The biodegradation of lupanine from *P. putida* LPK411 achieved both resolution of the racemic mixture to elevated e.e. values of L-(-)-lupanine as well as the production of novel alkaloid structures more prone to chemical modification. Thus,

the specific microorganism presents great potential for application in lupanine-based valorisation processes for the production of biopharmaceuticals. Different temperatures and pH values have been tested at LPK411 cultures aiming to determine the best conditions for growth on lupanine (Fig. 4). The results demonstrate that 31 ^{o}C and pH values of 6 and 7 enabled the highest removals of lupanine that reached 63% and 78% respectively using initial lupanine content of approximately 1.5 g L^{-1}. Future research will focus on optimising the growth conditions of LPK411 pertinent to varying initial lupanine concentrations and the use of immobilised cells.

4. Conclusions

The development of a lupanine valorisation 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 strains enable both the development of biological treatment and lupanine valorisation processes through resolution of the racemic lupanine contained in the waste and formation of useful intermediates for chemical conversion into biopharmaceuticals.

Acknowledgements

This work was supported by the M-ERA.NET collaborative project Biorg4WasteWaterVal+ co-funded by the Research Promotion Foundation (RPF, Cyprus) [grant number ΚΟΙΝΑ/ΠΚΠ-WATER/0315/14] and by Fundação para a Ciência e a Tecnologia (FCT, Portugal) [grant numbers WaterJPI/0001/2014, WaterJPI/0002/2014 and WaterJPI/0003/201].

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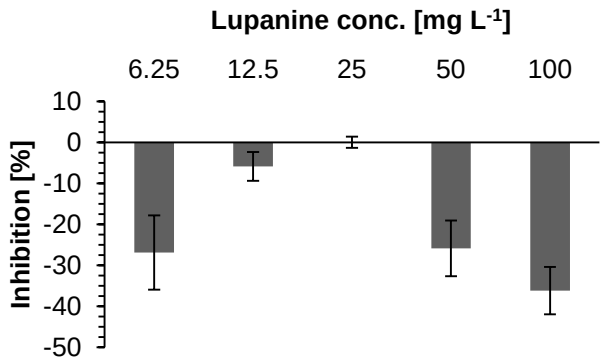
Figure Captions

Fig. 1 Ecotoxicological assessment of lupanine on plants. Inhibition of root growth for: **a)** *S. alba* and **b)** *S. saccharatum*

a)



b)

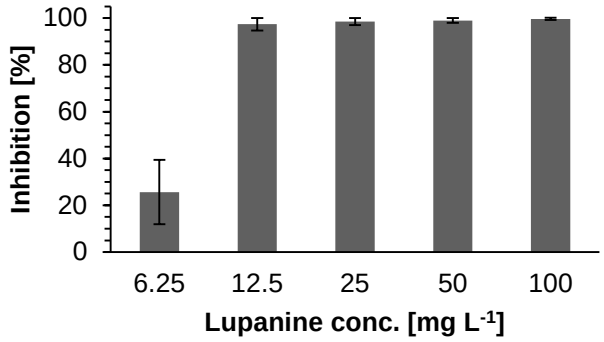


Fig. 2 Chemical structure of final metabolic products from lupanine bioconversion. **a)** lupanine-N-oxide; **b)** 17-oxolupanine; **c)** and **d)** novel structures

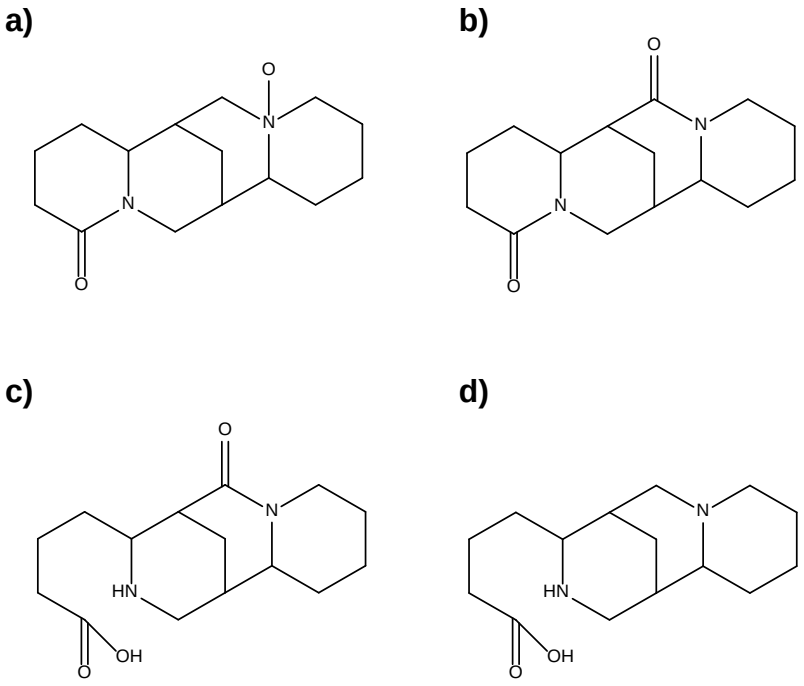


Fig. 3 L-(-)-lupanine e.e. achieved using microbial degraders. ♦ : *P. putida* LPK411; ■ : *R. ruber* LPK11; ● : *R. rhodochrous* LPK211; ▲ : *Rhodococcus* sp. LPK311

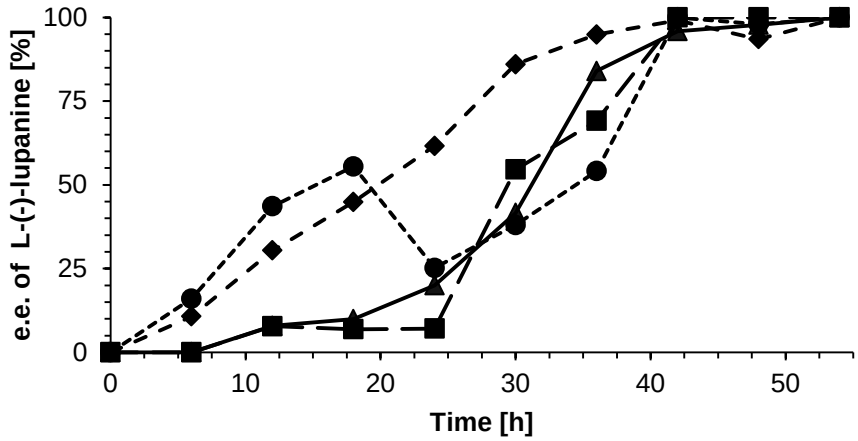
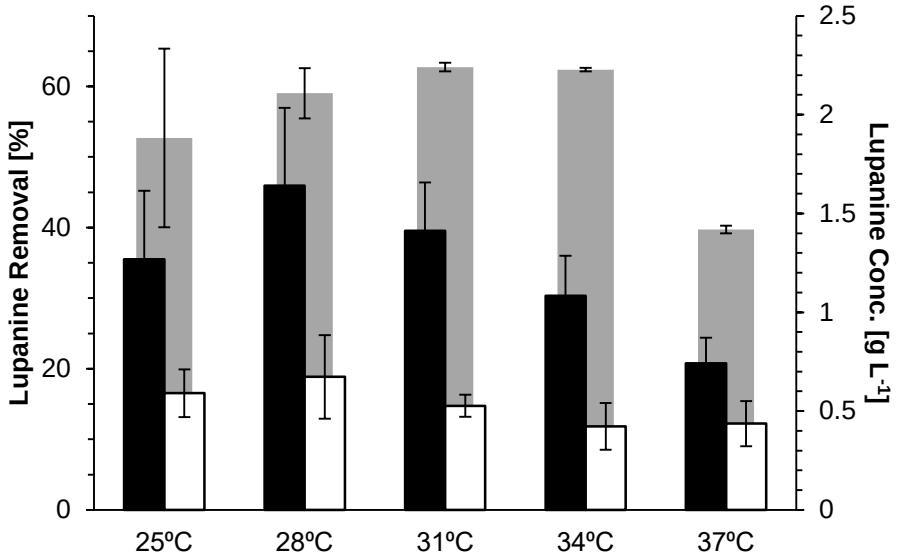
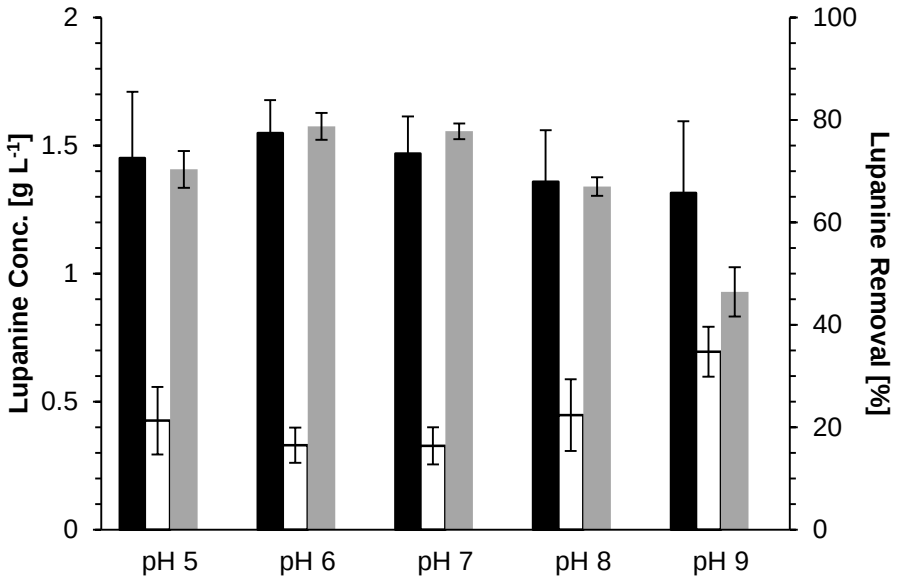


Fig. 4 Biodegradation of lupanine from *P. putida* LPK411 under different: **a)** temperatures and **b)** pH values. Grey columns: % removal of lupanine; black columns: concentration of lupanine at 0 h; white columns: concentration of lupanine at 12 h in a and at 9 h in b

a)



b)


Lupanine Conc. [g L-1]
Lupanine Removal [%]
2
1.5
1
0.5
0
100
80
60
40
20
0
pH 5
pH 6
pH 7
pH 8
pH 9