Bacterial Removal of Quinolizidine Alkaloids and Other Carbon Sources from a *Lupinus albus* Aqueous Extract

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Two Gram-negative bacterial strains capable of using lupanine, the predominant quinolizidine alkaloid in *Lupinus albus*, as a sole carbon source were isolated from soil in which *L. albus* and *L. luteus* had been grown [Santana, F. M. et al. *J. Ind. Microbiol.* **1996**, *17*, 110–115]. In the present study, we present results suggesting that these isolates are of potential interest for removing lupanine and other quinolizidine alkaloids (QA) from the effluent resulting from the wet processing of *Lupinus* seeds, at temperatures within the range 20–34 °C. Growth in *L. albus* aqueous extract was diauxic, with a first period of rapid growth leading to the simultaneous consumption of a significant part of the initial concentration of QA (3 g L^{-1}, being 2 g L^{-1} lupanine) and amino acids (1.5 g L^{-1}). This period was followed by a second period of slower growth corresponding to the subsequent partial utilization (25%) of the carbohydrates (initial concentration of 20 g L^{-1}) together with further removal of QA and amino acids. Despite the differences detected in the susceptibility of the two strains to lupanine toxicity, in particular at supraoptimal temperatures, and in the efficiency of lupanine catabolism, their performance on *L. albus* extract did not vary significantly.

KEYWORDS: Lupanine; quinolizidine alkaloids; *Lupinus albus*; biodegradation of quinolizidine alkaloids

INTRODUCTION

Quinolizidine alkaloids (QA) (**Figure 1**) are toxic compounds (*1–4*) present in plants, mainly within the Leguminosae (*5–7*). In particular, QA are present throughout plants of the genus *Lupinus*, imparting a bitter taste and constituting a defense mechanism against herbivory of high protein tissue (*8–10*). *Lupinus* seeds have been used as a protein source since antiquity, although the seeds of many varieties of agronomic interest show moderate to high alkaloid content. *Lupinus albus* (the white lupin; also lupine) is endemic in the Iberian Peninsula. This can be construed into an argument in favor of the processing of wild bitter lupins (*Lupinus* spp.) rather than massive sowing of relatively unstable sweet cultivars, now desirable as a result of modern breeding efforts (*11*).

Alkaloid removal is traditionally accomplished by leaching these substances out of the seeds of bitter varieties under running water. This debittering process also leads to the removal of part of the soluble protein, and it is feasible only on a small scale because of its high cost. This has prevented the introduction of bitter white lupins (*L. albus*) as an agricultural commodity for feed or food on a world scale. Any alternative industrial wet processing of seeds must assume the recovery of the leached components into useful fractions (*12*) in order to achieve reasonable costs. One of these fractions consists of a concen-

trated alkaloid-containing water-extract named Lupinex which also contains oligosaccharides, amino acids, and proteins (Mittex Anlagenbau GmbH, Weingarten, Germany, personal information). However, marketing of this byproduct depends on its application as a biological growth stimulator in agriculture and horticulture (*13*) or on the extraction of pure alkaloid fractions for pharmacological purposes (*14*). Unless it can be proved that such a rich alkaloid byproduct is of economic value, the effluent of the industrial wet processing of seeds will have a negative environmental impact.

Because lupanine (**Figure 1**) and other QA are biodegradable (*15–21*), the biological removal of QA from wastewater is possible. In previous work, two Gram-negative, rod-shaped bacteria capable of using lupanine, the predominant quinolizidine alkaloid in *L. albus* (**Figure 1**), as a sole carbon source were isolated from soil in which *L. albus* and *L. luteus* had been grown (*15*). The metabolic fingerprints of these isolates were similar but the Microlog2 system failed to identify them; the closest genus was *Acidovorax*, although with very low similarity index values (*15*). These bacterial strains are of interest for the biotechnological debittering of *Lupinus* flours (*16*). In the present work we have examined the potential of these two lupanine-catabolizing strains to remove the QA from a lupin seeds extract complex medium (LPX) with a composition similar to that of the effluent of an industrial plant of the company Mittex Anlagenbau GmbH that performed *L. albus* fractionation until 1994. The removal of QA and of other nutrients present

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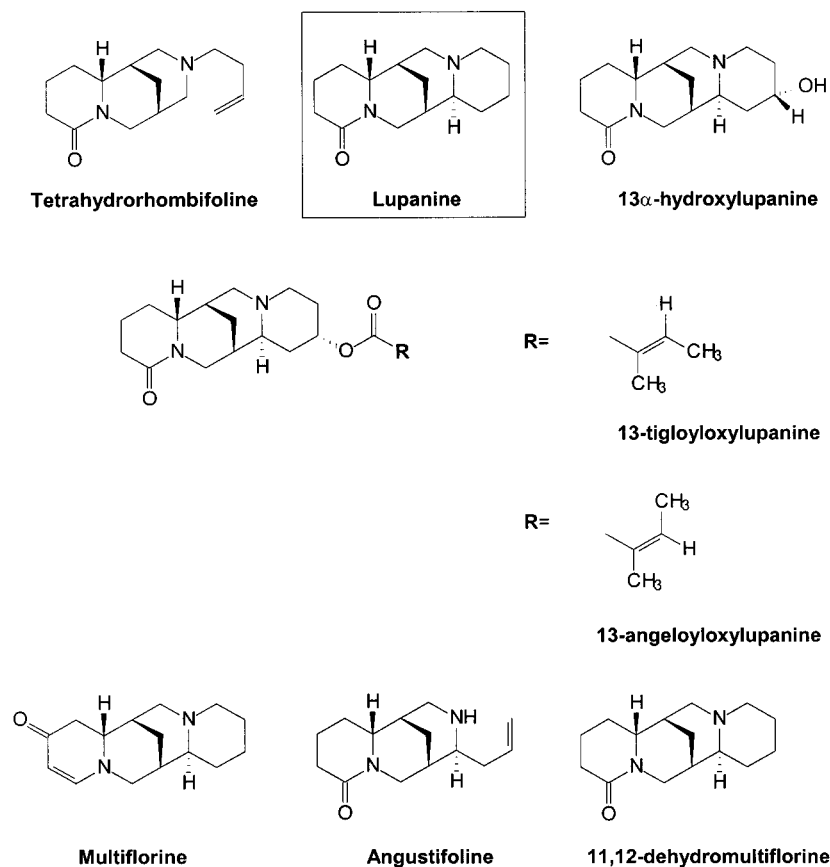
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Figure 1. Chemical structure of lupanine and other quinolizidine alkaloids present in *Lupinus albus* seeds.

in the LPX medium, and the consequent growth of the two bacterial strains, were examined during batch cultivation at different temperatures within the range 20−34 °C.

MATERIALS AND METHODS

Growth Media. Growth medium LUP1 (pH 7.0), containing (g L^{-1}): KH_2PO_4 0.3, K_2HPO_4 0.3, $MgSO_4$ 0.5, NaCl 0.5, $(NH_4)_2SO_4$ 0.25, and lupanine 1.4, was used to prepare the inocula. Basal LUP1 medium, lacking lupanine, was sterilized in an autoclave for 15 min at 121 °C, and lupanine was added as a concentrated solution, filter sterilized (Millipore Filter, Molsheim, France, 0.22-μm). The LUP2 medium was similar to LUP1 medium but had a higher concentration of lupanine (2 g L^{-1}). The lupanine (90% pure, free base form) was a gift from Mittex Anlagenbau GmbH (Weingarten, Germany). The LPX medium was prepared by diluting 1:10 an aqueous *L. albus* seed extract (Lupinex) in a buffered solution of KH_2PO_4/K_2HPO_4 (0.1 M, pH 7.0). This LPX medium had a final lupanine concentration of 2 g L^{-1} and a final total alkaloid concentration of 3 g L^{-1}. Lupinex was also provided, as an aqueous solution with a 36% (w/w) of dry material content and pH 3.5 (with H_3PO_4), by Mittex Anlagenbau GmbH. It had the following composition (analysis from the Fraunhofer Institut for Process Engineering and Packaging IVV, Freising, Germany) as percent dry weight: protein 4.1, quinolizidine alkaloids 8.6 (as lupanine), lipids < 0.1, carbohydrates 54.3, and ash 20.3. This aqueous lupin (*L. albus*) extract called Lupinex was filter-sterilized through filters of decreasing porosity (Gelman Sciences, Ann Arbor, MI, 0.45- and 0.2- μm pore size), diluted with buffered salt solution (pH 7.0), and sterilized in an autoclave.

Lupanine Catabolizing Bacterial Strains. Bacterial strains IST 20B and IST 40D, capable of using lupanine as a sole carbon source (*15*), were used. Cultures were grown in LUP2 medium and maintained frozen at −70 °C in sterile 50% glycerol. When in use, they were maintained at 4 °C on LUP2 and LB (Sigma, St. Louis, MO) plates and they were monthly subcultured onto fresh media.

Bacterial Cultivation in a Synthetic Medium with Lupanine and in a *Lupinus albus* Aqueous Extract. Liquid exponential pre-cultures

of IST 20B or IST 40D were prepared in synthetic media LUP1 or LUP2 until an optical density at 600 nm [OD_{600}] = 1.3 ± 0.1 was reached. They were used to inoculate (initial culture OD_{600} = ±0.1) 25 mL of fresh LUP1 or LUP2 media, respectively, in a 100-mL Erlenmeyer flask. Cultures were incubated in an orbital shaker at 27 °C (230 rev min^{-1}). Growth was followed based on culture OD_{600} and converted into concentration of dry biomass using standard curves prepared for each strain. Specific growth rates were calculated by least-squares fitting to the linear part of the semilog growth plots. Samples were taken at regular intervals for the determination of QA concentration. Biomass yields were calculated as the ratio of the concentration of the dry biomass produced to the concentration of lupanine consumed in the early stationary phase.

Cultivation of strains IST 20B and IST 40D in Lupinex-derived medium was carried out in 250-mL Erlenmeyer flasks containing 100 mL of LPX medium, at 20, 27, or 34 °C (±0.1) with orbital agitation (230 rev min^{-1}). Inocula were prepared in LUP1 medium, and cultivation was carried out overnight with orbital agitation, at the same temperature used in the main growth experiment. Cells of the inoculum were harvested at culture optical density of 600 nm (OD_{600}) = 0.85 ± 0.05 by centrifugation, washed twice with 0.9% NaCl, and resuspended in LPX growth medium. During growth at different temperatures, culture samples were taken at regular intervals for the determination of soluble protein, amino acids, total carbohydrates (CH), total QA, and lupanine concentrations in the supernatant. Growth was also followed by culture OD_{600}. The maximum specific rates of substrate consumption (q_{max}) were the maximum values determined, for short periods of cultivation ($t_{n-1} - t_n$), using eq 1:

$$q_{max} = -\frac{1}{x}\frac{S_{n-1} - S_n}{t_{n-1} - t_n} \tag{1}$$

where x is the average biomass concentration (g L^{-1}), and S_n and S_{n-1} are the substrate concentrations (mmol L^{-1}) for the corresponding incubation times t_n and t_{n-1}, respectively.

Analytical Methods. Alkaloid analysis, during growth of the two selected strains in LUP2 and LPX media, was carried out in 0.5 mL of
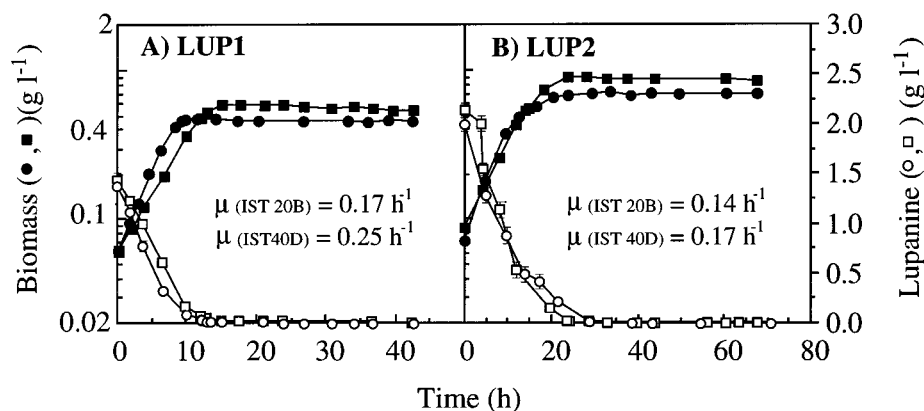
Figure 2. Decrease of lupanine concentration (○, □) during growth (●, ■) of strains IST 20B (■, □) and IST 40D (●, ○) at 27 °C in (A) LUP1 (with 1 g L^{-1} of lupanine) or (B) LUP2 (with 2 g L^{-1} of lupanine) media. Cells used as inoculum were previously adapted to growth in the same medium. Experimental values are the mean of at least two independent growth experiments which led to identical growth curves. Standard deviations were below 8% of the experimental values. The specific growth rates (μ) calculated for the two strains in the different media are shown.

supernatant obtained after centrifugation of 2 mL of culture samples. The supernatant was homogenized in 5 mL of 0.5 M HCl and held at room temperature for 30 min. The homogenate pH was increased to 12 with 25% ammonium hydroxide and applied onto a standard extralut column (Merk, Darmstadt, Germany). The alkaloids were eluted with 90 mL of methylene chloride. Elutates were evaporated to dryness and then taken up in methanol for GLC analysis. Separation of the alkaloids was performed using a fused silica capillary column (25 m × 0.25 nm i. d., 0.25-μm film) coated with SE 54 (J & W Scientific, Folsom, CA) and a Carlo Erba Gas Chromatograph (Vega 6000 model, Milan, Italy) with a nitrogen detector and integrator (Spectra Physics). The GLC conditions were the following: carrier gas, helium 25 KPa; split injection, 1:20; injector and detector temperature, 310 °C. The oven temperature was held at 150 °C for 2 min, then programmed from 150 °C to 250 °C at 15 °C min^{-1}, then to 300 °C at 30 °C min^{-1}, and held 15 min. Caffeine (Sigma-Aldrich, Madrid, Spain) was used as an internal standard for quantification purposes. The QA concentrations were expressed as lupanine equivalents.

Soluble protein was analyzed by the method of Bradford (*22*), using bovine serum albumin fraction V (Merck) as standard. Total carbohydrates were determined, as sucrose (BioRad, Hercules, CA) equivalents, by the method described by Dubois et al. (*23*). Amino acids concentration was determined as arginine (Sigma-Aldrich) equivalents by using the ninhydrin spectrophotometric method (*24*).

RESULTS AND DISCUSSION

Bacterial Removal of Lupanine from the Synthetic Medium. Bacterial strains IST 20B and IST 40D, capable of using lupanine as a sole carbon source, were grown in the same basal medium with 1 g L^{-1} (LUP1) or 2 g L^{-1} (LUP2) of lupanine at 27 °C (**Figure 2**). In LUP1 medium, with the lower initial lupanine concentration, strain IST 40D exhibited a specific growth rate higher than that of strain IST 20B (0.25 h^{-1} compared with 0.17 h^{-1}). However, in LUP2 medium, with double the initial lupanine concentration, the specific growth rates were closer (0.17 h^{-1} and 0.14 h^{-1}, respectively) (**Figure 2**). These results suggest that the two isolates may exhibit different susceptibility to lupanine toxicity (*25, 26*). Strain IST 40D appears to be more susceptible to lupanine than strain IST 20B, as suggested by the higher reduction in the specific growth rate (30% compared with 20%) due to doubling of the lupanine concentration in the growth medium. However, in the stationary growth phase, the percentage of lupanine removed by both strains from the two media was identical and was nearly complete (99%). The biomass yield for strain IST 20B (0.4 g dry biomass/g lupanine removed) was higher than the biomass yield for strain IST 40D (0.3 g/g). This indicates that lupanine

catabolism in strain IST 20B was more efficient. Differences in the efficiency of lupanine catabolism of the two bacterial strains is consistent with the detection of distinct QA as intermediate metabolites during their exponential growth. Indeed, gas chromatography and gas chromatography−mass spectrometry of chloromethane extracts of the IST 20B culture filtrates (from the LUP2 cultures) revealed the presence of 3-hydroxylupanine, 13-hydroxylupanine, and 17-oxosparteine as the three major lupanine intermediate metabolites. The two major metabolites identified during IST 40D growth were 3,4-dehydrolupanine and α-isolupanine. With the exception of α-isolupanine, which had a maximal concentration in the early stationary growth phase, the concentrations of the other intermediate metabolites increased during the first 8 h of exponential growth and then decreased to undetectable levels (Santana, F. M. C., Sá-Correia, I., and Empis, J. M. A., unpublished results). However, both intermediates and final products of degradation may have been overlooked because the GLC approach used can only detect alkaloids which are extracted into chloroform.

The two bacterial strains examined were also able to grow in LUP1 medium lacking ammonium. They exhibited similar growth kinetics and energetics (results not shown); therefore, they are capable of using lupanine as their only carbon and nitrogen source.

Bacterial Removal of Alkaloids and Other Nutrients from Lupin (*L. albus*) Aqueous Extract. The two lupanine-degrading bacterial strains, previously adapted to growth with lupanine, were cultivated in lupin (*L. albus*) aqueous medium LPX at 20, 27, and 34 °C (**Figure 3**). The initial concentration of total quinolizidine alkaloids (3.0 ± 0.2 g L^{-1}), in particular of lupanine (2.0 ± 0.2 g L^{-1}), decreased following LPX medium inoculation with the bacteria. During the first hours of cultivation, the initial concentration of free amino acids also rapidly decreased, while the concentration of carbohydrates maintained their initial levels during the active catabolism of amino acids and QA. Bacterial growth corresponding to the simultaneous consumption of QA and amino acids showed a first phase of rapid exponential growth (period I) that was followed by a second phase of slower growth (period II) (**Figure 3**, **Table 1**). This apparently corresponds to the subsequent partial utilization of the carbohydrates in the medium. Differences in the specific growth rates calculated for periods I and II (**Table 1**) of the diauxic growth depend on growth temperature, an environmental parameter that significantly affects growth kinetics, and on

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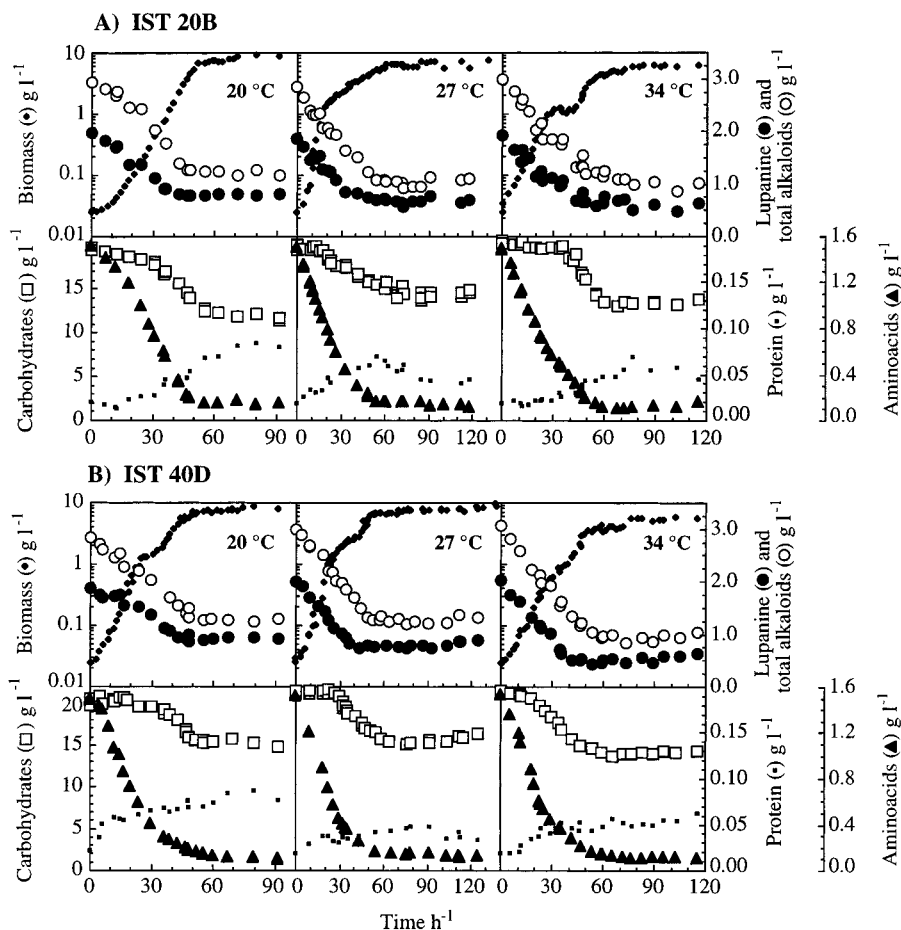
Figure 3. Concentrations of lupanine (●), total quinolizidine alkaloids (○), amino acids (▲), total carbohydrates (□), soluble protein (small ■), and biomass (small ◆) during growth of bacterial isolates (A) IST 20B or (B) IST 40D in a buffered aqueous *L. albus* extract medium (LPX medium) at 20, 27, or 34 °C. Cells used as inoculum were previously adapted to grow in a medium with lupanine (1 g L^{-1}) as the sole carbon source and the initial biomass concentrations were identical (0.025 ± 0.004 g dry biomass L^{-1}). Values are the mean of at least two independent growth experiments which led to identical data. Standard deviations were below 9% of the experimental values.

Table 1. Specific Growth Rates during the First (μI) and Second (μII) Phases of Diauxic Growth and Maximal Specific Rates of Substrate Consumption (q_{max}) during the First (I) and Second (II) Growth Phases of Bacterial Strains IST 20B and IST 40D in *L. Albus* Aqueous Extract Medium, Calculated Using the Data Shown in **Figure 2**

|				q_{max} (mmol g$_{biomass}$$^{-1}$ h^{-1})			
strain	T (°C)	μ_I (h^{-1})	μ_{II} (h^{-1})	lupanine (I)	quinolizidine alkaloids (QA) (I)	amino acids (I)	carbohydrates (CH) (II)
IST 20B	20	0.13	0.13	4.5	4.2	5.5	1.3
	27	0.37	0.04	4.7	5.5	6.4	1.3
	34	0.21	0.12	6.4	6.2	7.4	1.6
IST 40D	20	0.18	0.08	3.6	4.4	5.6	0.6
	27	0.26	0.06	4.2	5.0	6.0	0.6
	34	0.13	0.10	6.5	6.3	7.1	2.1

bacterial catabolic performance toward the various substrates available in the complex medium.

According to the metabolic fingerprints of the strains examined (*15*), they are both capable of growing by using as a carbon source the following amino acids, which are present in lupin (*Lupinus*) seeds: glutamic acid, alanine, leucine, phenylalanine, proline, serine, and treonine (*15, 27, 28*). The simultaneous catabolism of amino acids and lupanine in LPX medium, with a specific rate of amino acid consumption above QA consumption (**Table 1**), led to specific growth rates during the first period of growth in this complex medium at 27 °C (0.37 h^{-1} for IST 20B and 0.26 h^{-1} for IST 40D; **Table 1**) which were higher than those possible in the synthetic medium with 2 g L^{-1} of lupanine as the only carbon source, at the same

temperature (0.14 h^{-1} for IST 20B and 0.17 h^{-1} for IST 40D; **Figure 2**). Differences in bacterial growth kinetics in the complex and synthetic medium with lupanine were clearer for IST 20B. This strain, although it exhibited a specific growth rate that was slightly below the specific growth rate of IST 40D in the synthetic medium with lupanine at 27 °C, in the LPX medium, at the same temperature, it showed a higher specific growth rate (**Table 1**, **Figures 2** and **3**).

The specific growth rates calculated during the initial period of rapid exponential growth in LPX medium at 20, 27, and 34 °C, during which the two strains used QA and amino acids simultaneously (**Figure 3**), are compared in **Table 1** and **Figure 4**. The optimal temperature for both strains was 27 °C. This is consistent with the fact that the lupanine catabolizing strains

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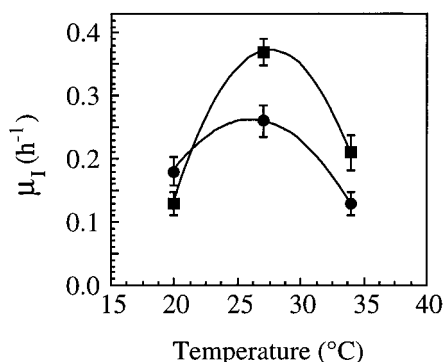
Figure 4. Temperature profiles of growth kinetics of strains IST 20B (■) and IST 40D (●) in complex LPX medium. Specific growth rates (μ_I) were calculated based on the first phase of exponential growth of the diauxic growth curves shown in **Figure 3**.

tested were isolated from soil. The comparison of the temperature profiles for growth of the two isolates during the first growth period (**Figure 4**) indicates that, although the specific growth rate of strain IST 40D was higher than that of IST 20B, at 20 °C, this relationship was reversed at higher temperatures. The observed differences in the two temperature profiles for growth are consistent with the indications obtained which suggest that IST 40D may be more susceptible to growth inhibition by lupanine than is IST 20B. Indeed, it is known that growth inhibitory compounds reduce specific growth rates and depress the optimal and the maximal temperatures for microbial growth by enhancing thermal death (*29, 30*). Because LPX included 2 g L^{-1} of lupanine and a concentration of 3 g L^{-1} of total QA, these compounds could be expected to affect bacterial growth (*25, 26*). As strain IST 40D is the most lupanine susceptible isolate, a more drastic joint effect of QA and high temperature is expected in this strain. Consistently, the specific growth rates of IST 40D at 27 and 34 °C were below those in IST 20B. However, this strain exhibited more favorable growth kinetics at the suboptimal temperature of 20 °C (**Figure 4**).

Almost one-half of the Lupinex extract dry weight was carbohydrates which were essentially soluble oligosaccharides [W. Jaeggle, Mittex Anlagenbau GmbH, Eisenbahnstrass 11 D-88250 Weingarten, personal communication]. Lupin (*L. albus*) seed cotyledons, from which Lupinex was derived, contain α-galactosides of sucrose on which the glucose moiety is substituted, on C$_6$, by a variable number (*n*) of α-(1→6)-linked galactose molecules. The predominant sucrose α-galactosides in *L. albus* are raffinose (*n* = 0), stachyose (*n* = 1), and verbascose (*n* = 2) (*31*). Only part of the carbohydrates (CH) present in the LPX medium (around 25%) were catabolized by both isolates. Their consumption during cell growth occurred only after removal of the majority of the metabolized alkaloids and amino acids. During the second growth period, the catabolism of CH was slow, as observed with QA and amino acids consumption, and the specific growth rates of the two strains (μ_{II}) at the different temperatures were, generally, much lower.

The concentration of soluble protein in the LPX medium was maintained or increased during bacterial growth (**Figure 3**). This was possibly the result of protein excretion by growing cells.

The soil bacterial isolates studied proved to be of potential interest in reducing QA and other organic compounds from the effluent derived from the industrial wet processing of lupin (*Lupinus*) seeds. The bacterial performance with regard to the percentage of the initial organic compounds that can be removed did not vary greatly within the range of temperatures tested (20–

34 °C). Nevertheless, at 27 °C, the duration of the biological removal process was shorter. There was a lower biomass yield at 34 °C; this was consistent with an increase of energy for cell maintenance at this supraoptimal temperature. Although an almost complete removal of 2 g L^{-1} of lupanine in the synthetic medium LUP2 was possible (**Figure 2B**), the maximum removal of the initial lupanine present in LPX medium was only 77%. This contrasted with the complete metabolization of the amino acids in the lupin (*L. albus*) extract, while the removal of the carbohydrates was only 25%. In the LPX medium, the presence of a mixture of carbon sources led to a high biomass concentration in the stationary phase of diauxic growth (close to 8 g dry biomass L^{-1}). This might have affected the percentage of QA and carbohydrates that could be removed under the experimental conditions examined. It is possible that the bacterial cultures, when they entered the stationary growth phase, had left a significant percentage of the carbon sources unused. This may be due to nitrogen source limitation, as suggested by the exhaustion of the amino acids, even though the bacteria are capable of using lupanine as a sole carbon and nitrogen source in a simple synthetic medium. Premature entrance into the stationary growth phase can also result from the accumulation of an unidentified toxic intermediate metabolite of lupanine catabolism, at high cell density. Further work is needed to optimize the biotechnological use of strains IST 20B and IST 40D for QA removal from a typical effluent resulting from the wet processing of bitter lupin (*Lupinus*) seeds. The higher dilution of Lupinex and/or growth medium supplementation with nutrients, the optimization and control of culture pH and the concentration of dissolved oxygen during growth are probable successful modifications.

ABBREVIATIONS USED

QA, Quinolizidine alkaloids; LPX, *Lupinus albus* aqueous extract Lupinex medium; LUP1, basal medium with lupanine 1 g L^{-1}; LUP2, basal medium with lupanine 2 g L^{-1}; OD$_{600}$, optical density at 600 nm; CH, carbohydrates; GLC, gas liquid chromatography.

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