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Isolation of bacterial strains capable of using lupanine, the predominant quinolizidine alkaloid in white lupin, as sole carbon and energy source

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Seven Gram-negative bacterial strains, capable of using lupanine, the predominant quinolizidine alkaloid in white lupin, as sole carbon and energy source, were isolated from soil in which *Lupinus albus* and *L. luteus* had been grown. A metabolic profile system (BIOLOG) identified only three of the seven isolates, two as *Xanthomonas oryzae* pv *oryzae* E and one as *Gluconobacter cerinus*. The maximum specific growth rates of the seven isolates when incubated at 27°C in a medium containing as sole carbon source 2 g L^{-1} of lupanine, ranged from 0.05 to 0.13 h^{-1} and the concentration of dry biomass at the stationary phase ranged from 0.7 to 1.1 g L^{-1}. Unidentified strains IST20B and IST40D exhibited the highest maximum specific growth rates (0.13 h^{-1}), removed 99% of the initial lupanine after 30 h of incubation, and the dry biomass yields did not exceed 0.4 g per g lupanine consumed. Strain IST20B is of potential use for *L. albus* debittering because, after 32 h growth in aqueous extracts of *L. albus*, 85% of initial alkaloids were removed while the concentration of soluble protein was only reduced by 8%.

Keywords: lupanine; quinolizidine alkaloids; BIOLOG; bacterial isolation; biodegradation; lupin debittering

Introduction

Lupanine is the predominant quinolizidine alkaloid (QA) of many lupin species including *Lupinus albus*. Seeds from plants of the genus *Lupinus* have high protein concentrations matching the levels found in seeds of the soybean *Glycine max* at 30–40% of ash-free dry weight levels [10,21]. The *L. albus* (white flowered lupin) seeds are traditional foodstuffs for populations that inhabit Mediterranean areas. Most varieties with good agronomic characteristics invariably contain moderate to high alkaloid content in their seeds. *Lupinus* alkaloids are present throughout the plant and impart a bitter taste in what is thought to be a genetically dominant trait, constituting a physiological defense against predation of high protein tissue [19,20]. The Iberian Peninsula is a region that enjoys the endemic presence of, among others, *L. albus*. This may be construed into an argument in favour of the processing of native bitter lupin rather than massive planting of unstable sweet cultivars.

Traditional debittering is performed in Portugal by boiling and leaching of lupin seeds with water/brine and this results in the separation of the undesirable alkaloids, but also removes part of the soluble protein in addition to oligosaccharides and mineral salts. Alternative physicochemical processing, more adequate for large-scale operation, must include the recovery and fractionation of the leached components into useful fractions, and must utilize the alkaloids as fine chemicals. The present work embodies a novel approach to lupin debittering, which is based upon the removal of the QAs by selected bacterial strains. Previous authors have reported that strains of *Pseudomonas* sp isolated from soil were capable of using QAs [5,6,11,13–15] as their only source of carbon and nitrogen. Wink [19] suggested that specific bacterial strains might be used to reduce *Lupinus* alkaloid levels in silage.

In this work the microbial debittering alternative has been assessed in a preliminary way with the aim of either producing an improved food or feed component or for removal of alkaloids from the effluent of a debittering plant. For this purpose, bacterial strains capable of using lupanine as sole carbon and energy source were isolated from soil in which *L. albus* and *L. luteus* had been cultivated. The metabolic profiles of the isolates were compared and their identification attempted by using the commercial identification system BIOLOG. Growth of a selected isolate in a lupin extract medium was carried out in order to assess its potential use to reduce alkaloid levels.

Materials and methods

Growth media

Culture medium LUP1 used for bacterial strains isolation was prepared with (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 1.0. Phosphate-buffered salts base growth medium was sterilized in the autoclave for 15 min at 121°C, and lupanine was added (as a 10 g L^{-1} solution) after filter sterilization (Millipore filter, Molsheim, France, 0.22-μm pore size). As an agar medium, LUP1 medium was solidified by the addition of agar (2% w/v of agar, Iberagar, Portugal). LUP2 medium is similar to LUP1 medium but with the lupanine concentration raised to 2 g L^{-1}. CXM medium, contained

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128 g L^{-1} of the freeze-dried lupin aqueous extract (equivalent to a final alkaloid concentration of 1 g L^{-1}). CXM had, as its base, a buffered salt solution with (g L^{-1}): KH_2PO_4 0.3, K_2HPO_4 0.3, $MgSO_4$ 0.5 and NaCl 0.5. The lupin extract was added (as a 10-fold concentrate) to buffered salts solution (sterilized in autoclave) after filter sterilization through filters of decreasing porosity (Gelman Sciences, MI, USA, 0.45- and 0.2-μm pore size).

Isolation of lupanine catabolizing bacterial strains

Samples of soil (1 g), taken from the top 10 cm of two parcels of a farm (Herdade de Almada located in Benavente, Ribatejo, Portugal) that had recently served for the cultivation of *L. albus* or *L. luteus*, were added to 10 ml of LUP1 liquid medium in a 100-ml Erlenmeyer flask and incubated on an orbital shaker (230 rpm) at 30°C for 24 h. After repeated liquid subculturing, 100 μl of diluted cultures (10^{-1} to 10^{-6} in NaCl 0.9%) were spread onto LUP1 agar plates. Morphologically distinct colonies were repeatedly transferred to LUP1 plates and incubated until pure cultures were obtained. Bacterial strains were maintained at 4°C on LUP1 and LB (Sigma, St Louis, MO, USA) plates and subcultured onto fresh media every month. Cultures of the isolates in LUP2 medium were also maintained frozen at –70°C in sterile 50% glycerol.

Attempted identification of isolates

The identification of seven Gram-negative bacterial isolates was attempted using commercially available BIOLOG Gram-negative (GN) microplates (Biolog, Inc, Hayward, CA, USA) based on the aerobic utilization of 95 different substrates. This method relies on the use of a redox dye (tetrazolium violet) to detect respiration due to utilization of various carbon sources. The 96-well GN microplate is comprised of 95 substrate-containing wells and a control well without a carbon source. Substrate, dye and nutrients are supplied in each well in a dried-film form which are reconstituted upon addition of sample [3]. As specified by the manufacturer, bacterial isolates were grown from single colonies on TSA (Difco, Detroit, MI, USA) plates for 48 h at 30°C and cells were suspended in 20 ml of sterile 0.85% NaCl. GN-microplate wells were inoculated with 150 μl of a cell suspension that had been adjusted to final optical density at 600 nm (OD_{600}) between 0.4 and 0.5 by comparison with the turbidity standards supplied by Biolog Inc. Microplates were incubated at 30°C and absorbance was read, after 4 and 24 h of incubation, at 595 nm in a BIO-RAD (model 3550, Richmond, CA, USA) microplate reader. As suggested by the Biolog manufacturer [2], absorbance at least 40% greater than the control well was used to distinguish positive from negative results; some values were considered as borderline. Microlog™2 software (release 3.50, 1993, version DE) was used to identify nearest species and to calculate a 'similarity index', which gives an indication of the acceptability of identification.

Growth in lupanine synthetic medium and in a lupin aqueous extract medium

Dense LUP2 liquid cultures of bacterial isolates (OD_{600} = 1.3–1.4) were used to inoculate 25 ml of fresh LUP2 medium to 100-ml Erlenmeyer flasks (the initial OD_{600} was around 0.1), which were incubated on an orbital shaker at 27°C (230 rpm). Growth was followed by culture OD_{600} and converted into concentration of dry biomass per liter by means of standard curves prepared for each strain. Specific growth rates were calculated by least-square fitting to the linear parts of the semi-log growth plots. Samples were withdrawn at regular intervals for the determination of QA concentration, and biomass yields were calculated as the ratios of the early stationary phase concentrations of dry biomass per amount of lupanine consumed.

Strain IST20B was also grown at 27°C with orbital shaking (230 rpm) in 25 ml of CXM medium in 100-ml Erlenmeyer flasks, inoculated with an exponential-phase liquid inoculum prepared in the same medium. Samples were withdrawn at regular intervals for the determination of QAs (expressed as lupanine equivalents) and soluble protein concentrations. Soluble protein analysis [4] was carried out on supernatants obtained after centrifugation of 1 ml culture. Growth was also followed by culture OD_{600}.

Preparation of lupanine and L. albus extract

Lupanine as well as other QA standards were obtained from methanol extracts of *L. albus* and *L. mutabilis* as follows: methanol extracts were evaporated to dryness under reduced pressure and purified by dissolution in 0.5 N HCl followed by multiple extraction (at least three times) with methylene chloride to remove lipid-like material. The aqueous phase was adjusted to pH 12 with 6 N NaOH and extracted several times with methylene chloride. Organic phases were pooled and evaporated to dryness in a rotary evaporator. The crude alkaloid extract was fractionated by elution from a column of silica gel 60 with cyclohexane/dichloromethane (70 : 30) to which a gradient concentration of diethylamine increasing from 1% to 20% was added. For each of the eluate fractions, qualitative TLC analysis of QA was carried out on silica gel F_{254} aluminofolium plates (Riedel-de-Häen, Seelze, Germany) with cyclohexane-diethylamine (70 : 30) as solvent. QAs were visualized with Dragendorff's reagent [12]. Fractions with the same alkaloid were pooled and the eluent was evaporated to dryness under reduced pressure at 40°C in a rotary evaporator. The individual alkaloid extracts were redissolved in a small volume of methylene chloride and transferred quantitatively to small sample vials. Solvent was evaporated by sparging the sample with a stream of nitrogen gas, and the samples were analysed by GLC and subsequently stored at 4°C in darkness.

The aqueous extract of *L. albus* was obtained by batch leaching of portions of 50 g of *L. albus* flour in 200 ml of water at pH 4.5 for 4 h (45°C) with stirring. The resulting extract was freeze-dried. This material, containing 7.9% total alkaloids by weight (expressed as lupanine equivalents) and 4.3% total nitrogen, was used to prepare CXM medium (as reported above).

Alkaloid analysis

Alkaloid analysis, during growth of two selected strains in LUP2 medium, was carried out in 0.5 ml of supernatant obtained after centrifugation of 2 ml of culture samples taken at regular intervals. The supernatant was homogen-

ized in 5 ml of 0.5 N HCl and held at room temperature for 30 min. The homogenate was made alkaline (pH 12) with 25% ammonium hydroxide and applied to a standard extrelut column (Merck, Darmstadt, Germany). The alkaloids were eluted with 90 ml methylene chloride. Eluates were evaporated to dryness and then taken up in methanol for analysis by GLC. Separation of the alkaloids was performed using a fused silica capillary column (25 m × 0.25 mm i.d., 0.25 μm film) coated with SE 54 (J & W Scientific, Folsom, CA, USA) and a Carlo Erba Gas Chromatograph (Vega 6000 model, Milan, Italy) with a nitrogen detector and integrator (Spectra Physics). GLC conditions were: carrier gas, helium 25 kPa; split injection 1 : 20; injector temperature, 250°C; detector temperature, 300°C. The oven temperature was programmed from 150°C, 2 min, to 250°C at 15°C min^{-1}, then to 300°C at 30°C min^{-1} and held 15 min. Multiflorine was used as an internal standard for quantification purposes. QA concentrations were expressed as lupanine equivalents.

During growth of strain IST20B in CXM medium, QA was determined (as lupanine equivalents) by the rapid spectrophotometric method of von Baer *et al* [18].

Results and discussion

Isolation of lupanine-catabolizing bacterial strains

Fifteen morphologically different colonies were observed among colonies on LUP1 plates. Most of the colonies were obtained from soil samples which had been cultivated with *L. albus* which possibly can be related with the predominance of lupanine in *L. albus* plants whereas the predominant alkaloid in *L. luteus* is lupinine [9].

The fifteen apparently different isolates were inoculated, as pure cultures, in LUP2 medium and incubated with orbital agitation at 27°C. At least seven of the fifteen bacterial isolates were capable of growth in pure culture using lupanine as sole carbon and energy source (Table 1). Maximum specific growth rates of the seven isolates at 27°C in lupanine medium ranged from 0.05 to 0.13 h^{-1}, and the concentrations of dry biomass at the stationary phase ranged from 0.7 to 1.1 g L^{-1}. Strains IST20B and IST40D, both isolated from *L. luteus* soil, exhibited the highest maximum specific growth rates under these growth conditions.

Table 1 Maximal specific growth rates (μ) and final concentration of dry biomass of bacterial strains isolated from soil in which *Lupinus albus* and *L. luteus* had been cultivated

Plant	Isolate	μ (h^{-1})	Dry biomass (g L^{-1})
L. albus	IST10A	0.05	1.1
	IST30C	0.07	1.1
	IST40E	0.08	0.8
	IST50E	0.10	0.8
	IST90A	0.08	0.8
L. luteus	IST20B	0.13	0.9
	IST40D	0.13	0.7

Growth was carried out in LUP2 medium at 27°C with orbital agitation. Dry biomass was determined at the early stationary phase of growth.

Metabolic fingerprints of bacterial isolates

All seven isolates were Gram-negative, rod-shaped bacteria. Oxidation of individual carbon sources was detected indirectly by observing the reduction of tetrazolium dye in GN-Biolog microplates using the MicroLog™2 system [7]. Table 2 lists representative results for each of the seven isolates. The following compounds gave negative results with all the isolates under study: glycogen, N-acetyl-D-galactosamine, adonitol, *i*-erythritol, gentiobiose, α-lactose, lactulose, melibiose, β-methyl-D-glucoside, xylitol, cis-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-glucosaminic acid, D-glucuronic acid, *p*-hydroxy phenylacetic acid, itaconic acid, malonic acid, D-saccharic acid, succinamic acid, L-aspartic acid, glycyl-L-aspartic acid, D,L-carnitine, γ-amino butyric acid, inosine, uridine, thymidine, phenylethylamine, D,L-α-glycerol phosphate, glucose 1-phosphate and glucose 6-phosphate.

The metabolic profiles of isolates IST20B and IST40D were very similar to each other but quite different from those obtained with the other strains. Both strains catabolized most of the amino acids, amines and alcohols and a large number of carboxylic acids. As suggested by the phenotypic dendrogram (Figure 1), MicroLog system analysis of results obtained for the seven isolates yielded three different clusters, designated I–III. Strains IST20B and IST40D could not be identified but the closest genus found for these strains using MicroLog™2 was *Acidovorax* although with very low similarity index values (0.162 and 0.165 respectively). Strain IST40E was related to the genus *Xanthomonas* with a similarity of 0.180 and isolates IST10A and IST50E were assigned to *Xanthomonas oryzae* pv *oryzae* E with similarities of 0.580 and 0.850 respectively. Subgroup E is an unofficial designation of BIOLOG used to indicate that some of their strains of *Xanthomonas oryzae* pv *oryzae* do not match closely the characteristics of the type strain, which is in subgroup A. Strain IST30C was related to *Gluconobacter cerinus* with a similarity index of 0.543. The isolate IST90A was assigned to the genus *Pseudomonas* based on results after 4 h of incubation (similarity of 0.731) but, when the incubation time was extended to 24 h, it was weakly related to *Deleya marina* (similarity of 0.253). The calculated similarity value in the MicroLog software is used as a calling criterion to judge the reliability and confidence of the identification. Bacterial identification is acceptable for similarity indices greater than 0.75 for 4 h of incubation and greater than 0.5 for 24 h of incubation, being considered excellent for values between 0.7 and 0.9 [2]. As reported for the BIOLOG and for other identification systems [1], this system failed to identify four (IST20B, IST40D, IST90A and IST40E) of the seven environmental isolates. Although IST10A, IST50E and IST30C were identified according to this system, it would be appropriate to confirm this with other evidence.

Growth of the selected strains on lupanine

Strains IST20B and IST40D, which exhibited the highest specific growth rates under the experimental growth conditions used, were grown in LUP2 medium and the decreases in the concentrations of lupanine and total alkaloids were compared (Figure 2). In the fresh LUP2 medium

Table 2 Oxidation of carbon sources

Isolates									
IST40E	IST10A	IST50E	IST90A 4 h	IST90A 24 h	IST20B	IST30C	IST40D		Carbon source
–	–	–	–	–	–	–	–	A1	water
–	–	–	–	–	–	/	–	A2	α-cyclodextrin
–	–	–	–	+	–	–	–	A3	Dextrin
+	+	+	–	+	+	+	+	A5	tween 40
–	–	–	–	–	+	–	+	A6	tween 80
–	–	–	–	–	+	–	+	A8	N-acetyl-D-glucosamine
+	–	–	–	–	–	+	–	A10	L-arabinose
–	–	–	–	–	+	–	+	A11	D-arabitol
+	–	–	–	–	–	–	–	A12	cellobiose
+	+	+	+	+	+	+	+	B2	D-fructose
–	–	–	–	–	+	–	–	B3	D-fucose
–	–	–	–	+	–	+	–	B4	D-galactose
–	+	–	+	+	+	+	+	B6	α-D-glucose
–	–	–	–	–	+	–	+	B7	*m*-inositol
–	–	–	+	+	–	/	–	B10	maltose
–	+	–	+	+	+	–	+	B11	D-mannitol
–	–	–	+	+	–	–	–	B12	D-mannose
+	–	+	+	+	+	+	+	C3	psicose
–	–	–	–	–	–	–	–	C4	D-raffinose
+	–	–	–	–	–	+	–	C5	L-rhamnose
+	+	+	–	+	+	+	+	C6	D-sorbitol
–	+	–	+	+	+	+	–	C7	sucrose
+	–	–	–	–	+	–	–	C8	D-trehalose
+	–	–	/	+	–	+	–	C9	turanose
–	–	+	+	+	+	–	+	C11	methylpyruvate
–	+	+	–	+	+	–	+	C12	monomethylsuccinate
–	–	–	–	–	+	–	+	D1	acetic acid
–	–	–	–	–	–	/	–	D6	D-galacturonic acid
–	+	–	/	+	+	/	+	D7	D-gluconic acid
–	–	–	–	–	+	/	+	D10	α-hydroxybutyric acid
+	+	–	–	+	+	+	+	D11	β-hydroxybutyric acid
–	–	–	–	–	+	–	–	D12	γ-hydroxybutyric acid
+	+	–	–	+	+	+	+	E3	α-ketobutyric acid
–	–	–	–	+	–	–	–	E4	α-ketoglutanic acid
–	–	–	–	–	+	–	+	E5	α-ketovaleric acid
–	–	–	–	–	+	–	+	E6	D,L-lactic acid
+	–	–	–	–	+	–	+	E8	propionic acid
+	–	–	–	–	+	–	+	E9	quinic acid
–	–	–	–	–	+	–	+	E11	sebacic acid
–	–	–	–	–	+	–	+	E12	succinic acid
–	–	–	–	–	+	–	+	F1	bromosuccinic acid
–	–	–	–	–	–	/	–	F3	glucuronamide
–	–	–	–	–	+	–	+	F4	alaninamide
–	–	–	+	+	+	–	+	F5	D-alanine
–	+	–	+	+	+	–	+	F6	L-alanine
–	–	–	–	+	+	–	+	F7	L-alanyl-glycine
–	–	–	+	+	+	–	+	F8	L-asparagine
+	–	–	–	+	–	–	–	F10	L-glutamic acid
–	–	–	–	–	–	/	–	G1	L-histidine
–	–	–	–	+	+	–	+	G3	L-leucine
–	–	–	–	–	+	–	+	G5	L-phenylalanine
–	–	–	–	+	+	/	+	G6	L-proline
–	–	–	–	+	–	–	–	G7	L-pyroglutamic acid
–	–	–	–	–	+	–	+	G8	D-serine
–	–	–	+	+	+	–	+	G9	L-serine
–	–	–	/	+	+	/	+	G10	L-threonine
–	–	–	–	–	+	–	+	H1	urocanic acid
–	–	–	–	–	+	–	+	H6	putrescine
–	–	–	–	–	+	–	+	H7	2-amino ethanol
+	–	–	–	–	+	/	+	H8	2,3-butanediol
+	–	–	–	–	+	+	+	H9	glycerol
							+		

For strain IST 90A results obtained after 4 h and 24 h are given; for the other strains the assay time was 24 h.
+, Positive reaction; /, borderline; –, negative.

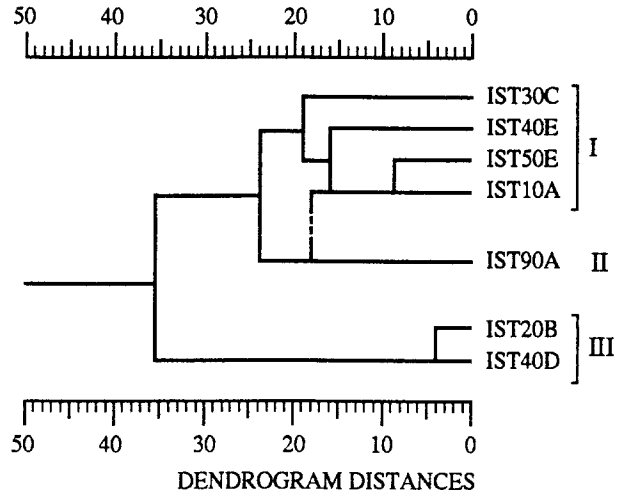

Figure 1 Dendrogram prepared from phenotypic data analysed by MicroLog™2 system representing the relationships between the seven isolates. The scale above and below is in units of taxonomic distance, which is a measure in MicroLog software that gives a uniform and consistent way to compare the similarity of any pattern versus any reference. The dashed-line cross link shown in the dendrogram, between the neighbouring groups I and II, means that the true distance is less than the distance between the dendrogram branches.

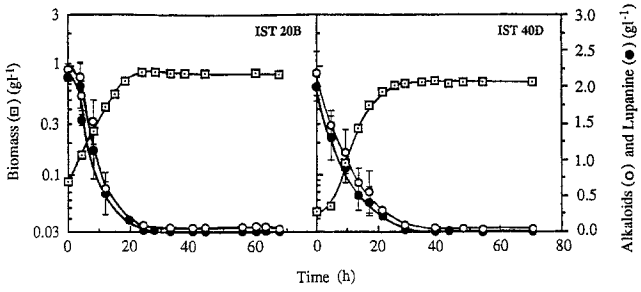

Figure 2 Decrease of the concentrations of lupanine (●) and total alkaloids (○) during growth (□) of strains IST 20B and IST 40D at 27°C in LUP2 medium.

some difference was detected between the concentration of total alkaloids and that of lupanine. This difference was attributed to the presence of other alkaloids besides lupanine, which remained as impurities after the lupanine purification process.

Strains IST20b and IST40D exhibited identical specific growth rates (0.13 h^{-1}) and the percentage of lupanine removed at the stationary phase (99%) was also identical (Figure 2). However, the biomass yield of strain IST40D (0.33 g dry biomass g^{-1} lupanine removed) was slightly less than the biomass yield of strain IST20B (0.40 g g^{-1}), suggesting that the two strains catabolize lupanine with different efficiencies. GC and GC/MS analysis of the alkaloid composition of LUP2 medium during growth were consistent with this presumption (Santana FMC, Sá-Correia I and Empis JMA, unpublished results). Distinct levels of intermediate compounds and distinct QA were detected for each strain during the first 8 h of exponential growth. Further investigation is needed in order to understand the mechanisms involved in lupanine catabolism by the strains.

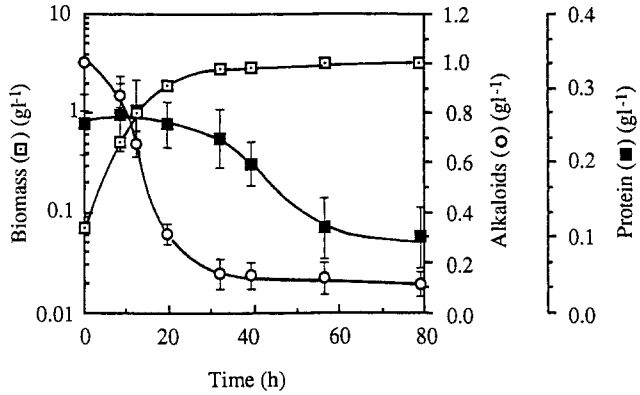

Figure 3 Decrease of the concentrations of alkaloids (○) (as lupanine equivalent) and protein (■) during batch growth (□) at 27°C of strain IST20B in CXM medium prepared with an aqueous lupine extract.

Removal of alkaloids from a lupin aqueous extract by strain IST20B

The performance of strain IST20B in the removal of QAs from an aqueous lupin medium was assessed. The composition of CXM medium in alkaloids and protein was designed to simulate that of waste streams from a lupin-debittering plant [8,16,17]. The concentration of alkaloids during growth was analysed by the rapid spectrophotometric method of von Baer *et al* [18] instead of by gas-liquid chromatography. When the stationary phase of batch growth was reached, after 32 h, at 27°C with orbital shaking, the initial concentration of soluble protein was reduced by only 8% while 85% of alkaloids were removed (Figure 3). From the kinetics it appears that protein was only used significantly when metabolizable alkaloids were limiting growth. By that time, the concentration of dry biomass reached 3.0 g L^{-1}, which suggested that strain IST20B had used other carbon sources such as carbohydrates present in the lupin extract. A high percentage of alkaloids were removed from the lupin extract by IST20B while the initial protein concentration was maintained. This result suggests that strain IST20B might be of practical use for debittering lupin seeds wet milled for feed purposes.

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