FERMENTABLE SUGARS FROM *Lupinus rotundiflorus* BIOMASS BY CONCENTRATED HYDROCHLORIC ACID HYDROLYSIS

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It is of general interest to produce fermentable carbohydrates from plant biomass. However, obtaining monosaccharides requires some effort, due to the intricate structure of the cell wall lignocellulosic complex. The aim of this study was to apply a simple two-stage hydrolysis process, using only concentrated hydrochloric acid, to generate fermentable carbohydrates from *L. rotundiflorus* biomass. First and second stage acid concentrations were 32% and 42.6%. Total monosaccharide yields with respect to dry matter after the first stage, second stage, and the overall process, were 27.5%, 21.0% and 48.4%, respectively. Xylose was the main first stage carbohydrate in the hydrolysate, followed by glucose, arabinose, and galactose. After the second stage only glucose and a small amount of xylose were detected. The polysaccharide hydrolysis was eased by overall low lignin content. Some advantages of this method were the use of a single hydrolyzing agent and that most of the polysaccharides were hydrolyzed in reasonably high yields. The acceptable yield, relative simplicity, the use of most of the biomass along with the wide availability, low cost of the chemicals, and the ample supply of lupines, would facilitate the scaling of these laboratory studies to pilot and industrial levels.

Keywords: Lupinus; Carbohydrate; Hydrolysis; Morphology; Holocellulose; Fermentable

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INTRODUCTION

Lignocellulosic biomass is an abundant, inexpensive and renewable resource, which can be utilized for the production of alcohols at a reasonable cost. Conversion of this biomass into alcohols is a difficult process due in part to the complexity of the plant´s fiber cell wall. The cell wall is composed of cellulose (38-50%), hemicelluloses (17-32%), lignin (15-30%) as well as proteins, pectins, and extractives (Kamm et al. 2006; Moxley and Zhang 2007; Silverstein et al. 2007; Ritter 2008). Hydrolysis of lignocellulosic materials, especially the enzymatic hydrolysis, is affected by substrate characteristics such as mechanical properties, morphology, crystallinity, and chemical structure (Lee et al. 1982). Morphological analysis of the plant material may be of

practical use in helping to predict the accessibility to hydrolysis of the polysaccharides comprising the plant tissues, particularly that of fibers and parenchyma from which fermentable carbohydrates could be released. Thus, anatomical structural analysis of the material is of help in qualitatively predicting how easily the acid will penetrate these structures, by observing if the fibers are closely bound to each other, by the distribution of the fibers, by the ratio and distribution of parenchyma in relation to fibers, etc. It is assumed that parenchyma will be easier to hydrolyze, since it is made up of amorphous non-fibrous cellulose and hemicelluloses with little or no lignin, in contrast to fibers wherein cellulose is present within a lignocellulosic complex, in which lignin is very refractive to hydrolysis. Also, the cellulosic fibers have a clearly higher degree of crystallinity (Fengel and Wegener 1989), making them somewhat impervious to acid penetration. Both parenchyma and fibers have similar monosaccharide composition, as has been reported elsewhere (Jones et al. 1979).

For the reasons just described, pretreatment and hydrolysis of this raw material for subsequent fermentation by yeast are the main hurdles in the production of alcohols from lignocellulosic feedstocks (Sánchez and Cardona 2005). Pretreatment alters the lignocellulosic matrix, thus allowing the subsequent hydrolytic degradation of polysaccharides to monosaccharides (saccharification). These processes may be carried out by either biotechnological methods preceded by physical or physicochemical treatments, or by chemical methods with dilute acids at high temperature (Iranmahboob et al. 2002), concentrated acids at low temperatures (Pessoa et al. 1997), or a combination of both (Kamm et al. 2006). Mineral acid hydrolysis has been described at least since 1819 (Harris and Beglinger 1946). Acid hydrolysis may be divided into two general approaches (Bergius 1937; Ladisch 1979; Taherzazeh and Karimi 2007; Galbe and Zacchi 2002): A) Concentrated acid at low temperatures, and B) dilute acids at high temperatures, which can be performed with different acids, such as sulphurous, sulphuric, hydrochloric (Israilides et al. 1978; Goldstein et al. 1983), hydrofluoric, phosphoric (Israilides et al. 1978), nitric and formic, using low solids loadings (5-10% w/w) (Brennan et al. 1986; Converse et al. 1989) or high solids loadings (10-40% w/w) (Esteghlalian et al. 1997). There are several concentrated HCl processes. The earliest one was used in Germany from 1935 to 1948, where full scale plants were built; it is known as the Bergius-Rheinau process. This process uses one 41% HCl hydrolysis stage with a 3:1 acid:wood ratio. The modified Bergius process known as Udic-Rheinau, consists of three stages; a pre-hydrolysis with 35% HCl at 20ºC, a main hydrolysis with 41% HCl, and a post-hydrolysis with dilute acid. Most of the acid is recovered by azeotropic distillation at 36 ºC, and the residual acid is removed from the syrup by drying it in a hot air spray diffuser (Fengel 1984; Ladisch 1979). Nevertheless, an improved and efficient HCl recycling process is necessary to make the Bergius process competitive. Attaining this goal has stimulated a lot of research. Recently the Israeli company HCl CleanTech, has developed a proprietary HCl recovery process, recovering HCl in gaseous form directly from aqueous solutions by using an immiscible extractant, such as trilaurylamine-dinonylnaphtalene sulfonic acid in a hydrocarbons diluent having a boiling range starting at 210°C (Eyal and Baniel 2010), which makes the modified Bergius-Rheinau process economically attractive and "clean". A pilot plant running this process was built at Southern Research Institute, North Carolina, U.S. and is working since June

2010. The plant takes in 1.25 tons per day of cellulosic feedstock. It is claimed that the cost of the sugars produced is at least 17% lower than the cost of corn mill sugars and of similar quality (HCl Clean Tech 2010).

On the other hand the Fabaceae (Leguminosae) is a plant family amply distributed around the world and economically very important as a food source. The *Lupinus* genus belongs to this family and consists of around 300 species, with some 2000 synonymia (Gladstones 1998); it is widely distributed in the Americas and Mediterranean regions with *ca.* 80 native species described for Mexico (McVaugh 1987; Rzedowski and Rzedowski 2001). *L. rotundiflorus* is a wild lupine (sometimes spelled "lupin") that grows in Mexico at altitudes of 1500 to 2000 m above sea level in the Pacific mountains and the western part of the volcanic axis, in pine and oak forest clearings, and near roadsides. It is an herbaceous plant; its branched basal crown consists of a small stem that underlines a cluster of many shoots. It is an available and fairly common wild species, easy to collect and with a potential to be established as a commercial crop (Ruiz et al. 2000). Elsewhere other lupines have been investigated and developed to be used as commercial crops. Thus, for example, Alaska-lupine today is grown in Iceland, where it was introduced in 1945, with yields (dry mass) ca. 7-8 ton/ha/year (Kamm 2006). Some of this production using the modified "Bergius/Rheinau" process is aimed at the biofuels and biorefinery (ethanol and lignin) markets. Also lupines are grown and processed extensively in Australia, Andean countries, and some European countries (Bañuelos et al. 2006). Although wild lupine species are widely distributed in Mexico, their utilization as animal feed or for human consumption is limited by their high alkaloid content without further processing. Production of fermentable sugars from this biomass can be an alternative use for this Mexican wild species. Therefore, the objective of this study was to evaluate the yield of fermentable soluble carbohydrates from *Lupinus rotundiflorus* biomass, choosing a proven and industrially successful 2-stage concentrated hydrochloric acid process, followed by a post-hydrolysis with diluted acid. This method is based on the modified Bergius process (Kamm 2006).

EXPERIMENTAL

The whole experimental procedure is schematized as shown in Fig. 1.

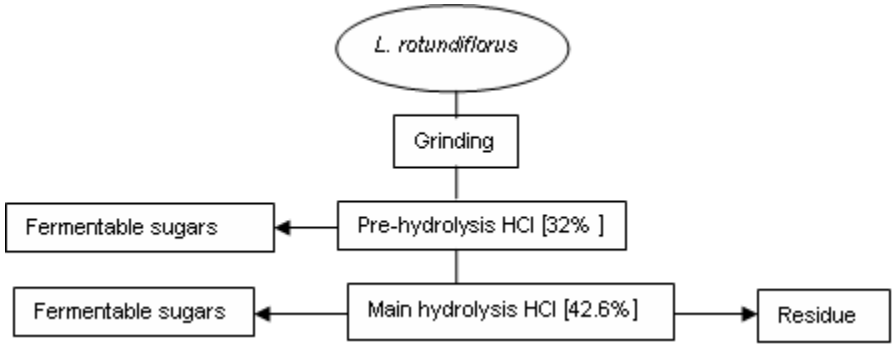

Fig. 1. Schematic presentation of the acid saccharification of *L. rotundiflorus*

Plant Collection and Sample Preparation

Whole plants of *L. rotundiflorus* were collected in the wild form during their flowering stage in October, 2008, at 2320 m above sea level in the vicinity of Atemajac de Brizuela, a town situated in western Mexico, 105 Km SW of Guadalajara, Jalisco. They were air dried and separated into stems, branches, roots, and leaves. Each part was then ground in a Wiley mill with 0.5 mm screen and stored in plastic bags at -4°C. Holocellulose (hemicelluloses and cellulose) (Wise et al. 1946), acid insoluble lignin (TAPPI T222), and extractives (TAPPI T264) content of each part were determined as described in the literature. Particularly, in the Wise procedure for the determination of holocellulose, each extractive-free ground sample is treated with one percent (1%; w/v) sodium chlorite followed by glacial acetic acid in a water bath at 70ºC. The reaction is allowed to continue for 4 h, adding each hour acetic acid and sodium chlorite. The sample is filtered through a medium porosity filter glass previously weighed and is dried in a vacuum oven at 40°C until constant weight.

These analyses were carried out to evaluate which plant parts had a higher content of hydrolysable carbohydrates, hence making it possible to decide which parts would be desirable to process and which to discard. Thus the foliage was cast aside.

Anatomy

Transversal histological sections, 20 µm thick, were prepared from the basal stem using a Leica Microtome. Some of the sections were stained with safranine and astral blue. Both stained and non-stained sections were placed on microscope slides and fixed in euparal mounting medium (Burger and Richter 1991).

The maceration study was carried out according to Franklin (1937). Sections of external cortex, internal cortex, and xylem were placed in test tubes, then a mixture of glacial acetic acid and 30% hydrogen peroxide at a 1:2 (v/v) ratio was added and heated to 60°C until the tissue was soft. The softened tissues were removed, drained, and then stained and fixed in the same manner as the transversal sections. The histological observations were done using a Wild compound light microscope at 10X and 40X magnification. The length and diameter of 30 fibers were measured using a certified scale attached to the microscope ocular.

Acid Hydrolysis

All chemicals used were of reagent grade and the water was deionized. 20 g of ground plant material (o.d. basis) were hydrolyzed with 100 mL of 32% (w/w) hydrochloric acid in a 250 mL Erlenmeyer flask sealed with a rubber stopper for 24 h in an orbital shaker at 20°C. After this, the suspension was diluted tenfold with water, heated for 30 min at 100°C, filtered, and then washed twice with 50 mL of water. The combined filtrate and washings were concentrated to around 15 mL using a rotary evaporator (T= 40ºC, p = 200 mbar) until a thick syrup was formed, this evaporation eliminated most of the acid. The syrup was taken to near neutrality by removing the residual HCl by addition of ethanol (4x100 mL) with subsequent distillation to dryness, after which the residue was fully dried in an exsiccator. The solid residue from the first hydrolysis was digested likewise for 24 h at 20 ºC, using 100 mL of 42.6% (w/w) hydrochloric acid (produced by bubbling pure gaseous HCl into concentrated reagent

grade HCl) (Kirk 1984), filtered, and rinsed twice with 50 mL of water. The liquid portion was concentrated and dried as described above (Kamm et al. 2006). Sugars recovered from the liquid fractions of the first and second hydrolysis were weighed. The solid residues remaining in both stages of hydrolysis were dried at 40ºC for 48 h, weighed, and analyzed to quantify lignin and holocellulose content. This procedure was carried out in duplicate.

The yield of fermentable sugars was evaluated by the difference in holocellulose content on each hydrolytic stage, and verified directly by the weight of the thick syrup (concentrated sugars), as a simple alternative to the DNS method (Miller 1959) commonly used in the evaluation of total reducing sugars.

Sugar Analysis

Monosaccharides were determined using a Varian HPLC (Vista 5500) equipped with a refractive index detector (Varian model 350), a polymeric Hamilton HC-75 Ca (305 x 7.8 mm) column at 80°C, and HPLC-grade water as the mobile phase (0.6 mL/min). Sugars were quantified using D-glucose, D-xylose, D-galactose, D-arabinose, and D-mannose as external standards. Chromatograms were processed using the Star Varian software.

Solid samples from untreated raw material were totally hydrolyzed with sulfuric acid (72% w/w), then neutralized with barium hydroxide (ASTM Method 1758-01), filtered through a 0.45 µm Millipore nylon membrane, and passed through cationic, anionic, and C_{18} cartridges. In order to assay the relative amounts of different monosaccharides in the hydrolizates (liquid filtrates), one milliliter of the concentrated (after rotary evaporation) first and second acid filtrates, was diluted to 1 L in water and then an aliquot filtered and passed through cartridges and HPLC analyzed as has been described above for the solid samples.

Sugars conversion as percentage of the theoretical glucose yield obtained from the equation for the hydrolysis process of cellulose to sugar

$$(C_6H_{10}0_5)_n + H_20 \longrightarrow (C_6H_{12}O_6)_n \quad (1)$$

was calculated as follows (Mandels et al. 1976),

$$glucan\ conversion\ efficiency = \frac{weight\ of\ glucose\ formed\ x\ \left(162/180\right)}{dry\ weight\ of\ cellulose\ used} x100 \quad (2)$$

where a factor of 162/180 was applied due to the difference in mass between the anhydroglucose ring and glucose. Similarly, a factor of 132/150 was applied due to the mass change in the hydrolysis of xylans to xylose.

The fermentable sugars yield was also calculated with respect to the input dry raw material, and as grams per 100 g raw material. This helps to give an idea about of the amount of fermentable sugars that could be obtained from the material (Sassner et al. 2008).

RESULTS AND DISCUSSION

Anatomic Characteristics

All weight and percent compositions are given on an oven-dry basis. The plant percent weight composition was 12% roots, 57% stem and branches, 29% leaves, and 2% flowers and pods. Upon microscopic examination (Fig. 2) of a basal stem transversal section, the external cortex, internal cortex, and xylem of *L. rotundiflorus* contained fibers, some were gelatinous, with a density of 90 fiber/mm^2, xylem having the highest density. Fibers showed little lignification, with thick cell walls and sharp angular ends. The fiber´s average length, diameter, and cell wall thickness were 1.1 mm, 19 µm, and 4.9 µm, respectively. The xylem´s fibers were shorter than those of the external and internal cortex. *L. rotundiflorus* fibers were longer than those reported for *L. meridanus or L. eromonomos* (0.5 mm for the stem fibers and 0.78 mm for the root fibers) collected respectively in Venezuela at 3000 and 4200 m above sea level (Briceño 2000).

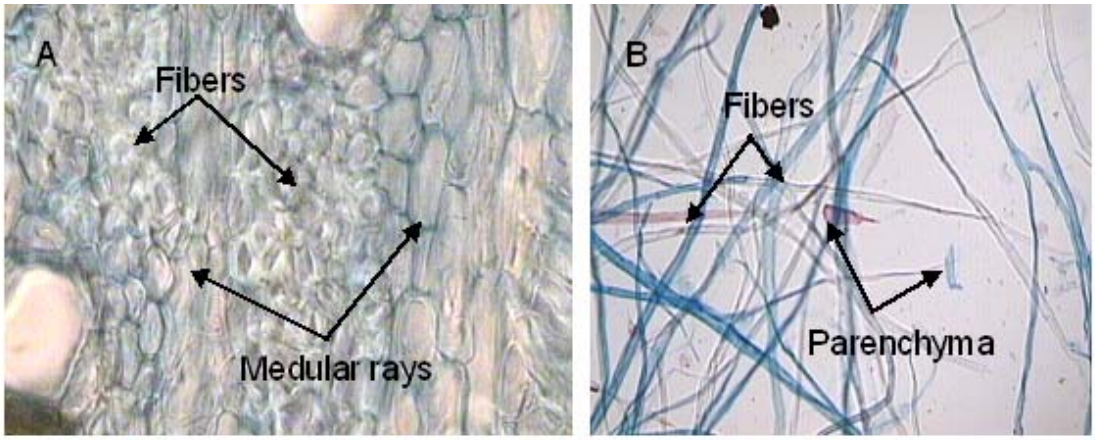

Fig. 2. Optical microscope micrographs of *L. rotundiflorus*:
A. Basal stem transversal section, 40X. B. Macerated material, 10X

Chemical Composition

The holocellulose content (Table 1) found in the stem, root, and branches was within the normal range (53-82%), although lignin content was lower than the 15 to 30% reported in common plant material (Ritter 2008). This difference could be due to the phenological stage of the plant at the time of collection. Leaves (petioles included) contained the lowest levels of holocellulose and lignin. Nevertheless, their lignin content (10.3%) was similar to those reported for the leaves of some leguminous plants such as soybean (10.7%) (Johnson et al. 2007).

Table 1 indicates the monosaccharide content of the four plant parts analyzed. In general, glucose was found in the highest relative concentration followed by xylose, galactose, arabinose, and mannose in that order. In contrast, leaves contained relatively higher concentrations of arabinose, galactose, and mannose in comparison with the other plant parts. The arabinose and mannose content was less than 7%, with roots having the lowest levels (0.4% and 0.5%).

Table 1. Chemical Composition and Monosaccharide Content of *L. rotundiflorus* Plant Parts (%)

Plant Part	Holocellulose	Lignin	Extractives	Ara[1]	Gal[1]	Glu[1]	Xyl[1]	Man[1]
Stem	72.2	14.0	15.4	4.0	13.0	61.2	20.7	1.1
Branches	65.6	13.2	25.6	3.2	12.3	63.8	19.6	1.1
Roots	69.7	11.7	19.4	0.4	12.5	61.4	25.2	0.5
Leaves	47.0	10.3	37.4	6.2	16.0	61.4	11.2	5.2

Ara=Arabinose; Gal=Galactose; Glu=Glucose; Xyl=Xylose; Man= Mannose
[1] Relative percent composition

Plant without foliage (Table 2), which was used for acid hydrolysis, contained 62.6% holocellulose, 15.8% lignin, and 22.6% extractives; while monosaccharide content was similar to that found in each plant part.

Table 2. General Chemical Composition and Monosaccharide Content in *L. rotundiflorus* Without Foliage and After Each of the Stages of HCl Hydrolysis (%)

	Holocellulose	Lignin	Extractives	Ara[1]	Gal[1]	Glu[1]	Xyl[1]	Man[1]
Whole plants without folliage	62.6	15.8	22.6	3.3	11.0	61.0	24.0	0.7
First step hydrolysis (32% HCl)	31.4 (s.f.)	14.8 (s.f.)	nd	14.8 (l.f.)	12.7 (l.f.)	22.5 (l.f.)	46.5 (l.f.)	tr
Second step hydrolysis (42.6% HCl)	8.0 (s.f.)	14.5 (s.f.)	nd	nd	nd	97.7 (l.f.)	2.3 (l.f.)	nd

Ara=Arabinose; Gal=Galactose; Glu=Glucose; Xyl=Xylose; Man= Mannose
s.f. = solid fraction; l.f. = liquid fraction
[1] Relative percent composition.
nd, not detected
tr, trace

Acid Hydrolysis

After the two-step hydrolysis only 8.0% of the original carbohydrates (62.6% holocellulose) remained (Table 2). The difference of 54.6 percent units (48.4 considering the hydrolysis factor) with respect to the original carbohydrates (Table 3) was conceived as hydrolyzed carbohydrates; 31.2 percent units (27.5) and 23.3 percent units (21.0) of the total available sugars were recovered in the first and second phase, respectively. These data are consistent with those obtained when the purified hydrolyzates (thick syrup) were weighed, assuming they are comprised only of hydrolyzed carbohydrates.

The insoluble residue (22.5%) contained 14.5% lignin and 8% undigested carbohydrates.

Table 3. Hydrolyzed Sugars Yield After the Different Saccharification Steps of *L. rotundiflorus* with HCl, Insoluble Residue and Lost (unrecovered) Material (%)

Sugars yield first hydrolysis	Sugars yield second hydrolysis	Sugars overall yield	Insoluble residue	Extractives	Total	Lost material[1]
27.5	21.0	48.4	22.5	22.6	87.7	12.3

Data are the average of two replicates
Insoluble residue = 14.5% lignin + 8.0% undigested carbohydrates
[1]Lost material = 100 – %Total

Although the yield from *L. rotundiflorus* was lower than those that can be achieved from biomass sources such as starch (100%), it represents an important source of fermentable carbohydrates. The conversion of lupine biomass into fermentable sugars would not compete with materials such as starch that are destined for human or animal consumption.

Figure 3 depicts the changes in lignin and holocellulose content with respect to the total original weight of the plant material after each acid hydrolysis stage. As expected, there was a negligible change in the lignin content. Lignin is known to be refractory to acid hydrolysis. However, contrary to lignin, the holocellulose content decreased in proportion to the amount of fermentable sugars obtained in each acid hydrolysis step. Almost all of the hemicelluloses and of the extractives were solubilized by the acidic conditions used in the first stage.

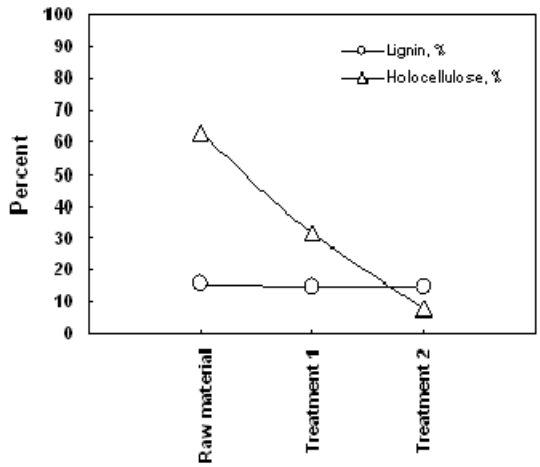

Fig. 3. Holocellulose (sugars) and lignin content variation in raw material (before hydrolysis) and in the insoluble residues after the two stage hydrolysis of *L. rotundiflorus* without leaves

Also shown in Table 2 are the initial content and the monosaccharides released during the first and second hydrolysis stages. The main carbohydrate present in the hydrolyzate after the first stage was xylose (46.5% relative composition), followed by glucose, arabinose, and galactose, while in the second phase glucose (97.7%) and xylose (2.3%) were the only two carbohydrates present.

The total hydrolysis process used in this study produced, per 100 g of dry matter, 26.8 g of glucose (70% glucan conversion efficiency), 13.2 g of xylose (88.1% conversion), and 7.6 g of other sugars such as arabinose and galactose (81% conversion). This is probably due to the fact that pentoses such as xylose and arabinose are easier to hydrolyze from polysaccharides in acidic media than are the hexoses glucose, galactose, and mannose (Moxley and Zhang 2007).

The results from this study are similar to those reported for *Lupinus nootkatensis* (Kamm et al. 2006). Using the same process they reported 55% yield in total fermentable carbohydrates, 30% in the first phase, and 25% in the second phase. Xylose was 60 to 80% of carbohydrates present in the first phase with only small quantities of arabinose, glucose, galactose, while in the second phase glucose was 80% of the carbohydrates, and only traces of xylose, mannose, and galactose were found.

Fermentation processes can be utilized to transform glucose and xylose into alcohols, i.e. ethanol and n-butanol, as well as other chemicals such as furfural. These compounds are or might be the basis for many industrial organic chemicals and substitutes of petrochemical products, including polymers, pharmaceutical, pesticides, dyes, etc.

CONCLUSIONS

Before and during the flowering stage *Lupinus rotundiflorus* fibers in the xylem as well as in the external and internal cortex contain little lignin. This facilitates the release of fermentable sugars by acid hydrolysis. Hydrolysis of plant materials released mainly xylose and glucose in the first step and mostly glucose in the second step. It is well known that xylose-based hemicelluloses are more prone to acid hydrolysis in comparison to glucose-based cellulose.

One advantage of this method is the use of a single hydrolyzing reagent in both stages to release fermentable sugars from the plant material. This is combined with the fact that *L. rotundiflorus* is a wild plant that may be easily grown even in poor soils and different climates. The acceptable yield and relative simplicity of this saccharification process, the use of most of the biomass, including roots, but excluding foliage, along with the wide availability, low cost of the chemicals, more efficient acid recovery and the ample supply of lupines, particularly after the development of commercial growing of *L. rotundiflorus*, would facilitate the scaling of these laboratory studies to pilot and industrial levels.

However, hydrochloric acid is a corrosive and dangerous chemical that requires special handling and recycling procedures. The use of enzymes is a well known alternative to acid hydrolysis in obtaining fermentable sugars; however their use may carry along some disadvantages as well, such as relative complexity of the process, as well as concerns about the availability and cost of suitable enzymes.

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