Bioprocessing of Lupin Cotyledons (*Lupinus mutabilis*) with *Rhizopus oligosporus* for Reduction of Quinolizidine Alkaloids

Eduar Ortega-David* and Aida Rodriguez-Stouvenel

Grupo de Investigación en Procesos Agroalimentarios y Biotecnológicos GIPAB, Universidad del Valle, Calle 13 No 100-00 Meléndez, Cali-Colombia

**Abstract**

Lupin cotyledons are good source of nutrients such as proteins and lipids and around of 3.8% of toxic quinolizidine alkaloids difficult to extract. Previously it had reported by Tempeh fabrication was possible to achieve 90% of detoxification. Never before had been reported the incidence of variables of fermentation in the detoxification that could be useful for industrial scale up of process. In this investigation we studied the influence of moisture and particle size in elimination of alkaloids in a process of solid state fermentation with *Rhizopus oligosporus*. Wet material was fermented without forced aeration during 48 h testing two particle sizes, whole (W) and broken (B) particles. Moreover three moisture levels were evaluated at 40% (H-40), 50% (H-50) and 60% (H-60). Results showed maximum detoxification of 70.55% and 67.71% in broken cotyledons for H-60 and H-50 respectively. In whole cotyledons were achieved 64.26% and 61.08% for same moisture levels. In H-40 lower levels of degradation were showed with 47% and 52% for whole and broken particles respectively although water activity was over 0.9. Moisture over 50% did not cause a proportional increase in removal of alkaloids. Both factors, moisture and particle size influenced degradation; however less size had major incidence than moisture when water content was over 50%. Fermentation process could be useful as partial way to eliminate main toxins of Lupin.

**Keywords:** Solid state fermentation; Detoxification; Bioprocess; Andean legume; Biodegradation

**Introduction**

Lupin (*Lupinus mutabilis*) is a plant native from Andes noted for its resistance to adverse environmental conditions. It has seeds with protein and oil in quantities around of 44.86% and 13.91% respectively [1], besides of diversity of nutrients and beneficial substances [2,3]. The industrial development of seeds has been limited because quinolizidine alkaloids are difficult to remove and confer toxicity and contribute at formation of a strong bitter taste [4].

These substances are part of mechanisms of plant for defense. These are derived from Lysine, formed for two heterocyclic rings, soluble in water, with a molecular weight around of 250 g/mol [4]. Type and proportion of alkaloids change depending of species, planting site, environmental conditions, among others. In complete Andean Lupin seeds were quantified an average amount of 35 mg of alkaloids per gram of dry seed. The 90% of these substances are compound for lupanin, spartein, tetrahydroxirombifolin, 4-hydroxylupanin and 13-hydroxyrupanin [1]. Toxicity is higher in insects and reduced in vertebrates with different doses for each substance. These do not have psychotropic effects and some alkaloids present in lesser proportion such as anagirin and ammodendrin are teratogenic [5]. Its formation is carried out in chloroplasts and is stored within vacuoles of cells [6].

The Incas made a method of processing used until present based on multiple aqueous soakings to detoxify or extract bitter taste of the seeds. Although this method is very effective, is expensive, because hard work to extract substances cause damage to seed besides the strong environmental impact by toxicity of leachates. Other methods have also been tested to detoxify such as soaking with addition of chemicals, heat treatments [7], germination [8], among others. It has also been proven genetic selection being preferred seed for sowing which have fewer alkaloids [9]. In *L. mutabilis* detoxification in vivo caused a new plant variety lost its resistance, making it unattractive for cultivation.

Fermentation also has been explored for detoxification of Lupin seeds. Jimenez et al. [10], showed that making Tempeh was possible to achieve a reduction of 91% in concentration of alkaloids. Santana et al. [11], identified and characterized microorganisms capable of degrade quinolizidine alkaloids. Therefore a process of fermentation with microorganisms safe for foods could be applied to detoxify cotyledons in industrial scale. In this sense a process with fungus *R. oligosporus* which is used in oriental food industry could be implemented for this purpose.

Fungi are well suited for solid fermentation medium with advantages such as reduction in water usage, increased production of enzymes, and synthesis of beneficial metabolites, among others [12,13]. Implementation and operation of bioprocess with *Rhizopus oligosporus* require establishing conditions of medium and environment. The PH is related with production of enzymes of degradation, and studies showed that an initial value of 5.5 favors growth and removal of alkaloids [14]. Water content is related at growth and diffusive phenomena for alkaloids and enzymes inside particle. Particle size is related at area for growth and penetration of fungal mycelium. Both properties in addition to their interrelation have not been studied. The purpose of this study was to establish impact of these issues and determine most appropriate conditions to reach highest level of detoxification.

**Materials and Methods**

**Vegetal material**

The seeds were obtained from experimental crops established in the city of Pasto (Narino-Colombia). These were peeled to remove seed

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**Corresponding author:** Eduar Ortega DA, Grupo de Investigación en Procesos Agroalimentarios y Biotecnológicos GIPAB, Universidad del Valle, Calle 13 No 100-00 Meléndez, Cali-Colombia, Tel: 572-3212392; E-mail: eduarhortegad@gmail.com

**Received** March 19, 2014; **Accepted** May 02, 2014; **Published** May 08, 2014


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Microorganism and inoculum

We used a strain of Rhizopus oligosporus ATCC 22959 obtained from ATCC collection. Potato dextrose agar (PDA) was prepared in slant tubes to produce spores. Tubes were inoculated, incubated at 30°C and allowed to sporulate for 9 days. Spore scraping was made adding 6 ml of sterile distilled water to each tube. Suspension was standardized at quantity of cells of 10^7 spores / ml counted in Malassez camera. Each experimental unit was inoculated with 1% of suspension in base of total mass, and finally mixed.

Preparation of medium

These were standardized in three moisture levels of 40, 50 and 60% referred as H-40, H-50 and H-60 respectively, and two particle sizes, whole and broken. Medium was prepared calculating mass balance and using vegetal material, distilled water and hydrochloric acid solution 0.1 N. The 545.43 g of medium H-40 was prepared with 332.01 g of cotyledons, 205.92 g of water and 7.5 grams of acid solution. The 666.67 g of medium H-50 was prepared with 332.01 g of vegetal material, 205.92 g of water and 7.5 grams of acid solution. Finally we prepared 857.14 g of medium H-60 with 332.01 g material, 205.92 g of water and 11.76 grams of acid solution.

We used glass flask containers of 43 mm diameter by 95 mm deep as reactors. It was introduced aluminum foil in each reactor filled with 36.36, 44.44 and 57.14 grams of wet vegetal material for H-40, H-50 and H-60 respectively. Finally experimental units were put to heat treatment at 121°C at 15 psi for 5 min, after depressurized and cooled to room temperature. Subsequently these were immediately inoculated then incubated at 25°C for 48 h. Three reactors were randomly taken for analysis at final time of fermentation to make degradation kinetics.

Determinations in experiments

We recorded weights of flask, dry foil and wet matter in each experimental unit. At final time of fermentation we recorded weight of reactor with fermented material. These were introduced into an oven to determine dry weight at 105°C for 72 h. The variations in dry matter content were determined by difference between weights of wet and dry matter. The change of pH was measurements by potentiometric analysis according to AOAC 943.02/90 methodology. Samples were taken for a proximal analysis at begin and end of process [15].

Alkaloids quantification was performed by gas chromatography according to technique of Muzquiz et al. [16]. Sample (0.5 g) was homogenized with 5 ml of trichloroacetic acid (5%). Mixture was centrifuged at 10000 x g for 15 min repeating this procedure twice more. The collected supernatants were neutralized with 1 ml of sodium hydroxide (10 M). Extraction of alkaloids was performed in funnel adding 15 ml of chloroform (Merck Uvasol) for three times. Chloroform collected was evaporated at 30°C until dryness. Residue was dissolved in 1 ml of methanol for inject alkaloids to chromatography. Quantification was performed in a gas chromatograph Shimadzu GC-17A equipped with a FID detector at temperature of 280°C. We used a DB-5MS column 18W Scientific (30 m × 0.25 mm ID x 0.25 mm) and helium as carrier gas at rate of 1.0 ml min-1. The temperature program started at 150°C increasing at rate of 5°C min-1 to reach 235°C. Caffeine standard was used as internal standard. Identification of peaks was performed by gas chromatography coupled to mass spectrometry, on a Shimadzu GC-MS QP-2010 under same conditions used in chromatography.

Statistical analysis

The analysis of variance was made using statistical package MINITAB 14. We used a significance level of p <0.05. All measurement was made for three times.

Results

Observed growth

Development of mycelium was lower and slower in both particle sizes in H-40 treatment. In first 24 hours growth was only visible with small mycelium emerging from surface. Until 48 h was observed small mycelium completely covered but a lesser amount. Probably it was due at water was not sufficient to sustain growth of fungus although water activity was 0.96 ± 0.001.

Growth had not visible differences in treatments H-50 and H-60, showing uniform mycelia development longitudinal and top of bed (Figure 1). There were not visible differences with regard to particle size, although theory report growth into a bed with higher surface will be greater. In particle sizes experienced no noticeable differences were observed, so that we can say that both treatments showed optimal conditions for growth of R. oligosporus.
growth in experimental conditions. The differences in treatment of broken cotyledons H-60 could be due to a limited supply of oxygen and CO₂ due to tendency of particles to agglomerate; however no conclusion we can to do about it.

Sporulation was after 40 h mainly in upper part of beds. The spores were observed in a higher proportion in H-50 than H-60 and were very low in H-40. With respect to particle size was not tendency observed therefore not possible to make statements about this.

Variation in pH

The variation in pH of medium showed low intensity in H-40 compared at process performed at higher moisture. After 24 h, there was an increase of pH being slightly higher for broken seed, reaching 6.6 at final of 48 h in both sizes. The measurement of pH showed a greater intensity for H-60 although no differences were visible mycelia growth with H-50. Therefore metabolic activity of microorganism was greatest in media of high moistures.

There were also differences between whole and broken particles showing a lower intensity in medium with big particles (Table 1). The major particle size could reduce growth area and decreasing aeration causing reduction of metabolism. Microbial activity was evident after 12 h and between 36 to 48 h pH variations was performed at lower rate, assuming the growth phase is reducing velocity. The behavior of pH was similar at observed in soybean fermentation, that increasing due at production of ammonia substances derived from proteolysis [17].

Changes in dry matter and moisture

One of greatest change undergone in the material was decreased in dry matter content, whose major change was for H-50 and H-60 than H-40. In H-60 and H-50 with whole particles was determined a decrease of 4.1% and 3.8% respectively. However in H-40 showed a low dry matter intake with rates of 2.1% and 2.5% for whole and broken. These values are lower than reported by Ruiz-Teran and Owens in soybeans, who report a 10% of reduction in 32h. Nevertheless in other experiments were registered an average of 3% in the same fermentation [18]. This behavior confirms the reduction in dry mater content, but to calculate a precise value of reduction could be difficult because variety of methods used increase mistakes.

Decrease of dry matter content in experiments H-50 and H-60 began after 12 h and subsequently started exponential growth phase. Process continues at constant rate until 48 h without a stationary phase can be observed (Table 2). A different behavior had H-40 than other treatments, despite an Aw of 0.96 showed a slow exponential phase observed between 24 and 48 h. Low water content caused a lower dry matter intake than most hydrated treatments, reflecting the importance of optimal level of moisture to obtain good fermentation. For other side, moisture was increased to insignificant rates in all media above to initial values. This behavior was independent of particle size because we did not find differences between whole and broken particles.

Changes in organic matter

The variation was measured by difference of weight of the ashes (Figure 2). Significant differences were identified between H-40 and media with higher moisture. In broken material H-60, H-50 and H-40 were determined maximum consumption of organic matter of 5.05%, 4.68% and 2.75% respectively. In whole cotyledons H-50, H-60 and H-40 the consumption was of 4.45%, 4.15% and 2.14% respectively. These values confirm major consume of organic matter in media with high moisture.

Particle size reduction resulted in an increased in the consumption of organic matter for three media. Between whole and broken H-40 particles there was an increase in the consumption of organic matter of 28.4% plus for small particles. Between sizes in media H-50 and H-60 the increase was 12.73% and 13.4% respectively plus in broken particles. The values of consumption observed for small sizes were majors than moisture; therefore it appears that particle size has more influence than water content.

The most significant changes were observed in lipids and proteins (Table 3), because R. oligosporus prefers to take these substances as carbon, energy and nitrogen sources [13]. Consumed in nutrients followed the same trend showed before with respect to moisture and particle size. In whole particles the reduction in ether extract was of 2.29% for H-60 and 1.3% for H-50. Similarly reduction in broken particles reaches 3.27% and 2.47% for H-60 and H-50 respectively. A small reduction of 0.49% of ether extract was achieved in broken H-40 treatment. On the contrary, no reduction was observed in media whole H-40 but decreases the content of free nitrogen extract. This would contribute at change in proportion of components in each medium.

Protein was increased proportionately when more water was present in the medium (Table 3). For broken particles H-60 and H-50 the increase were of 2.82% and 1.89%, moreover 2.24% and 1.12% for whole particles respectively. The value of increase for H-40 is less than the above, showing an increase of 0.28% and 0.73% for whole and broken particles respectively. Although this is a proportional increase is important because improve nutritional qualities of material.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>H-40 Whole (%)</th>
<th>H-50 Whole (%)</th>
<th>H-60 Whole (%)</th>
<th>H-40 Broken (%)</th>
<th>H-50 Broken (%)</th>
<th>H-60 Broken (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.52 ± 0.004</td>
<td>5.51 ± 0.017</td>
<td>5.52 ± 0.009</td>
<td>5.490 ± 0.005</td>
<td>5.499 ± 0.010</td>
<td>5.492 ± 0.006</td>
</tr>
<tr>
<td>12</td>
<td>5.57 ± 0.004</td>
<td>5.489 ± 0.012</td>
<td>5.556 ± 0.010</td>
<td>5.499 ± 0.002</td>
<td>5.482 ± 0.013</td>
<td>5.508 ± 0.005</td>
</tr>
<tr>
<td>24</td>
<td>5.592 ± 0.005</td>
<td>5.936 ± 0.005</td>
<td>6.260 ± 0.002</td>
<td>5.407 ± 0.011</td>
<td>5.974 ± 0.009</td>
<td>6.001 ± 0.012</td>
</tr>
<tr>
<td>36</td>
<td>6.190 ± 0.004</td>
<td>6.735 ± 0.015</td>
<td>6.725 ± 0.009</td>
<td>6.334 ± 0.015</td>
<td>6.757 ± 0.038</td>
<td>6.830 ± 0.016</td>
</tr>
<tr>
<td>48</td>
<td>6.600 ± 0.018</td>
<td>7.059 ± 0.053</td>
<td>7.116 ± 0.013</td>
<td>6.660 ± 0.009</td>
<td>7.112 ± 0.036</td>
<td>7.358 ± 0.011</td>
</tr>
</tbody>
</table>

Table 1: Variations in pH of medium during fermentation process.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>H-40 Whole (%)</th>
<th>H-50 Whole (%)</th>
<th>H-60 Whole (%)</th>
<th>H-40 Broken (%)</th>
<th>H-50 Broken (%)</th>
<th>H-60 Broken (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.05 ± 0.00</td>
<td>0.14 ± 0.02</td>
<td>0.07 ± 0.01</td>
<td>0.08 ± 0.02</td>
<td>0.23 ± 0.04</td>
<td>0.29 ± 0.03</td>
</tr>
<tr>
<td>12</td>
<td>0.90 ± 0.12</td>
<td>1.59 ± 0.21</td>
<td>2.09 ± 0.16</td>
<td>1.07 ± 0.35</td>
<td>1.73 ± 0.15</td>
<td>2.60 ± 0.12</td>
</tr>
<tr>
<td>36</td>
<td>1.78 ± 0.09</td>
<td>3.21 ± 0.44</td>
<td>3.58 ± 0.33</td>
<td>2.23 ± 0.30</td>
<td>3.57 ± 0.42</td>
<td>3.96 ± 0.39</td>
</tr>
<tr>
<td>48</td>
<td>2.10 ± 0.61</td>
<td>3.82 ± 0.33</td>
<td>4.11 ± 0.51</td>
<td>2.57 ± 0.49</td>
<td>4.22 ± 0.71</td>
<td>4.52 ± 0.69</td>
</tr>
</tbody>
</table>

Table 2: Dry matter consume during 48 h of fermentation with R. oligosporus.

Figure 2: Percentage of organic matter in whole and broken Lupin cotyledons H-40, H-50 and H-60 after 48 h of fermentation.

Changes in the alkaloids

Detoxification is related with water in system, because concentration of alkaloids decreased while moisture content increased. Between whole cotyledons H-40 and H-60 were observed a difference in concentration of alkaloids of 16.75% after 48 h of fermentation. In H-40, all other measurements showed changes with low fungal metabolism, and moreover with a minor decrease in the concentration of alkaloids. Detoxification in H-40 is apparently proportional at fungal growth, with a small amount of enzymes produced to degrade few quantities of alkaloids (Table 4).

A different tendency was showed in treatments H-50 and H-60 where there was a difference of only 3.2%. Here demonstrate the importance of high level of moisture but also indicate that water excess was not contributed proportionally reducing concentration of alkaloids. A similar trend was also observed in broken cotyledons showing a difference of 17.14% between H-40 and H-60, and only 2.83% for H-50 and H-60. In treatments H-60 between whole and broken particles, we calculated a difference in reduction of alkaloids of 6.32% in 48 h of fermentation. In treatments H-50 the difference was of 6.69% and between H-40 was of 5.93%. Reduction in particle size in all moisture levels had a significant effect in degradation of alkaloids.

The concentration of alkaloids between H-50 and H-60 showed a difference of only 3.0%, showing high moisture in environment affected slightly reduction of alkaloids. Contrary at before, between media of whole and broken particles H-40 and H-50 the difference was around of 17.0%. Between H-40 and H-50 the effect of water content was greater than particle size, which means that water in sufficient quantity is imperative to have the greatest possible reduction in concentration.

Compared to results obtained by Jiménez et al. [10], there were several differences. First, profile of alkaloids of Lupinus mutabilis used in experiments was different. Although these are same species of Lupins, experiments could be performed with different ecotypes. Plants change composition of secondary metabolites depending of place of growth [1]. Second, preparation of media could also responsible for variations. The 91% reported by Jiménez et al. [10], was calculated since previous processes of conditioning of cotyledons (soaking and cooking). In our experiments, reduction of alkaloids by conditioning of medium was imperceptible and it only was due at fermentation. These treatments resulted in cotyledons with physicochemical differences which changed the relationship between microorganism and environment.

The amount of total alkaloids in each experimental unit was calculated multiplying dry matter in each point with respective total alkaloid concentration. Thus, it was observed for whole and broke particles a removal of 400.53 and 457.44 mg per experimental unit (flask) for H-40 and 521.37 and 594.59 mg flask-1 for H-50 respectively. In whole particles increase of moisture since H-40 to H-50 represented a 30.17% plus of degradation, similar at observed in broken particles where increase managed was of 29.98%. In H-60 treatments were observed greatest degradation with 569.93 and 596.15 mg flask-1 for whole and broken particles respectively. Difference between H-50 and H-60 for entire cotyledons was in average 4.5% plus for H-60. Moisture over than 50% had a limited impact in removal of alkaloids. By itself particle size reduction improved detoxification in 5.93%, 6.69% and 6.32% for H-40, H-50 and H-60 respectively. Degradation of alkaloids was more significant increased by effect of particle size than higher moisture levels (Figure 3).

Changes of each alkaloid

Lupinin was most degraded alkaloid with a 77.91% in broken
cotyledons and less a difference of 5.22% for whole particles. Similar at observed in totals, the increase in degradation of lupanin between H-50 and H-60 was presented in a proportion of 4.51% to 0.04% plus for whole and broken respectively. In H-40 degradation occurs in similar proportion with respect at higher moistures, being higher for broken cotyledons with a value around of 61% (Table 5).

Similar behavior was presented for spartein whose maximum removal was in broken particles H-60 with a 69.35% and 63.05% for whole particles. In this alkaid low degradation was observed in both treatments with detoxifications averaging 40% for H-40. The difference of degradation between H-50 and H-60 for spartein did not exceed 1.3%.

The tetrahydrorombifolin (THR) reached a maximum of 68.33% removal in broken material H-60. Equal at observed with other alkaloids in THR also showed low removal of H-40 that did not exceed an average of 30%. The 4-hydroxylupanin in general showed low degradation in comparison with other alkaloids in all treatments. Maximum removal was 48.89% in H-60 with broken cotyledons. The low degradation may be due to degradation of lupanin because hydroxylation reaction increases continuously content of hydroxylupanin.

The minority alkaloids showed low degradation in all treatments. Unlike of other treatments degradation of this alkaid was higher in broken cotyledons H-40. This presented a 49.52% of removal with 9.94% less for whole material. Degradation of H-50 and H-60 in broken material was around 35% and 40% and whole particles were of 28% and 32% respectively. Low reduction of these substances could be due at intermediate degradation reactions of formation of 4-hydroxylupanin or simply may be difficult for fungus removal. Given the lack of data to support any of these hypotheses, we cannot conclude about this phenomenon.

### Discussion

The observed growth over material let conclude about good physicochemical composition to maintain fungal development. Previous studies of Lupin seed showed good molecular composition and optimum mineral content to support nutritional demands of any organism [1]. Although in H-40 was observed lower and slower development until 48 h there was growth. Therefore nutrients of cotyledons are adequate to maintain fungal growth at least the genera Rhizopus. The good growth observed in treatments H-50 and H-60 let conclude medium did not show sign of inhibition, and there was not substances that affect fungal development.

Sporulation is an unwished phenomenon observed in elaboration of Tempeh [13]. Spores could be result of stress of microorganism and/ or expression of ripeness of fungal development. This phenomenon was also observed in fermentation of Lupin after 40 h in upper part of beds. Considering good microbial growth measured in previous works [14] and optimal growth observed in the medium without signals of death, carbon dioxide levels were not sufficient to affect fungal growth (Figure 1). Therefore we saw optimal development then we can conclude sporulation was due at ripeness for complete fungal development.

Similar at growth, pH of medium showed less intensity in media with minus moisture, but while water content is in enough quantity pH

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Whole cotyledons</th>
<th>Broken cotyledon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H-40 (mg A flask⁻¹)</td>
<td>H-50 (mg A flask⁻¹)</td>
</tr>
<tr>
<td>Lupanin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>453.43 ± 34.92</td>
<td>428.48 ± 10.37</td>
</tr>
<tr>
<td>24</td>
<td>313.29 ± 2.30</td>
<td>285.94 ± 19.83</td>
</tr>
<tr>
<td>48</td>
<td>168.42 ± 10.88</td>
<td>136.33 ± 0.35</td>
</tr>
<tr>
<td>Spartein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>323.07 ± 6.21</td>
<td>254.00 ± 2.64</td>
</tr>
<tr>
<td>24</td>
<td>155.91 ± 0.81</td>
<td>155.06 ± 11.26</td>
</tr>
<tr>
<td>48</td>
<td>130.09 ± 10.51</td>
<td>96.50 ± 0.17</td>
</tr>
<tr>
<td>Tetrahydroombifolin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>51.98 ± 1.04</td>
<td>51.17 ± 0.63</td>
</tr>
<tr>
<td>24</td>
<td>47.44 ± 3.37</td>
<td>28.53 ± 2.41</td>
</tr>
<tr>
<td>48</td>
<td>42.93 ± 4.45</td>
<td>18.12 ± 2.29</td>
</tr>
<tr>
<td>4-Hidroxyupanin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>49.53 ± 1.66</td>
<td>42.11 ± 1.21</td>
</tr>
<tr>
<td>24</td>
<td>42.51 ± 2.67</td>
<td>35.39 ± 1.05</td>
</tr>
<tr>
<td>48</td>
<td>34.47 ± 1.48</td>
<td>25.86 ± 2.27</td>
</tr>
<tr>
<td>Minor alkaloids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>89.51 ± 7.53</td>
<td>77.81 ± 3.33</td>
</tr>
<tr>
<td>24</td>
<td>71.00 ± 6.06</td>
<td>58.81 ± 3.59</td>
</tr>
<tr>
<td>48</td>
<td>54.08 ± 2.02</td>
<td>55.39 ± 3.77</td>
</tr>
</tbody>
</table>

Table 5: Quantity of each alkaloid present in the medium of fermentation during 48 h.
did not show big differences. We observed similar trend before with experiments in Lupin material [14], but with cotyledons in a solid state fermentation process, control of pH was also impossible. This property is extremely important for fungal growth [19] and increase observed only was around of 7.0. Mainly in low moisture media the increase of pH should be major than ones more moisture, but no significant differences were observed. Perhaps proteins in medium could act as buffer reducing effects of ammonia increasing the pH.

Fermentation also caused a diminution in dry matter which average exceeds 4% in broken media H-50 and H-60. In comparison of average of H-40 around of 2.3%, dry matter intake confirms the variation between media with different moisture levels. Despite different values reported in similar fermentations [18,20] we can conclude good adaptation of fungus at nutrients presents in medium, and capability of enzymes to break molecules present in cotyledons. With respect to water presence in media, dehydration occurs commonly in beds with forced aeration [21] but without aeration and high relative humidity, moisture increase by formation of metabolic water proportionally in a reduction of dry matter. In conclusion moisture remains constant during process in static aeration and ensures the quantity of water along fermentation. Although initial water activity was higher than 0.9 in H-40 treatments, the amount of water was not enough to sustain growth. In fact, literature wide report values over 0.7 for fungal growth but we can saw that not was true for this case. Thus, water in medium is a limiting factor for development of fermentation and it must to be near of maximum capability of retention of solid material to ensure acceptable fungal growth.

Organic matter changed producing energy and fungal biomass moreover carbon dioxide and ammonia [19,22]. Consumption registered of organic compounds confirms the importance of water content and incidence of size of broken particles that show major superficial area of fermentation. The increase of area of growth improved availability of water and nutrients for the fungus and the changes caused for fermentation in compounds are widely known [13]. The behavior observed in fermentation studied had been present in similar fermentations with soybeans, which lipid intake of the fungus is around of 24 h to achieve roughly 6% until 72h [23]. This would contribute to the reduction of lipid and increase of total protein content [24], therefore consolidating lupin cotyledos as source of proteins. The increase in protein content could show similar values and behavior at fermentation of soybeans [25]. It was evident that increase was proportional and is caused in Lupin for hydrolysis of other components, that increase total protein content, and moreover free amino acids and soluble proteins as had been reported [26].

Water plays important role in detoxification because could observed major level of elimination in medium with more water. The difference between treatments was due to increase in water content that surely improves the diffusion of alkaloids and enzymes of degradation into particle [27]. While water was enough quantity low difference reduction was observed, hence high level of moisture is need for process but except did not affect proportionally reducing alkaloids. Despite particle size had similar behavior showing low difference between treatments with high moisture, these properties had more relation with detoxification.

The purpose of less size in cotyledons was to improve scope of mycelium into particle, but we observed the tendency of material to compact mainly with high moisture, then, we did not consider making further reductions in size. Reduction of size had significant effect in degradation of alkaloids for all moisture levels. The effect was more significant when environmental water content was above 50%, because it was in H-50 and H-60 where particle size had more importance than moisture level. Therefore with good moisture level fungal growth will be adequate and hence greater reduction in concentration of alkaloids will be achieved. The influence of combined effects of moisture and size reveal a significant reduction in concentration of alkaloids. Particle size had a greater effect than moisture when water content was sufficient quantity to maintain growth. In conclusion, if water is in adequate quantity and particle size let optimal fungal development, detoxification will carry out without big differences.

The experiments performed for this job were different at previous with Lupin made around the world but never before had been tested specifically these properties in Lupin fermentation. Jimenez et al. [10], tested Lupin whole beans using different process of preparation of media achieving reduction in alkaloids concentration of 91%. Other fermentation process was evaluate for Santana et al. [28], in Lupin flour to obtain a mass free of alkaloids, achieved a reduction roughly of 90%. A previous job made with Lupin flour in shape of agar as medium achieved a maximum detoxification of 63.23% [14]. As conclusion, reduction of alkaloids changes depending of preparation of media, microorganism and way of fermentation, whose variations are directly related with, develop of process.

Equally at reported in previous job lupanin was the most degraded alkaloid in the essays with cotyledons [14]. This was for spartein and tetrahydrorombifolin (THR) with major values than reported previously. Moreover of these substances we could measure the 4-hydroxy lupanin showing low degradation in relation to other alkaloids in all treatments. Possibly low degradation may be due at degradation of lupanin like had been reported for [29]. The hydroxylation of lupanin increases continuously the content of hydroxylupanin. The minority alkaloids showed similar behavior that could due at intermediate degradation reactions of formation of other components or simply may be difficult for fungus removal. There is not detailed information about these phenomena. Biochemical aspects of this fermentation are interesting topics of study in this process in the future.

In conclusion degradation of quinolizidine alkaloids is affected by both factors studied. The reduction of particle size has a positive effect in degradation, with adequate growth without observing signs of stress in fungi. The moisture improves degradation when it is in optimum quantity for growth and excess of water do not cause a commensurate benefit. When moisture is adequate, particle size has a major effect in the detoxification. Fermentation process in a bed without forced aeration could be useful as partial way to eliminate toxins of Lupin and could be useful as complementary treatment for detoxification of material.

Acknowledgments

The authors express their acknowledgment to the Departamento Administrativo de Ciencia y Tecnología COLCIENCIAS for financial support. To Dr. Ángel Zamora Burbano research group ASINDETEC and Dra. Cristina Ramirez Toro research group GIPAB for logistical support.

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ISSN: 2157-7110 JFPT, an open access journal Volume 5 • Issue 5 • 1000323


