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# Obtaining microorganisms with cellulolytic activity in different regions of Ecuador

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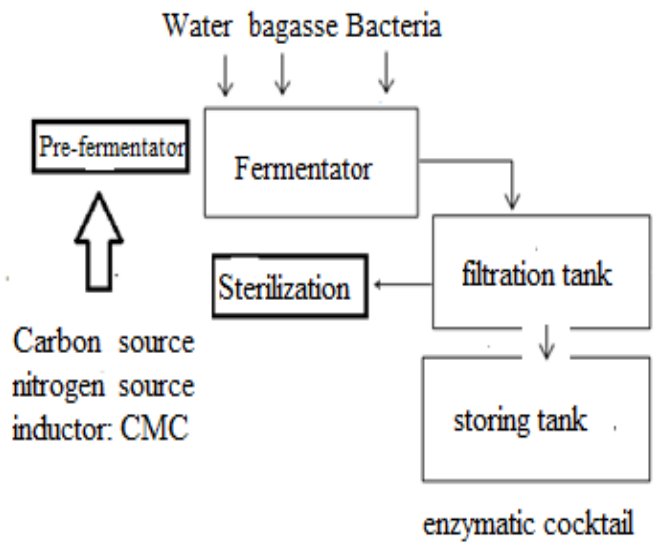



**Abstract.**
It is estimated that in developing countries 60% of waste is from lignocellulosic material that can be used in recycling processes. The purpose of this research is to find enzymatic cocktails from bacteria native to Ecuador that allow degradation of the lignocellulosic material, by searching native microorganisms collected in Los Andes, Amazonia and Antartida, which can be introduced in a process of bagasse enzymatic hydrolysis on an industrial scale. For this, qualitative and quantitative tests were carried out to measure the endoglucanase, exoglucanase and filter paper activity of the microorganisms and their enzymatic cocktails. Enzymatic hydrolysis tests were also performed on sugarcane bagasse. In addition, the value of the investments for the production of enzymatic cocktail of bacteria *Bacillus sp*. and the total cost of production was calculated.
It was identified that *Peniccillium sp*. was the species with the highest activity of filter paper,

showing 0.0073 UFP and a glucose yield of 11,96 in 100 grams of bagasse, which opens the possibility of its use, in industrial processes. From this best microorganism strain, the equipment was sized on an industrial scale and it was concluded that the investment cost would benefit the country.
The study shows that it is possible to generate a suitable technology for the production of cellulolytic enzyme crudes in Ecuador.

Keywords: Cellulases, Bacillus, enzymatic cocktail, bagasse, sugar cane

## Introduction

Microorganisms play an important role in the carbon cycle as well as cellulolytic enzymes are essential for the degradation of the cell walls of plants. However, there are limitations to understanding the functioning of this process: the vast diversity of microorganisms in the environment and the complexity to cultivate them (Gomashe et al., 2013).

The most studied microorganisms for industrial purposes are fungi and bacteria, because generally, they are groups that present extracellular enzymes secreted in the medium in which they are cultivated (Gobat Jean & Willy, 1998).

Enzymes are biological catalysts that come from different environments, but most of them are of microbial origin (Prabha et al., 2007). There are two types of enzymes: extracellular and intracellular. The extracellular or exoenzymes, are synthesized inside the cell, forming a coctkail or crude enzyme that are subsequently secreted by the metabolism that allows the cell to make exchanges with the environment in which it lives. The intracellular ones are synthesized and used inside the cell or they are linked to subcellular particles or intracellular membranes, and their extraction is more difficult.

To elucidate the mechanism in which they act is complex, since they do it through the contact of the substrate with the enzyme through the aminoacids of the active site. To collect these enzymatic cocktails, releasing processes are carried out, such as cell lysis for the extraction or collection of the enzymatic crude from the medium in which the microorganism is found (Gobat, 1998).

Vegetable biomass is the most abundant renewable resource on the planet; this is an attractive source for obtaining bioenergy and biochemical products. It is composed of lignin, cellulose and hemicellulose (Gonzalo de Gonzalo, 2016). The raw material, in which these studies are carried out, does not compete with the food biomass.

The largest fraction of organic matter in terrestrial ecosystems is made up of cellulose. Soil microorganisms are the main responsible for transferring carbon from cellulose and other compounds to the atmosphere, but their contribution is not exactly known (Hairchar, 2007). One of the agro-industrial products with a large amount of cellulose is the sugarcane bagasse, which is a residue of the

stems after juice extraction, which is generally used in sugar factories as boiler fuel (Pandey et al. ., 2000).

Due to the potential of biomass as a renewable source for the production of fuels and chemical products, with the modern metagenomic techniques, several studies have been carried out to understand the cellulose-degrading microorganisms. However, the mechanism of degradation of cellulose is still not clear and what microbial communities carry out this work. Those are important reasons for increasing research in these areas (Wilson, 2011).

In addition to reducing the volume of agro-industrial or urban lignocellulosic waste by 40%, the cellulose-degrading microorganisms diminish the problems of treatment, management and risk of urban, agricultural and forestry waste.

The objective of the study was to find microorganisms native to Ecuador capable of producing cellulolytic enzymes that can be used on an industrial scale for bagasse degradation and to perform the analysis of the cost of investment and total production.

## **Materials and Methods** *(optional), no page limit*

### Collection of lignocellulosic material

Remnants of lignocellulosic material were supplied from an artisan *panela*[1] factory in Balzapamba (foothills of Los Andes), in the reforestation program of Petroproduction (Amazonia) and around Pedro Vicente Maldonado Station (Antartida). The samples collected in the aforementioned sites were washed with distilled water, in the same way as for the study of microbiota in other species (Lauer et al., 2007). The microbiota was then analyzed using techniques that are commonly used for the analysis of cellulolytic microorganisms (Salvador et al., 2012). For this, 100 grams of lignocellulosic substrate was placed in 1000 ml of peptone water, at 400 rpm, for 20 minutes. The solution with the microorganisms was heated at 80 ° C, for sporulation and were sown in petri dishes. Dilutions of 1:10 were made seven times.

### Obtaining microorganisms

40 µl of dilutions 4,5 and 6 from the stock solution were sown, they were observed for seventeen days in ACP medium (Agar Count Plate) and PDA (Potatoe Dextrose Agar). The number of colony forming units (CFUs) per cm2 was counted. They were also sown in nutritious broths under the temperature conditions of ± 37˚C for bacteria and at ± 25˚C for fungi until strains growth was observed. The colonies obtained by exhaustion were isolated and purified. Pure strains were stored in cryo tubes at -80 ° C in the strains collection at the University of the Americas in Quito, Ecuador.

### Production of cellulase enzyme

The isolated sporulating strains were cultured in CMC medium (Carboxymethylcellulose, which is a derivative of cellulose). Petri dishes that had halos of degradation (which are clear zones), were

[1] It is a sweetener derived from the sugarcane juice

searched using the Congo Red Test. For this, 1% carboxymethylcellulose was placed as the sole carbon source (Sherief et al., 2010). The boxes were incubated at 25°C for 36 hours.

**Obtaining the enzymatic cocktail**

The supernatants with the cells were harvested at 84 hours, by centrifugation at 4000 rpm for 10 min and finally used in the cellulolytic tests (Shi et al., 2011). LB Broth was not put as indicated by Shi (2011) but another source of carbon, which is crystalline cellulose, was added, because the substrate, recommended by the author, had problems when using the 0.45 μm membrane to filter and obtain the enzymatic cocktail and separate it from the microbial biomass. In addition, because both were added in the qualitative and quantitative tests to establish their performance as a microbial cell or their role as an enzymatic cocktail, and to select whether it was better to work with the whole microorganism in the degradation process or to work with the cocktails that it produced.

**Tests with the bacteria and with the enzymatic cocktail. Qualitative and quantitative cellulolytic activity**

The test was carried out according to the principles of microbiology (Andro et al., 1884), in which colonies were transferred with a calibrated loop, after 48 hours of colonies incubation the Petri dishes were imbibed with Congo red at 1 h. % for 15 minutes. The result of the incubation was washed with a 1M NaCl solution and clear areas were evidenced that indicated the hydrolysis of the cellulose.

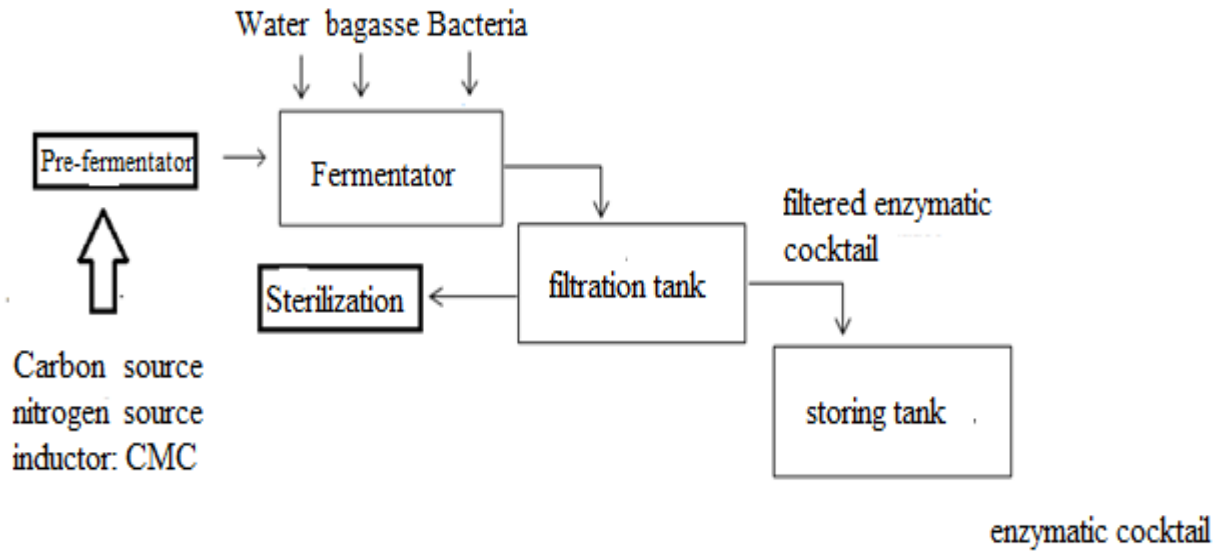
This test shows only the presence or absence of the activity. The strains that presented the halo of degradation were selected (Figure 3). For the quantitative tests, an approximate concentration of 5.67 * 10 EX9 CFU / g of total microorganisms was inoculated in each of the tubes prepared with the substrates to measure endo, exo glucanase and filter paper (Lin et al., 2009). The reaction contained 50 μl 0.5% CMC (Sigma), 100 mM citrate buffer (pH 4.8) and 50 μl of solution collected with enzyme. In addition, filter paper activity was determined and calculated according to the methodology established by Adney and John Baker (1996). All the tests described to determine endoglucanase, exoglucanase and filter paper activity showed the presence of glucose with the dinitro salicylic acid method (to show glucose). According to these tests, a microorganism was selected that was evaluated for the enzymatic hydrolysis of bagasse.

**Obtaining the strains in a pre-fermenting flask**

The *Bacillus sp.* strain was inoculated into the 500 ml pre-fermenting flask, placed in a medium with the following carbon sources: 3.39 grams of bagasse, 1 gram of Carboxymethylcellulose and 1.46 grams of whey. It was filled with 333.3 ml of drinking water. The medium was sterilized.

**Obtaining strains in the fermenting flask**

The 1: 1: 1 ratio of the bacteria produced in the pre-fermentor, sugarcane bagasse and drinking water was put in the 1000 ml fermentor flask. The medium was sterilized.
The diagram showing the scheme for obtaining the enzyme is shown below (Figure 1)




Block diagram of the production of the enzymatic cocktail of *Bacillus sp* bacteria, Source: Elaborated by the author (2018)

**Enzymatic hydrolysis in bagasse**

**Pretreatment of lignocellulosic material**

**Raw material**

Sugarcane bagasse (60% w / w) was collected in Puyo-Ecuador. It was crushed to a size of 1.5mm to be used in the experiments. The composition of the material in relation to the percentage of dry matter was glucan, 49.0%; xylan, 15.6%; lignin, 24 27.2%.

**Acid hydrolysis**

In this treatment, 500 grams of bagasse, 1.25% of sulfuric acid (w / w) were used and autoclaved for 40 minutes at 134 ° C and 2 atm pressure. The ratio of bagasse and sulfuric acid was 1:10. The liquor was collected and the sample was washed with water in a 1: 1 ratio and was filtered.

**Basic hydrolysis-organosolv**

After filtration, the cake was obtained to which 30% ethanol and 7% NaOH concentration was placed in dry fiber (Dimitrios et al., 2015). The bagasse-Na OH ratio is 1: 7, and it was placed in the autoclave at 174ºC for 90 minutes. The pretreated solid was washed with water to remove the ethanol and alkali, dried for four hours at 40 ° C, and the sample was analyzed to see the remnants of glucose, xylose and lignin content (Sidiras and Sapala, 2015; Mesa et al. al., 2010)

**Enzymatic hydrolysis**

The microorganism that had been previously selected was evaluated in soluble natural substrate (sugarcane bagasse) in an Erlenmeyer flask containing 1:2 ratio of bagasse pretreated in citrate buffer with the bacterial enzymatic cocktail and 0.5 g of tween 80 ( Donglin et al., 2017); the pH of 4.8 was maintained. For the hydrolysis, a temperature of 35°C and agitation speed of 200 rpm was used.

**Glucose measurement**

After the hydrolysis, the content of glucose and other sugars was measured by HPLC through the Sugar Pack 2017 method and the filter paper units as described above.

**Molecular Identification**.-The species were determined through the study of DNA that was extracted from approximately 2X10Exp 9 bacteria (in 1 ml of distilled water) that were in nutritious broth (Difco) using the PureLink Genomic DNA Kits for purification of genomic DNA (Invitrogen). 50 µl of final volume per reaction, containing 10 µl of DNA at a concentration of 5ng, were amplified in a Thermal cycler Multigene Gradient (Labnet International, Inc.). 50 µl of final volume per reaction, containing 10 µl of DNA at a concentration of 5ng of each first species, 0.20mM of dNTPs of 1.5mM MgCl2 and 1.25 U Taq Polymerase (Invitrogen). The first used for the sequencing of the 16SDNAr region are 8F AGAGTTTGATCCTGGCTCAG Universal Turner et al. 1999and 1492R (s) GGTTACCTTGTTACGACTT Universal Lane et al. 1991

The amplified fragments were verified running by electrophoresis in agarose gels. PCR was purified using the Pure Link PCR Purification Kit (Invitrogen). Forward and Reverse were aligned using the Heracle Biosoft program (2010) and Bioedit (2009). The sequences present in the organisms were aligned to organisms present in the Database GenBank database (January 2018), using the Basic Local Alignment Search Tool (Blast) of the National Center for Biotechnology Information

**Economic technical analysis**

It was considered to produce 50,000 L of ethanol per day to perform the technical-economic analysis of the scaling. The conditions were the same as those that were maintained in this research in the pre-fermentor and fermentor stage. From these data, it was raised to produce 2,320 liters of bacteria per day, with an enzymatic cocktail production of 1.72 tons in 15 hours.

## Results and Discussion

Isolation of sporulating microorganisms

The amount of microorganisms in the sample obtained in Los Andes comes from a site with high anthropogenic activity, since there is a factory producing *panela* in this area, which is why the species found are characteristic of this site and do not have pathogenicity. The sample obtained in Amazonia has an ecological niche with much defined functions, therefore it required more specific conditions for its growth, while the sample from the Antartida might possibly arrived through the tides, and was attached to an alga. This could reach Antartida by dragging all the content of the material from the continent. The growth of the microorganisms in the sample was slow. The sporulating species (interesting for the industry) are shown in the table below (Table 1):

Table 1. Amount of sporulating microorganisms (that tolerate stress conditions in the industry). Source: elaborated by the author (2018)

| Región | Total quantity of microorganisms | Organisms esporulate |
|---|---|---|
| Andes | 2,8 exp 5 | 2,2 exp 5 |
| Antártida | 1,3 exp 5 | 0,8 exp 3 |
| Amazonía | 1,3 exp 4 | 1,2 exp 4 |

Source: elaborated by the author (2018)

After the sporulation tests were carried out, it was observed that the strains of bacteria and fungi collected in the foothills of Los Andes and Amazonia grew, after 24 and 36 h of incubation respectively; however, microorganisms in Antartida grew at 13 days. The waiting time to observe the growth of the Antartida strains is a negative factor to be considered while working with these strains because of the waiting time of the activation of the metabolism and the possible variability of it. The other strains of the continent environments showed a normal expected behavior in relation to the growth time.

**Qualitative tests for the production of cellulases**

Each of the 17 sporulating strains analyzed was evaluated three times to determine which of them degraded more the medium containing CMC and its association with the degradation rate. According to the expressed data, the *Trichoderma* fungus produces a greater degradation, but records a slower degradation rate. Then the strains of bacteria from the Amazonia (Y3), (Y2) and Balzapamaba (BAL3) with high degradation rates are degraded. In the particular case of Y3 and BAL3, there is a high degradation but greater variability in the results is observed (Figure 2).

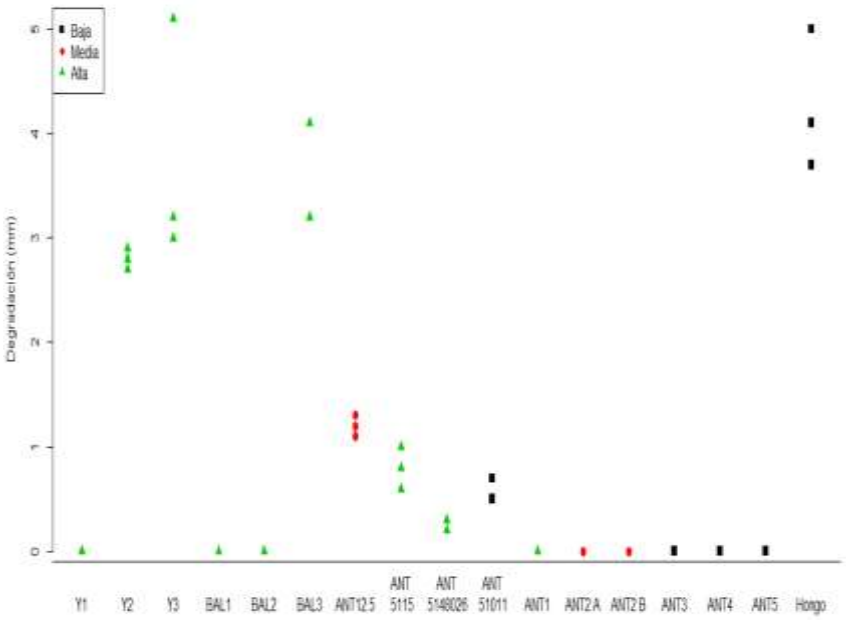



Figure 2. Degradation of cellulolytic strains
Source: Elaborated by the author (2018)

To evaluate the differences, the 95% confidence intervals for average degradation were constructed. Figure 4 shows how the greatest degradation is registered in *Trichoderma sp*., followed by the bacterial strains Y3 and BAL3, but in fact, the variability of these last strains shows heterogeneous results.

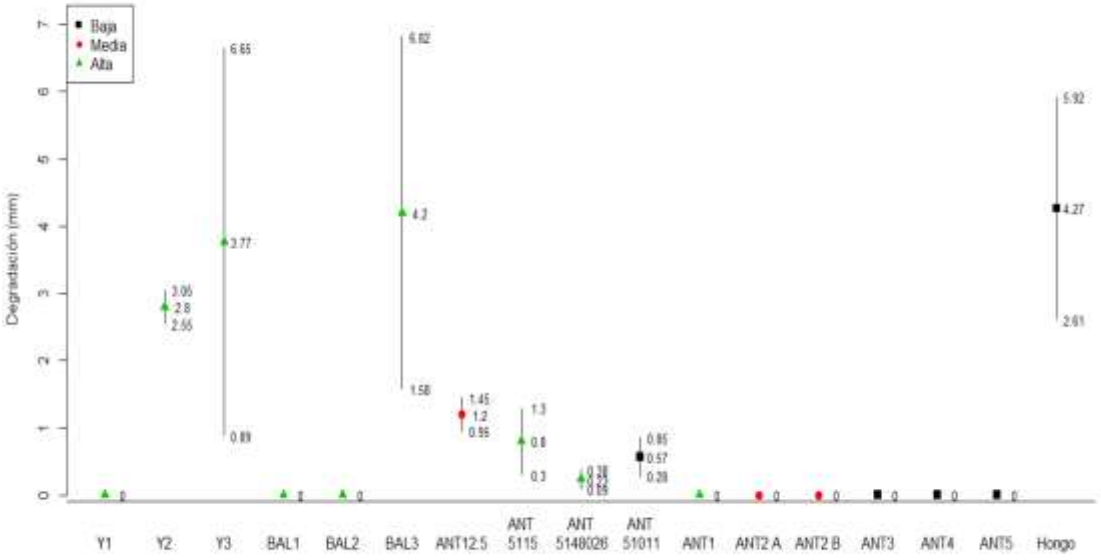



Figure 3. Average strain degradation and 95% confidence intervals
Source: Elaborated by the author (2018)

It is shown that the best strains in relation to degradation are those from Amazonia (Y3) and Balzapamba (BAL3 and H1).

**Quantitative tests for the production of cellulases**

It was found that enzymatic cocktails have higher endoglucanase, exoglucanase and filter paper activity than microorganisms. The results obtained for UFP are 0.0073 UFP for *Bacillus* bacteria (Bal 3); 0.0041 UFP for *Trichoderma* sp., and 0.0012 UFP for *Actinomycete* (Ant 12). The glucose concentration produced in the endoglucanase tests are: 3,45 mg / ml for *Bacillus sp*. Bacteria (Bal 3); 2,076 mg / ml for *Trichoderma sp*., and 1,53 mg / ml for Actinomycete.

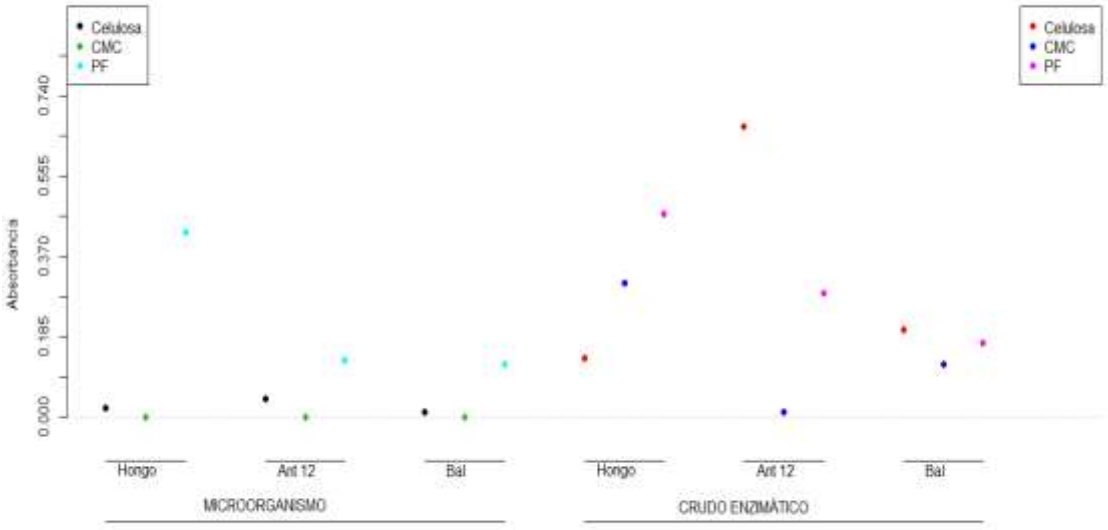



Figure 4. Average strain degradation and 95% confidence intervals.
Source: Own elaboration (2018)

### 3.4 Enzymatic hydrolysis

The bagasse that was used for the hydrolysis had a cellulose percentage of 37%. On this basis, the yield was calculated, 11.9% for Bacillus bacteria (Bal3) from a factory that produces *panela*; 0.81%, for *Trichoderma sp*., (Fungus) and 0.27% for the Actinomycete from Antartida (Ant 12). These results are promising considering that the biotechnological conversion of different carbohydrates, including glucose, are the basis of the production of ethanol, carbohydrates and aromatics (Kawaguchi et al., 2016).

Enzymatic cocktails that have cellulolytic enzymes can be isolated from autochthonous microorganisms, which demonstrates that this would allow to hydrolyze the bagasse cellulose to be applied in situ in a process of environmental industrial degradation, such as for the production of second-generation ethanol.

### Economic Technical Analysis of *Bacillus sp*.

The economic technical analysis of *Bacillus sp.* was achieved by scaling up a technological laboratory process with an approximate production cost. For the Scale –Up, the conditions produced by the *Bacillus* species (Bal 3) were used.

The cost estimates of the investments were made by the methods recommended by Peter and Timmerhauus, 1981)

Table 2. Values for investments for production.

| **Calculating the value of investments for the production of enzyme cocktail of bacteria Bacillus sp.** | | | |
|---|---|---|---|
| N | Concept | estimated USD | amount USD |
| I | Direct costs (CD) | A + B + C + D | 448796 |
| | A. Equipment + others | S (1 to 5) | 398651 |
| | 1. Acquisition costs of equipment (CE) | 571,776.00 | 250,724.00 |
| | 2. Installation including insulation and paint. | 35% CE | 87753 |
| | 3. Installation of instrumentation and control | 6% EC | 15,043 |
| | Pipe 4.Installation | 10% EC | 25,072 |
| | 5. Electrical installations | 8% CE | 20,058 |
| | B. Buildings | 10% EC | 25,072 |
| | C. Facilities and Services | 10% EC | 25,072 |
| | D. Land | 0% | 0 |
| II | indirect costs (CI) | A + B + C | 80311 |

| | | | |
|---|---|---|---|
| | A. Engineering and supervision | 5% CD | 22,440 |
| | B. Construction and contracting costs | 7% CD | 31416 |
| | C. Contingency | 5% IFC | 26455.3408 |
| III | Invested Capital fixed (IFC) | I + II | 529107 |
| IV | Working capital | 10% CTT | 58,790 |
| V | Working Capital Total (CTT) | III + IV | 587896 |

Source: Own elaboration (2018)

**Determination of investment values**

For the calculation of equipment production costs, real values of industrially installed equipment with similar characteristics were used, updating the values through the annual cost indexes for equipment, which are included in technical scientific literature (Peters and Timmerhauss, 1981). All the values obtained have also been updated (Peters and Timmerhauss, 1981). In addition, they were estimated with the help of the Rule of Point 6 (Peters -Timmerhaus, 1981), with which they were adjusted to the year 2018, using the idea proposed by Aden et al. (2002), to predict the annual cost index for that year by adjusting the annual data since 1957 (González and Castro, 2012), as shown in Figure 6.

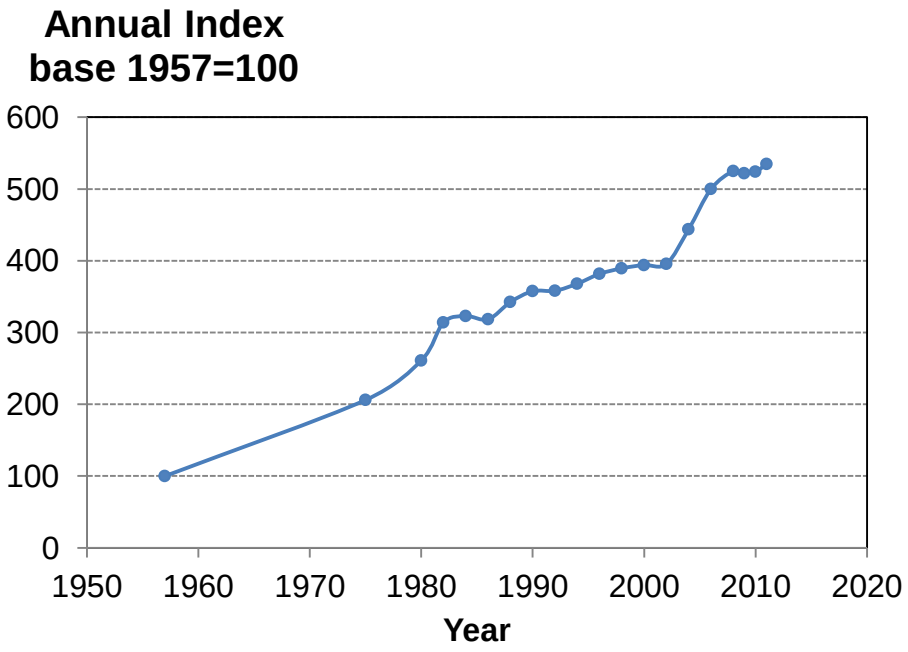



Figure 6. Graph of the evolution of the Chemical Engineering Cost Annual Index 1975-2011 and forecast. (González and Castro, 2012).

Table 3. Total Cost of Bacterial Enzyme Production

| Total Production Cost | Price $ / UM | Quantity | UM | Cost M $ / year |
|---|---|---|---|---|
| Manufacturing costs I (A + B + C) | | | | 444289 |
| A Direct Costs | | | | 296789 |
| Prima 1.Materia | | | | 175414 |
| chaff | twenty-one | 317515 | Kg / a | 7260 |
| nutrient broth | 500 | 3062.4 | Kg / a | 165000 |
| Bacterium | 1654 | 765.6 | The | 1654 |
| Water | 0,001 | 1405800 | The | 1500 |
| 2. Work operation | 10% CTP | | | 85000 |
| 3. Direct supervision work operation 10% | 10% 2 | | | 8500 |
| 4.Utilidades and services | | | | |
| Water | 0,001 | 16000 | The | 240 |
| 5.Mantenimiento and repair CFI% | | | | 10,582.13632 |
| 6. Supplies of 5% | | | | 52.9106816 |
| 7. Charges laboratory% 2 | | | | 8500 |
| 8. Patents% CTP | | | | 8500 |
| B Fixed Charges | | | | 85000 |
| 1. Depreciation 10% IFC | | | | 52910.6816 |
| 2. Local rate 01.04% IFC | | | | 52911 |
| 3. Tax 0.4-1% CFI | | | | 2116.427264 |
| C. 5-15% CTP INDIRECT COSTS | | | | 42,500 |
| II. Overheads (A + B + C) | | | | 403289 |
| A .. Distribution and sales% CTP | | | | 17000 |
| B. Administration% | | | | 17000 |

| | | | | |
|---|---|---|---|---|
| CTP | | | | |
| C. Research and Development% CTP | | | | 17000 |
| D. Financial Interests, IFC 0-7% | | | | 5291.06816 |
| III. Total production cost (I + II) | | | | 850000 |

Source: Elaborated by the author (2018).

It is observed in Table 3 that the production cost is $ 850,000 per year is divided by 50,000 liters of ethanol per day and for 330 days, and then the cost of enzyme production per liter of ethanol is $ 0.0515. In relation to the commercial enzyme, its cost is $ 0.059 per liter of ethanol (Salvador et al, 2018), which means a saving of $ 0.0075 per liter of ethanol produced. This is a saving of $ 123750.00 per year.

In addition savings are achieved in investment of foreign currency expenditures for unnecessary purchases because the necessary supplies are achieved in Ecuador of $517,325.12 (Table 4, Table 5), which are also relevant because it is not the same expense inside the country than abroad (Cunningham, 2005).

Table 4. Investment savings

| **Investment for the production** | **Cost in USD dollars** |
|---|---|
| Total Investment | 587896 |
| Expenses | 70570,88 |
| Savings | 517325,12 |

Raw materials are saved by using local production inputs for a value that is reflected in Table5.

Table 5. Savings of raw materials.

| **Raw material** | **Cost in USD dollars** |
|---|---|
| Bagasse | 7260,00 |
| Bacteria | 1654,00 |
| Water as raw material | 1500,00 |
| Water for the general use | 240,00 |
| Whey and sugarcane bagasse | 77220,00 |
| **TOTAL** | 87874,00 |

Overall, an annual import saving of $ 691,649 is achieved, which allows determining the recovery of the investment in relation to expenses abroad (Table 6).

Table 6. Values of investments for production.

| | Price in USD dollars | Percentage % |
|---|---|---|
| Suplies | 850.000 | 100 |
| Imported | 158.351 | 18,63 |
| Domestic | 691.649 | 81,37 |

Source: Elaborated by the authors (2018)

**Conclusions**

1. To obtain the degradation of lignocellulosic residues in the enzymatic hydrolysis stage, extracellular enzymatic cocktails are better, unlike the use of microorganisms in the substrate in which the culture medium hydrolysis usually takes place.
2. A bacterium of the genus *Bacillus* that showed the cocktails with high levels of degradation was isolated from the 17 analyzed microorganisms. This shows that it is feasible to investigate microorganisms that produce a cocktail of autochthonous enzymes for the production of cellulolytic enzymatic crudes in Ecuador.
3. Biodiversity is a sustainable source that generates value-added products that can provide technological independence.
4. It was determined through technical economic analysis that in the production process of native *Bacillus* enzymes, savings are achieved in the costs of ethanol production and investment, which have a positive impact on the Ecuador national economy.

**References**

1) Adney Bill y John Baker, 1996. Measurement of Cellulase Activities. ChemicalAnalysis and TestingTaskLaboratoryAnalyticalProcedure.
2) Andro T, Chambost JP, Kotoujansky A, Cattano J, Barras F (1984) Mutants of Erwiniachrysanthemidefective in secretion of pectinase and cellulase. J Bacteriol., 160:1199-1203.
3) Brevick, EC; cerdá, A; J Mataix-Solera:Pereg, L;Quinton, JN:et al.,Soil Gottingen Arquitectura de los suelos..cuantificación delos microorganismos.
4) Cunningham Bosco, R., M. A. Laborde Dominguez, E. Gonzalez Suarez. (2002) La gestion deproyectos en lagerencia de conocimientos para eluso de la biomasa como fuente deproductos químicos y energia.I Encuenrro Internacional de Gestion de Tecnologias.Caracas.2002
5) Dimitrios K. Sidiras. Y Ioanna S. Salapa, 2015. Organosolv Pretreatment as a major step of lignocellulosic biomass refining. Engineering Conferences International ECI Digital Archives. Biorefinery I: Chemicals and Materials From Thermo-Chemical Biomass Conversion and Related Processes. Follow this and additional works at: http://dc.engconfintl.org/biorefinery_I Part of the Chemical Engineering Commons.
6) Donglin Xin , Ming Yang, Xiang Chen, Ying Zhang, Li Ma, and Junhua Zhang Improving the Hydrolytic Action of Cellulases by Tween 80: Offsetting the Lost Activity of Cellobiohydrolase Cel7A. 2017.College of Forestry, Northwest A&F University, 3 Taicheng Road, Yangling 712100, Shaanxi, China*ACS Sustainable Chem. Eng., 5* (12), pp 11339–11345. DOI: 10.1021/ acssuschemeng.7b02361Publication Date (Web): October 19, 2017. http://pubs.acs.org/doi/abs/10.1021/acssuschemeng.7b02361

7) Gobat, J., ;M. Aragno and. W. Matthey. 1998. Le sol vivante. Bases of pedolologie. Biologie des sols. Laussanne.
8) González Suárez, E y Castro Galiano, E. (2012)
9) Gomashe ,Gulhane PA and Bezalwar PM. Isolation and Screening of Cellulose Degrading Microbes from Nagpur RegionSoil.Int. J. of Life Sciences, 2013, Vol. 1(4): 291-293 ISSN: 2320-7817| eISSN: 2320-964X.
10) Gonzalo de Gonzalo, Dana I. Colpa. 2016. Mohamed H.M. Habib, Marco W. Fraaije. 2016. Bacterial enzymes involved in lignindegradation. Journal of Biotechnology 236 (2016) 110–119.
11) Hairchar Fz. 2007. Identification of cellulolyticbacteria in soil by stable isotope probing. Environ Microbiol. Mar;9(3):625-34.
12) Kawaguchi, H., Hasunuma, T., Ogino, C., Kondo, A., 2016. Bioprocessingofbio-basedchemicalsproducedfromlignocellulosicfeedstocks. Curr. Opin. Biotechnol. 42, 30–39.Kim, S.J., Shoda, M., 1999.
13) Lauer A, Simon MA, Banning JL, Andre E, Duncan K, Harris RN(2007) Common cutaneous bacteria from the eastern redbacked salamander can inhibit pathogenic fungi. Copeia 3:630–640.
14) Liming Xia y Peilin Cen. Cellulase production by solid state fermentation on lignocellulosic waste from the xylose industry_Department of Chemical Engineering, Zhejiang Uni6ersity, Hangzhou 310027, People'sRepublic of China.
15) Lin, L.; Meng, X.; Liu, P.; Hong, Y.; Wu, G.; Huang, X.; Li, C.; Dong, J.; Xiao, L. and Liu, Z. (2009). Improved catalytic efficiency of Endo-β-1,4-glucanase from Bacillus subtilis BME-15 by directed evolution. Applied Microbiology and Biotechnology, vol. 82, no. 4, p. 671-679.
16) Mesa, L., Gonzáles, E., Cara, C., Ruiz, E., Castro, E., Mussatto, S. (2010). An approach to optimization of enzymatichydrolysisfromsugarcanne bagasse base don organosolvpretreatment. Journal TechnicalBioetchnology. 85: 1092-1098.
17) Mesa,L., E. Gonz´alez, C. Cara, M. Gonz´alez, E. Castro, S.I. Mussatto. 2010. The effect of organosolv pretreatment variables on enzymatic hydrolysis of sugar cane bagasse, *Chemical Engineering Journal* (2010),doi:10. 1016/j.cej.2011 .02.003.Cellulase production is the most important step in the economical production of ethanol, single cell protein and other chemicals from renewable cellulosic materials
18) PandeyAshok , Carlos R. Soccol a, PoonamNigam b, Vanete T. Soccol. 2000. Biotechnological potential of agro-industrial residues. I: sugar cane bagasse. Bioresource Technology 74 (2000) 69±80

19) Prabha, M., I. Jayaraaj and R. Jeyaraaj. 2007. Comparative studies on the digestive enzymes in the gut of earthworms, Eudriluseugeniae and Eiseniafetidaeugeniae .Indian Journal of Biotechnology. Vol 6, October 2007, pp 567-569 Vol 6, Octobre. P.p 567-569

20) Salvador Carmen Amelia, González Erenio, Mesa Leyanis, Rojas Maira, Batallas Fernando, Pérez-Martínez Amaury, Concepción Diana. (2018). Revista FIGEMPA. Empleo de biocatalizadores en la degradación de material lignocelulósico: Principales Impactos.

21) Salvador Pinos C. A. Mesa Noval, F. Batallas Merino, J. Villavicencio Montoya, L. Mesa Garriga, E. González Suárez.(2018). Evaluación de las mejores condiciones de operación en la hidrolisis enzimática del bagazo pretratado para etanol de bagazo. Ponencia. MOL2NET, International Conference Series on Multidisciplinary Sciences http://sciforum.net/conference/mol2net-03

22) Sherief, A. A., El-Tanash, A. B., and Atia, N. 2010. "Cellulase production by *Aspergillus fumigatus* grown on mixed substrate of rice straw and wheat bran," Research Journal of Microbiology. 5(3):199–211.

23) Shi, Q.Q.; Sun, J.; Yu, H.L.; Li, C.X.; Bao, J. And Xu, J.H. (2011). Catalytic performance of corn stoverhydrolysis by a new isolate Penicillium sp. ECU0913 producingboth cellulase and xylanase. AppliedBiochemistry and Biotechnology, vol. 164, no. 6, p. 819-830.

24) Wilson DB. Microbialdiversity of cellulose hydrolysis, CurrOpinMicrobiol (2011), doi:10.1016/j.mib.2011.04.004