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Selection of *Bacillus* Strains Producing Enzymes with a Potential for Animal Nutrition

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ABSTRACT

Background: The ever-growing awareness of environmental problems has encouraged the implementation of ecological alternatives in industrial production. Cellulases and hemicellulases are the most commonly used enzymes in industry, guaranteeing the effectiveness of production, with a reduction in chemicals that pollute the environment. **Aim.** To select *Bacillus* spp. strains that produce cellulases, β-mannanases, and xylanases for use in animal nutrition. **Materials and methods:** Overall, ten strains were studied in plates with minimal medium supplemented with beechwood xylan, algarroba gum, and carboxymethylcellulose for the production of xylanases, β-mannanases, and endocellulases, respectively. The plates were incubated at 37 ºC for 24 hours and were coated with 0.5% Congo red solution. The diameter of the hydrolysis area, and that of the colony, were determined to calculate the potency index. **Results:** All the strains showed the capacity to produce study enzymes. As a result, 23.3% of enzymatic activity was considered good. *Bacillus subtilis* E-44 was the strain with the best enzymatic activity in the three substrates evaluated (3.01 ± 1.18; 3.82 ± 0.31, and 4.22 ± 0.23) for cellulases, β-mannanases, and xylanases, respectively. **Conclusions:** *Bacillus subtilis* E-44 was picked as the best cellulase and hemicellulase-producing strain, increasing their possibilities in animal nutrition and the food industry.

Keywords: β-mannanases, bacterium, endonucleases, hemicellulase, xylanases (*Source: MeSH). MESH*)

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INTRODUCTION

Awareness of environmental threats has favored the implementation of ecologic strategies in industrial processes (Angural *et al*., 2020). Technology that conceives the utilization of enzymes ensures these precepts since they encourage the efficient use of raw materials with minimum residue generation, and a reduction of contaminating chemical compounds (Danilova and Sharipova, 2020).

The demand for enzymes and their application in industry is constantly growing. In 2021, the world enzyme market produced 6.4 billion dollars. This value is expected to grow up to 8.7 billion by 2026, with an annual growth rate of 3.6% (Benatti and Polizeli, 2023). Cellulases and hemicellulases (mannases and xylanases) are the most commonly used enzymes worldwide, particularly in the food industry, with a remarkable demand accounting for over 30% of the market (Cann *et al*., 2020).

Cellulases, mannnases, and xylanases can degrade glycosidic bonds between the carbohydrates present in their corresponding polymers. They belong to the family glycosyl hydrolases, according to the site of carbohydrate-active enzymes (Carbohydrate- Active Enzymes Database CAZy: http://www.cazy.org/) (Gonzalez-Gonzalez and Miranda- Lopez, 2022). They are also known as fibrinolytic enzymes or carbohydrases (Gusakov *et al*., 2011; Mousa *et al*., 2022).

Cellulolytic and hemicellulolytic enzymes are mainly obtained from microorganisms, particularly *Bacillus* strains, by biotech companies (Su *et al*., 2020). The bacteria from this genus are Gram-positive bacilli that form non-synthesizing endotoxin endospores, a reason why many species are considered safe. These microorganisms have an efficient enzymatic secretion system that allows them to degrade a variety of substrates that help them survive in different settings, making them excellent industrially important enzymes. These fast-growing bacteria have shorter fermentation times than other hydrolase-producing microorganisms (Blibech *et al*., 2019). Approximately 50% of the total enzymes in the market are obtained from de *Bacillus* (Sulistiyani *et al*., 2021).

However, the production of these catalyzers depends on the environmental conditions that host these microorganisms, so it is important to mention that every *Bacillus* does not always synthesize the same type of enzymes. Hence, it is important to make the proper selection of microorganisms depending on the substrate. Accordingly, this study aims to select *Bacillus* strains that produce cellulases, β-mannanases, and xylanases for use in animal nutrition.

MATERIALS AND METHODS

Bacterial culture and pre-inoculum

The study used ten strains from the genus *Bacillus*, which were isolated at the Microbiology Laboratory, the Faculty of Agricultural Sciences at the University of Matanzas. The samples were stored at -30 °C in nutrient broth at 20%, then Erlenmeyers containing 50 mL nutrient broth were

inoculated and incubated at 37 °C in an orbital shaker at 110 rpm for 16 h with 600 nm optical density to reach 0.8^{-1}.

Culture medium

The assay was performed on Petri dishes containing minimal medium (MM) including NaCl (0.1%), KH_2PO_4 (0.3%), K_2HPO_4 (0.6%), $MgSO_4$ (0.12%), peptone (0.5%), yeast extract (0.3%), and agar (1.5%). The plates were supplemented with carboxymethylcellulose (1%), algarroba gum (0.5%), or beechwood xylan (1%). The mean pH was adjusted to 7.5 using KOH (1 mol·L^{-1}) and was sterilized at 121 °C for 15 min. The reagents were purchased from Sigma-Aldrich.

In vitro determination of enzymatic production of xylanases, mannanases, and cellulases

The strains were inoculated using a sterile loop on the culture medium' s surface, then the plates were incubated for 24 h at 37 °C. Cellulase and hemicellulase synthesis was performed by bacteria, adding a Congo red solution (0.5%) to each plate, and left to rest at room temperature for five minutes, and washed with $NaCl^{-1}$ mol L^{-1}. The potency index (PI) was estimated from the ratio between the hydrolysis area and the diameter of the colony measured in millimeters. According to the PI, the microorganisms evaluated were considered excellent (IP > 5.0), good (2.0 > IP < 5.0), and poor (IP < 2.0) (Latorre *et al*., 2016). A Vernier gauge caliper (Stuertekcap, ± 0.02 mm) was used to measure the diameters under a completely randomized design with four repetitions for every enzyme.

Statistical analysis

The data were processed through Statgraphics Plus 5.0. A one-way analysis of variance was performed to determine the presence of statistically significant differences between the strains and the substrates evaluated. The Student-Newman-Keuls test was performed for mean contrast. Significance was set for $p < 0.05$. The data were shown as the mean ± standard deviation.

RESULTS

Ten strains of *Bacillus* were studied to evaluate the capacity to produce hydrolytic enzymes for research and industry. The results showed that 100% of the bacteria evaluated could produce endocellulases, β-mannanases, and xylanases. According to the results of the analysis, strains E-44 and C-31 were classified as good producers of the three enzymes. Likewise, strain 45 BP was considered a good producer of β-mannanases. The other bacteria in the study showed lower results in terms of relative enzymatic activity, thus classifying as poor. TTable 1 shows the enzymatic activity profile of the strains studied.

Table 1. **Values of potency indexes of *Bacillus* sp. strains evaluated for xylanase, β-mannanase, and cellulase production.**

***Bacillus* strains**	**Cellulases**	**β -Mannanases**	**Xylanases**
5 BP 1	1.31 ± 0.05^{de}	1.48 ± 0.08^{de}	1.44 ± 0.14^{de}
23 BP4	1.74 ± 0.05^{c}	1.51 ± 0.20^{de}	1.63 ± 0.17^{cd}
45 BP5	1.49 ± 0.06^{cd}	$2.03 \pm 0.09^{c*}$	1.90 ± 0.07^{c}

48 BP6	1.48 ± 0.09^{cd}	1.66 ± 0.04^{de}	1.68 ± 0.09^{cd}
54 BP7	1.16 ± 0.11^{e}	1.63 ± 0.10^{de}	1.41 ± 0.11^{de}
55 BP8	1.69 ± 0.21^{c}	1.57 ± 0.16^{de}	1.68 ± 0.12^{cd}
E-44 9	3.01 ± 0.18^{a*}	3.82 ± 0.31^{a*}	4.22 ± 0.23^{a*}
C-31 10	2.41 ± 0.22^{b*}	2.55 ± 0.25^{b*}	2.55 ± 0.25^{b*}
12 BCm11	1.13 ± 0.07^{e}	1.34 ± 0.11^{e}	1.60 ± 0.15^{cd}
6 BCm12	1.12 ± 0.14^{e}	1.76 ± 0.14^{d}	1.22 ± 0.14^{e}

The data represent the mean ± standard deviation with four repetitions. Different scripts in a column represent statistically significant differences according to the Student-Newmn-Keuls for $p< 0.05$. Strains marked (*) are considered good producers of the enzyme.

The potency index calculated for the enzymes produced by *Bacillus* sp. E-44 was higher than the other strains studied. The analysis of variance that compared this factor showed statistically significant differences among them, depending on the strains studied, so it showed the highest activity of the three enzymes against the substrates evaluated and therefore was the best. The assay' s results to determine the enzymes produced by *Bacillus* sp. E-44 (**¡Error! No se encuentra el origen de la referencia.**) shows light areas around the microorganism in the plates containing xylan, algarroba gum, and CMC as substrate, indicating that the bacterium expressed enzymes that depolymerize the substrate present in the culture medium.

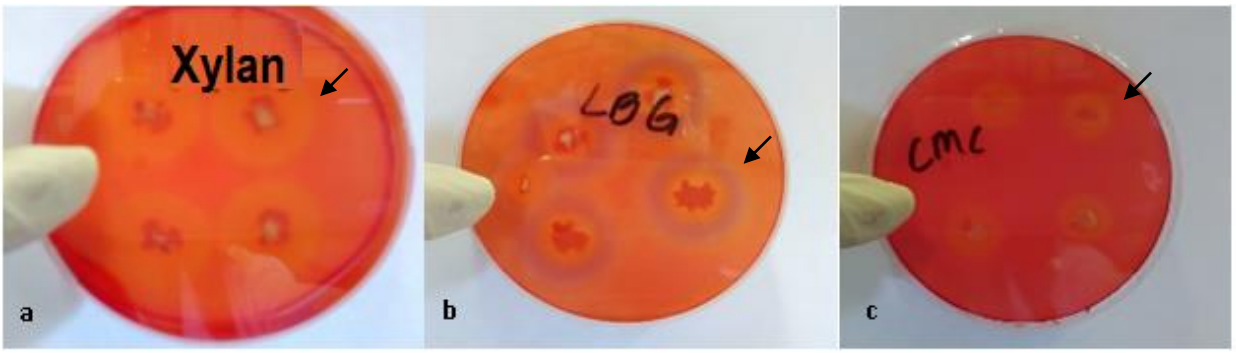

Figure 1 Hydrolysis halo made through the growth of *Bacillus* sp. E-44 on the media with (a) beechwood xylan, (b) algarroba gum, and (c) carboxymethylcellulose The arrows indicate the pale areas around the microorganism growth

DISCUSSION

The bioprospection of microorganisms with the capacity to produce enzymes with biotechnological and industrial interest is a constant concern of science. To make this search effective, fast, sensitive, replicable, and cost-effective work methods are necessary. The most commonly used qualitative technique is the one suggested by Teather and Wood (1982), based on the growth of microorganisms with a minimum of salts in the substrate of the enzyme to be evaluated and the presence of a depolymerizing reporter colorant (Moreno and Vélez, 2011).

This study used beechwood xylan, algarroba gum, and carboxymethylcellulose as the only source of carbon in the selective medium used, in combination with Congo red dye. This colorant interacts with the polymers in the medium and produces disaccharides, monosaccharides, and organic acids from degradation when the enzymes are excreted. The formation of a transparent halo around the colony results from a drop in the pH (Li *et al*., 2020).

As mentioned previously, the capacity of several species of microorganisms to degrade complex substrates is caused by the production of extracellular enzymes and largely depends on the carbon source used (Rodrigues *et al*., 2020). Cellulases, xylanases, and β-mannanases are inducible in the natural conditions by their action. Some authors say that they are expressed at low levels so they favor the production of low molecular weight fragments acting as their inducers. The regulation of these enzymes is strictly controlled by catabolic mechanisms of activation and repression, and would only secrete in the presence of a specific substrate and the existence of easily assimilated sugars will depress its production (Behera *et al*., 2017; Chauhan and Gupta, 2016).

Several authors use the potency index (PI) to evaluate the expression of microbial enzymes like cellulases (Li *et al*., 2020), xylanases (Latorre *et al*., 2015), and mannanases (Riaz *et al*., 2019). The relative enzymatic activity is like the potency index, according to Latorre *et al*. (2015). These authors used the same scale to select *Bacillus* sp. strains that synthesize cellulases and xylanases. The results favored their classification as excellent and good producers of the enzymes.

This indicator was also used to select the strain of *Bacillus velezensis* that excreted the largest amount of cellulases in a study that evaluated ten strains (Li *et al*., 2020). The results matched the findings of the current study, even higher. The potency index for the production of cellulases from *Bacillus* sp. E-44 was higher than the one reported by Ma *et al*. (2020). Likewise, the strain selected for this study showed higher potency indexes than *Bacillus subtilis* US 191 for mannanase production (1.60). (Blibech *et al*., 2019). However, the values recorded in this study were lower than the ones obtained by Zhang *et al*. (2018) for xylanase production in *Bacillus velezensis* ZY-1-1. Nevertheless, these data are similar to the previous authors in that xylanase activity was higher than that of cellulases, which according to them, is caused by the regulation mechanisms of genes codifying lignocellulosic enzymes, which require further investigation.

The *Bacillus* species are characterized by synthesizing diverse extracellular enzymes, but not all of them can do it with the same magnitude. Consequently, it could be a specific characteristic of the strain, regardless of the species, which largely depends on the origin of the microorganism (Latorre *et al*., 2016). Moreover, the literature collected shows that during growth, temperature and pH affect the final production of extracellular enzymes directly. Hence, various culturing conditions are optimized for the selection of biomolecules, confirming that the expression of these catalyzers is uniquely associated with growth adjustments (Liu *et al*., 2023).

The bacterium showing the best results in cellulase and hemicellulase synthesis is known as *Bacillus subtilis*, subspecies *subtilis*, and it was isolated from rotten tomato juice (Milián *et al*., 2014). This strain revealed features with the potential to be used as an additive with a probiotic

effect on animals with zootechnical interest (Milián *et al*., 2017). The results reported expand the possibilities of using *Bacillus subtilis* E-44 for the production of enzymes that improve the quality of fiber-rich foods, enhancing their probiotic effects (Luise *et al*., 2022). Cellulases, mannanases, and xylanases are commonly used in animal production (Sathitkowitchai *et al*., 2022).

In addition to the possible benefits for the nutrition of animals with zootechnical interest using the enzymes produced by this strain, they could also be used in other food items. It would increase the possibilities of industrial use. For instance, to improve the quality of fruit and vegetable juice, raise the production of instant coffee, soften the dough for bread, and diminish food rotting (Behera *et al*., 2017; Chauhan y Gupta, 2016; Kaur *et al*., 2021; Marimuthu *et al*., 2019).

Furthermore, complete hydrolysis of complex carbohydrates present in lignocellulosic demands mixed enzymatic activity. Accordingly, the inclusion of enzymatic cocktails is more suitable for purified enzymes, since it reduces costs, and processes are more efficient. In terms of animal nutrition, enzymatic cocktails increase the nutritional value of foods, affecting the neutral detergent fiber values (NDF) and acid detergent fiber (ADF), and increasing the total digestible nutrients (Weschenfelder *et al*., 2023). One of the most widely accepted strategies for the production of enzymatic cocktails is the utilization of microorganisms with the capacity to secrete two or more cellulolytic and hemicellulolytic enzymes (Angural *et al*., 2020). In that sense, it would be convenient to evaluate the potentiality of *B. subtilis* E-44 to produce enzymatic cocktails in the face of complex lignocellulosic substrates. Additionally, further research should tackle the quantification of enzymatic activities and the determination of enzymatic stability at different pH and temperatures. The findings of this study constitute a preliminary study and the first step to select a strain with the potential to produce biotechnological and industrial interest enzymes.

CONCLUSIONS

The evaluation of the capacity to produce cellulases and hemicellulases favored the selection of two strains as good producers. However, *Bacillus subtilis* E-44 showed higher secretion of cellulases, β-mannanases, and xylanases. It increases the potentialities of this enzyme for animal nutrition and its perspectives in the food sector.

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AUTHOR CONTRIBUTION STATEMENT

Research conception and design: YRF, MMMT, ALVA, YPH; data analysis and interpretation: YRF, MMMT, ALVA, YPH, ZRA; redaction of the manuscript: YRF, ALVA, MMMT, and ZRA.

CONFLICT OF INTEREST STATEMENT

The authors state there are no conflicts of interest whatsoever.