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PRODUCTION AND EVALUATION OF EXTRACELLULAR ENZYMES FROM *BACILLUS LICHENIFORMIS* IN DIFFERENT RAW MATERIALS USED IN ANIMAL FEED

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Abstract

The production of different enzymes, including amylase and protease by Bacillus licheniformis ATCC 21424 were tested on different raw materials and compound feed, which were used as a substrate in process fermentation: soybean meal, peas, sorghum flour, corn and compound feed (FC). The bacterial growth and enzyme production were done in a fermentative medium (Erlenmeyer 100 ml flash in a shaking incubator) and enzyme activity was registered after 24, 48 and 72 h, pH 7 ± 2). The inoculum strain presents 10.19 log CFU/ml at 37°C, 24 h, 150 rpm. The screening showed a capacity of amylase and protease strain production. The highest amylase activity was obtained when the strain was cultured in corn fermentation medium (19.43 U/ml), followed by soybean meal (18.31 U/ml), sorghum (17.52 U/ml), peas (19.43 U/ml), and FC (6.63 U/ml) at 72 h. Great protease activity was noticed in FC (97.75 U/ml), soybean meal (94.67 U/ml), sorghum (89.36 U/ml), corn (78.6 U/ml), peas respectively (75.91 U/ml). The observation of this study suggested that Bacillus licheniformis ATCC 21424 could be capable of producing protease and amylase enzymes, particularly in fermented medium contained soybean meal or corn, and can be administrated in animal nutrition as source of feed additive.

Key words*: Bacillus* spp.*, extracellular enzymes, enzymatic activity, raw materials.*

INTRODUCTION

The European Commission (EC) decided to stop the utilization of feed antibiotics as growth promoters in the animal live cycle (2006) due to their capacity to transfer resistance genes between bacteria decreasing the beneficial intestinal flora (Hmani et al., 2017).

Exogenous feed enzymes occur as an essential solution in animal nutrition by acting against antinutritional factors (e.g., β-glucans, pentosans, phytate) with positive effects on dietary components digestion (Slominsky, 2011; Ciurescu et al., 2020), digesta viscosity, nutrients utilization, pathogens inhibitor by equilibrating the host intestinal microflora (Chen et al., 2013; Dumitru et al., 2020a), improving, in the end, the productive animal performance (Ravindran, 2013).

Bacteria from *Bacillus* group can involve beneficial biotechnological products, with industrial applications, including a source of probiotics in animal nutrition (Dumitru et al., 2020a, 2020b; Ciurescu et al., 2020). Probiotics were well-defined as live cultures of bacteria or yeasts with positive actions in the host (Chen et al., 2013).

Due to their ability to synthesize enzymes, *Bacillus* spp. are known for the production of amylases and proteases, together representing more than 70% of the total important enzymes (Mukhtar & Haq, 2012). *Bacillus* spp. are used in industrial animal production, enhancing the absorption of the nutrients and reducing the *Salmonella* spp. (Ghorban Hosseini et al., 2018) and *Escherichia coli* biotype β-hemolysis infections during the gastrointestinal tract (GIT) (Dumitru et al., 2020a).

Blanco et al. (2016) affirmed that optimization of the fermentation process is based on the selection of a suitable culture medium including substrate composition, incubation time, temperature, pH medium (most enzymes have the optimum pH between 4 to 6), carbon and nitrogen source type, and agitation conditions. Regarding the high rate of sporulation and growth, bacteria as *Bacillus amyloliquefaciens, Bacillus subtilis, Bacillus licheniformis,* and *Bacillus stearothermophilus* (Far et al., 2020) have been shown the

capability of producing quantitatively α-amylase for industrial applications, due to their property to resist at high temperatures. Generally, enzyme activities increase up to 40°C, but quickly, a zone of decline is recorded due to the denatured and loss of their structure recorded with an inactivation for the enzyme of interest.

The current study is based to test the potential of *Bacillus licheniformis* ATCC 21424 for evaluating the capacity to produce and secrete hydrolytic amylase and protease enzymes in the different fermented medium as a new criterion for use as probiotics in animal feed.

MATERIALS AND METHODS

The *in vitro* assessment was performed at the Biotechnology Laboratory of IBNA Balotesti and all procedures complied with the experimental protocol.

Bacterial culture

Bacillus licheniformis (BL) was acquired from the American Tissue Culture Collection (ATCC 21424) as a freeze-dried form. After revitalized in nutrient Merck broth medium (g/l: tryptone 10; meat extract 5; sodium chloride 5; pH medium 7.2 ± 2 before autoclaving) and incubated at 37°C for 24 h in aerobic conditions, the stock culture was maintained at 4°C on Merck nutrient agar slants (g/l: tryptone 5; meat extract 3; bacteriological agar 5; distilled water). For long preservation, the strain was stored at -80°C with 20% sterile glycerol.

Medium preparation, inoculum and fermentation process conditions

Inoculum was prepared as follows: some colonies of a 24 h old slant *BL* culture were transferred with a sterile inoculation loop into a tube containing 9 ml of sterile broth medium under aseptic conditions.

The amylase was produced in **submerged fermentation medium (SFM)** consists of (g/l): glucose 6%, $CaCl_2$ x $2H_2O$ 0.04%, $MgCl_2$ 0.02% with the addition of 2% raw materials (soybean meal, peas, sorghum flour, corn and combined feed).

The inoculated culture was incubated at 37°C, 24 ± 2 h, 150 rpm (in a shaker-incubator) follow by inoculation (1:10, v/v) into 250 ml Erlenmeyer flasks containing 100 mL of autoclaved (121°C, 15 min) SFM. After an incubation time of 24, 48 and 72 h at 37°C under continuous shaking (150 rpm), each fermentation medium with the addition of corresponding raw materials was centrifugated at 8000 rpm, 20 min, 4°C in a cooling centrifuge. The supernatants obtained were used for quantitative estimation for enzymatic activity.

Each fermentation process was done in triplicate and optical density (OD) was measured following the enzymatic method.

In vitro assessment of amylase activity

Qualitative method

BL strain was tested for amylase activity by starch hydrolysis test using the Iodine method. A nutrient agar medium with the addition of 1% (w/v) starch following by sterilization at 121°C for 15 min was prepared. The inoculated plates were incubated at 37°C for 24 h. After incubation, the starch hydrolysis zone was detected by inundating the plates with 3-5 ml of iodine solution (Lugol Grams straining or 0.4% KI + I_2, w/v). The blue color development showed the presence of starch, while the areas around the hydrolytic bacteria appeared clear (Dumitru et al., 2018).

Quantitative method

Enzymatic activity. For evaluation of amylase activity (AA), DNS (3,5-dinitrosalicylic acid) spectrophotometric assay was used. As an alkaline reagent, DNS attaches to the reducing sugars, following be measurements of the color changes by UV absorbance at 546 nm with a BioSpectrometer Basic Eppendorf. The cell-free supernatant recovered represents the crude enzyme extract. The reaction mixture containing: crude enzyme extract (0.5 ml) with 0.2 M phosphate buffer (0.5 ml) for pH 7.0 and 1% (w/v) soluble starch prepared in 0.2 M phosphate buffer (1 ml) previously maintained at 30°C/10 min. The reaction was stopped immediately adding 2 ml of DNS following by 5 min of boiling. After cooling, the sample was diluted with distilled water (up to 12 ml/tube) and absorbance was measured at 546 nm.

Standard curve of maltose

A maltose standard curve was done to determine the quantity of reducing sugars in the

reaction mixture. One unit of amylase activity (enzymatic units, U/ml) was defined as the amount of enzyme/ 1 ml of culture supernatant that released 1 µmole of reducing sugars as maltose/min under the assay conditions.

In vitro assessment of protease activity

Qualitative method

Bacillus licheniformis ATCC 21424 was screened for protease-producing bacteria. After inoculated on the nutrient agar with addition of skim milk (1% w/v), the plates were incubated at 37°C, for 48 h, in anaerobic conditions. After incubations, the strain protease capacity was evidential by clear zones around colonies. Zone of hydrolysis was done by flooding agar plate with 25% TCA (trichloroacetic acid) solution and incubated for 15 min, at 45°C (Dumitru et al., 2018; Siddalingeshwara et al., 2010).

Quantitative method

Enzymatic activity. The production of protease in culture filtrate was estimated by the Anson method. Method is based on the determination of tyrosine, resulting from the action of proteases on the casein substrate.

The sample test consists of 1 ml of casein (1% enzymatic substrate prepared in 0.2 M phosphate buffer, pH 7) added to 0.5 ml crude enzyme solution. The reaction mixture was well homogenized and incubated for 10 min at 35°C. Then 2 ml of TCA reagent (5%, w/v in distilled water) was added, shaken to mix very well and filtrate with filter paper. In another tube, was taken 1 ml from filtered solution over which was added 2 ml 0.5 N NaOH and 0.5 ml Folin-Ciocalteu (FC, 1:3 v/v in distilled water) reagent, mixed and incubated 10 min at 35°C. To enhance the amylase production of *BL*, the fermented medium was incubated at 37°C, 150 rpm followed by measuring the enzymatic activity at 24, 48 and 72 h. OD of the solutions was determined at 660 nm.

Standard curve of tyrosine

A tyrosine standard curve was effectuated to quantify the amount of protein in the SFM. One unit of protease was defined as the enzymatic activity that releases 1 µmole of tyrosine from 1 ml of SFM in one minute.

Statistical analysis

All results were performed in triplicates and the results were done using analysis of variance (one-way ANOVA) as a completely randomized design, 2011). The results are expressed as mean values and standard error of the mean (SEM). Data were analyzed by STAT VIEW for Windows (SAS, version 6.0), the differences between means were considered statistically significant at $P<0.05$, using Fisher's PLSD test for the untitled compact variable.

RESULTS AND DISCUSSIONS

Bacterial culture

Before testing the enzymatic capacity of *Bacillus licheniformis* ATCC 21424 to synthesize extracellular enzymes as amylase and protease on various raw materials feed (soybean meal, peas, sorghum flour, corn and combined feed), the present strain was subjected to several tests for evaluating their potential as a source of probiotics in animal nutrition. Morphological and biochemical characterrization done by Dumitru et al. (2019) indicated the strain potential with other assays as growth rate, percentage of survivability at low pH, bile salts concentrations, high temperatures, hemolysis activity etc. involving positive results. Furthermore, the active 24 h culture *BL* was tested for their carbohydrate fermentation using API 50 CHB system kit (BioMerieux, France) respecting the manufacturer's instructions. After a visual examination at 24, 48 and 72 h, Dumitru et al. (2019) reported the API 50 CHB, results obtained after the color change from red to yellow and their capacity to synthesize enzymes. As could be seen in the report of Dumitru et al. (2019), *BL* has the capacity to ferment the starch and lactose substrate present in API 50 CHB kit as confirms enzymatic status to produce amylase and protease.

Amylase activity assay

Qualitative method

During the present study, *BL* secrets amylase in agar medium in the presence of 1% starch, property observed by the development of a hydrolysis zone around colonies.

Optimization of culture conditions is an essential aspect for enhances bacteria growth and enzyme production. The enzymatic system of *BL* to secrete amylases can be observed by discoloration of the nutrient agar medium supplemented with soluble starch at the addition of

Iodine solution. It is very important to know what is the strain capacity to secretes enzymes and to do that, the substrate-specificity must be selected carefully.

The introduction of starch into the nutrient medium accelerated the *BL* strain system for amylase secretion that decomposes the highly specific substratum.

Amylase enzyme was observed by a hydrolysis zone around colonies developed on agar medium. Another study conducted by Deb et al. (2013), reported similar results in case of *Bacillus amyloliquefaciens* P-001 as a source of amylase production.

Quantitative method

To determine the amylase activity of our strain, a standard curve of maltose was done (Figure 1). The extracellular amylase activity of *BL* strain was carried out in shake-flask fermented medium using different raw materials used in animal-based diet: soybean meal, peas, sorghum flour, corn and combined feed (FC) in a percentage of 2%. The results can be observed in Table 1.

As can be observed, in the first 24 h of fermentation, *BL* when grown in fermented media with corn flour exhibited a higher capacity to secrete amylase, following by soybean meal, sorghum, peas and FC, registering statistically differences ($P < 0.05$) between all substrate used as a carbon source. This finding is very helpful in animal feed.

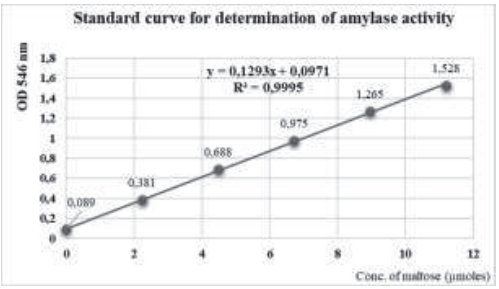

Figure 1. Maltose standard curve for determination of amylase activity

The highest *BL* amylase activity was at 72 h in FC fermented medium, while the strain capacity produces 19.43 U/ml.

Because the presence of starch in raw materials encourages the production of amylase, during the entire period of fermentation, AA was synthesized by *BL* strain to hydrolyze the starch present in the medium and to produce more easily assimilable sugars (dextrins and progressively smaller polymers composed of glucose units and maltose, Abdel-Fattah et al., 2013; Blanco et al., 2016).

Table 1. Extracellular amylase production from *Bacillus licheniformis* ATCC 21424 in shake-flask cultivation

Item	Amylase activity [U/ml]						
	[S]					*SEM**	P-value
T	**Soybean meal**	**Peas**	**Sorghum**	**Corn**	**FC**		
24h	13.11^{aA}	6.96^{bA}	11.34^{aA}	14.75^{dA}	3.54^{cA}	1.10	0.0001
48h	16.77^{aB}	10.05^{bB}	$14.^{43bB}$	5.09^{dB}	17.88^{eB}	1.26	0.0001
72h	18.31^{aC}	11.60^{bC}	17.52^{cC}	6.63^{dC}	19.43^{eC}	1.30	0.0001
Effect							
[S] × T							
SEM**	0.77	0.68	0.89	0.44	0.69		
P-values	0.0001	0.0001	0.0001	0.0001	0.0001		

Where: FC, combined feed; *BL, Bacillus licheniformis* ATCC 21424; [S], raw material used as substrate; T, time (h); SEM, standard error of the means in a row; $^{a-c}$Means within same rows with different superscript letters are significantly different ($P < 0.05$); $^{A-C}$Means in same collumns between rows with different superscript uppercase letters are significantly different ($P < 0.05$).

After 48 h, the amylase production in corn fermented medium was decreased comparatively with soybean meal. For us, an important place consists in AA evolution in FC medium, the formula which will be given to the animal as a feed source. Considering that, at 72 h, *BL* strain had a considerable growth rate in FC medium, soybean meal, sorghum, peas and corn, AA increasing in parallel with time of incubation ($P < 0.05$).

In the present work, *Bacillus licheniformis* was found as an effective enzyme producer through submerged fermentation process.

As source of energy, corn represents the main ingredient in animal diet, and together with sorghum, are known as no viscous cereals (Ravindran, 2013). In our study, corn has 3.353 kcal/kg of metabolizable energy (ME), 87.63% of it is dry matter, 7.11% crude protein and 3.86 cellulose (Habeanu et al., 2017). For example, NRC (2012) presented from the ME of corn, 62.6% of it is starch and 9.7% non-starch polysaccharides (NSP) as indigestible components. The addition of enzymes as amylases aims to degrade the presence of indigestible components and can contribute to better digestion of starch and therefore to intensify the nutritional value of feed and energy in animal nutrition (Hmani et al., 2017).

It is known that sorghum is a non-viscous grain with high protein content (9.91%, Habeanu et al., 2017; Shargie, 2020) and antinutritional factors (i.e., tannins, phytate) that may form stable complexes with proteins and minerals which reduces digestibility and nutritional value (Schons et al., 2012). Addition of exogenous amylase (derived from *Bacillus* spp. as affirmed Mahagna et al., 1995) and protease in sorghum-based broiler diets enhanced the total tract digestibility of amino acids. Of relevance of the present study is that the amylase production in sorghum fermented medium at 72 h of incubation was higher than corn substrate due to the *BL* activity to degrade starch into maltodextrins and simple sugars. Between all raw materials used, corn medium realised the maximum level of glucose in early stage of fermentation (24 h) due to starch-hydrolysing effect of activated amylase.

Soybean meal (SBM) is one of the commonly used protein source for animal feed with a high NSP content, factors which diminish its utilization (Mukherjee et al., 2015). Incorporation of *BL* as a microbial enzymatic source in fermented medium with soybean meal involved a strong interaction. *BL* as can be observed in Tables 1 and 2, has the capability to improve nutritional value of SBM

registering 18.31 U/ml amylase, but with a significant level of the protease of 94. 66 U/mL in 72 h of fermentation. Hong et al (2004) confirmed that the addition of microbial fermentation using bacteria or fungi efficiently improves the nutritional value of SBM by eliminated the anti-nutritive compounds. Besides, Han et al. (2001) and Yang et al. (2007) affirmed that *Bacillus* spp. are preferred to produce fermented soy-based feed.

Peas are usually utilized in nonruminant diets as excellent source of protein. For swine, field peas present average of 23% crude protein and 3.435 kcal/kg digestible energy compared to corn (NRC, 1998). Peas contain anti-nutritional factors (i.e. galactosidase, trypsin inhibitors, resistant starch, pectin, tannins, lectin, phytic acid) and NSP which significantly damage the digestive process especially in young chicks (Goodarzi Boorojeni et al., 2017). The addition of microorganisms in fermentation processes could provide several benefits with enzymatic probiotic effects of animal GIT. The use of microbial strains with enzymatic properties as an additive in animal feed could inactivate anti-nutritional factors, enhance the digestion process and nutrient availability (Bedford, 2000).

In GIT of poultry, the retention time of feed is very short (~2 to 4 h) and this interval can fluctuate in function of the chemical and physical characteristics (i.e. particle size, feed form administration, NSP concentration, etc.).

On the other hand, the optimum pH of most exogenous enzymes is between 4 and 6. Therefore, the GIT pH and the fact that enzymes can be exposed to hydrolysis by endogenous proteolytic enzymes in the GIT, the degradation activity of exogenous enzymes seems mainly limited in the crop, proventriculus and gizzard (Ravindran, 2013).

The inclusion of *BL* in peas fermented medium improves the level of amylase and protease enzymes (Tables 1 and 2) where were registered significant enzymatic activities (72 h).

Several studies confirmed that younger animals, in the first days of life, due to an immature digestive system, do not produce sufficient endogenous digestive enzymes to degrade NSP present in cereals which involves a negative effect and decreases the nutrients digestibility (Adami dos Passos & Kim, 2014; Song et al., 2010). The addition of amylase enzyme catalyses the presence of endohydrolysis linkages NSP in simple units as glucose, form which can be absorbed by the animal body. As an energy source of growth, amylases complement bird endogenous enzyme secretion, increase starch digestibility and

diminish the availability of glucose as a potential substrate for non-beneficial bacteria in the latter part of the animal GIT (Anguita et al., 2006). It was observed that supplementation with *Bacillus licheniformis* ATCC 21424 (Dumitru et al., 2020a) in piglets diet based on 33.48% corn, 25% sorghum, 17% peas, 13% soybean meal improved growth performance and intestinal microflora population. Furthermore, positive modifications of the entire study were observed with significantly results on diarrhoea incidence of piglets. In most cases, studies with addition of *Bacillus* as a source of microbial enzymes may be a suitable alternative and have been found to involve high benefits in the reduction of bacterial gastrointestinal diseases in animal feed (Ibrahim et al., 2012; Lattore et al., 2016).

Protease activity

Qualitative method

On solid medium supplemented with skim milk, the Petri plate revealed a clear zone around colonies sign the capacity of strain to produce protease. Protease enzyme is very important in the process of digestion due to the capacity to hydrolase proteins and anti-nutritional factors (Hmani et al., 2017).

Quantitative method

With regard to protease production, a standard curve of tyrosine was done (Figure 2).

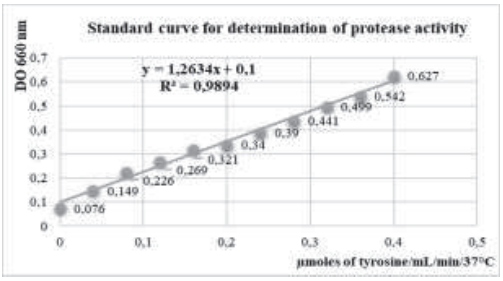

Figure 2. Tyrosine standard curve for determination of protease activity

The strain capacity to secrets proteases enzymes was followed in fermented media with different sources of raw materials used as substrates (Table 2). The protease activity (PA) was determined by quantifying the amounts of tyrosine released from casein hydrolysis by incubating the fermented media time of 24, 48, and 72 h.

Table 2. Extracellular protease production from *Bacillus licheniformis* ATCC 21424 in shake-flask cultivation

Item	Protease activity [U/ml]					*SEM**	P-value
	[S]						
	Soybean meal	Peas	Sorghum	Corn	FC		
24 h	2.57^{aA}	2.19^{bA}	3.46^{cA}	2.69^{adA}	3.02^{eA}	0.11	0.0001
48 h	72.50^{aB}	68.46^{bB}	73.06^{acB}	70.68^{abdB}	94.27^{eB}	2.52	0.0001
72 h	94.66^{aC}	75.91^{bC}	89.36^{cC}	78.60^{dC}	97.75^{eC}	2.13	0.0001
Effect							
[S] × T							
SEM**	13.87	11.71	13.17	15.51	12.04		
P-values	0.0001	0.0001	0.0001	0.0001	0.0001		

Where: FC, combined feed; *BL, Bacillus licheniformis* ATCC 21424; [S], raw material used as substrate; T, time (h); SEM, standard error of the means in a row; $^{a-e}$Means within same rows with different superscript letters are significantly different ($P < 0.05$); $^{A-C}$Means in same collumns between rows with different superscript uppercase letters are significantly different ($P < 0.05$).

Protease production increased gradually. In all fermentation cultures, the evolution of protease synthesis was improved from 24 to 72 h of incubation. Individually, each raw material due to the presence of proteins induced the production of proteases during the fermentation process at the addition of *BL*; the strain acted and degraded them in small components (proteins to peptides and amino acids). *BL* showed the highest protease production at 72 h of incubation in fermented medium with FC, followed by soybean meal, sorghum, corn, and peas used as enzymatic substrates ($P < 0.05$). Production of protease varied among raw materials used with *BL* addition. A littlest decrease in protease enzymatic activity at 72 h was found for peas (< 28.77%) and corn (< 24.36%) compared to FC fermented medium ($P < 0.05$). These results correlate with Blanco et al. (2016) report. Hmani et al. (2017)

affirmed that protease-amylase combination determined significant improvements in the animal growth performance, body weight gain (Garcia et al., 2008), total protein digestibility and reduction in gas emissions (Bundgaard et al., 2014). Enzymes such amylase, protease, cellulose etc. are generally produced by *Bacillus* spp.; an addition to an animal-based diet is known to involve beneficial effects on digesta viscosity, body weight, feed intake, nutrient absorption (Schallmey et al., 2004).

CONCLUSIONS

The results obtained showed that the present raw materials could be used for enzyme (amylases and proteases) production by *Bacillus licheniformis* ATCC 21424. The addition of 10% (v/v) inoculum in different fermented medium gives appreciable results at 72 h to produce and secrete significant quantities of extracellular enzymes as amylase (corn, soybean meal, sorghum, peas and FC) and protease (FC, soybean meal, sorghum, corn and peas). Hence its *BL* can be recommended as an enzymatic source for animal nutrition.

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