

# Evaluation of Enzymatic Potentialities of *Bacillus subtilis* using as Substrate Different Animal Raw Materials Feed

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## Abstract

In the present investigation, the production of amylase, cellulase, and protease activity by *Bacillus subtilis* ATCC 6051a was evaluated on different raw materials (soybean meal, peas, sorghum flour, corn) and combined feed (CF). The effect of various fermentation conditions (24-72 h) on enzymatic production through shake-flask culture (Erlenmeyer 100 mL) in optimum conditions (150 rpm, pH 7.0±0.2, 37°C) was investigated. The inoculum strain presents 1.907 optical density (OD) 600 nm with a concentration of  $1.6 \times 10^9$  CFU/mL. The screening showed a capacity of amylase, cellulase, and protease strain production. The maximum amylase level was obtained at 72 h when the strain was cultured in CF fermentation medium (13.19±0.15 U/mL), followed by corn (11.72±0.15 U/mL), peas (9.22±0.11 U/mL), soybean meal (7.29±0.19 U/mL) and sorghum (6.31±0.2 U/mL). For production of cellulase by DNS method, the activity was noticed in corn (4.39±0.04 U/mL), sorghum (4.05±0.02 U/mL), peas (2.94±0.06 U/mL), soybean meal (2.87±0.04 U/mL) and CF medium (1.41±0.02 U/mL). Maximum protease activity was observed after 24 h in CF (4.91±0.08 U/mL), with minimum enzyme production in the rest of fermentation media, both at 48 h and 72 h. In conclusion, qualitative analysis revealed that *Bacillus subtilis* ATCC 6051a could be successfully used for high scale production of amylase, cellulase and to less extend protease, particularly in fermented medium containing CF or corn, and may be considered a potential candidate for supplementation of animal feed.

**Keywords:** enzymatic activity, shake flask fermentation, probiotic

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## 1. Introduction

On an industrial scale, microorganism are known to play a vital role in technology for the production of intracellular and extracellular enzymes [1].

The microbial enzymes are very important and occur an essential solution in animal nutrition. Most bacteria are grown in selective media under optimum conditions and can be further used as sources of enzymes production which are essential for life. A group of bacteria with high enzymatic properties was occurred by *Bacillus*. Due to their capacity to produce spores, species from *Bacillus* group involve resistance and can grow in harsh conditions with positive actions in the host [2].

The major advantages of spores over vegetative cells are their stability to withstand harsh body environments (low pH, bile salts, high temperature, antibiotics resistance etc.) and extended shelf life without losing viability [3]. Besides *Bacillus* spp. has the capacity to produce exogenous enzymes (protease, cellulase, amylase, lipase, and xylanase) and to improve nutrients utilization from different feedstuffs [4] Further, the informations about the enzymatic nature of *Bacillus* probiotics in order to explore their potential as feed additive are few. Microbial amylase hydrolyzes the starch molecules in dextrin, maltose, and finally glucose units [5]. It is well known that *Bacillus* genus is very famous for alpha-amylase production [6]. Some *Bacillus* strains as *B. subtilis*, *B. licheniformis*, *B. cereus*, *B.*

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*stearothermophilus*, and *B. amyloliquefaciens* are isolated for amylase production and their utilization are involved in raw starch degradations [7-11]. In addition, solid-state fermentation (SSF) can be used as a carbon and energy source for cultivation of microorganisms on moist solid supports [12]. Different studies have reported that the production of enzymes should be carried out at a low cost by using economically and accessible culture media (i.e. food wastes or agro-industrial residues) for optimization of the fermentation process [13].

Cellulose represents one of the most abundant materials, which can be produced by many bacteria and plants [14]. Cellulase is an enzymatic complex which hydrolyze cellulose to fermentable mono- and oligo-saccharides (glucose units) that can be more easily assimilated in the host [15].

Cellulase enzyme can be produced by several microorganisms as bacteria and fungi. Major attention has been given to lignocellulose as a substrate for the production of cellulase enzymes. The component of lignocellulose materials is cellulose, hemicellulose and lignin [16]. Fibers can not be completely digested by animals and around 20-70% of cellulose is lost. Thus, for improving utilization of dietary carbohydrates and enhancing digestion, it is very important to use cellulolytic bacteria due to their contribution to the energy supply for foraging animals [14,15].

Also, the bacteria are employed for the production of proteases enzymes due to their capacity to hydrolyze the proteins by degradation of peptide bonds from different material sources. The most commonly used microorganisms for protease include several species from *Bacillus* group, *Aspergillus*, *Penicillium*, and *Rhizopus* [17]. Animal feed supplementation with enzymatic bacteria products determines an increase of the nutritional value from feed ingredients [18]. Moreover, different *Bacillus* strains due to their capacity to germinate, and survive in the gastrointestinal tract (GIT) of different animal species [19,20,9] are considered part of the host [21].

In this context, the possibility to use enzymatic probiotic feed supplements to reach better digestibility of feed and a significant animal performance has gained considerable interest.

In conclusion, the results obtained in this study based on qualitative analysis revealed that *Bacillus subtilis* ATCC 6051a was found to possess amylase, cellulase and protease activities,

particularly in fermented medium with CF or corn, and may be considered a potential candidate for supplementation of animal feed.

## 2. Materials and methods

The research was carried out at the Laboratory Animal Nutrition and Biotechnology of IBNA Balotesti, Romania.

### ***Bacterial strain, culture preparation and growth conditions***

*Bacillus subtilis* (*Bs*) was acquired from the American Tissue Culture Collection (ATCC 6051a) as a freeze-dried product. After revitalized in nutritive medium (Merck KGaA, Darmstadt, Germany, g/L: tryptone 10; meat extract 5; sodium chloride 5; pH medium  $7.2 \pm 0.2$ ) and incubated at 37°C for 24 h in aerobic conditions, the stock culture was maintained at 4°C on nutrient agar slants (g/L: tryptone 5; meat extract 3; bacteriological agar 5; distilled water).

For a long preservation, the strain was stored at -80°C with 20% sterile glycerol addition and can be found in the intern Bacterial Collection of IBNA Balotesti, Animal Nutrition and Biotechnology Department, code IBNA 74.

### ***Determination of colony-forming units***

The growth rate of *Bs* was determined from overnight culture and the result was expressed as  $\log_{10}$  CFU per mL. The strain contains at least  $1.6 \times 10^9$  CFU mL<sup>-1</sup>.

### ***Culture medium and inoculum preparation***

Inoculum was prepared as follows: colonies from 24 h old slant *Bs* culture were transferred with a sterile inoculation loop into a tube containing 9 mL of sterile nutrient medium under aseptic conditions.

### ***Fermentation process conditions***

The enzyme activity was produced in submerged fermentation medium (SFM) consists of (g/L): glucose 6%, CaCl<sub>2</sub> x 2H<sub>2</sub>O 0.04%, MgCl<sub>2</sub> 0.02% with the addition of 2% raw materials: soybean meal (*Glycine max*), peas (*Pisum sativum*), sorghum flour (*Sorghum*), corn (*Zea mays*) and combined feed [7]. Combined feed (CF) is a concentrate consisting of soybean meal, peas, sorghum flour, and corn in different percentages.

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

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

#### ***Amylase 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 the 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 followed 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.

#### ***Cellulase activity***

It is based on the enzymatic hydrolysis of carboxymethylcellulose (CMC, Roth) and the dosing of reducing sugars released with 3,5-dinitrosalicylic acid (DNS) colorimetric method.

#### ***Standard curve of glucose***

A glucose standard curve was done to determine the amount of reducing sugar in the reaction mixture. A cellulolytic unit activity (U) represents the amount of enzyme able to release from a CMC

solution, an amount of reducing sugars, which form with the DNS reagent, the same optical density, equivalent to a milligram of glucose [22].

#### ***Protease activity***

The production of protease in culture filtrate was estimated by the Anson method. The 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 7.0) 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 protease production of *Bs*, 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***

The results were expressed as mean values and standard error of the mean (SEM). The graphics for the present study were generated using SigmaPlot V.14.5 software (San Jose, CA, USA).

### **3. Results and Discussion**

#### ***Bacterial strain, morphological characteristics, and growth conditions***

Morphologically, on agar medium, *Bs* generated irregular colonies, with a diameter of 1.2-5.0 mm, at 24 h of incubation, type R, opaque, whitish, with a Gram positive staining. Results obtained by API 50 CHB reported in Dumitru et al. [8] indicated a percentage of *Bs* identification around 99.4% ID (very good percentage identification). The strain

fermented D-glycerol, salicin, D-cellobiose, D-maltose, D-arabinose, D-ribose, D-melibiose, D-xylose, D-saccharose (sucrose), D-trehalose, D-raffinose, D-glucose, starch, D-fructose, glycogen, D-mannose, gentiobiose, D-turanose, inositol, D-mannitol, D-sorbitol, methyl- $\alpha$ D-glucopyranoside, amygdalin, arbutin and esculin. In addition, an absent fermentation was found for D-arabinose, D-lactose, L-xylose, D-adonitol, methyl- $\beta$ D-xylopyranoside, D-melezitose, D-galactose, xylitol, L-sorbose, L-rhamnose, dulcitol, D-lyxose, D-tagatose, D-fucose, L-fucose, methyl- $\alpha$ D-mannopyranoside, D-arabitol, L-arabitol, N-acetylglucosamine, potassium gluconate, potassium 2-ketogluconate, and potassium 5-ketogluconate substrate.

### Fermentation process

Generally, the inoculum culture and fermentation substrate influence the production of enzymes. In our study, various raw materials feed (soybean meal, peas, sorghum flour, corn, and CF) were used to evaluate the production of the enzymes *Bacillus subtilis* ATCC 6051a.

Utilization of the present materials feeds as substrate of the fermentation process accelerate the enzymatic system of *Bs* strain and maximize the capacity to synthesize extracellular enzymes at 150 rpm, 37°C during the incubation time. Shaking is a very essential condition, together with temperature, and time of incubation, which accelerates cell growth and enzymes productions [5]. In line with our results, Dumitru and Habeanu [7] reported the correlation between the time of incubation and enzymatic activity which can maximize the strain enzymatic secretion. In addition, after an interval, Blanco et al. [23] affirmed that the enzymatic activity gradually decreased due to the consumption of nutrients from the fermentation medium.

### Amylase activity

Triplicate incubation were carried out under the optimized conditions for determine the enzymes activity.

To estimate the amylase activity of *Bs*, a standard curve of maltose was done (Figure 1).

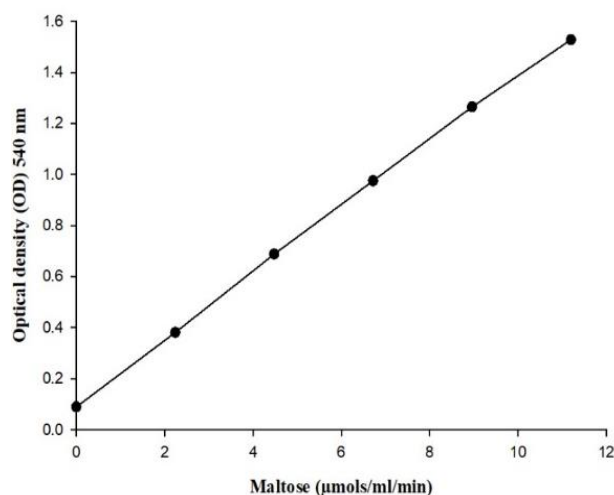


Figure 1. Standard curve of maltose

The extracellular amylase activity of *Bs* strain was carried out in shake-flask fermented medium using different raw materials administrated in an animal based diet: soybean meal, peas, sorghum flour, CF, and corn in a percentage of 2% (Figure 2). Over all fermentation process, the amylase production of *Bs* manifests growth trends. As can be observed, in the first 24 h of fermentation, *Bs* started the secretion of amylase in CF medium, followed by corn, soybean meal, sorghum, and peas.

The amylase production continued to be monitored at 48 h, respectively 72 h. Figure 2 shows the maltose production and significant amylase activities of SFM over 72 h of fermentation in CF ( $13.19 \pm 0.155$  U/ml), corn ( $11.72 \pm 0.155$  U/ml), peas ( $9.22 \pm 0.118$  U/ml), soybean meal ( $7.29 \pm 0.195$  U/ml) and sorghum ( $6.31 \pm 0.205$  U/mL).

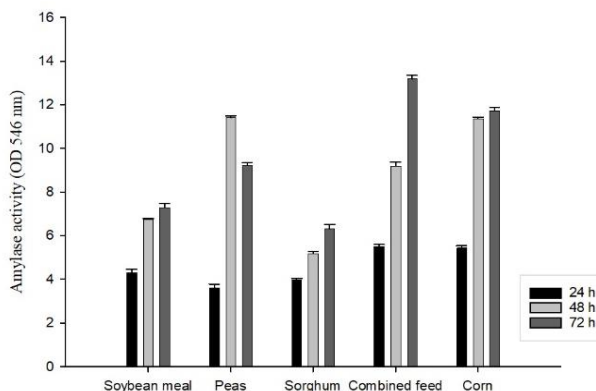


Figure 2. Extracellular amylase production from *Bacillus subtilis* ATCC 6105a during 72 h

Enzyme production was maximal when the cell bacteria are in the growth stationary phase [6].

According to Blanco et al. [23] report, the biomass production of *B. subtilis* UO-01 increased until 15 h of incubation and remained constant in some fermentation processes until the total sugars were not totally consumed. In our case, the evolution of amylase synthesis during the entire 72 h and contradictory, in peas fermentation medium for example, after 48 h, the amylase secretion decreased slightly.

The amylase production by *Bs* is influenced by different fermentation parameters (time of incubation, substrate concentration, pH, temperature, nitrogen sources and inorganic nutrients). Generally, any fermentation type depends on the substrates. In order to achieve maximum  $\alpha$ -amylase production, these parameters need to be adjusted. For example, the presence of starch in the fermentation medium induces the production of amylase [23]. During 24 h, amylase activity was synthesized by *Bs* to hydrolyze the starch present in fermentation medium, producing assimilable sugars as glucose and maltose to accelerate the growing strain.

However, other researchers ascribed the decreasing of amylase activity to a possible denaturation of the enzyme due to the interaction with other compounds in the fermented medium [24].

### Cellulase activity

To estimate the cellulase activity of *Bs*, a standard curve of glucose was done (Figure 3).

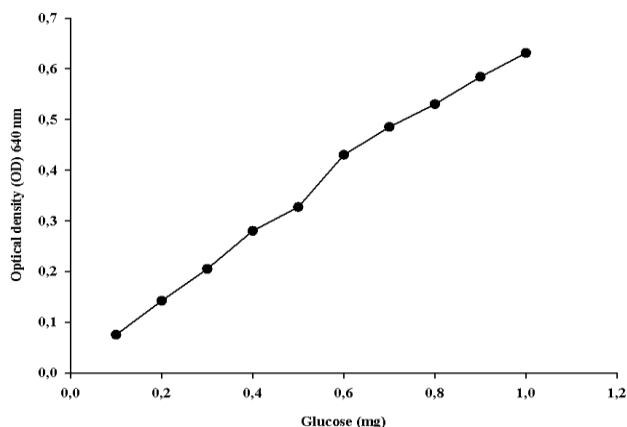


Figure 3. Standard curve of cellulase

During 24-48 h, *Bs* did not involve the capacity to synthesize cellulase enzyme (Figure 4).

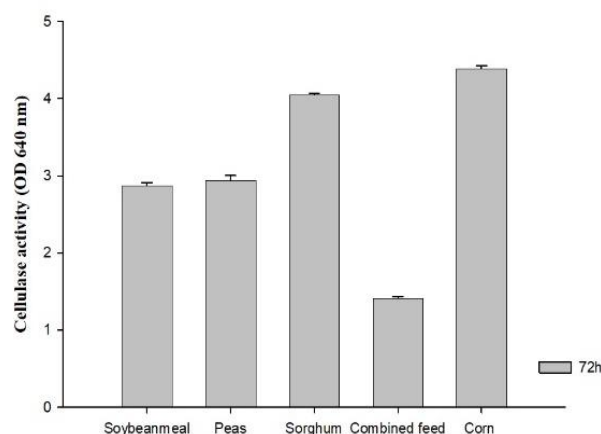


Figure 4. Extracellular cellulase production from *Bacillus subtilis* ATCC 6105a at 72 h

It was observed that 2% of raw material used as substrate in fermentation medium yielded maximum cellulase activity after 72 h as followed: peas ( $4.39 \pm 0.040$  U/ml), sorghum ( $4.05 \pm 0.021$  U/ml), peas ( $2.94 \pm 0.063$  U/ml), soybean meal ( $2.87 \pm 0.041$  U/ml) and CF ( $1.41 \pm 0.021$  U/ml).

Generally, *Bacillus* species are attractive sources of various extracellular hydrolytic enzymes, which may help in nutrient digestion and utilization of feed [25]. Additionally, cellulase production depended on the nature of the carbon source and other vital nutrients existing already in the substrate.

The dietary fiber in animals consists of non starch polysaccharides such as arabinoxylans, cellulose, many other plant components including resistant dextrins, inulin, lignin, chitins,  $\beta$ -glucan and oligosaccharides, which can act as anti-nutritional factors for several animals [26]. In addition, cellulase enzyme occurs an important place in animal nutrition, improving the nutritional value of feed or by degrading certain cereal components [27].

### Protease activity

To estimate the protease activity of *Bs*, a standard curve of tyrosine was done (Figure 5).

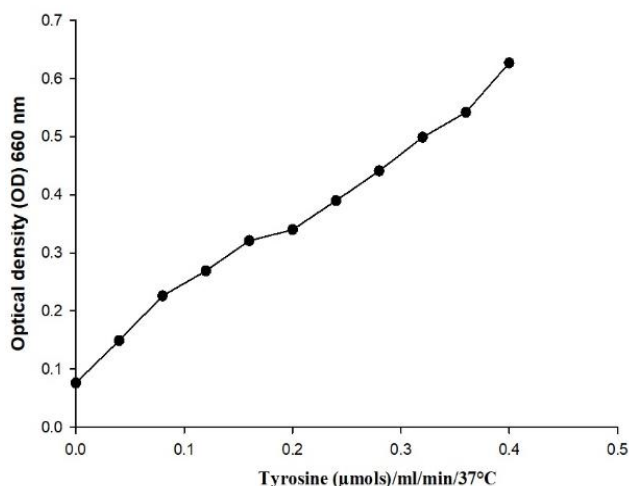


Figure 5. Standard curve of protease

In the present study, as can be observed in Figure 6, the maximum activity of protease was noted in the enzyme extract harvested at 24 h in CF fermented medium ( $4.91 \pm 0.085$  U/ml), followed by soybean meal ( $2.18 \pm 0.008$  U/ml) and peas ( $1.82 \pm 0.022$  U/ml).

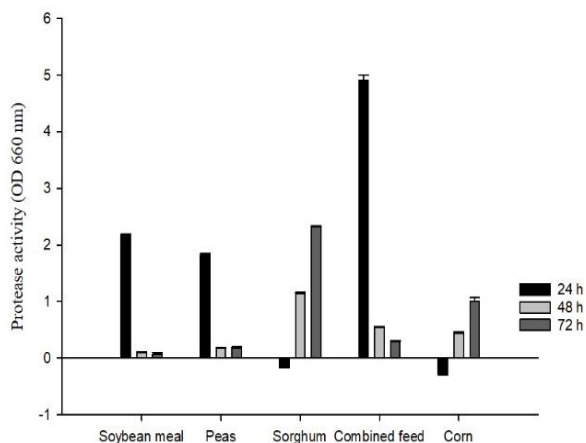


Figure 6. Extracellular protease production from *Bacillus subtilis* ATCC 6105a during 72 h

After 48 h, the protease production in CF fermented medium, peas and soybean meal were decreased comparatively with sorghum and corn fermentation media where secretion was in progress and the enzymatic system of *Bs* was activated after this time of incubation. Considering that, at 72 h, *Bs* involved the protease secretion in sorghum, corn, CF, peas and soybean meal.

The scientific report of Santoso et al. [28] shows that the inclusion of *Bs* in broiler chicks diet, which involves the capacity to secrete protease enzyme, improves growth performance.

Due to the viscosity of fermented medium (soybean meal, peas and CF) the capacity of protease production by *Bs* declined. This can be attributed to the high viscosity of culture medium of fermentation which interferes with  $O_2$  and leading as reported Rukhaiyar and Srivastava [29] limitation of dissolved  $O_2$  for the bacteria growth. Soares et al. [30] reported that *Bs* shows a high biotechnological potential for protease production in solid-state fermentation by using different substrates sources as culture medium. In our study, *Bs* was able to produce extracellular protease in soybean meal, peas and CF fermented medium, results which are in concordance with Zhu et al. [2008], respectively Zhang et al. [2014] when was used as substrate soybean meal and chickpeas.

#### 4. Conclusions

In conclusion, qualitative analysis revealed that *Bacillus subtilis* ATCC 6051a could be successfully used for high scale production of amylase, cellulase and to less extended protease, particularly in fermented medium containing CF or corn, and may be considered a potential candidate for supplementation of animal feed.

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Conflict of interest statement: we declare that we have no conflict of interest.

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