Hydrolytic enzymes production by *Bacillus licheniformis* growth on fermentation media formulated with sewage sludge

Alberto Renato Inca-Torres[1, 2], Anabell Del Rocío Urbina-Salazar[1, 2], Gonzalo Falcón-García[1], Pilar Carbonero-Aguilar[1], Bruno Rodríguez Morgado[1], Juan Parrado[1], Juan Bautista[1, *]

[1]Department of Biochemistry and Molecular Biology, University of Seville, C/ Professor García González nº2, 41012-Seville, Spain. [2]Polytechnical High School of Chimborazo, Faculty of Science, Panamericana Sur km 1 1/2, EC060155- Riobamba, Ecuador.

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In this work, the production of hydrolytic enzymes by *Bacillus licheniformis* grown in media formulated with sewage sludge as the main carbon and nitrogen source with induction by keratins has been studied. The three main types of enzymes of industrial interest produced were proteases, lipases and cellulases. The addition of an inductor, keratin in our case, improves the production of these enzymes to reach a productivity of 16.89 mU/mL·day, 0.25 mU/mL·day, and 0.51 mU/mL·day for proteases, lipases and cellulases, respectively. The secretion of proteins and enzymes into the fermentation media was studied by electrophoretic and proteomic methods, which revealed the presence of proteases, lipases, and cellulases in the fermentation media, among other excreted proteins. Our results show that the growth of *B. licheniformis* in fermentation media formulated with sewage sludge as the main carbon and nitrogen sources, supplemented with keratin from feather meal as an inductor could be used for the industrial production of these enzymes, particularly of proteases.

Keywords: Hydrolytic enzymes; *Bacillus licheniformis*; sewage sludge; proteases; lipases; cellulases.

Abbreviations: GRAS: generally regarded as safe; CDDGs: corn dried distillers grains with solubles; TCA: trichloroacetic acid; DNS: dinitrosalicylic acid; EA: enzymatic activity; p-NPG: p-nitrophenyl-β-glucopyranoside; MUB: Modified Universal Buffer; CBB: Coomassie Brilliant Blue.

[*]**Corresponding author:** Juan Bautista, Department of Biochemistry and Molecular Biology, University of Seville, C/ Professor García Gonzalez nº2, 41012-Seville, Spain. Phone: +34 954556854. E-mail: jdbaut@us.es.

Introduction

Industrial application of enzymes began in the mid-sixteen centuries, although its stable implementation was not achieved until the mid-twenty centuries, between 1950-1960 [1]. The main industrial applications of enzymes are in the production of food, textiles and detergents, representing approximately 90% of the market volume. Other applications, such as organic synthesis, medical and pharmaceutical applications, agronomic uses, etc., are much less used [2]. However, recently, the application of enzymes in agricultural and environmental applications has experienced a significant increase, mainly due to the development of new production methods using cheap culture media derived from agricultural products and urban residues [3].

Most industrial enzymes are bacterial enzymes, although fungal enzymes have lately become increasingly important, both quantitatively and qualitatively. Bacterial enzymes produced by the genus *Bacillus* are of special interest for both research and industrial applications due to the

high rate of growth of these bacteria and their ability to secrete a high volume of enzymes into culture medium (20-25 g/L) [4]. In addition, these microorganisms are "generally regarded as safe" (GRAS). The *Bacillus* strains considered of industrial interest are the species *B. licheniformis* and *B. subtilis*.

One of the major bottlenecks for the industrial application of enzymes is the high production cost, while the cultivation medium may represent 30-40% of total production costs [5]. This has produced great interest in the development of efficient and cheap cultivation media. Among the media developed to address this issue, those derived from agroindustrial byproducts (whey, brewers grains, rice bran, defatted sunflower flour, and corn dried distillers grains with solubles (CDDGs)) and urban wastes (water and sewage sludge) are two types of promising substrates [6]. In addition to the economic benefit associated with the use of agroindustrial byproducts and urban waste, another important advantage is the resulting contribution to environment maintenance [7, 8]. In industrialized countries, the high rates of production of household and industrial liquid waste results in a high polluting effect [9, 10]. The treatment of these wastes in depuration plants generates two main products: purified water and sewage sludge. While the purification of water has received great attention, much less attention has been devoted to the management of sewage sludge, which is a dangerous source of environmental pollution if not treated properly. However, it has recently been shown that sewage sludge, when adequately processed, may serve as a useful substrate in the treatment and regeneration of soil and/or as an inexpensive cultivation media for the production of microorganisms and enzymes [11]. Sewage sludge is one of the most abundant and inexpensive substrates available for the culture of microorganisms and has practically zero cost.

Sewage sludge may be processed to modify and/or adjust its physical, chemical, and biological properties to yield acceptable material for use as fertilizer and/or culture medium for the production of substances of high added value [12, 13]. Currently, sewage sludge is transformed mainly into bio-fertilizer, while only a small portion is used for the production of high value-added products such as enzymes. The goal of this work was to study the use of sludge from water treatment plants as a cultivation medium for the production of hydrolytic enzymes (proteases, cellulases, lipases, acid phosphatase, and β-glucosidase) using *Bacillus licheniformis*.

Materials and methods

Microorganisms

Bacillus licheniformis strain 21415 provided by the American Type Culture Collection (ATCC) (Manassas, VA, USA) was used for the production of enzymes in this study. The bacteria were stored frozen at -80˚C and refreshed in LB medium (10 g/L peptone, 5 g/L yeast extract, and 10 g/L NaCl, adjusted to pH 7) 24 h before inoculation. Briefly, after thawing at 4˚C overnight, 2 mL of bacteria was inoculated into a flask containing 30 mL of LB medium at pH 7 and 37˚C, and then grown until the transmittance at 620 nm reached 20%.

Substrates: Sludge samples, medium composition, and fermentation

Sludge samples were provided by the experimental treatment plant of Carrión de los Céspedes (Sevilla, Spain) of the CENTA Foundation. Thickened sludge with a humidity of 90±1.5% was used both alone and in the preparation of cultivation medium (table 1). Culture medium was sterilized at 121˚C for 30 min prior to inoculation to eliminate pre-existing sludge bacteria. Six hundred mL sterile media was inoculated with 30 mL inoculums and grown over 14 days at 37˚C with constant agitation (150 rpm). During growth, 10 mL samples were taken each day and stored at -80˚C until use for measurement of biomass and enzymatic activities, including protease, cellulase, lipase, acid phosphatase, and β- glucosidase.

Table 1. Compositions of culture media formulations.

Media	Sludge (g)	Inductor (g) Feathers	Inoculum (mL) *B. licheniformis*
M1	600	0	0
M2	600	0	30
M3	588	12	30

Sample preparation

Samples were thawed at 4˚C and either used directly for biomass determination or centrifuged at 12,000 × g for 20 min to obtain the supernatant used for enzyme activity assays.

(1) Determination of biomass.

Biomass was determined indirectly by measuring the protein content of samples at different growth times using the Bradford method [14]. Biomass concentration was determined in µg/mL by interpolation from a standard curve plotting biomass (µg/mL) against protein concentration (µg/mL) (Figure 1).

(2) Enzymatic assays

The supernatant obtained after centrifugation was used for determination of enzyme activities.

Protease activity assay

The total extracellular protease activity was determined following the method described by Beynon and Bond [15]. Briefly, 250 µL of the sample was mixed with 250 µL of a reaction mixture containing 0.1 g azocasein and 0.2 mL of ethanol dissolved in 4.8 mL of 0.1 M phosphate buffer at pH 7. Samples were then incubated for 10 min at 40˚C. The reaction was then terminated by adding 2.5 mL of 5% (w/v) TCA solution. The reaction mixture was centrifuged at 10,000 × g for 2 min. The absorbance of the supernatant at 440 nm was measured. One unit of proteolytic activity was defined as the amount of enzyme required to produce an increase in optical density of 0.001.

Cellulase activity assay

The total cellulase activity was determined by the method described by Galindo [16], modified for application to cellulolytic enzymes. Briefly, 250 µL of a 2% soluble microcrystalline cellulose (Avicel PH-101, Sigma- Aldrich, Barcelona, Spain) solution in 0.1 M acetate buffer (pH 5) was incubated with 250 µL of the sample for 2 hours at 37˚C. The reaction was stopped by adding 1 mL of DNS reagent, and the mixture was heated to 95˚C for 10 min. The reaction mixture was then cooled to room temperature and the absorbance at 575 nm was measured. A standard curve was obtained using glucose for used in the calculation of cellulase activity. One unit of cellulase activity was defined as the amount of enzyme required to liberate 1 mg/mL of reduced sugars into the test solution.

Lipase activity assay

The total extracellular lipase activity was determined using a modified version of the method described by Kilcawley et al. [17]. Briefly, 1.75 mL of buffer (0.1 M sodium phosphate pH 7, 0.15 M NaCl, and 0.5% (v/v) Triton-X) was mixed with 0.25 mL of sample that had been previously centrifuged for 30 min at 12,000 × g, along with 20 µL of 50 mM p-nitrophenol laurate in acetonitrile. This mixture was then incubated for 30 min at 37˚C. Afterward, the mixture was cooled in an ice bath for 5 min, then centrifuged for 1 min at 9,000 × g. The supernatant absorbance at 400 nm was measured. Activity was quantified using the molar extinction coefficient of p-nitrophenol (14,800 /M·cm) at 400 nm. The activity units were defined as one unit corresponding to the release of 1 nmol of p-nitrophenol/min·mg protein under the test conditions.

β-glucosidase activity assay

The β-glucosidase activity was determined using the colorimetric method described by Eivazi and Tabatabai [18] with slight modifications. Into 250 µL of the sample, 2 mL of modified universal buffer (MUB, pH 6) and 0.5 mL of p-nitrophenyl-β-glucopyranoside 25 mM (7.53 mg/mL) were added. The mixture was then incubated at 37˚C for 30 to 60 min. The reaction was stopped by placing the mixture in an ice bath. Subsequently, 2 mL of 0.5 M NaOH was added and the solution absorbance at 400 nm was measured. The

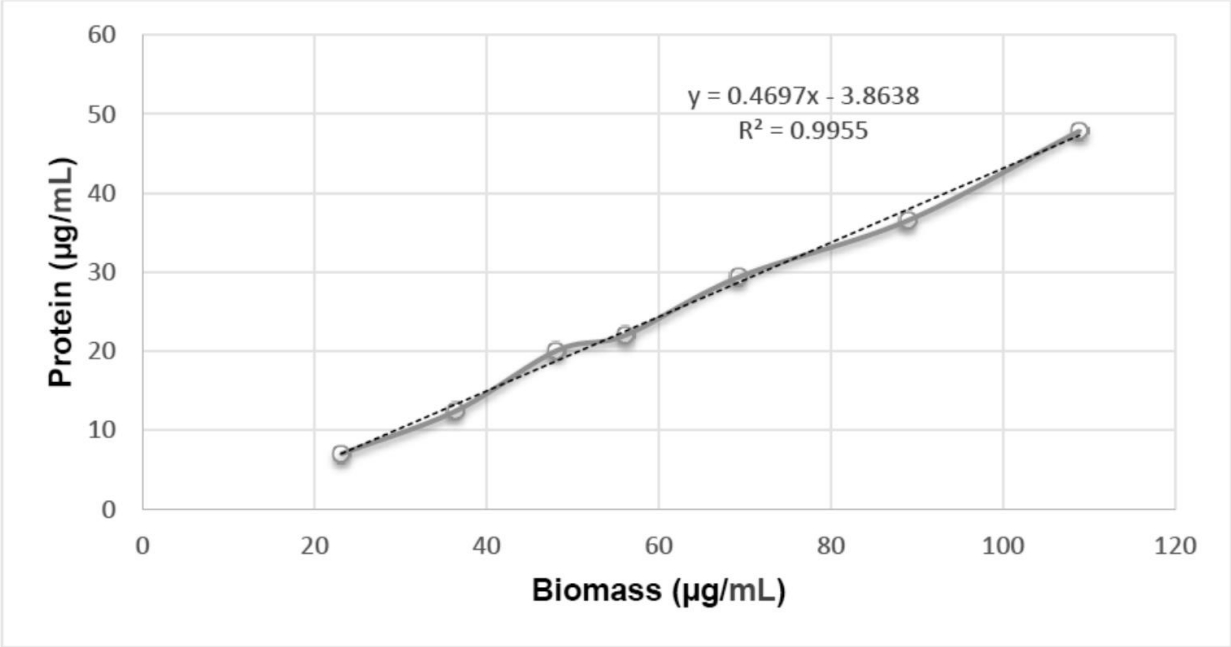

Figure 1. Standard curve of biomass versus protein concentration for *Bacillus licheniformis* grown in a defined medium.

enzymatic activity (EA) was expressed in µmol/g of sample·min and was calculated by the following formula:

$$EA = C \times V / T \times G$$

where C is the concentration of liberated p-nitrophenyl (mM); V is the sample volume (mL); T is the incubation time (min); and G is the sample weight (g). One β-glucosidase unit of activity was defined as the amount of β-glucosidase needed to release 1 nmol p-nitrophenol from p-nitrophenyl-β-glucopyranoside in 1 min under the specified conditions.

Acid phosphatase activity assay (APHA)

Acid phosphatase activity was determined by the method established by Tabatabai and Bremmer [19] with slight modifications. The procedure was initiated by creating a mixture containing 250 µL of sample, 2 mL of MUB, pH 6, and 0.5 mL of p-nitrophenyl-phosphate 25 mM (9.28 mg/mL). This mixture was incubated at 37˚C for 30-60 min. Afterward, the samples were cooled in an ice bath to stop the reaction. Then, 2 mL of 0.5M NaOH was added to the sample and the solution absorbance at 400 nm was measured. The EA was expressed in µmol/g of sample·min and was calculated by the following formula:

$$EA = C \times V / T \times G$$

where, C is the concentration of liberated p-nitrophenyl (mM); V is the sample volume (mL); T is the incubation time (min); and G is the sample weight (g). One phosphatase unit of activity was defined as to the amount of acid phosphatase needed to release 1 nmol p-nitrophenol from p-nitrophenyl-phosphate in 1 min under the specified conditions. Spectrophotometric detection of the artificial molecule p-nitrophenol manifesting yellow as a product of acid phosphatase activity was used to quantify the enzyme activity in these samples.

Protein assay

Protein concentration was quantified by the method described by Bradford, using bovine serum albumin as the protein standard [14].

SDS-PAGE protein profiling

The extracellular protein profile was investigated by SDS-PAGE analysis. After acid-acetone

precipitation of the supernatant obtained after fermentation broth centrifugation at 12,000 x g, the pellet was solubilized in sample preparation buffer (1 mM Tris–HCl, pH 6.8) containing 5% (w/v) SDS and 7% (v/v) β-mercaptoethanol. The samples were kept at room temperature for 2 h and then centrifuged at 12,000 × g for 15 min. The supernatant was collected and run on 12% SDS-PAGE gel in triplicate, with each run containing 20 µL of supernatant (approximately 50 µg of protein) and 5 µL of loading buffer (0.5 M Tris-HCl, pH 6.8, 50% (v/v) glycerol, 10% (w/v) SDS, and 5% (v/v) bromophenol blue). The samples were boiled for 5 min, centrifuged at 10,000 × g for 10 min, and cooled before being loaded on the gel. Electrophoresis was carried out at constant intensity (25 mA) using a Tris–glycine buffer system containing 0.1% SDS until the bromophenol front ran off of the gel. After completion of electrophoresis, the gels were stained with Coomassie Brilliant Blue (CBB) G-250 (Bio-Rad, Hercules, CA, USA) and/or silver staining solution (GE Healthcare, Barcelona, Spain) and digitized on a Gel Doc™ XR + Imaging system (Bio-Rad, Hercules, CA, USA).

Zymogram analysis

Proteolytic activity was detected via electrophoresis on 12% polyacrylamide gels prepared with 1% SDS and 0.1% (w/v) gelatin. After completion of electrophoresis, the gels were incubated in 0.05 M Tris–HCl buffer (pH 8.0) containing 2% (v/v) Triton X-100 at 35°C for 1 h, and then again in the same buffer without detergent at 35°C for 3 h. After CBB staining and subsequent destaining, proteolytic activity was determined from the presence of non-stained zones and bands on the stained background composed of nondigested gelatin [20].

Proteomics

A proteomics study was carried out according to the procedure described in Parrado et al. [21]. Briefly, to remove contaminants such as cells and debris in suspension, the samples were centrifuged at 12,000 × g for 20 min and the supernatants were recovered. Then, 10 µL of the supernatant was treated with 40 µL of methanol and vortex mixed for approximately 1 min. 10 µL of chloroform was added and the mixture was shaken again for 1 min before 30 µL of Milli-Q water was added and mixed well. The mixture was centrifuged at 14,000 × g for 5 min and the supernatant was removed. 30 µL of methanol was added to the pellet and the mixture was vortexed again for 1 min. Samples were centrifuged at 16,000 × g for 5 min. The supernatant was discarded, and the pellets were dried by lyophilization. The pellet proteins were resuspended in Milli-Q water. A total of 30 µg of resuspended protein was added into 30 µL of 6 M urea, 200 mM ammonium bicarbonate, 10 mM DTT and allowed to remain at room temperature for 30 min for protein reduction. 10 µL of 100 mM iodoacetamide was added to promote protein alkylation, and the samples were kept at room temperature in the dark for 30 min. Samples were then dialyzed against Milli-Q water until the urea concentration was below 0.1 M and subsequently treated with trypsin (Promega, Madison, WI, USA) in a protein-to-enzyme ratio of 50:1. Digestion was carried out at 37°C overnight. The mixture was then acidified with TFA and concentrated using a Speed C18/18 column (Applied Separations, Allentown, PA, USA). Peptides were eluted in 400 µL of 70% acetonitrile containing 0.1% TFA. After the samples were completely dried, they were resuspended in 15 µL of 5% acetonitrile containing 0.1% formic acid for use in tandem liquid chromatography–mass spectrometry (LC–MS) analysis. LC–MS analysis was performed in a Surveyor HPLC system in tandem with a Finnigan LTQ mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA). A total of 5 µL of sample was injected into a C18 PepMap100-Precolumn Cartridge (Dionex, Netherlands) for pre-concentration and washing, then resolved in a Biobasic C18 75 µm × 10 cm column (ThermoFisher Scientific, Waltham, MA, USA). Peptides were eluted at a nominal post-split flow rate of 250 L/min using a 120-min gradient of 5% acetonitrile with 0.1% formic acid to 40% acetonitrile with 0.1% formic acid. The LTQ mass spectrometer was run in positive-ion mode using the nanospray source. The spray voltage was set

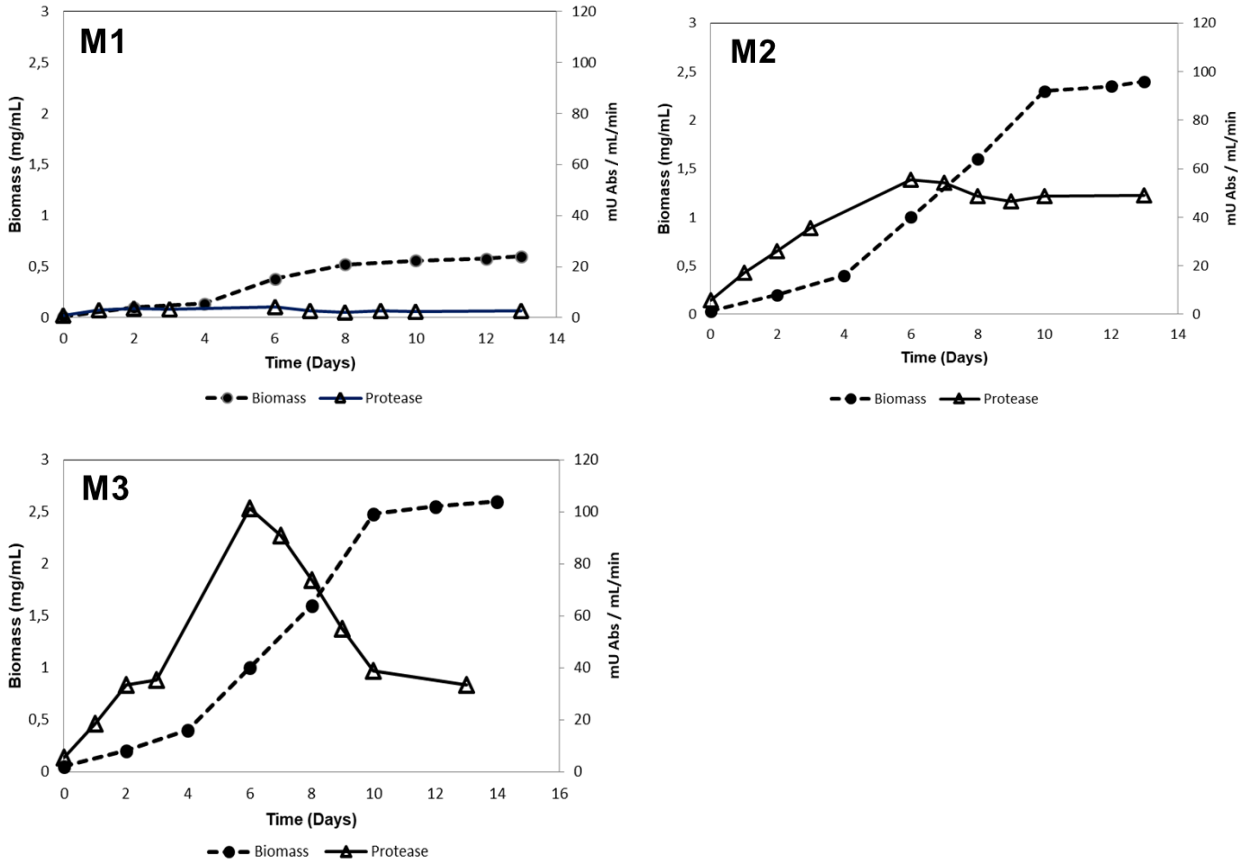

Figure 2. Biomass and protease production by *B. licheniformis* grown in media formulations M1, M2, and M3.

at 2 kV, and the capillary temperature was set to 170°C. The samples were scanned in the range of 400–1500 m/z using the full scan mode, and data-dependent MS/MS analysis with collision-induced dissociation (CID) was performed on the top five ions with dynamic exclusion. The data was converted to SEQUEST format (DTA) and compared against the NCBI database (version 11/10/2007) using an in-house MASCOT (Matrixscience, London, UK) search engine with taxonomy restrictions set to Firmicutes and with carboximethylated cysteine as a fixed modification.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). All experiments were completed in triplicate, and the mean of the three data sets is presented for each experiment. A level of significance of $\alpha < 0.05$ was used to calculate significant differences. One-way ANOVA analysis revealed that the results of the triplicate analyses for each experiment did not differ significantly.

Results and discussion

In an attempt to find an abundant and cheap cultivation medium for the production of hydrolytic enzymes using *Bacillus licheniformis*, we present in this work the results obtained from the use of sewage sludge as the main fermentation source in the culture of *B. licheniformis*. As observed in figures 2 to 4, measurement of the protein production indicated that the microorganism grew well, although the biomass production was not high (2.76 µg/mL). Biomass estimation was carried out by protein determination using the Bradford method. As figure 1 shows, a linear relationship (R2 = 0.995) between protein concentration and

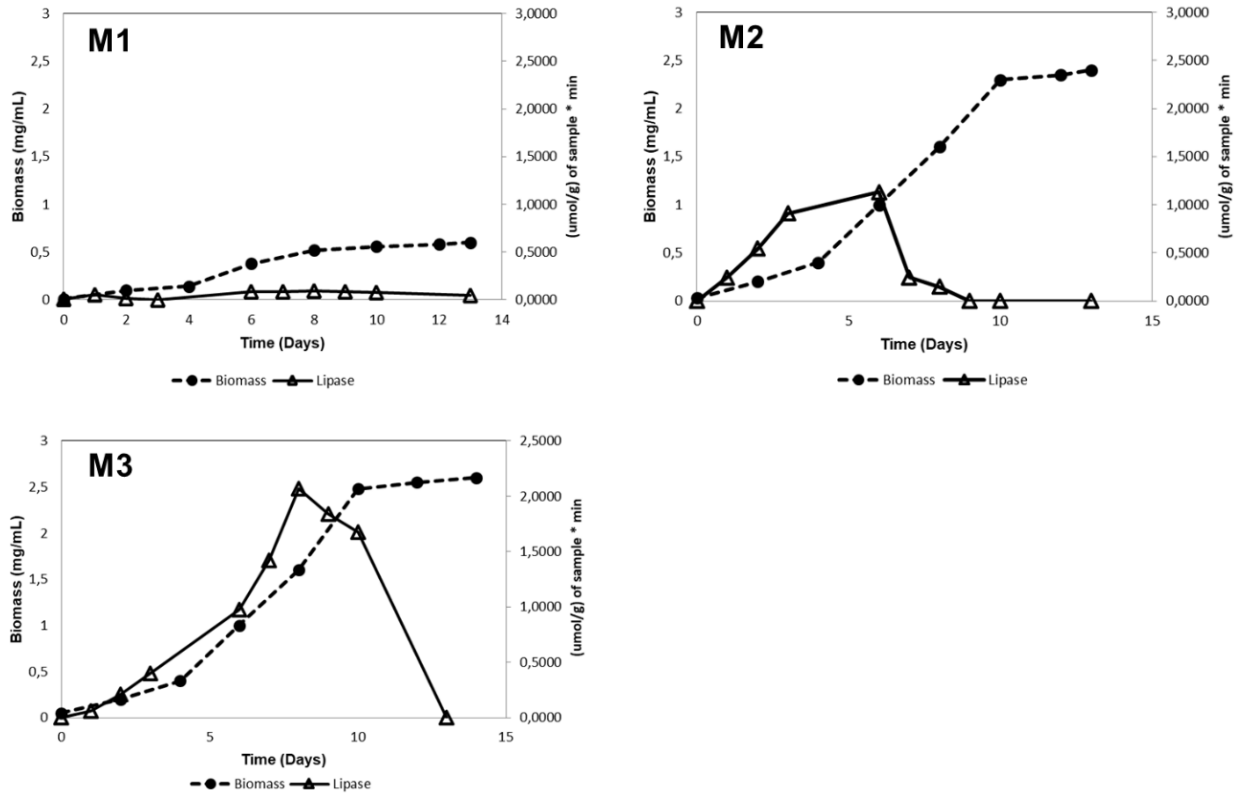

Figure 3. Biomass and lipase production by *B. licheniformis* grown in media formulations M1, M2, and M3.

dry biomass was observed, from which biomass concentration can be estimated by interpolation. Figures 2 to 4 also show hydrolase production by *B. licheniformis* grown in media M1, M2, and M3, specifically, proteases, lipases, and cellulases. Among these hydrolases, proteases are of the highest industrial interest. Proteases (EC 3.4.21-24 and 99) are industrially useful hydrolytic enzymes that cleave peptide bonds between amino acid residues and are the dominant enzyme in the worldwide market. Two-thirds of the proteases produced annually are used in the detergent industry. Microbial proteases, especially from *Bacillus* species, have traditionally comprised the predominant portion of industrial enzymes on the market. The major application of microbial proteases is in the formulation of various detergents, which constitutes a significant share of worldwide enzyme sales [22]. Many bacteria in the genus *Bacillus* excrete large amounts of enzymes into culture medium. Alkaline serine protease, one of the most important enzymes industrially, is excreted into culture medium by strains of *B. licheniformis* or *B. pumilus* [23]. These alkaline proteases (i.e., subtilisin) operate in a high alkaline pH range of 8.5-10 and are thus optimal for use in the formulation of detergents. In addition to their use in the detergents industry [24], these alkaline proteases have several other industrial applications, including in the production of foods, pharmaceuticals, leathers, and diagnostic reagents [25]. Proteases are also used for the bioconversion of chitinous materials as a waste-treatment alternative for the disposal of shellfish wastes [26, 27]. As shown in figure 2, *B. licheniformis* produces extracellular proteases during exponential growth and at the start of the stationary growth phase [28], after which point the production decreased. The analysis of the results obtained in medium M2 (without the addition of feather meal) and M3 (with added feather meal) show that the production of proteases by *B. licheniformis* is induced by the

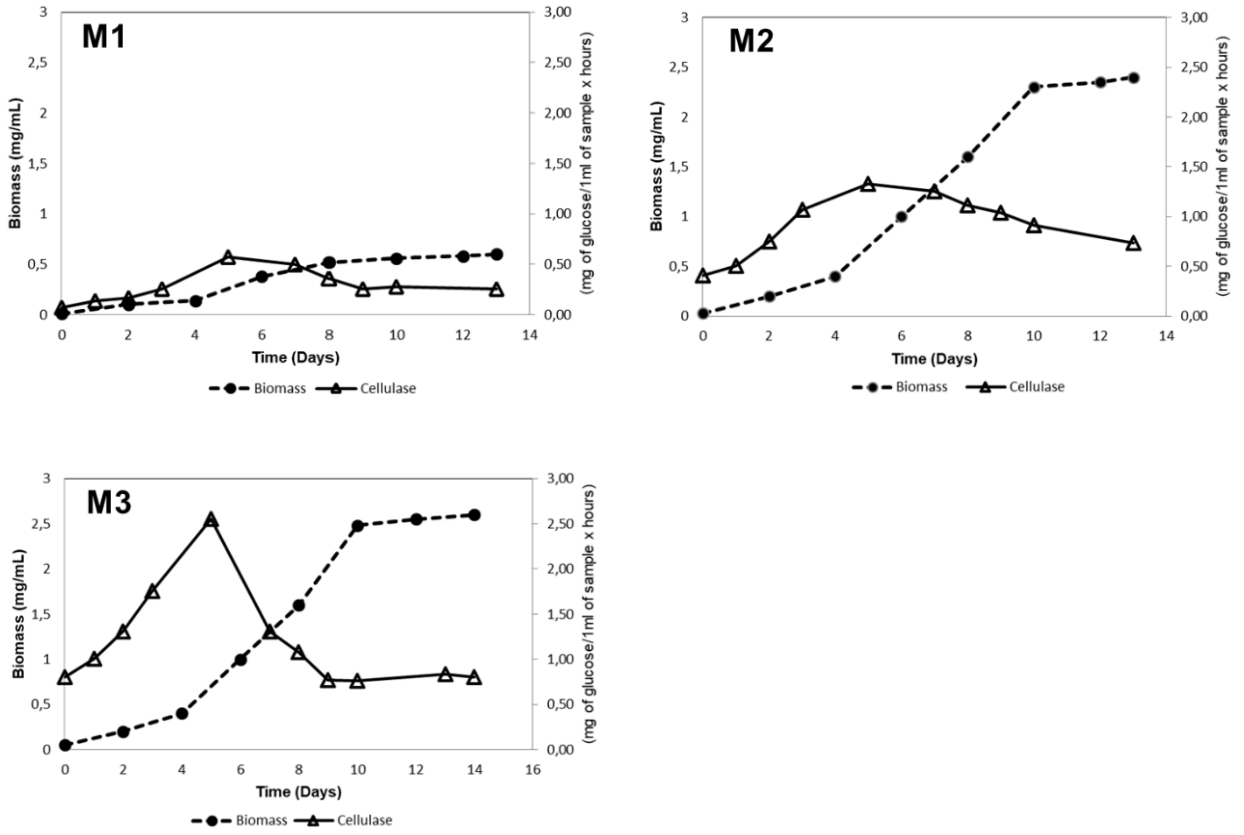

Figure 4. Biomass and cellulase production by *B. licheniformis* grown in media formulations M1, M2, and M3.

presence of feather meal. In both cases, it was also observed that the protease production activity became decreased if the stationary growth phase was prolonged. Therefore, we harvested the fermentation after 6 days of growth. The productivity at the point of harvesting was 16.89 mU/mL· day in medium M3 and 9.22 mU/mL· day in medium M2. Our results are in accordance with those previously reported by other authors [21, 28, 29, 30]. Extracellular protease production in microorganisms is highly influenced by media components, including variations in the carbon/nitrogen ratio, presence of some easily metabolizable sugars such as glucose [28, 29], and presence of metal ions [30]. In addition to these factors, several other properties such as aeration, inoculum density, pH, temperature, and incubation time were also found to affect the amount of extracellular protease produced [31, 32]. Glucose and peptone were found to be important factors in enhancing the formation of alkaline protease, specifically (figure 2). Protease production is only of industrial interest if the microorganisms that produce substantial amounts of extracellular enzymes can be grown well in an easily prepared and low-cost medium. The use of sewage sludge amended with the soluble sugar fraction of agroindustrial by-products (beer bagasse, sugar bagasse, rice bran, etc.) as fermentation media, such as media sample M2 and M3 in this study, to achieve these requirements could substantially improve the industrial production of proteases (Bautista et al., unpublished results). The recovery of proteases from fermentation broth was carried out by continuous centrifugation of the fermentation broth on a non-foaming disc centrifuge and subsequent recovery of the filtrate and discarding of the cake. The clear filtrate was then concentrated by ultrafiltration using a 50 kDa ultrafiltration membrane, and the resulting

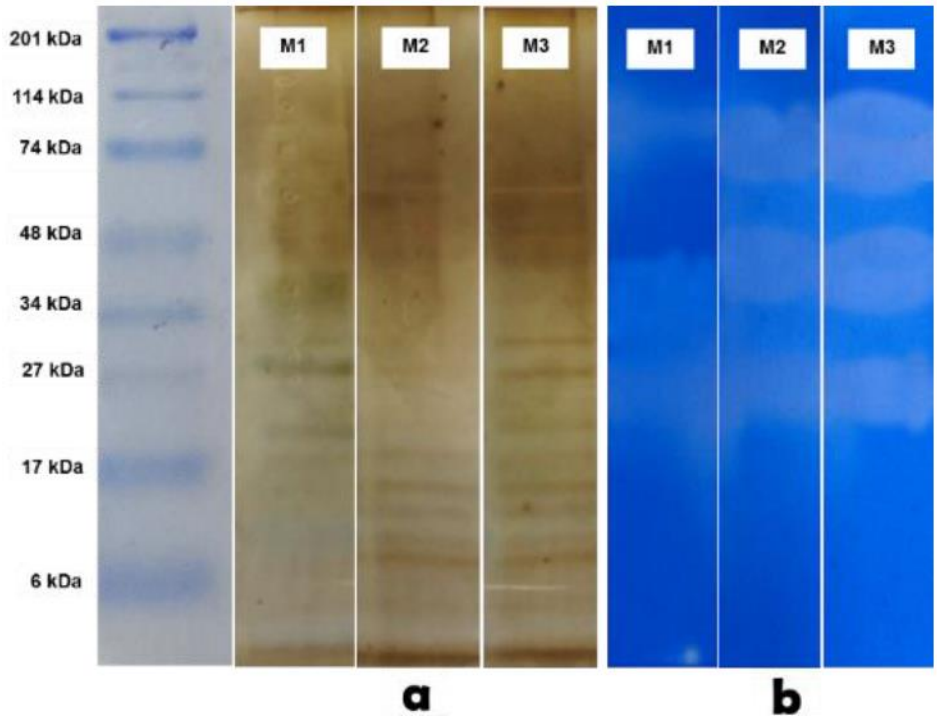

Figure 5. Electrophoretic separation by SDS-PAGE analysis of protein secreted by *B. licheniformis* grown in media formulations M1, M2, and M3. (a: silver staining; b: activity staining (zymogram)). (Molecular weight standards: Myosin 201 kDa, β-Galactosidase 114 kDa, BSA 74 kDa, Ovalbumin 48 kDa, Carbonic anhydrase 34 kDa, Soybean trypsin inhibitor 27 kDa, Lysozyme 17 kDa, Aprotinin 6 kDa).

ultraconcentrate was precipitated with the addition of 80% ethanol. The precipitate was then liophylized or dried with air at 30-40°C. Lipases are a group of enzymes of which the main function is the hydrolysis of triacylglycerol in a lipid-water interphase. This group of enzymes is of special interest in many industrial sectors, such as the detergent industry, food-industry and chemical and pharmaceutical industry [33]. *B. licheniformis* has been described as a major producer of lipases; therefore, we also studied the production of lipases by this microorganism in the media formulated with sewage sludge used in this work. Figure 3 shows the production of lipases in the three tested media formulations. These results show that the addition of feather meal also induced the production of lipases. This induction could be attributed to the relatively high fat content of the feather meal (8.4%). However, in this case an important difference was observed in lipase production by *B. licheniformis* in the two media formulations. In medium M2, the production of lipase was observed from the beginning of bacteria growth, while in medium M3, the production increased drastically after 4 days of growth. In both formulations, the lipase production level was maintained between days 5-9, after which point the lipase concentration in the medium decreased sharply, probably due to the actions of proteases. Therefore, the lipase productivity at day 8 was used to evaluate lipase production and was estimated as 0.25 mU/mL· day (figure 3). The production of enzymes that degrade cellulosic material, such as cellulases, is of great importance in the development of new biorefinery approaches to produce biofuels and high value-added products through fermentation. Therefore, the development of abundant and cheap sources of cellulose-degrading enzymes is necessary. For this reason, we also tested the production of cellulases by *B. licheniformis* in media formulated with sewage sludge. Figure 4 shows the production of

cellulases by *B. licheniformis* grown in media formulations M1, M2, and M3. The production of cellulase in medium M2 started at the beginning of bacteria growth, likely due to the presence of cellulosic materials in the sewage sludge. Cellulase production reached a maximum after 5 days of bacteria growth, with a productivity of 0.51 mU/mL· day. Surprisingly, the addition of feather meal also induced the production of cellulolytic enzymes, although this induction was less pronounced than that seen for proteases and lipases. The cause of this induction remains obscure. In addition to protease, lipase and cellulase activities, other enzyme activities, such as those of β-glucosidase and acid phosphatase, were also tested in the supernatant from the growth of *B. licheniformis* in media formulations M1, M2, and M3. No enzymatic activities beyond those of proteases, lipases, and cellulases were detected (figure 4).

Proteases, lipases and cellulase are inherently produced by *B. licheniformis*, but in the presence of feather meal, the production of these enzymes was clearly enhanced, particularly in the case of proteases and lipases [21]. Induction of the production of these enzymes with hydrolytic activities suggests that these enzymes are involved in the utilization of insoluble C- and N-sources for *B. licheniformis* survival when soluble C- and N-sources are low. Other enzymes are likely also involved in this adaptation process; therefore, we analyzed the set of proteins excreted (secretome) by *B. licheniformis* using electrophoretic and proteomic techniques.

The proteins produced by *B. licheniformis* grown in the media formulations M1, M2, and M3 were separated by SDS-PAGE analysis and are shown in figure 5. As these results show, the excretion of proteins/enzymes into the growth medium was greater in mediums M3 and M2 than in M1 as a result of the enhanced growth of *B. licheniformis*. Zymographic analysis showed that in both M2 and M3, three main protease zones can be detected—one approximately 75 kDa and other two approximately 34 and 27 kDa. In order to study the set of proteins secreted by *B. licheniformis* grown in media formulated with sewage sludge (M2 and M3), we used a proteomic approach based on the shotgun procedure. In the presence of a culture medium that includes feather meal as an inductor, *B. licheniformis* is capable of excreting various kinds of enzymes in order to degrade the components of the medium and, thus obtain nutrients required for survival [34-37] (figure 5). Most *Bacillus sp*. initiate a series of transitional responses that are designed to maintain or restore growth under different environmental conditions, including the induction of macromolecular hydrolases, such as proteases, lipases, and polysaccharidases. Enzymatic induction through addition of materials to the substrate has been described for *Bacillus*. For example, xylane addition induces the excretion of xylanases and other glycoside hydrolases [38], and substrates formulated with olive oil, an important inductor and source of lipids, induces the synthesis and excretion of lipases [34]. The results obtained in this study show that feather meal can be used as a N-source for the growth of *B. licheniformis* and can also act as a strong inducer of the secretion of hydrolytic enzymes, specifically, proteases, lipases, and to a lesser extent, cellulases, and other proteins and enzymes. The proteins and enzymes secreted by *B. licheniformis* grown in medium M3 was characterized by a proteomic approach using the shotgun procedure, and the results are shown in table 2. The analysis of exocellular proteins excreted by *B. licheniformis* in medium M3 showed a relatively low diversity of proteins compared to that found in other fermentation media, such as dextrose broth [21] (table 2). The main proteins and/or enzymes secreted were hydrolases (> 80%), and of these, the protease keratinase (KerA) is of special relevance. This enzyme is typically produced when *B. licheniformis is* grown in a media with primarily insoluble N-sources, such as keratins. In addition to promoting the hydrolysis of keratins, keratinase is capable of hydrolyzing a broad range of protein substrates and, therefore, has many potential agricultural and industrial

Table 2. Identified extracellular proteins/enzymes secreted by *Bacillus licheniformis* grown in medium M3.

Protein name	Mass (Da)	Score	Queries Matched	emPAI*
KerA, Subtilisin precursor	31,266	640	18	1.02
Chain A, Chitosanase	435	468	16	0.44
Glycoside hydrolase family 14 protein	79,565	419	9	0.22
Glycoside hydrolase family 18 protein	66,111	273	5	0.10
Gamma-glutamyltranspeptidase (Ggt)	54,048	221	3	0.10
Flagellin	3,201	218	5	0.21
Extracellular serine protease	85,573	184	5	0.08
Hypothetical protein BL00275	14,050	173	9	0.24
Putative acylaminoacyl-peptidase YuxL	73,549	109	2	0.04
Chitosanase	50,616	348	11	0.37
Intracellular serine protease	33,888	232	7	0.45
Superoxide dismutase	22,530	187	11	0.51
Metalloregulation DNA-binding stress protein	17,909	157	4	0.19
Metal-dependent hydrolase	24,831	122	2	0.10
Chitosanase	19,722	116	3	0.17

applications. Gamma-glutamyl transpeptidase (GGT, E.C. 2.3.2.2) was another minor protein found in the fermentation solutions examined. This protein catalyzes the transfer of the γ-glutamyl moiety from γ-glutamyl compounds to a variety of amino acids and dipeptide acceptors [39, 40]. The hydrolytic and glutaminase activities involved in this process are used in the food industry for debittering amino acids. GGT has also been suggested to assist subtilisin during its action on keratin [41]. *B. licheniformis* was found to secrete extracellular GGT and keratinase during keratin degradation processes. Keratinase in the presence of GGT exhibits an extended substrate spectrum, cleaving all types of keratins. The complex thus exhibits better catalytic properties when GGT is present, a characteristic which can be exploited in various biotechnological applications [42].

Conclusion

These results demonstrate the successful culture of *B. licheniformis* in a medium formulated with sewage sludge as the main source of carbon and nitrogen with added feather meal (keratin) as an inductor. This formulation is a cheap and abundant fermentation medium that can be used for the regular production of hydrolases, particularly proteases and lipases, on a large scale in relatively short culture periods of 5 and 8 days. The subsequent recovery of these enzymes through continuous centrifugation and concentration by ultrafiltration, followed by fractionation with ethanol (actually underway), could be a promising process for the preparation of products with high proteolytic and/or lipolytic activity for inclusion in enzymatic formulations for food production and agro-industrial uses.

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