

High-energy ball milling treatment of soybean for *Bacillus thuringiensis* culture media

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Soybean meal has been intensively used as a substrate in culture media for several microorganisms. However, the fermentable sugar containing the soybean needs to be released from the solid matrix through different processes. Against this backdrop, the present study explores the use of high-energy ball milling as a one-step treatment method for expedited production of fermentable sugars of textured soybean. The best result is observed after only 5 min of milling, obtaining 34.1 times more fermentable sugars than untreated textured soybean, and 2.5 times more than commercially used soybean meal. Notably, the textured soybean ball-milled has been used as a substrate for *Bacillus thuringiensis* var. *kurstaki* HD-73 fermentation. The cell and spore production is also compared with a standard Rowe media. The maximum cell concentration obtained in the entire fermentation process using ball-milled textured soybean media is found to be higher than the concentration obtained using the standard Rowe media. In addition, it is observed that there is a direct correlation between maximum cell production and reducing sugar concentration generated by the high-energy ball milling treatment. No fermentation inhibitors or by-products are generated during the physical treatment.

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[**Key words:** Biomass treatment; High-energy ball milling; Reducing sugar; Soybean meal; Textured-soybean]

Soybean meal (SM) is a co-product after oil is extracted from seeds. It is rich in protein and it has been used to supplement plant protein for animal, chicken, and pet feeds (1). In addition, SM has been used as a substrate in culture media for several microorganisms, such as *Bacillus* sp. (2,3), *Bacillus subtilis* (4), and *Thermomucor indicae-seudaticae* N31 (5), among others. Hydrolyzed SM, obtained with an enzymatic treatment, has been used as a substrate in culture media for *Clostridium butyricum* TK2 (6), *Rhizopus oryzae* (7), *Saccharomyces cerevisiae* NRRL Y-2233, *Zymomonas mobilis* subsp. *mobilis* NRRL B-4286 (8), and *B. subtilis* CICC 10025 (9), among others. Obtained with acid hydrolysis, hydrolyzed SM has been used as a substrate for *Bacillus thuringiensis* var. *kurstaki* HD73 (10–12). *B. thuringiensis* produces Cry1Ac protein, which is one of the most sold bioinsecticides available in the market (13).

SM contains 44–49% of protein and 30–32% of carbohydrates on a dried basis (14). Half of the total carbohydrates are structural and the remainder constitutes of low-molecular weight sugars, oligosaccharides and starch in relatively small quantities (15). It may also be noted that around 17% of low molecular weight sugars consist of a mixture of glucose, arabinose, galactose, fructose, and sucrose (16). Although SM can be used as a substrate, fermentable sugars must be

released from the solid matrix, by hydrolysis with the involvement of dilute acid, by enzymatic hydrolysis, or by other methods (8). Hydrolysis is a reaction that entails the breaking of bonds in a molecule. In this regard, acid hydrolysis is one of the most frequently used methods for breaking starch and cellulose into glucose (17). The major advantages of acid hydrolysis include a fast reaction rate and the fact that it facilitates continuous processing. However, the low glucose yield is a significant shortcoming.

Several physical pretreatments that change the physicochemical properties of substrate have been reported; these include microwave (18), high pressure (19), or ball milling (20,21). Meanwhile ball mills can be divided into two groups: low energy and high energy ball mills. Low energy ball mills are usually used as the first step to reduce the particle size, create a higher accessible surface area and decrease crystallinity. In contrast, high-energy ball mills are normally used in chemistry or material science in order to activate matter (mechanical activation) or to promote reaction (mechanochemistry) (22–26). For this reason, if the acid hydrolysis breaks the chemical bonds in the soybean, thereby reducing sugar and amino acids, the energy gets transferred from the balls during the high energy ball milling process, which is strong enough to trigger chemical reactions and hydrolyze biomass within a single step. Therefore, the present study explores the use of high-energy ball milling as a one-step treatment to release reducing sugar from textured soybean (TS). Additionally,

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due to the fact that SM is extensively used in microbiological culture media, this study also explores the use of TS at different stages of high-energy ball milling (TS-tmin) as a substrate for *B. thuringiensis* var. *kurstaki* HD-73 fermentation.

MATERIALS AND METHODS

Soybean (glycine max) Two different soybean samples were used in this study: TS and SM. Notably, SM was used only as a reference in this study. The SM used was donated from the Bioprocess Laboratory (Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, CINVESTAV, Mexico) and has been used and reported before (12). On the other hand, the TS used was purchased in a health food store.

High-energy ball milling A planetary high-energy ball mill (PM 400/2, Retsch GmbH, Haan, Germany) was used to physically treat the TS. The treatment was carried out in air (in hermetically sealed vials) with vials (250 mL) and balls (9 of 2 cm) of stainless steel (Fe, $\rho = 7.9 \text{ g/cm}^3$). A rotation rate of 400 rpm and a weight ratio of 1:54 sample: milling balls (5.5 g: 297 g) were used. The milling times tested were noted as 2, 5, 8, 10 and 20 min. While the temperature during milling was not measured, the vials could be touched directly after the milling. Besides, the milling was conducted in cycles of 5 min milling and 5 min of pause for cooling in order to avoid overheating.

Reducing sugar quantification Reducing sugars are mono and oligosaccharides with an aldehyde group or a free carbonyl group that can be easily assimilated by microorganisms (27). The reducing sugar quantification was undertaken with the dinitrosalicylic acid technique, albeit with some adjustments (28,29). A calibration curve was obtained using D-glucose as the standard. The dinitrosalicylic acid solution was prepared by dissolving 1 g of 3,5-dinitrosalicylic acid into 10 mL of distilled water, after which the solution was transferred into a 100 mL volumetric flask. Subsequently, 1.6 g of NaOH and 30 g of sodium potassium tartaric were added into the solution, and the volume of the solution was fixed to the mark with distilled water. On the other hand, the sample was prepared by dissolving 1 g of sample into 100 mL of distilled water. For the purpose of reducing sugar determination, an aliquot of 0.25 mL of sample was placed in a test tube; 1.5 mL dinitrosalicylic acid solution was then added into it. The mixture was heated at 100°C for 5 min in a water bath. Thereafter, the sample was cooled at room temperature and its absorbance was recorded at 540 nm using a UV-visible spectrophotometer Jenway model 6700 (Jenway, Staffordshire, UK). The concentration of reducing sugar was calculated by substituting the absorbance of the sample into the line equation that was obtained from the standard curve.

***B. thuringiensis* var. *kurstaki* HD-73 fermentation** Rowe medium (30) and two modifications of this medium were used for *B. thuringiensis* var. *kurstaki* HD-73 fermentations. Rowe medium contains 3.2 g/L casein peptone, 10 g/L glucose, 3.2 g/L yeast extract, 0.106 g/L CaCl₂·2H₂O, 0.5 g/L KCl, 0.3 g/L MgSO₄·7H₂O, 0.05 g/L MnSO₄·0.0075 g/L ZnSO₄·7H₂O, 0.0045 g/L CuSO₄·5H₂O, 0.075 g/L C₆H₅FeO₇, 1.856 g/L Na₂HPO₄, and 1.153 g/L NaH₂PO₄·H₂O. For the modified media, casein peptone was replaced by 3.2 g/L SM (R-SM) and ball-milled TS (R-TS-tmin). All media were sterilized at 120°C and 1.3 kg/cm² for 20 min. Fermentations were carried out in 1000 mL shake-flasks with 200 mL medium, seeded with a spore disk, and cultured at 30°C at 200 rpm within a New Brunswick G-25 rotary shaker (New Brunswick Scientific Co., Inc., Edison, NJ, USA). The end-of-fermentation criterion was set at 90% released spores and determined microscopically. Cell and spore counts were microscopically determined in as many as four replicates with a Neubauer chamber (ISOLAB Laborgeräte GmbH, Wertheim, Germany). Serial dilutions were also used to avoid cell or spore clumping. It may be noted that the fermentation parameters were defined on the basis of previous reports (10,12).

Characterization techniques The morphology and characteristics of the milled TS samples' surface were observed by scanning electron microscopy (SEM) using a JEOL JSM-6390 LV (JEOL, Tokyo, Japan) and an FEI-Helios Nanolab 600 (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, these samples were placed on a 30 nm carbon electrically conductive tape or covered with a gold layer. X-ray powder diffraction patterns were then collected in air at ambient temperature in a Bruker D-8 Advance diffractometer (Bruker, Billerica, MA, USA) with CuK α radiation. The 2 θ -range explored was 10°–80° with 0.05° step size, 10s counting time, continuous mode, and spinning of 15 rpm. Thermogravimetric (TGA) and differential thermal analysis (DTA) were conducted using a Perkin Elmer STA 6000 simultaneous thermal analyzer (PerkinElmer, Inc., Waltham, MA, USA). Subsequently, 15 mg of sample was placed within an open alumina pan and analyzed with N₂ atmosphere in the temperature range of 30–700°C at 10°C/min.

RESULTS

Soybean reducing sugar content The correlation between the reducing sugar concentrations of SM, TS and TS-tmin can be

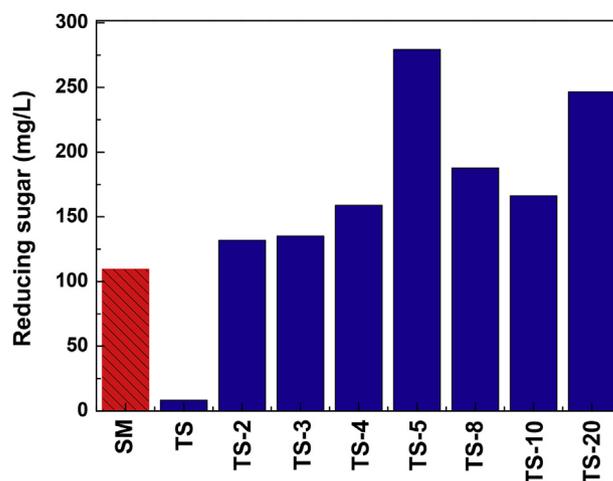


FIG. 1. Reducing sugar concentration of soybean meal (SM), textured soybean (TS) and TS at different stages of high-energy ball milling (TS-tmin; e.g., TS-2 indicates textured soybean with 2 min of milling).

illustrated in Fig. 1. The reducing sugar concentration in all the milled samples is found to be higher than in TS (Fig. 1). When compared to TS, the reducing sugar concentration of TS-tmin after 2, 3 or 4 min of milling increases 17.2 (1.2) times, while it increases 34.1 times after 5 min (3406.2%, 279.3 mg/L). As compared to the reducing sugar concentration of SM, which is extensively used in microbiological culture media, the reducing sugar concentration of TS-tmin increases 1.3 (0.1) times after 2, 3 or 4 min of milling, while it increases 2.5 times (254.6%) after 5 min of milling. The concentration of reducing sugar decreased after more than 5 min of milling.

***B. thuringiensis* var. *kurstaki* HD-73 cultures** The SM and TS-tmin samples were used in the growth media for *B. thuringiensis* var. *kurstaki* HD-73 in order to determine whether the reducing sugar generated during the treatment can indeed be assimilated for the microorganism. However, TS was not tested due to its lack of solubility.

Cell and spore production are compared between Rowe media, Rowe media with SM (R-SM) and TS (R-TS-tmin), as depicted in

TABLE 1. Cell and spore production of *Bacillus thuringiensis* var. *kurstaki* HD-73 from different media.

Medium	X_{\max} ^{a,b}	Sp_{\max} ^{c,b}	$t_{X_{\max}}$ (h) ^d	$t_{Sp_{00}}$ (h) ^e	Increment related to R		Increment related to R-SM	
					X_{\max}	Sp_{\max}	X_{\max}	Sp_{\max}
R ^f	6.7	2.9	26	40	–	–	–	–
R-SM ^g	6.0	12.5	72	72	–	–	–	–
R-TS-2 ^h	18.5	29.0	40	40	2.8	10.0	3.1	2.3
R-TS-3	17.0	35.3	32	32	2.5	12.2	2.8	2.8
R-TS-4	20.5	41.5	32	40	3.1	14.3	3.4	3.3
R-TS-5	28.3	31.5	40	40	4.2	10.9	4.7	2.5
R-TS-8	22.0	36.0	32	40	3.3	12.4	3.7	2.9
R-TS-10	18.8	31.5	24	32	2.8	10.9	3.1	2.5
R-TS-20	26.3	36.8	32	40	3.9	12.7	4.4	2.9

^a Maximum cell concentration (X_{\max}).

^b 1×10^8 /mL.

^c Maximum spores concentration (Sp_{\max}).

^d Time where the maximum cell concentration is detected.

^e Time where the first spore is detected.

^f R, Rowe medium.

^g R-SM, Rowe medium modified with soybean meal.

^h Rowe medium modified with textured soybean with time of high-energy ball milling treatment (R-TS-tmin) (e.g., R-TS-2 indicates Rowe medium modified with textured soybean with 2 min of milling).

Table 1. The maximum cell concentration (X_{\max}) obtained in the entire fermentation process using R-TS-tmin is found to be higher than for Rowe media or R-SM media. **Table 1** shows that the maximum X_{\max} is obtained in the medium R-TS-5 ($2.83 \times 10^9/\text{mL}$), where X_{\max} increases by 4.2 and 4.7 times as compared to the X_{\max} obtained for Rowe media and R-SM, respectively. Besides, there is a direct correlation between the maximum cell concentration and the reducing sugar concentration generated by the high-energy ball milling treatment (Fig. 2A,B). Notably, a correlation analysis between the cell production and reducing sugar concentration gives a Pearson's correlation coefficient of 0.977 (Fig. 2B). These results confirm the generation of reducing sugar from TS through the high-energy ball milling treatment. Additionally, no fermentation inhibitors are detected after the physical treatment.

All media with TS (with and without milling) produces a higher spore concentration (Sp_{\max}) than Rowe media. The maximum Sp_{\max} is obtained in the medium R-TS-4 ($4.15 \times 10^9/\text{mL}$), where Sp_{\max} increases by 14.3 and 3.3 times as compared to the Sp_{\max} obtained for Rowe media and R-SM, respectively (Table 1). Importantly, the maximum spore concentration and the reducing sugar concentration generated by the high-energy ball milling are not directly proportional (Table 1). Moreover, $t_{X_{\max}}$ indicates the moment during the culture where the maximum concentration of cells is attained, and $t_{\text{Sp}0}$ indicates the moment at which the first spore is detected (i.e., the sporulation phase onset). The $t_{X_{\max}}$ for R-TS-tmin are found to be smaller than for R-SM from 32 to 48 h. Therefore, the maximum cells concentration is obtained 32–48 h earlier in R-TS-tmin than for R-SM (Table 1). Moreover, the sporulation phase onset occurs 32–40 h earlier in R-TS-tmin than for R-SM, which implies a decrease in culture duration, as shown in Table 1. These findings confirm that no fermentation inhibitors are generated after the physical treatment and that this physical treatment generates reducing sugar that can be easily assimilated for the microorganisms.

Characterization of TS-tmin samples Once it was established that the reducing sugars of TS-tmin do indeed support the growth of *B. thuringiensis* var. *kurstaki* HD-73, the TS-tmin samples were further characterized. SEM images of some TS-tmin samples are illustrated in Fig. 3. The average TS particle size was 4 ± 1 mm. After 2 min of milling the average TS-2 particle size was 35 ± 22 μm , which then increased to 38 ± 25 μm at 5 min; meanwhile it was 24 ± 18 μm at 10 min. One of the early uses of ball milling has been reducing the particle size, but the particles begin to agglomerate after reaching a critical particle size (31). The agglomeration can be detected after long milling times in low energy mills, but this stage is reached quickly in high-energy mills. Thus, the particle size of TS decreases rapidly at short milling times (up to 2 min). At this milling time, the particle surfaces are flat, which is typically associated with fragile

fractures. At 5 min, the particles are found to be heterogeneous in size and particle aggregates can be seen everywhere; it also becomes evident that the particle surfaces are no longer flat. The aggregates were broken again at a higher duration than 10 min, at which point a heterogeneous and amorphous material is observed. High-energy ball milling generates both physical and chemical transformations of substances induced by mechanical energy. Put succinctly, the effect is very well documented; however, that is not the case in complex organic matrices. Importantly, energy dispersive X-ray spectroscopy analysis from five spots of different areas of TS and TS-20 reveals that the samples were not contaminated from the grinding material (Fe) during the high-energy ball milling treatment.

Fig. 4 illustrates the powder diffraction patterns of SM, TS, and TS-tmin. A typical diffraction pattern of an amorphous material is observed on these samples. This behavior was expected since the SM composition mainly comprises of protein, carbohydrate, and moisture.

TGA and DTA curves of SM, TS, and TS-tmin are illustrated in Fig. 5. The TGA curves reveal a similar behavior. Importantly, two weight loss regions can be observed. The first one, at around 100°C , is associated with water loss at around 8% weight loss. This is confirmed by the endothermic peak observed at the same temperature in the DTA curves. Meanwhile there is an association of the second region, between 220°C and 400°C , with the degradation of the soybean (32). The weight loss in this region increases up to 60%. The DTA curves depict an increasing endothermic heat flow that consists of the energy required to degrade the soybean. After the soybean degradation process, just the inert carbonaceous residues remain, which needs higher energy to keep on degrading. The effect of ball milling seems to be the increment of soybean thermal stability. No crystallization or phase transition processes were observed in this process. The first derivative of the TGA curve allows the observation of commencement and end of each event of mass loss, indicating the temperature range where the decomposition reaction occurs. The first derivative of the TGA curves (Fig. 5) clearly illustrates the two regions observed in TGA curves. Particularly in the second region, it can be seen that the major weight loss is associated with two different events, one at around 250°C and the other at above 310°C . As mentioned above, the soybean composition mainly consists of protein, carbohydrate, moisture, and oil. The moisture is lost at around 100°C while the oil is lost during the texturing process. Thus, the event at 250°C could be correlated with the degradation of protein, while the event at 310°C is associated with the degradation of carbohydrate. It has been reported that starch is one of the main components of carbohydrates in soybean, while the degradation of starch was observed between 300°C and 340°C (33). Therefore, the weight loss observed at 310°C is consistent with the carbohydrate degradation.

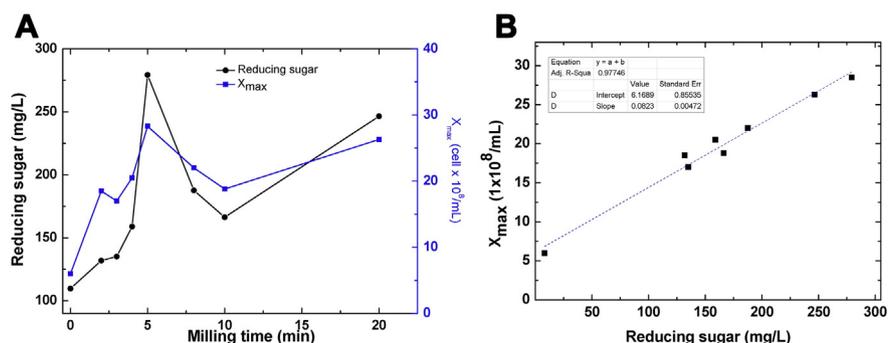


FIG. 2. (A) Reducing sugar concentration and maximum cell concentration of different media used in the growth of *Bacillus thuringiensis* var. *kurstaki* HD-73. (B) Correlation between the cell production and the reducing sugar concentration.

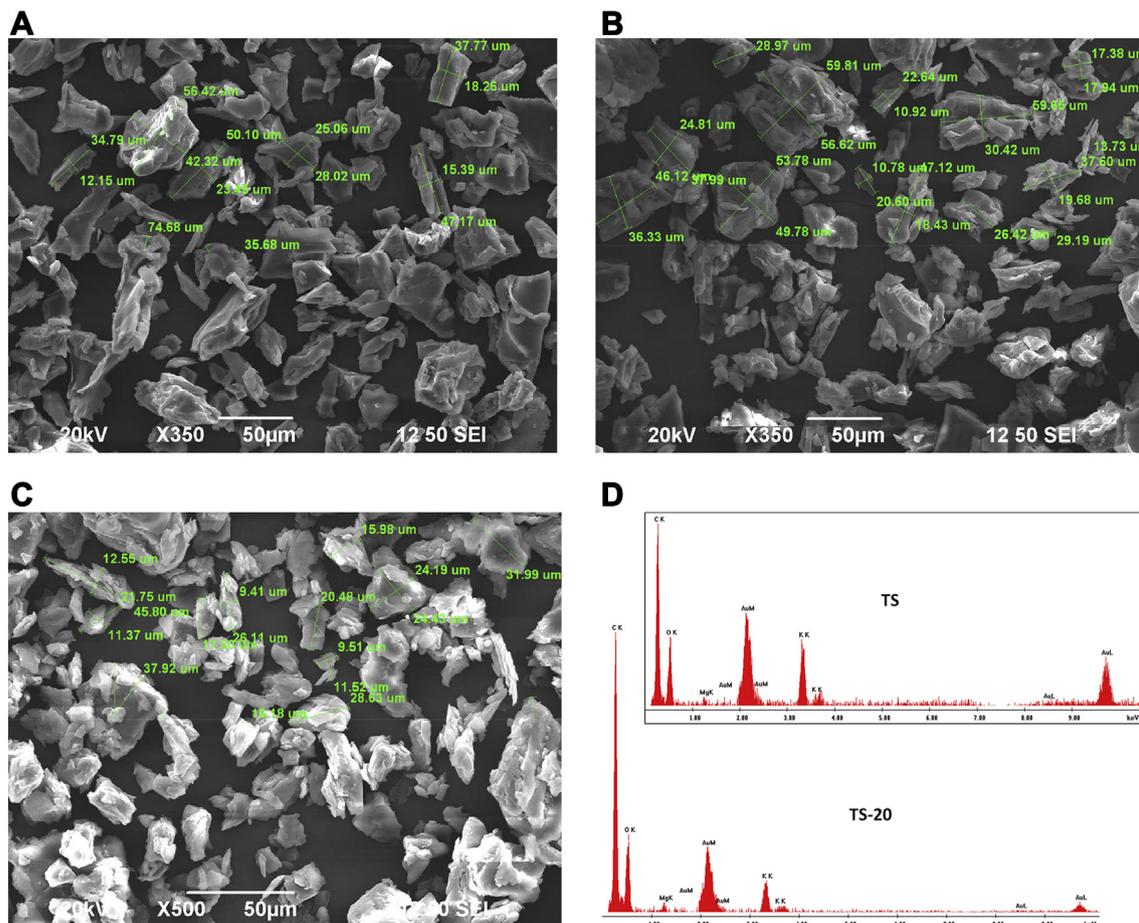


FIG. 3. Scanning electron micrographs of textured-soybean (TS) at different stages of high-energy ball milling: (A) TS-2, (B) TS-5, and (C) TS-10. (D) EDS analysis of TS and TS-20.

DISCUSSION

As compared to TS and SM, the reducing sugar concentration of TS-5, without any other further treatment, increases by 34.1 and 2.5 times, respectively. This increment can be correlated with the re-organization of the chains of the saccharides.

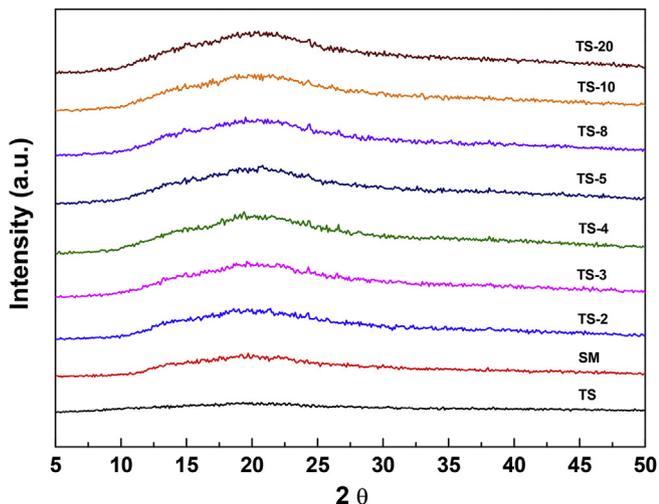


FIG. 4. X-ray diffraction patterns of soybean meal (SM) and textured-soybean (TS) at different stages of high-energy ball milling (TS-tmin).

In general, reducing sugar generation from biomass is a very energetically expensive process. Therefore, various efforts have been made to seek less expensive pretreatments or explore the usage of different substrates. The pretreatments reported mainly consist in a combination of two or more types of treatments in order to increase the reducing sugar content and lower the associated economic cost. For example, Yoo et al. (34) analyzed the combination of enzymatic hydrolysis, thermo-mechanical extrusion and acid/alkali hydrolysis of soybean hulls. They reported that as compared to untreated soybean hulls, the glucose yield from enzymatic hydrolysis increased by 0.7, 1.3 and 1.3 times when the soybean hulls were pretreated with dilute acid, alkali, and extrusion, respectively. Correspondingly, Huang et al. (35) undertook an analysis of the combination of physicochemical pretreatments with enzymatic hydrolysis in order to convert lignocellulose to reducing sugar. According to their results, the pretreatment used increases the reducing sugar content by 2.3 times. Analogously, Xu et al. (36) reported the combination of lime pretreatment with enzymatic hydrolysis of switch-grass. They observed that the best conditions (50°C, 24 h, 0.1 g Ca(OH)₂/g raw biomass) for the yields of glucose, xylose, and total reducing sugars reached was 3.15, 5.78, and 3.61 times than those of untreated biomass. Therefore, comparing those results with the findings of the present study demonstrates that high-energy ball milling is an effective one-step treatment to generate reducing sugar from TS. Besides, this physical treatment not only increases the reducing sugar concentration, but also reduces the treatment time and the associated reagents cost.

In conclusion, high-energy ball milling has been established an effective one-step treatment to generate reducing sugar from TS.

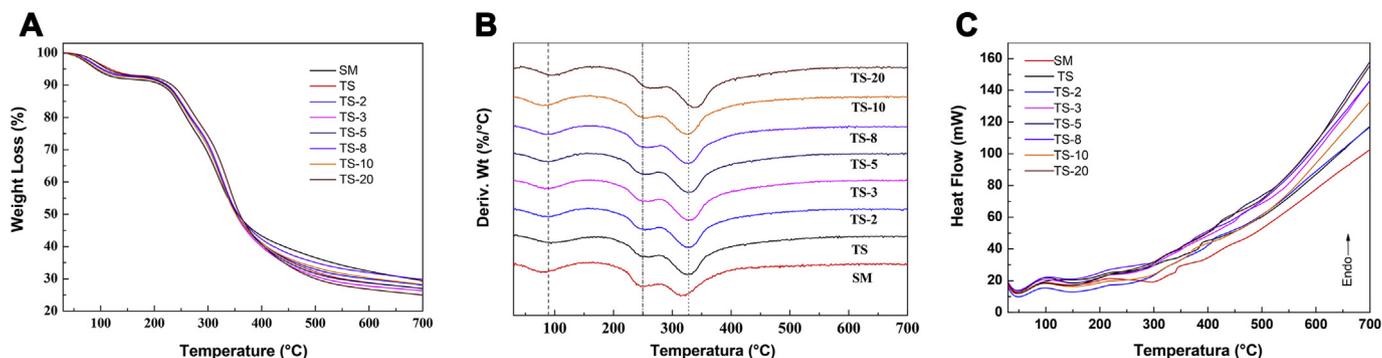


FIG. 5. (A) Thermogravimetric analysis (TGA), (B) first derivative of the thermograms and (C) differential thermal analysis (DTA) curves of soybean meal (SM) and textured-soybean (TS) at different stages of high-energy ball milling (TS-tmin).

This one-step physical treatment can be very useful not only in culture media, but also in any fermentation base industry. More specifically, 5 min of high-energy ball milling of TS is sufficient enough to increase the production of *B. thuringiensis*.

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