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Soybean-based media for the cultivation of *Bacillus subtilis* 105 as a bio-fungicide against *Sclerotinia sclerotiorum*

Medio a base de soya para el cultivo de *Bacillus subtilis* 105 como bio-fungicida contra *Sclerotinia sclerotiorum*

L.G. Sarmiento-López[1], M. López-Meyer[2], I.E. Maldonado-Mendoza[2], G. Sepúlveda-Jiménez[3], M. Rodríguez-Monroy[3]*

[1]Unidad de Investigación en Ambiente y Salud, Universidad Autónoma de Occidente Unidad Regional Los Mochis, Sinaloa, México. [2]Departamento de Biotecnología Agrícola, Instituto Politécnico Nacional. Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional, Unidad Sinaloa, Guasave, Sinaloa, México. [3]Departamento de Biotecnología, Instituto Politécnico Nacional. Centro de Desarrollo de Productos Bióticos, Yautepec, Morelos, México

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Abstract

Bacillus subtilis 105 is a bacterium with biocontrol potential against *Sclerotinia sclerotiorum,* a plant pathogen. To achieve the mass production of *B. subtilis*, this study proposes the use of agro-industrial byproducts to prepare an alternative medium to the microbiological Luria Bertani (LB). Three different media were prepared: Soybean flour, yeast extract, and NaCl (SYM); Pea flour, yeast extract, and NaCl (PYM); Solulys, yeast extract, and NaCl (SolYM). SYM was selected for use in a shake flask as a suitable alternative to the microbiological LB medium because it results in the highest cell density. The selected SYM was evaluated for its ability to grow *B. subtilis* in a stirred tank bioreactor, and the effects 400 and 700 rpm in a bioreactor were assessed. The growth of *B. subtilis* grown at 400 rpm was similar in SYM media and microbiological LB, but oxygen limitation was observed. At 700 rpm, oxygen limitation was improved. Notably, the highest antagonistic activity against *S. sclerotiorum* was observed with *B. subtilis* cultures grown in SYM at 400 rpm. In conclusion, SYM is an innovation to use of microbiological LB medium for the production of *B. subtilis* in bioreactors while preserving its antagonistic activity against *S. sclerotiorum*.

Keywords: Stirred tank bioreactor, *Bacillus subtillis*, *Sclerotinia sclerotiorum*, Low-cost medium, Soybean.

Resumen

Bacillus subtilis 105 es una bacteria con potencial para el biocontol de *Sclerotinia sclerotiorum*, un patógeno de plantas. Para cultivar *B. subtilis* a gran escala, este estudio propone el uso de subproductos agroindustriales para preparar un medio alternativo al medio Luria Broth (LB). Tres diferentes medios fueron preparados con: harina de soya, extracto de levadura y NaCl (SYM), harina de chícharo, extracto de levadura y NaCl (PYM), Solulys, extracto de levadura y NaCl (SolYM). El medio SYM fue seleccionado en matraces, porque genera el mayor crecimiento celular. Para hacer crecer *B. subtilis* en un biorreactor de tanque agitado, se empleó el medio SYM y los efectos de dos velocidades de agitación (400 y 700 rpm) fueron determinados. El crecimiento de *B. subtilis* a 400 rpm fue similar en los medios SYM y LB, pero se observó una limitación por oxígeno. A 700 rpm, la limitación de oxígeno fue superada. Los cultivos de *B. subtilis* cultivados en SYM a 400 rpm presentaron la mayor actividad antagónica frente a *S. sclerotiorum.* En conclusión, el medio SYM representa una innovación para la fermentación a escala piloto o industrial de *B. subtilis*, ya que mantiene su actividad antagónica contra *S. sclerotiorum.*

Palabras clave: biorreactor tanque agitado, *Bacillus subtillis*, *Sclerotinia sclerotiorum*, Medio de bajo costo, Harina de soya.

*Corresponding author. E-mail: mrmonroy@ipn.mx ;
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1 Introduction

Pathogens, including viruses, bacteria, and fungi, can induce plant diseases that disrupt plant growth and development. Diseases potentially decrease crop yield and quality, thereby posing a significant threat to global food security (Strange and Scott, 2005; Fones *et al*., 2020). Globally, crop yield losses caused by these biological agents are estimated to reach 30%, resulting in an annual economic loss of hundreds of billions of dollars (Geffersa *et al*., 2023).

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic fungal pathogen with a global distribution, capable of infecting more than 400 plant species (Smolińska and Kowalska, 2018). This pathogen infects the leaves, flowers, fruits, and stems of economically important crops such as *Phaseolus vulgaris*, *Solanum lycopersicum*, *Lactuca sativa*, *Helianthus annuus*, and *Daucus carota* (Na *et al*., 2018; Sabaté *et al*. 2018; Lobo *et al*., 2000; Macioszek *et al*., 2023). This pathogen causes white mold disease, particularly in *Phaseolus vulgaris*. Additionally, *S. sclerotiorum* can persist in the soil in the form of sclerotia. Under suitable environmental conditions, these sclerotia can germinate to form apothecia, releasing millions of ascospores into the air, which facilitates their dispersal and recurrence during successive crop cycles (O'Sullivan *et al*., 2021). There are various strategies for controlling *S. sclerotiorum*, such as moisture management, crop rotation, and the use of resistant cultivars. However, the application of chemical fungicides has been the most effective control strategy (Mueller *et al*., 2002; Deising *et al*., 2008). Despite its immediate effectiveness, the continuous use of fungicides poses risks associated with pathogen resistance and negative environmental impacts (Derbyshire and Denton-Giles, 2016). Therefore, the development of environmentally friendly strategies and technologies that ensure food security and are effective in the long term is urgently needed.

Bacillus species are Gram-positive bacteria commonly present in soil and the rhizosphere of crops. They produce diverse bioactive compounds that support plant growth and protect against biotic and abiotic stresses (Backer *et al*., 2018). These formulations are used both as microbial fertilizers to enhance crop productivity and as biocontrol agents effective against nematodes, bacteria, and pathogenic fungi (Backer *et al*., 2018; Yoshida and van Dijl, 2020; Vlajkov *et al.*, 2022; Ayaz *et al*., 2024; Correa da Silva *et al.*, 2024). The *B. subtilis* 105 strain was isolated from the rhizosphere of *Solanum lycopersicum* in Sinaloa, Mexico, where it was selected for its strong adaptation to the region's extreme environmental conditions and for its proven biocontrol activity against *Sclerotinia sclerotiorum*.

However, the high production costs of culture media represent an obstacle for scaling up a bioprocess. A promising strategy to mitigate these costs involves replacing costly microbiological media with more economical agro-industrial waste and byproducts, which have the potential to substantially lower production expenses (Lobo *et al*., 2019; Lima *et al*., 2020; Sarmiento-López *et al*., 2022). Several studies have shown that byproducts such as rice husks, sugarcane residues, soybean flour, pea flour, fruit pulps, and oil industry residues can be effectively used as sources of carbon, nitrogen, and other essential nutrients for bacterial growth (Gudiña *et al*., 2015; Sarmiento-López *et al*., 2022; Grigs *et al*., 2023; Naik *et al*., 2023). In fact, studies by Wang *et al*. (2008) demonstrated that the formulation of a culture medium based on soybean and potato promoted the growth and production of antifungal compounds in *B. subtilis* while maintaining its efficacy as a biocontrol agent against *Fusarium* sp. Similarly, Imandi *et al*. (2008) reported that replacing the synthetic medium with one formulated from pineapple waste increased both bacterial growth and citric acid production. Furthermore, Sarmiento-López *et al*. (2022) demonstrated that culture media formulated with pea flour as a substrate effectively induced the production of indole-3-acetic acid (IAA) by *Niallia circulans* E9 under bioreactor conditions. The use of agro-industrial substrates, such as soybean and corn flour, has been shown to reduce production costs for various bacterial strains significantly. For example, *Pseudomonas putida* Rs-198 can achieve high cell densities (10^{13} CFU mL^{-1}) within 48 hours, with an estimated cost reduction of 80-90% (Peng *et al*., 2014). Similarly, soybean-based media promote a vigorous growth and metabolite production, including antifungal compounds and indole-3-acetic acid, in *N. circulans* E9 and *B. mojavensis*, resulting in a 20 times cost reduction compared to LB medium. These results demonstrate that low-cost, plant-derived media can maintain functional performance across diverse plant growth-promoting rhizobacteria (PGPR) strains, highlighting their potential for cost-effective, industrial-scale biofertilizer production (Hammami *et al*., 2018; Sarmiento-López *et al*., 2022).

Additionally, in large-scale production processes, the optimal growth of *Bacillus* and the production of its bioactive compounds are determining factors when replacing a conventional culture medium. Stirred tank bioreactors are used for bacterial mass cultivation because of their ability to maintain precise control over critical environmental variables for bacterial growth, such as oxygen supply, pH, culture volume, and nutrient concentration, resulting in increased production efficiency (Bhari *et al.*, 2021; Boruta *et al*., 2022; Páez *et al*., 2024). Furthermore, the use of bioreactors enables process scalability, which is essential for the commercial production of these compounds on a large scale while maintaining reproducibility and the quality of the final product (Li *et al*., 2015). However, the formulation of culture media based on agro-industrial waste and its optimization in stirred tank bioreactors is limited. We hypothesize that the use of agro-industrial substrates to formulate a low-cost medium can support the growth of *B. subtilis* 105 at the bioreactor level while preserving its biofungicidal properties, providing a viable alternative to conventional LB medium. Based on these hypotheses, this study aims to investigate the effect of using low-cost agro-industrial substrates for the formulation of a culture medium for *B. subtilis* 105 at the flask level. Additionally, we evaluated the effects of medium substitution on the growth of *B. subtilis* 105 in a bioreactor and its antagonistic activity against *S. sclerotiorum*.

2 Materials and methods

2.1 *Agro-industrial by-products and biological material*

Six agro-industrial substrates were used: soybean flour (ArcherDaniels Midland Company, Chicago, IL, USA), pea flour (Roquette, Lestrem, France), Solulys (Roquette), Pharmamedia (Archer Daniels Midland Company), yeast extract (Roquette) and NaCl (Roquette). Roberto Gutiérrez donated all substrates from Fermic Mexico S.A. de C.V.

B. subtillis 105 and *S. sclerotiorum*, were generously provided by Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional-Unidad Sinaloa (CIIDIR Sinaloa-IPN), Sinaloa, Mexico. *B. subtillis* 105 was stored in 1.5 mL cryovials containing 20% glycerol and kept at -70 °C until further experimentation. The strain was isolated from *S. lycopersicum* rhizosphere and selected because it exhibits antagonistic activity against *S. sclerotiorum in vitro*. *S. sclerotiorum* inoculum was cultured in potato dextrose agar (PDA) plates and incubated at 23 °C (Mendoza-Soto *et al*. 2022). For long-term preservation, *S. sclerotiorum* cultures were maintained as mycelial plugs in sterile distilled water at 4 °C and as PDA colonized agar blocks stored in 20% glycerol at –70 °C. Before use, both *B. subtilis* 105 and *S. sclerotiorum* were transferred to fresh media.

2.2 *Culture media and Erlenmeyer flask experiments*

In the present study, four different culture media were employed to evaluate their ability to promote the growth of *Bacillus subtilis* 105.

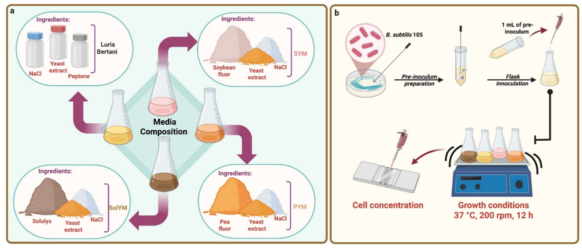

Figure 1. Schematic model representation of *Bacillus subtilis* 105 growth experiments. (a) Composition of the agro-industrial media formulated as an alternative culture medium for *B. subtilis* 105 cultivation. (b) Inoculation strategy and bacterial growth conditions in agro-industrial media at the flask level, outlining key parameters for optimal microbial proliferation. SYM: Soybean fluor, yeast extract and NaCl; PYM: Pea fluor, yeast extract and NaCl; SolYM: Solulys, yeast extract and NaCl.

The Luria-Bertani (LB) microbiological medium (Sigma-Aldrich) served as a control, and its chemical composition (g L^{-1}) is tryptone (10), yeast extract (5), and NaCl (5). Three alternative media were formulated using industrial substrates (g L^{-1}): SYM medium, composed of soybean flour (10), yeast extract (5), and NaCl (5); SolYM medium, containing Solulys (10), yeast extract (5), and NaCl (5); and PYM medium, made with pea flour (10), yeast extract (5), and NaCl (5) (Fig. 1a). These alternative media were explicitly designed to assess the potential of industrial byproducts as cost-effective substitutes for traditional growth media (Sarmiento-López *et al*. 2022). Before use, all media were autoclaved.

For the Erlenmeyer flask experiments, the inoculum preparation involved transferring a loopful of *B. subtilis* 105 cells from LB agar plates. After 24 h, a single colony was transferred to 15 mL polypropylene tubes containing 5 mL of sterile microbiological LB medium and incubated at 37 °C for 24 h at 200 rpm. A 1 mL aliquot of the pre-inoculum (1 x 10^6 CFU mL^{-1}) was then used to inoculate Erlenmeyer flasks. *B. subtillis* 105 was cultured in 500 mL Erlenmeyer flasks containing 100 mL of LB, SYM, SolYM, and PYM media and incubated on a rotary shaker at 37°C and 200 rpm for 12 h, following the methodology described by Sarmiento-López *et al*. (2022). Cell growth was assessed by quantifying the cell concentration per milliliter. A 1 mL aliquot from each culture was prepared through serial dilution, and a 10 μL sample containing cells was loaded into a Neubauer chamber (Brand 717810, Sigma-Aldrich, Darmstadt, Germany) for cell counting (Fig. 1b). The cells were obtained by examining five squares (four corners and the center) as described by Thunyaporn *et al*. (2021). Cell counts were performed using a microscope (Olympus SZX7, Olympus, Hamburg, Germany), and the results were expressed as the number of cells per milliliter at each time point during the growth kinetics.

Kinetic parameters, including the doubling time (T_d) and maximum specific growth rate (μ_{max}), were calculated using the equation provided by Zhang *et al.* (2017). Three independent experiments were performed for each culture medium, and consistent results were obtained across all trials.

2.3 Bioreactor cultures

B. subtilis 105 was cultured in a 2 L stirred tank bioreactor (Applikon Biotechnology, Delft, Netherlands), operated with 1 L of microbiological LB medium and SYM media. The SYM medium was chosen because of its superior performance in promoting the cell growth of *B. subtilis* 105 compared with that of other agro-industrial media formulations, as observed in flask experiments. Cultures from the late exponential growth phase (1 × 10^8 CFU mL^{-1}) were used as the inoculum for each media condition as described above. The bioreactor was equipped with a Rushton impeller (50 mm diameter) and operated at two stirring speeds (400 and 700 rpm) to investigate the effect of stirring on microbial growth. The aeration rate was set to 0.1 vvm, and the temperature was controlled at 37 °C, which is optimal for *B. subtilis* growth (Errington and Aart, 2020). The pH value was maintained at 7.0 using automatic acid/base addition, and the dissolved oxygen (DO) concentration was monitored and maintained between 20 and 40% air saturation throughout the experiment. Silicone M30 (Sigma-Aldrich, Darmstadt, Germany) at 10% in mineral oil was added as an antifoam agent to prevent excessive foaming. The pH and DO were continuously measured using electrodes connected to the bioreactor's automated control system (Applikon Biotechnology, Delft, Netherlands). Samples were collected every one h for biomass, including cell and spore concentration. Growth kinetics were recorded for 24 h, and kinetic parameters were determined. Growth kinetics were measured in triplicate on each medium to ensure statistical reliability.

2.4 *Sclerotinia sclerotiorum* inhibition assays

The antagonistic activity of *B. subtilis* 105 against *S. sclerotiorum* was evaluated using the dual culture method on PDA plates (Farzand *et al*. 2019). *S. sclerotiorum* was cultured on PDA medium, and a 5-day-old mycelial disc (5 mm in diameter) was placed at the center of a fresh PDA plate. Samples of exponentially growing *B. subtilis* 105 were harvested from the bioreactor (1 x 10^8 CFU mL^{-1}) grown in microbiological LB medium and SYM media at agitation speeds of 400 and 700 rpm. They were streaked on one side of the plate where *S. sclerotiorum* mycelial plug was set. Control plates were inoculated with only an *S. sclerotiorum* plug. After 5 days of incubation at 22 °C, the inhibition of mycelial growth was measured, and the percentage of growth inhibition was calculated using the formula described by Aeron *et al*. (2011).

2.5 Statistical analysis

The data were processed to calculate central tendency measures, including means and standard deviations (SD). Differences in the biomass concentrations and kinetic parameters of *B. subtilis* 105 grown in microbiological LB medium, PYM, SYM, and SolYM media in Erlenmeyer flasks were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test ($P< 0.05$). The differences in the kinetic growth of *B. subtilis* 105 grown in microbiological LB medium and SYM media in bioreactors were analyzed using Student's t-test, with a significance value of $P <0.05$. Kinetic parameters obtained from bioreactor experiments were subjected to one-way ANOVA, and the means were compared using Tukey's post hoc test ($P< 0.05$). One-way ANOVA with Tukey's post hoc test ($P< 0.05$) was used to test differences in *S. sclerotiorum* growth inhibition assays. The normality of all data was checked using the Shapiro-Wilk test before performing statistical analysis. All statistical analyses were conducted using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA). To identify the optimal biocontrol characteristics of *Bacillus subtilis* 105, a principal component analysis (PCA) was conducted on cultures grown in SYM at two agitation speeds (400 and 700 rpm) in the bioreactor. Variables included maximum specific growth rate (μ_{Max}), doubling time (T_d), pH, dissolved oxygen, cell and spore concentrations, colony area, and inhibition of *S. sclerotiorum*, as described by Lever et al. (2017). PCA was conducted on the SRPLOT platform (`https://www.bioinformatics.com.cn/en?keywords=PCA`), using a 68% confidence interval and the Singular Value Decomposition (SVD) algorithm by default.

3 Results

3.1 SYM medium enhances the growth of *B. subtilis* 105 in an Erlenmeyer flask

The growth of *B. subtilis* 105 exhibited a characteristic lag phase during the initial period of incubation, with different durations: SYM medium (2 hours), microbiological LB medium (3 hours), SolYM medium (4 hours), and PYM (5 hours). Maximal cell growth in the stationary phase in microbiological LB medium at 7 hours and in the other media at the same time (8 hours). Compared with the microbiological

Table 1. Kinetic parameters of *B. subtilis* 105 cultured in microbiological LB medium and agro-industrial media in an Erlenmeyer flask.

	LB	SYM	PYM	SolYM
Lag duration (h)	3	2	5	4
Exponential cell growth (10^8cell mL^{-1})	2.4 ± 0.20^b	4.9 ± 0.30^a	1.3 ± 0.04^d	1.7± 0.01^c
μ_{Max} (h^{-1})	0.85 ± 0.02^a	0.78 ± 0.02^b	0.45 ± 0.10^c	0.58 ± 0.07^c
T_d (h)	0.82 ± 0.02^c	0.88 ± 0.02^b	1.56 ± 0.30^a	1.21 ± 0.14^a

Data represents the mean ± standard deviation of three individual replicates. Different letters indicate a significant difference according to Tukey´s test (P<0.05). μ_{Max}: Maximum specific growth rate; T_d: Doubling time.

Table 2. Kinetic parameters of *B. subtilis* 105 cultured in SYM and microbiological LB medium under different agitation rates in a bioreactor.

	Bioreactor agitation rate (rpm)			
	LB-400	SYM-400	LB-700	SYM-700
Cell growth (10^8 cell mL^{-1})	1.3 ± 0.05^c	2.3± 0.01^b	2.0 ± 0.20^b	8.3 ± 0.60^a
Sporulation (10^8 Spore mL^{-1})	0.9 ± 0.2^c	0.9± 0.01^c	2.6 ± 0.20^b	5.5 ± 0.30^a
μ_{Max} (h^{-1})	0.30 ± 0.05^d	0.77 ± 0.03^a	0.55 ± 0.01^c	0.62 ± 0.07^b
T_d (h)	2.36 ± 0.38 ^{a}	0.90 ± 0.03^c	1.25 ± 0.01^b	1.12 ± 0.13^b

Data represents mean ± standard deviation of three individual replicates. Different letters indicate a significant difference according to the Tukey´s test (P<0.05). μ_{Max}: Maximum specific growth rate; T_d: Doubling time.

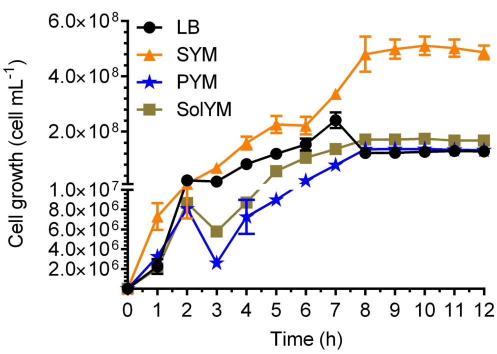

Figure 2. Kinetic profile of *B. subtillis* 105 cultured in microbiological LB medium and agro-industrial media in an Erlenmeyer flask. The cell growth data are represented as the mean ± standard deviation of three individual replicates.

LB medium, only SYM medium supported a 2.0-fold increase in cell concentration. Compared with the LB medium, the PYM and SolYM media resulted in lower cell growth (Fig. 2, Table 1).

The kinetic parameters indicated that the lowest specific growth rates (μ_{Max}) of *B. subtilis* 105 were observed in SolYM (0.45 h^{-1}) and PYM media (0.58 h^{-1}), corresponding to extended doubling times (Td) (Table 1.). In contrast, microbiological LB medium exhibited the highest μ_{Max} of 0.85 h^{-1} and the shortest Td of 0.82 h, indicating that it was the most favorable medium for rapid bacterial proliferation. The growth of *B. subtilis* 105 in SYM medium exhibited a μ_{Max} lower than the one obtained in microbiological LB medium; however, the bacteria reached the highest growth concentration (Table 1). These findings indicate that SYM medium is a suitable alternative to microbiological LB medium for cultivating *B. subtilis* 105, offering a viable option for cost-effective growth while still supporting substantial bacterial proliferation. Considering these results, the ability of the SYM medium to grow *B. subtilis* 105 in a stirred tank was evaluated.

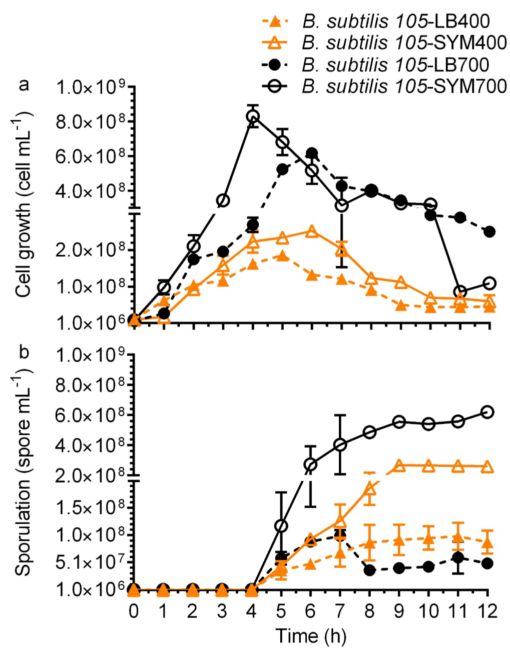

Figure 3. Growth kinetics of *B. subtillis* 105 under different agitation rates in a bioreactor. a) Bacterial growth at 400 (triangles) and 700 (circles) rpm in microbiological LB medium (Luria Broth; dashed

lines, solid figures) and SYM (soybean flour, yeast extract, and NaCl; continuous lines, open figures) media, b) Sporulation. Values represent the mean ± standard deviation of three individual replicates.

3.2 *Agitation speed in the bioreactor modulates bacterial growth in SYM medium*

The results of Fig. 3 and Table 2 indicate that the growth of *B. subtilis* 105 in the SYM medium was greater than that in the microbiological LB medium at both agitation speeds in the bioreactor (400 and 700 rpm). The highest growth of *B. subtilis* 105 (8.3 x 10^8 cells mL^{-1}) was obtained with the SYM medium at the agitation speed of 700 rpm after 4 h of culture. When the same SYM medium was used at 400 rpm, maximum growth (2.3 x 10^8) was achieved at 6 h of culture. The maximum growth of *B. subtilis* 105 in the microbiological LB medium occurred after 5 h of culture, resulting in the production of 1.3 × 10^8 cells mL^{-1} and 2.3 × 10^8 cells mL^{-1} at stirring speeds of 400 and 700 rpm, respectively. This finding is consistent with the effects of the culture medium and the agitation speed of the bioreactor on the growth of *B. subtilis* 105. Spore production began after 5 h of culture, when the maximum spore concentration was 5.5 × 10^8 spores mL^{-1}. The μ_{Max} of *B. subtilis* 105 was 2.6 times greater in SYM than in microbiological LB medium at a shaking speed of 400 rpm; consistently, the T_d of the culture in SYM was lower than that in microbiological LB medium. A similar trend was observed at 700 rpm, where both the μ_{Max} and T_d were more favorable in SYM than in microbiological LB medium (Table 2).

As shown in Fig. 4a, the initial pH of the *B. subtilis* 105 cultures in the bioreactor, using either SYM or microbiological LB medium, ranged between 6.5 and 7.2. Nevertheless, the pH of the culture tended to increase to values close to 8 at the end of the kinetic process. As shown in Fig. 4b, *B.subtilis* 105 cultures growing in a bioreactor at 400 rpm during their exponential growth phase (2-5 h) experienced a complete loss of dissolved oxygen levels from 100% to 0% in both SYM and microbiological LB medium in the bioreactor. These results suggest that the growth of the *B. subtilis* 105 cultures in the bioreactor at 400 rpm is limited by insufficient oxygen supply. When the *B. subtilis* 105 cultures were grown in SYM and agitated at 700 rpm, the dissolved oxygen level decreased to 2% during hours 2 to 4, and then increased to 100% as the cells reached their stationary growth phase. In this way, increasing the agitation speed of the bioreactor to 700 rpm allowed for an improvement in the oxygen supply and avoided the limitation of this vital element.

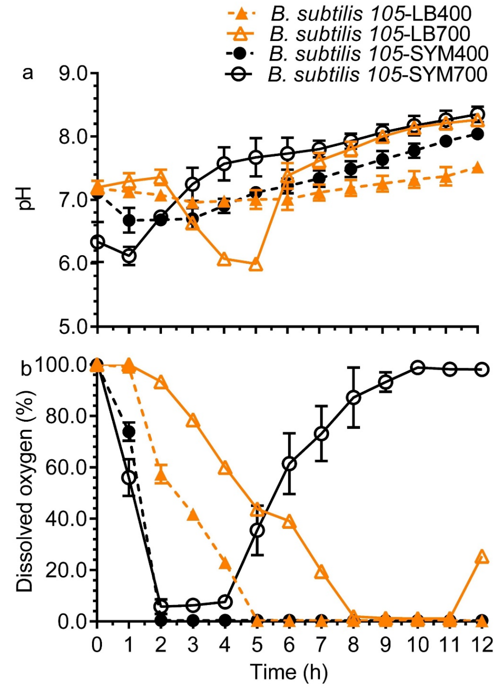

Figure 4. Dissolved oxygen and pH profile of *B. subtilis* 105 under different agitation rates and medium culture in a bioreactor. a) pH at 400 (triangles) and 700 (circles) rpm in microbiological LB medium (Luria Broth; dashed lines, solid figures) and SYM (soybean flour, yeast extract, and NaCl; continuous lines, open figures) media, b) Percentage of dissolved oxygen. Values represent the mean ± standard deviation of three individual replicates.

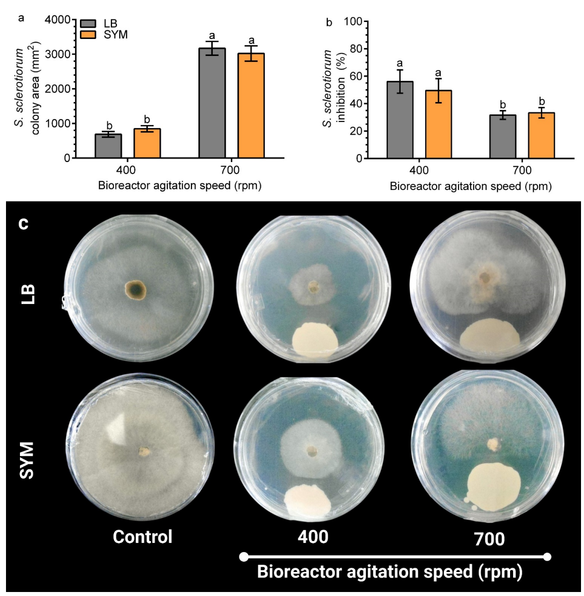

Figure 5. Antagonistic activity of *B. subtilis* 105 cultures grown under bioreactor conditions. (a) Colony growth area, (b) inhibition percentage, and (c) *in vitro* dual culture assay with bacteria cultivated in microbiological LB medium and SYM media at agitation speeds of 400 and 700 rpm in a bioreactor.

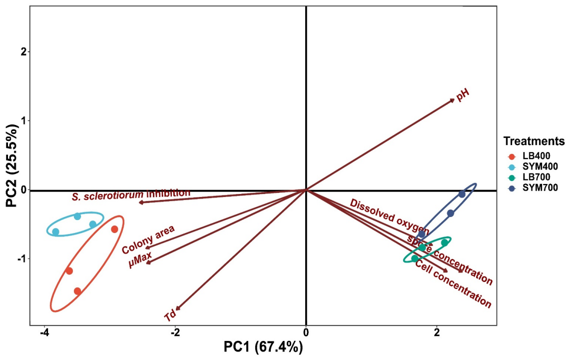

Figure 6. Principal component analysis (PCA) of biocontrol characteristics of *Bacillus subtilis* 105 cultivated in SYM and LB media at 400 and 700 rpm in a bioreactor.

3.3 *B. subtilis 105 cultured in SYM medium in the bioreactor maintained its antagonistic activity against S. sclerotiorum*

The in *vitro* dual culture assays conducted to assess the antagonistic activity of *B. subtilis* 105 against *S. sclerotiorum* demonstrated a significant inhibition of mycelial growth, with bacteria cultures from the bioreactor (Fig. 5). The results indicated that cultures from the bioreactor agitated at 400 rpm, and sampled during the exponential phase of growth, significantly reduced mycelial area, with 55% inhibition observed in both microbiological LB medium and SYM media (Fig 5a and 5b). In contrast, bacteria cultured at 700 rpm exhibited an inhibitory activity against *S. sclerotiorum* of 30 % inhibitory activity against *S. sclerotiorum*, regardless of the culture medium (Fig. 5a and 5b). These results indicate that replacing microbiological LB medium with SYM medium, prepared with less expensive components, does not significantly change the antagonistic activity of *B. subtilis* 105 against *S. sclerotiorum*. Both microbiological LB medium and SYM media showed similar antagonistic effects.

3.4 *Principal component analysis correlated antagonist activity from B. subtilis 105 at the bioreactor level*

Principal component analysis (PCA) was used to evaluate the effect of *B. subtilis* 105 broths growing in SYM and microbiological LB medium agitated at 400 and 700 rpm on antagonistic activity against *Sclerotinia sclerotiorum* (Fig. 6). The analysis revealed that the first principal component (PC1) explained 67.4% of the variance, while the second principal component (PC2) accounted for 25.5%, together capturing a large part of the dataset's variability. The PCA results revealed that parameters related to the antagonistic effect, as well as μ_{Max} and T_d of *B. subtilis* 105, were grouped with the microbiological LB medium and SYM media in bioreactors agitated at 400 rpm. These findings indicate that a lower agitation speed could promote the production of bioactive metabolites that inhibit the mycelial growth of *S. sclerotiorum*. In contrast, at 700 rpm, the PCA grouped parameters related to *B. subtilis* 105 growth and sporulation with the microbiological LB medium and SYM culture media. This implies that higher agitation speeds primarily increase bacterial proliferation and spore formation rather than the biosynthesis of antimicrobial compounds (Fig. 6). The observed trend supports the hypothesis that moderate agitation (400 rpm) creates a more favorable physiological environment for secondary metabolite production, thereby enhancing the antagonistic activity of *B. subtilis* 105 against *S. sclerotiorum* (Palmerín-Carreño *et al*., 2016).

Furthermore, PCA analysis revealed that SYM medium possesses nutrient properties similar to those of microbiological LB medium, making it a practical substitute under various bioreactor operating conditions. The suitability of SYM as an alternative depends on the specific biological focus of *Bacillus subtilis* 105 activities, such as growth, sporulation, or antagonistic activity (Fig. 6).

4 Discussion

The overuse of synthetic fungicides has been shown to damage the environment, harm soil health, and cause resistance in pathogenic microorganisms. (Deising *et al*., 2008; Derbyshire and Denton-Giles, 2016). *Sclerotinia sclerotiorum,* a highly destructive necrotrophic fungus, causes significant crop losses worldwide. *S. sclerotiorum* affects a wide variety of economically important crops, including oilseeds, vegetables, and legumes, thereby posing a threat to agricultural productivity and food security worldwide (O'Sullivan *et al*., 2021; Macioszek *et al*., 2023). These factors underscore the need to develop sustainable, eco-friendly alternatives to chemical fungicides, such as biological control agents. Furthermore, bacteria from the *Bacillus* genus have become a promising alternative to decrease dependence on synthetic fertilizers. These bacteria are especially notable for their enzyme abilities and their capacity to absorb nutrients from various substrates. (Latorre *et al*., 2016; Danilova and Sharipova, 2020).

In this study, three alternative culture media formulated from low-cost industrial substrates were evaluated to assess the growth of *B. subtilis* 105. Compared with the other media tested, the SYM medium effectively enhances the development of

B. subtilis 105, resulting in a higher final biomass concentration (4.8×10^8 cells mL^{-1}), making it a cost-efficient choice for large-scale cultivation. Although microbiological LB medium supported the highest specific growth rate (0.85 h^{-1}), SYM medium achieved superior bacterial proliferation, which is consistent with the finding of previous studies that highlighted the benefits of complex media containing soybean flour and yeast extract, due to their rich supply of amino acids and peptides (Wang *et al.,* 2008; Cheng *et al*., 2017; Correa da Silva *et al*., 2024). From a physiological perspective, the enhanced performance of *B. subtilis* 105 in SYM may be linked to its efficient assimilation of nitrogen-rich substrates present in soybean hydrolysates. Soybean-based ingredients supply not only readily available nitrogen but also short peptides that can be directly transported through the bacterial permease systems, which are highly expressed during exponential growth in *Bacillus* species. This nutrient profile minimizes the metabolic cost associated with de novo amino acid biosynthesis and supports accelerated biomass production. Moreover, the complex carbohydrates and residual lipids of soybean flour may provide a more sustained carbon supply than the simpler carbon sources in LB, thereby extending vegetative growth and enabling higher final cell densities. Comparable physiological responses have been documented in other bacteria, including *B. thuringiensis* HA1, *N. circulans* E9, and *Enterobacter soli*, where agro-industrial substrates rich in organic nitrogen significantly enhanced growth kinetics and metabolite biosynthesis by improving the carbon/nitrogen balance and increasing cofactor availability (Sarmiento-López *et al*., 2022; Castilla-Marroquín *et al*., 2024; Gayosso-Sánchez *et al*., 2024). These parallels reinforce the notion that soybean-based media offer not only an economical substitute for traditional microbiological formulations but also a physiologically advantageous environment for microbial growth and productivity. From bioprocess perspective, several studies have suggested that using low-cost industrial substrates instead of traditional microbiological media can significantly lower operational costs while supporting bacterial growth. (Lima *et al*., 2020; Sarmiento-López *et al*., 2022; Naik *et al*., 2023; Páez *et al*., 2024).

Scaling up fermentation processes from laboratory to commercial scale presents significant challenges due to the difficulty in assessing key factors that affect cultivation. Microorganisms are susceptible to extensive environmental changes, which can affect their growth and the production of metabolites. Critical parameters such as medium composition, pH, temperature, aeration, and agitation must be carefully controlled to optimize microbial activity and ensure consistency in large-scale bioprocesses. Stirred-tank bioreactors play a crucial role in the mass production of biological control agents for agricultural use, as they are the basis for large-scale manufacturing of microorganisms. Their efficient mixing system improves mass transfer rates, ensuring an even distribution of nutrients and oxygen while preventing sedimentation (Zhou *et al*., 2018; Machado *et al*., 2022). The results of this study indicate that agitation in a bioreactor significantly affected the growth of *B. subtilis* 105, which was 1.9 times higher in SYM medium than in microbiological LB medium at 400 rpm during the first 7 hours. At 700 rpm, growth reached 8.3×10^8 cells mL^{-1}, demonstrating improved nutrient availability and oxygen transfer efficiency. These results show that the rapid decline of dissolved oxygen to 5% in SYM medium at 700 rpm within 2 hours indicates a high metabolic rate, which is consistent with earlier research on *B. subtilis* oxygen uptake kinetics (Zhou *et al*., 2018; Sarmiento-López *et al*., 2022). Increased spore formation in SYM medium at higher agitation rates indicates a link between aeration, stress conditions, and sporulation efficiency, a phenomenon reported in other *Bacillus* strains such as *B. amyloliquefaciens* and *B. velezensis*, where high oxygen transfer rates favor sporulation and secondary metabolite synthesis (Richard and Margaritis, 2003; Dey, Bhunia, and Dutta, 2016; Biermann *et al*., 2023).

Comparative studies using alternative agro-industrial media, such as soybean, molasses, or corn steep liquor-based formulations, have demonstrated that medium composition strongly interacts with agitation and aeration to modulate growth and metabolite production in *Bacillus* species (Sabaté *et al*., 2018; Farzand *et al*., 2019; Naik *et al*., 2023). For instance, soybean-based media have been shown to support both rapid biomass accumulation and sustained production of lipopeptides and proteases in *B. subtilis* and *B. mojavensis*, respectively, often with lower costs than conventional microbiological LB medium (Hammami *et al*., 2018; Lima *et al*., 2020). More recently, *B. halotolerans* has been shown to produce surfactin when grown on potato peel and frying-oil wastes, illustrating the versatility of *Bacillus* species in converting diverse residues into valuable antimicrobial compounds (Abdelraof *et al*., 2024). In the present study, SYM medium not only achieved comparable kinetic parameters to LB but also maintained vigorous antagonistic activity at moderate agitation, demonstrating its potential for scale-up while preserving biocontrol properties.

Additionally, the metabolic activity under different agitation conditions suggests that oxygen levels play a crucial role in regulating the balance between vegetative growth and spore formation, which is essential for optimizing large-scale bioproduction. These findings align with kinetic parameters, demonstrating that an agitation speed of 700 rpm has a positive influence on the maximum specific growth rate (μ_{Max}) and doubling time (T_d) compared to 400 rpm in both culture media.

Interestingly, the antagonistic potential of *B. subtilis* 105 against *S. sclerotiorum* observed with microbiological LB medium was replicated using SYM medium. The inhibition of mycelial growth observed with the culture grown at 400 rpm (55%) was significant, although it decreased at 700 rpm. This trend aligns with PCA clustering, which links growth and sporulation parameters to higher agitation, suggesting that metabolic flux is redirected from secondary metabolite biosynthesis to primary growth under these conditions. Similar phenomena have been reported in other *Bacillus* strains, where suboptimal or moderate agitation promotes the production of bioactive metabolites while high shear favors rapid biomass accumulation (Palmerín-Carreño *et al*., 2016; Boruta et al., 2022; Machado *et al.,* 2022). These results suggest that *B. subtilis* 105 retains its antagonistic properties even when microbiological LB media are replaced with SYM medium, possibly because of the production of similar metabolites under bioreactor conditions. Furthermore, the production of antimicrobial compounds, such as lipopeptides and other bioactive molecules, is strongly influenced by fermentation conditions, highlighting the need for process optimization (Sabaté *et al*., 2018; Lima *et al*., 2020; Sarmiento-López *et al*., 2022; Naik *et al*., 2023). The PCA clustering further supports this, as parameters associated with antagonism grouped similarly across both media at 400 rpm, indicating that the biological mechanisms underlying metabolite production remain consistent despite changes in medium composition. Since agitation rates impact the production of antimicrobial compounds vital for biocontrol, optimizing fermentation parameters is crucial for balancing growth, sporulation, and biocontrol efficacy. Moreover, the ability of certain strains to sporulate provides an additional advantage, since spores ensure long-term survival, facilitate downstream formulation, and maintain antagonistic activity during storage and field application (Grigs *et al*., 2023). Similar biological responses have been observed in other *Bacillus* species against various phytopathogenic fungi, *including S. sclerotiorum*, demonstrating that bioreactor operational conditions and media composition significantly influence the antagonistic effects of *Bacillus* strains (Sabaté *et al*., 2018; Farzand *et al*., 2019; Vlajkov *et al*., 2022; Ayaz *et al*., 2024).

Overall, the PCA-based clustering, coupled with empirical antagonism data, demonstrates that *B. subtilis* 105 maintains strong biocontrol potential across media types, if agitation is carefully controlled. These findings reinforce the notion that microbial physiology is highly responsive to bioprocess parameters, and targeted optimization of culture conditions is essential for enhancing the production of antimicrobial compounds while maintaining viable, sporulating populations suitable for agricultural applications.

Taken together, these findings suggest that SYM medium is a practical alternative to microbiological LB medium for large-scale cultivation of *B. subtilis* 105, especially in agricultural applications where cost-effective biofungicide production is crucial. Future research should aim to optimize culture conditions to improve biocontrol effectiveness while maintaining efficient bacterial growth by using advanced bioprocess monitoring and control strategies.

Conclusions

In this study, a soybean-based medium (SYM) was chosen as a cost-effective alternative to replace the microbiological LB medium for large-scale cultivation of *B. subtilis 105* in a bioreactor. The growth rates of *B. subtilis* 105 in the SYM medium were similar to those in the microbiological LB medium, and the kinetic parameters were comparable. The results of this study revealed that a high agitation speed (700 rpm) led to the highest bacterial concentrations in SYM but caused a reduction in the antagonistic activity against *Sclerotinia sclerotiorum in vitro*. On the other hand, cultures grown at 400 rpm showed the strongest antagonistic response in both SYM and microbiological LB medium, reducing the mycelial growth of *S. sclerotiorum* by 55%. These results suggest that SYM is a viable alternative to microbiological LB medium for *B. subtilis* 105 cultivation with maintenance of its antagonistic properties. This study lays the foundation for developing a new culture medium formulation to grow biocontrol agents, such as *B. subtilis* 105, against *S. sclerotiorum*, thereby reducing media preparation costs and ensuring stable operational conditions in bioreactors. Notably, the soybean-based medium demonstrates strong potential for pilot or industrial-scale fermentation, offering a cost-effective platform for large-scale production of *B. subtilis* 105 with preserved biocontrol activity. However, more research is necessary to assess the effectiveness of *B. subtilis 105* in protecting plants under both controlled and field conditions.

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