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Using green waste as substrate to produce biostimulant and biopesticide products through solid-state fermentation

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ABSTRACT

Although the use of green waste as a substrate in different types of microbial bioprocessing has a major impact on improving green waste valorization, very little information has been provided on this issue. The purpose of this paper is to study the feasibility of using green waste to produce a biostimulant (Indole-3-acetic acid (IAA)) and biopesticide (conidial spore) through solid-state fermentation. *Trichoderma harzianum* was selected as the inoculum of the process and the green waste was a mixture of grass clippings and pruning waste. An experiment was designed to study the effect of tryptophan concentration, proportion of grass and pruning waste, and substrate moisture on IAA and spore production. The results show that washing and using phosphate buffer has a beneficial effect on green waste quality in terms of bioproduction. The maximum IAA and spore productions reported in the current study were 101.46 μg g^{-1} dry matter and 3.03×10^9 spore g^{-1} dry matter, respectively. According to the results, IAA production increases with a higher amount of tryptophan and grass. However, the number of spores increased with lower amounts of tryptophan and grass. The model suggested the following optimized parameters for the production of spores and IAA: tryptophan 0.45 %, grass 61 %, and moisture 74 %. The effect of fermentation time was also studied, and the results show that the maximum IAA and spore production was obtained on days 3 and 7, respectively.

1. Introduction

Sustainable agriculture needs innovative strategies to increase productivity within the framework of the circular economy. To this end, the use of plant wastes as the substrate to produce valuable agricultural bioproducts is a considerable step towards improving sustainability. Green waste (GW) is plant-based, and has become one of the most abundantly available forms of organic waste (Wang et al., 2021). It is categorized among municipal wastes and is generally considered to refer to the biodegradable waste collected from public parks and private gardens. It consists of wood, pruned parts of trees and shrubs, grass clippings, and leaves that are mixed with soil. As urban areas expand, so too does the number of municipal green spaces. This leads to a larger amount of GW that needs to be properly managed (Langsdorf et al., 2021; Reyes-torres et al., 2018).

For many years, GW has mainly been used for composting (Inghels et al., 2016; Liguori et al., 2013). More recently, it has also been used as a substrate for the production of biochar, biogas, and bioethanol (Jesus et al., 2017; Karami et al., 2011; Świechowski et al., 2019). However, GW has been also reported as a potential source of energy for microbial processes, which has led to the production of marketable products such as enzymes, polyaromatic hydrocarbons (PHAs), and lipids (Cerrone et al., 2015; Langsdorf et al., 2021). Recently, GW has even been used as a substrate to produce bio-hydrogen (Yue et al., 2021).

Considering the solid structure of GW, solid-state fermentation (SSF) can potentially be one of the primary options for bioproduction. SSF is a process that occurs in the absence or near absence of free water. To date, SSF has been utilized to generate marketable bioproducts such as enzymes, biosurfactants, biopesticides, biostimulants, and many other products that are of use to the industrial, agricultural, and pharmaceutical sectors (Ballardo et al., 2017; Jiménez-Peñalver et al., 2019; Mejias et al., 2018; Sala et al., 2021).

One of the major challenges for using GW as a substrate in SSF for microbial bioproduction is the heterogenicity of its composition. The type of plant source, season, and collection and preservation methods can change the composition of GW and consequently its potential for bioproduction. However, conditioning procedures can minimize the effects of GW heterogenicity and make the process easier to control

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(Inghels et al., 2016; Langsdorf et al., 2021).

Biostimulants have a promising role to play in making agriculture more sustainable. They stimulate plant growth by improving characteristics such as the efficiency of nutrient use, tolerance of (a)biotic stress, and availability of confined nutrients in the soil or rhizosphere (Baltazar et al., 2021; Carletti et al., 2021). A wide range of substances and also microorganisms can be utilized as biostimulants. Examples of non-microbial biostimulants include humic substances, protein hydrolyses, and seaweed extract. The most used microbial biostimulants are *Pseudomonas* spp., *Enterobacter* spp., *Bacillus* spp., *Rhizobium* spp., *Streptomyces* spp., *Trichoderma* spp. *Aspergillus* spp., and arbuscular mycorrhizal fungi (AMF) (Drobek et al., 2019; Visconti et al., 2020). The European Biostimulant Industry Council (EBIC) has estimated the annual growth of biostimulant products in the market at around 10 to 12 % (EBIC, 2020).

Trichoderma strains have predominantly been used as biostimulants due to their capacity to control fungal phytopathogens and improve plant tolerance against abiotic stresses. This fungus is a well-known biocontrol agent and has been successfully used as a biopesticide, especially in the form of conidial spores (Sala et al., 2020; Zhang et al., 2022). Moreover, some strains of *Trichoderma,* including *T. harzianum*, *T. virens*, and *T. atroviride* can affect a plant's root activity, and some of its metabolic pathways, by releasing metabolites to the rhizosphere. Auxin is a plant hormone that can be produced by plants and microorganisms. Specifically, it is one of the metabolites produced by fungi as a signal to interact with them. The plant produces organic compounds that fungi can use as nutrients to grow and in turn improve the plant's own growth by producing auxin (Keswani et al., 2020; López-Bucio et al., 2015; Visconti et al., 2020). Indole-3-acetic acid (IAA) is the most common form of auxin. This hormone plays an important role in many physiological processes in plants, including apical dominance, tropisms, shoot elongation, cambial cell division induction, and root initiation (Dong et al., 2022; Jahn et al., 2021). Indole-3-acetic acid can be synthesized by several pathways in microorganisms. However, its production generally occurs through tryptophan (Trp)-dependent or non-dependent pathways (Jahn et al., 2021).

The microbial production of IAA using a standard culture medium as the substrate and Trp as a precursor, if necessary, has been thoroughly investigated (Ikram et al., 2018; Numponsak et al., 2018; Shokri and Emtiazi, 2010). However, only a limited number of studies have addressed organic waste as the main substrate. Bose et al. (2013) used Jatropha seedcake as a substrate and a kind of white rot fungus to produce IAA through submerged fermentation (SmF). Also, in a more recent study, auxin was produced by SSF using different agricultural wastes and microorganisms (Zanoni do Prado et al., 2019). Fungal biopesticide production through fermentation has also been studied recently using *Trichoderma* spp. and different types of agricultural and biological wastes (de Rezende et al., 2020; Sala et al., 2021). However, there is a lack of information on the use of green waste as the substrate of fermentation.

This study aims to produce indole-3-acetic acid and conidial spores of *T. harzianum* using GW as substrate through solid-state fermentation. There are two main objectives. The first is to study the potential of green waste for use as the substrate for bioproduction. And the second is to analyze the factors involved in IAA and conidia production in order to optimize the amount of both IAA and spores in the process.

2. Materials and methods

2.1. Substrate

The green waste used in this study included grass clippings (G) and pruning waste (PW) collected from the campus of the Universitat Autònoma de Barcelona, Spain. The pruning waste originated from a mixture of different trees.

The grass used for all tests was first crushed into pieces of approximately 1 cm length washed with tap water. It was then dried in a 50 l reactor that was aerated by 5 l min^{-1} constant air at 22 °C for 9 days. The substrate was agitated manually once a day. After drying, all the substrates were preserved at −20 °C. The pruning waste was already crushed at the time of collection, and was directly transferred for storage at −20 °C.

Before fermentation, the substrates were sterilized twice at 121 °C for 30 min. The moisture was then adjusted using sterilized potassium phosphate buffer (pH = 6.8) to prevent high pH alteration.

2.2. Inoculum

Trichoderma harzianum (TH) (CECT 2929) provided by the Spanish Type Culture Collection (CECT) was the fungus used as inoculum in this study. *Trichoderma viride* (TV) was also used in the time course experiment as inoculum. This strain was provided by the Biological Control Subdirectorate (SCB) SENASA Laboratory in Peru.

The original strains were kept at −80 °C in sterile cryovials with 10 % glycerol. For inoculum preparation, in all tests, TH and TV were cultured on malt extract agar and potato extract agar plates, respectively at room temperature for 6–8 days and diluted to approximately 10^6 spores g^{-1} of dry substrate before inoculating.

2.3. Solid-state fermentation

2.3.1. Experimental setup

The experimental setup included a 0.5-l packed reactor consisting of an inlet and outlet to pass the air through the material inside the reactor with a constant flow rate (20 ml min^{-1}). The airflow was provided by a mass flowmeter (Mass-Stream D-6311, Bronkhorst, Netherlands). The reactors were kept in a water bath during fermentation to control the temperature. An electrochemical sensor (Alphasense, UK) was used to measure the oxygen content of the output gases. The airflow and oxygen consumption data were recorded and analyzed by a non-commercial self-made software based on Arduino®.

The consumed oxygen was calculated as a specific oxygen uptake rate (sOUR) to evaluate microbial activity. Cumulative oxygen consumption (COC) was also determined by calculating the area below the oxygen consumption curve. sOUR was determined using a formula described in equation (1).

$$sOUR = F \times (0.209 - yO_2) \times \frac{P \times 32 \times 60 \times 10^3}{R \times DW \times 10^3} \quad (1)$$

where, sOUR is the specific Oxygen Uptake Rate (g O_2 kg^{-1} DM h^{-1}); F is airflow (ml min^{-1}); yO_2 is the oxygen molar fraction in the exhaust gases (mol O_2 mol^{-1}); P is the constant pressure of the system (101325 Pa); 32 is oxygen molecular weight (g O_2 mol^{-1} O_2); 60 is the conversion factor from minute to hour; 10^3 is the conversion factor ml to l; R is ideal gas constant (8310 Pa l/K mol^{-1}); DW is the initial dry weight of solids in the reactor (g); 10^3 is the conversion factor g to mg.

2.3.2. Experimental design

To analyze the effect of different factors on spore and IAA production, a Box-Behnken design was conducted with 15 runs. Three numeric factors with two responses were selected for the design. The factors had three levels including a maximum, a minimum, and a center point. The selected factors were tryptophan concentration, G to PW ratio, and initial moisture, while the responses were the number of spores and the IAA concentration.

To set up the experiment, 15 reactors were loaded with 80 g of wet material. The substrate was conditioned as described above and the fermentation temperature was set at 25 °C following the study by Sala et al. (2020). The reactors were harvested after 7 days and a representative sample was taken for the following analysis: IAA analysis, spore count, and respiration indices. Dry matter, pH, and organic matter were also analyzed according to standard procedures (US Composting

Council, 2002).

2.3.3. Time course experiment

A time course experiment was carried out to investigate the different factors during a specific period. To perform a 10-day time course experiment, 10 reactors were filled with 80 g of the wet material that had been conditioned beforehand. All reactors were prepared according to the optimized condition estimated in the experimental design. The fermentation temperature was 30 °C. Every day, one reactor was sampled and then discarded. The sample was used for spore counts, IAA concentration, pH, dry matter, organic matter, and respiration indices.

2.4. IAA analysis

For IAA extraction, 10 g of the fermented sample was mixed with 50 ml of distillate water in a 500 ml Erlenmeyer and shaken for 20 min at room temperature at 150 rpm. The extract was separated using a fine sieve and centrifuged for 15 min at 4 °C and 10000 rpm. The supernatant was filtrated by a 0.22 μm filter and preserved at −20 °C for analysis.

For the analysis, a Dionex ultimate 3000 HPLC (Dionex, Idstein, Germany) was used equipped with an UltiMate 3000 Autosampler Column Compartment, UltiMate 3000 Photodiode Array Detector, and Chromeleon software. An LC Kinetex® 5 μm EVO C18 100 Å column (250 × 4.6 mm) was used. The eluent was isocratic and involved a solution of 2.5 % acetic acid in ultrapure water, and 80 % acetonitrile in ultrapure water. The column oven was set at 30 °C and the flow rate was 0.7 ml min^{-1}. The fluorometric detector was set at 280 nm. The total run was 15 min, and the peak was observed at 11 min. To determine the concentration of IAA in the samples, the area of the obtained peak was compared to that of the standard curve. All the IAA analysis tests were carried out in triplicate from one extract.

2.5. Spore count

To determine fungal spore production, 10 g of the fermented substrate was added to 50 ml of Tween 80 0.1 % (v/v), mixed manually for 30 s, and diluted according to the procedure reported by Cavalcante et al. (2008) with some modifications proposed in Sala et al. (2020). In all the tests, the spores were counted in triplicate using a Neubauer chamber (Brand™ 717805). The spore concentration per g of dry matter was calculated by the following formula:

$$SporeConcentration = \frac{N}{DF \times CV} \times \frac{EV}{SWW} \times \frac{SWW}{SDM} \quad (2)$$

where spore concentration is the number of spores per g dry substrate (spore g^{-1} dm); N is the number of spores counted in the Neubauer chamber; DF is the dilution factor; CV is the volume of sample in the Neubauer chamber (ml); EV is the extraction volume (ml); SWW is sample wet weight (g ww); and SDM is sample dry matter (g dm).

2.6. Statistical analysis

Design Expert 12 (Stat-Ease, Inc, USA) was used to analyze the results of the experimental design including ANOVA and to produce an optimization model. All the graphs were plotted using SigmaPlot 12.5 (Systat Inc. USA). Paired *t*-test ($p < 0.05$) was performed using SigmaPlot 12.5 software to compare the means between treatments when necessary.

3. Results and discussion

3.1. The effect of GW conditioning procedures on SSF

As mentioned earlier, the heterogenicity of the GW could be a problem for its valorization, especially when it is supposed to be used for bioproduction. A conditioning process can help to reduce the heterogenicity of the GW and improve the quality of fermentation (Inghels et al., 2016; Langsdorf et al., 2021). In this regard, a large enough amount of grass and pruning waste was collected for use in all the experiments in this paper. Also, different conditioning procedures were used for the grass and the way they affect the sporulation of TH was studied. The conditions were as follows: A) Wash the grass and dry it with airflow, B) Add phosphate buffer to the grass before starting fermentation, and C) Use conditions A and B together.

The results of using different conditioning procedures on TH sporulation are presented in Fig. 1A. As observed, after washing and drying the grass, no TH growth was detected. The initial and final pH were 6.42 ± 0.02 and 8.23 ± 0.01, respectively. However, after using buffer TH, growth reached (3.7 ± 2.4) × 10^8 spore g^{-1} dm and the final pH was 7.57 ± 0.39. The maximum number of conidia was (2.0 ± 1.1) × 10^9 spore g^{-1} dm obtained when the grass was washed, dried, and buffer added, and the final pH was 7.46 ± 0.36. The results of the pH measurement indicate that a pH value higher than 8 can negatively affect the growth of TH and the buffer could keep the pH in the tolerable range for TH. Zhang and Yang (2015) reported an optimum pH range of 6–7 for TH which is similar to our finding.

It seems that the conditioning also affected the sOUR profiles of the grass (Fig. 1B). When the grass was only washed, the maximum sOUR was almost 1.5 g O_2 kg^{-1} dm h^{-1} with a lag phase of 18 h. However, in the two conditions where the buffer was used, the maximum sOUR was between 2 and 2.5 g O_2 kg^{-1} dm h^{-1} although the lag phases were different. When using buffer alone, the lag phase was longer (35 h) while when washing and using buffer together the lag phase was 24 h. Here the higher sOUR could be related to the higher sporulation of TH.

In summary, these results indicate that washing and drying the grass and adding buffer can considerably improve the amount of conidia production.

3.2. Feasibility of TH growth and sporulation on grass and pruning waste

In order to study TH growth on G and PW, fermentation was conducted using G, PW, and a mixture of G and PW in a ratio 1:1 (w/w) for 9 days. In view of the occurrence of maximum spore production for TH in 5–6 days in a similar study (Sala et al., 2020), a larger span was chosen to be sure that the maximum spore production would be obtained during the run time.

Fig. 2A shows the spore production at different percentages of G and PW. On day 5, spore counts were the highest for PW ((9.03 ± 0.01) × 10^8. However, after 7 days of fermentation the number of spores increased on G and the mixture. On day 9, the spore counts reached the maximum on G and the mixture, namely (1.16 ± 0.16) × 10^9 and (1.22 ± 0.13) × 10^9 spore g^{-1} dm, respectively.

The sOUR profile of the SSF process of G, PW, and the mixture is presented in Fig. 2B. The maximum sOUR was higher for PW than for G and the mixture. However, COC for the mixture was 110.54 g O_2 kg^{-1} dm h^{-1}, which is higher than the COC of G and PW (80.98 and 93.09 g O_2 kg^{-1} dm h^{-1}, respectively). The time to reach the maximum respiration was between 24 and 30 h for all the substrates. There was no strong correlation between sporulation and microbial activity (r = -0.53). In a similar study, Sala et al. (2021) reported sporulation of TH with different agro-industrial substrates, and they did not find a correlation between spore production and the biodegradability of the substrate either.

Taken together, these results suggest that G and PW seem to be suitable substrates for spore production. As it was mentioned before, no evidence of using grass and pruning waste through solid-state fermentation was found. However, other lignocellulosic wastes especially agricultural wastes have been successfully used as substrate in solid-state fermentation (Marín et al., 2019; Tanruean et al., 2021; Wang et al., 2019). Moreover, regarding the low biological activity of the grass and the future scale-up of the process, the mixture probably provides more advantages in this case. Besides, PW can be used as a bulking agent

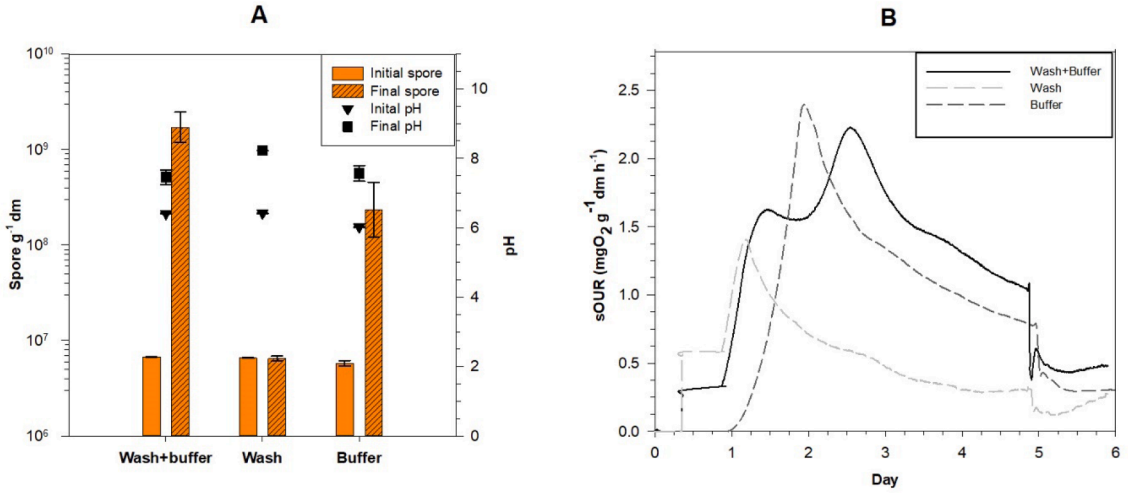

Fig. 1. Different conditioning procedures for the grass. A) The effect of conditioning on spore production and pH. B) Respiration profiles for the conditioning procedures.

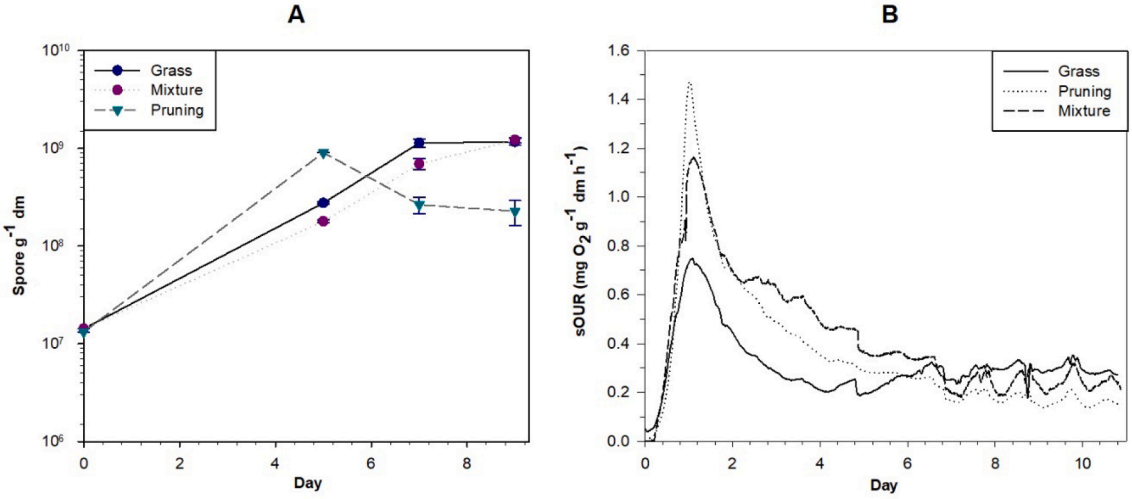

Fig. 2. A) Spore counts in different percentages of grass and pruning waste. B) Respiration profile of different percentages of grass and pruning waste.

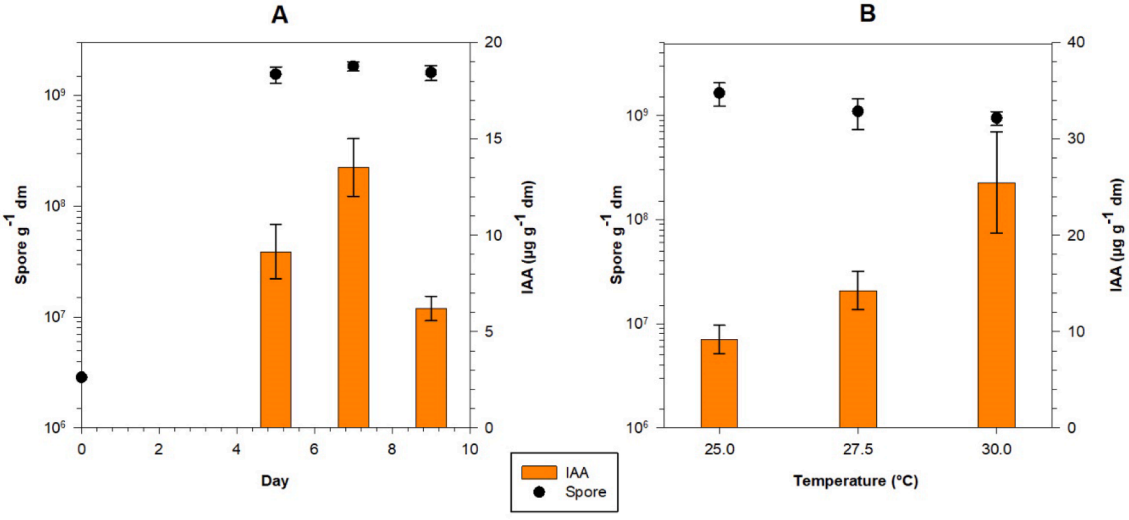

Fig. 3. A) The effect of Trp (0.5 %) on IAA concentration and spores. B) The effect of temperature on spores and IAA production.

that will make the mixture more porous (Sala et al., 2021; Ballardo et al., 2017; Mejias et al., 2018). So, in all the following experiments in this paper, the mixture of grass and pruning waste was used as the substrate. To the best of our knowledge, this is the first time that the use of grass or pruning waste to produce conidia by TH has been reported.

3.3. Effect of tryptophan on spore and IAA production

A preliminary experiment was carried out to test the feasibility of IAA production using GW and the effect of Trp concentration on spore and IAA production. Tryptophan in three different concentrations of 0.5, 1, and 1.5 weight % was added as a powder to the substrate (mixture of G and PW 1:1 mixing ratio (w/w)). The number of spores and IAA were checked after 5, 7, and 9 days. As shown in Fig. 3A, TH did not grow with 1, and 1.5 weight % of Trp. It appears that IAA was not produced by the fungi either. However, TH growth with 0.5 % of Trp was high. The IAA produced by TH were 9.2 ± 2.5, 13.5 ± 2.6, and 6.19 ± 1.12 μg g^{-1} dry substrate after 5, 7, and 9 days of fermentation, respectively. The numbers of spores were $(1.55 \times 10^9 \pm 4.45) \times 10^8$, $(1.83 \pm 0.30) \times 10^9$, and $(1.61 \pm 0.43) \times 10^9$ spore g^{-1} dm on days 5, 7, and 9, respectively. The results show that the highest concentration of IAA was obtained on day 7, while the number of spores peaked on day 9. However, spore counts on day 9 were not significantly different from the spore counts on day 7 (p-value = 0.52). Hence, these findings suggest that 7 days may be a desirable period of time for fermentation to produce spores and IAA together. The fermentation time used in the study was 5 days for IAA production. Sala et al. (2020) found that the maximum number of conidial spores produced by TH was obtained in 5–6 days. This inconsistency in fermentation time could be related to the different substrates and different lag phases in the fermentation. More information on this issue will be provided in the following sections.

Based on these results, it is proposed that a mixture of grass and pruning waste can be a good substrate to produce IAA and conidial spores by TH. It can also be inferred that a higher value of Trp can negatively affect TH growth and sporulation. It was recently reported that in several species of *Trichoderma*, growth and sporulation decreased in a media containing Trp. On the other hand, the results of previous studies have proposed that Trp can considerably increase the production of IAA in microorganisms that have a Trp-dependent pathway of IAA biosynthesis (Kumla et al., 2020; Mehmood et al., 2019; Reineke et al., 2008). Following these studies, it can be assumed that the *Trichoderma* strain used in the current study produces IAA through a Trp-dependent pathway. However, higher amounts of Trp decreased TH growth and sporulation. Therefore, an optimized concentration of Trp should be found to produce high amounts of spores and IAA together.

3.4. Optimization of IAA and spore production through SSF (experimental design)

As mentioned earlier, the effect of three factors on IAA and spore production was studied by conducting an experimental design. The selected factors and the tested levels were as follows: tryptophan concentration (0, 0.25 and 0.5 weight %), grass to pruning waste ratio (1:4, 1:1 and 4:1 (w/w)), and initial moisture (60, 70 and 80 %). The parameters and results of this experimental design are presented in Table 1. The maximum spore production was $(1.81 \pm 0.87) \times 10^9$ spore g^{-1} dm when tryptophan, G to PW ratio, and moisture were 0.25 weight %, 1:1 (w/w), and 70 %, respectively. However, the highest IAA production was the 75.77 ± 8.61 μg g^{-1} dm obtained with tryptophan 0.5 %, G: PW 4:1 (w/w), and moisture 70 %. Mehmood et al. (2019) produced 24.2 μg ml^{-1} of IAA *in vitro* by *Aspergillus awamori*. In a similar study, an SSF process set up in 250 ml Erlenmeyer flasks containing 10 g of the substrate produced 9.83 and 7.15 μg g^{-1} dm of IAA by *Bacillus subtilis* and *Trichoderma atroviride*, respectively through SSF (Zanoni do Prado et al., 2019). Considering the working volume in the current study, the amount of IAA produced is higher than that produced in Zanoni do Prado et al. (2019). Conidia production entailed the use of submerged liquid fermentation and produced 1.38×10^8 spore ml^{-1} by *Trichoderma* spp. In another study, Cavalcante et al. (2008) reported that *T. harzianum* produced 2.28×10^9 spore g^{-1} dm through SSF. These data show that the number of conidia obtained in this experiment is in a similar range to the other studies. However, there are reports of higher production of IAA using other microorganisms as inoculum. Luziatelli et al. (2021) set up a fermentation in a 2-l stirred tank fermenter using *Enterobacter* spp. P-36 strain and reported a production of 412.49 mg l^{-1} IAA. In another study, a yeast (*Rhodosporidium paludigenum*) produced 2,743.9 mg l^{-1} IAA through a fed batch fermentation (Nutaratat et al., 2016).

Table 1
The treatments and results of the Box-Behnken experimental design.

Tryptophan (%)	Grass (%)	Moisture (%)	Spore g^{-1} dm	IAA (μg g^{-1}dm)
0.25	20	60	$(6.14 \pm 0.76) \times 10^8$	16.40 ± 5.20
0	50	60	$(4.51 \pm 2.03) \times 10^8$	16.32 ± 5.65
0.25	80	60	$(4.10 \pm 1.37) \times 10^8$	27.61 ± 2.41
0.5	50	60	$(6.90 \pm 0.22) \times 10^9$	13.36 ± 5.37
0.25	50	70	$(1.44 \pm 0.19) \times 10^9$	16.08 ± 6.85
0.25	50	70	$(1.81 \pm 0.87) \times 10^9$	23.14 ± 7.67
0.25	50	70	$(1.64 \pm 0.20) \times 10^9$	18.30 ± 7.61
0	20	70	$(1.61 \pm 0.45) \times 10^9$	11.63 ± 4.22
0.5	20	70	$(1.63 \pm 0.45) \times 10^9$	13.97 ± 4.24
0	80	70	$(9.02 \pm 1.62) \times 10^8$	17.11 ± 1.60
0.5	80	70	$(6.50 \pm 6.50) \times 10^8$	75.78 ± 8.62
0.25	80	80	$(6.08 \pm 3.01) \times 10^8$	31.80 ± 8.53
0.25	20	80	$(7.94 \pm 0.20) \times 10^8$	41.07 ± 4.42
0	50	80	$(5.85 \pm 1.47) \times 10^8$	25.93 ± 10.50
0.5	50	80	$(5.24 \pm 1.45) \times 10^8$	36.14 ± 11.30

To optimize the production of IAA and spores, a prediction model was created using the results of the experimental design, a statistical analysis of which is shown in Table 2. For spore production, a modified quadratic model with basic 10 logarithm data transformation was used and the model was significant. The ANOVA analysis shows that the percentage of grass is the only significant main parameter for spore response. All the terms in the model were significant except for tryptophan and moisture. This means that although tryptophan and moisture content are not significant parameters in the model, their interactions significantly affected the response (spore production). The lack of fit was also not significant, and the model can explain 95 % of the variability of the response (R^2). The model produced the following regression equation for the response of spore counts:

$$\log^{10}(spore) = 9.21 + 0.0313X_1 - 0.0862X_2 + 0.0346X_{3-0.0996}X_1X_2 - 0.1268X_1^2 - 0.1005X_2^2 - 0.3394X_3^2 \quad (3)$$

where X_1, X_2, and X_3 are tryptophan%, grass%, and moisture%, respectively. The high intercept of the model suggests that the variables used in the model are not the most significant for the response (spore production).

Table 2
The p-value of the model and the parameters for spores and IAA. The model created for spore production is modified quadratic, and for IAA production was linear (p-value < 0.05 is significant).

Source	p-value Spore	IAA
Model	0.0005	0.0148
A-Tryptophan	0.2280	0.0545
B-Grass	0.0083	0.0339
C-moisture	0.1875	0.0320
AB	0.0206	
A^2	0.0083	
B^2	0.0235	
C^2	< 0.0001	
Lack of Fit	0.3500	0.3418

For analysis of IAA production, the data were best fitted in a linear model with the inverse transformation to predict the optimum condition. The regression equation was as follows:

$$\frac{1}{IAA} = 0.160878 - 0.041623X_1 - 0.000360X_2 - 0.001187X_3 \quad (4)$$

where X_1, X_2, and X_3 are tryptophan%, grass%, and moisture%, respectively.

The model was significant in terms of p-value with a non-significant lack of fit. R^2 for this model was 60.03 %. All the parameters in the model for IAA were significant.

The optimized condition to produce spores and IAA predicted by the model were as follows: tryptophan 0.45 weight %, G: PW 1.56:1 (w/w), and moisture 74 %. Fig. 4 shows that when moisture is 74 % and grass content is low, the number of spores increases by Trp in the range of 0.2–0.5 %. However, increasing G and Trp improves IAA production (Fig. 4). According to the obtained data, higher grass content (low lignin) in the substrate can increase IAA production. Zanoni do Prado et al. (2019) analyzed IAA production by different microorganisms using different types of agricultural wastes and also noted that *Trichoderma* sp. produced more IAA when the substrate has a low lignin content. On the other hand, increasing the concentration of Trp led to an increase in IAA while the number of spores decreased. Giri and Sharma (2020) optimized IAA production in a submerge fermentation using an experimental design and found that maximum production of IAA obtained with higher Trp concentration. Taken together, these results show that the values of the optimized parameters for spores and IAA are not in the same direction.

3.5. Effect of temperature on spore and IAA production

To study the effect of temperature on spore and IAA production, three fermentations were set up at different temperatures (25, 27.5, and 30 °C). The optimized condition predicted by the model was used for fermentation. The results indicate that at 30 °C, TH produced more IAA (25.5 ± 9.1 μg g^{-1} dm) (Fig. 3B). However, the number of spores peaked at 25 °C (1.64 ± 0.72) × 10^9 spore g^{-1} dm) and was (9.43 ± 2.47) × 10^8 spore g^{-1} dm at 30 °C. Napitupulu et al. (2019) reported that the optimum temperature range for *Trichoderma harzianum* growth is between 25 and 30 °C. However, for IAA production Zanoni do Prado et al. (2019b) set up a fermentation with *Aspergillus flavipe* at 30 °C. In another study, a temperature of 28 °C was used for *Trichoderma* spp. to grow and produce phytohormones including IAA (Illescas et al., 2021).

3.6. Effect of fermentation time on IAA and spore production

To obtain more information on the fermentation process, a 10-day time course experiment was performed. This involved setting up 10 reactors with the same conditions, which were selected according to the optimized parameters predicted by the model. Fig. 5A presents the profile of spore and IAA production by TH over a 10-day period. Interestingly, the maximum IAA production (101.46 ± 4.11 μg g^{-1} dm) was obtained after 3 days of fermentation whereas the number of spores peaked on day 7 ((3.03 ± 0.86) × 10^9 spore g^{-1} dm). The pH tended to increase until day 4–5, going from an initial value of 7.06 ± 0.04 to reach 8.22 ± 0.03. The pH figures were 8.05 ± 0.07 and 7.84 ± 0.05 respectively when the maximum amounts of IAA and spores were produced. From then they started to decline and by day 10 had fallen to 7.37 ± 0.06. The respiration profile showed that microbial activity peaked on days 1–2, the maximum sOUR value being1.46 g O_2 kg^{-1} dm h^{-1}.

A second time course experiment was carried out to see if a different strain of *Trichoderma* produces IAA and conidia similarly to TH using the optimized conditions predicted by the model. In this experiment, *T.viride* was used as inoculum with the same condition of the TH time course experiment (Fig. 5B). Here, the maximum IAA was obtained on day 1 and the maximum spore production was obtained on day 4, being 18.06 ± 5.23 and (4.27 ± 0.43) × 10^9 respectively. As can be seen from the data, TV produced more spores and a lower amount of IAA than TH. However, note that in both strains, IAA concentration decreased significantly when the number of spores started increasing, even though no contamination was detected. The pH increased over time, when the inoculum was TV, starting from 7.05 ± 0.03 on the initial day to 8.15 ± 0.04 on day 8. At the time of most spores, the pH was 7.52 ± 0.05 while when IAA reached its maximum, the pH was 7.20 ± 0.05. The maximum sOUR for TV observed on days 1–2 of fermentation is similar to that of TH (1.24 g O_2 kg^{-1} dm h^{-1}). The optimized time of fermentation to produce IAA was 5 days in the study of Nutaratat et al. (2017). They studied the effect of different factors including incubation time on IAA production by a strain of *Enterobacter* sp.

The results of the time course experiments for TH and TV showed that there was a similar interaction between IAA concentration and the number of spores produced for both strains. This observation might suggest a relationship between IAA and sporulation. The different values of the optimized parameters for spores and IAA obtained previously by the model also support the hypothesis. It was mentioned earlier that IAA acts as a signal for the interaction between plant-fungi and fungi-fungi (Tomberlin et al., 2017). Manzo-Valencia et al. (2016) described how in some fungal species a low concentration of IAA can stimulate sporulation, but high amounts of IAA inhibit it. A similar interaction was reported to occur between plants and fungi when IAA is the mediator (Keswani et al., 2020). It has also been reported that *Pseudomonas putida* and *T.atroviride* have the ability to degrade excess IAA to reduce the negative effects on plant growth (Gravel et al., 2007). So, the TH and TV in the current study might have also degraded excess IAA to increase their sporulation. However, the information and evidence on this issue are limited and more research is required to elucidate the reasons in

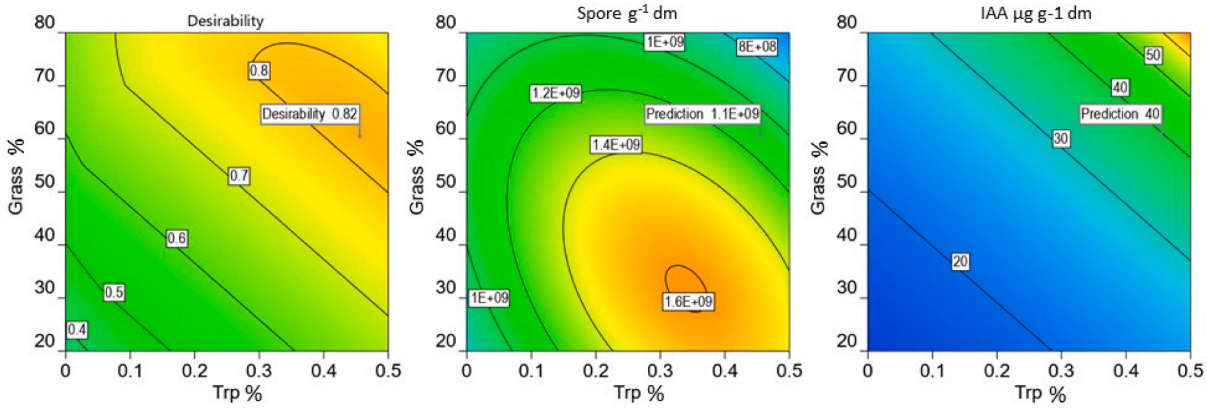

Fig. 4. Contour profile of the model's predicted optimized point. The moisture was set at 74 %.

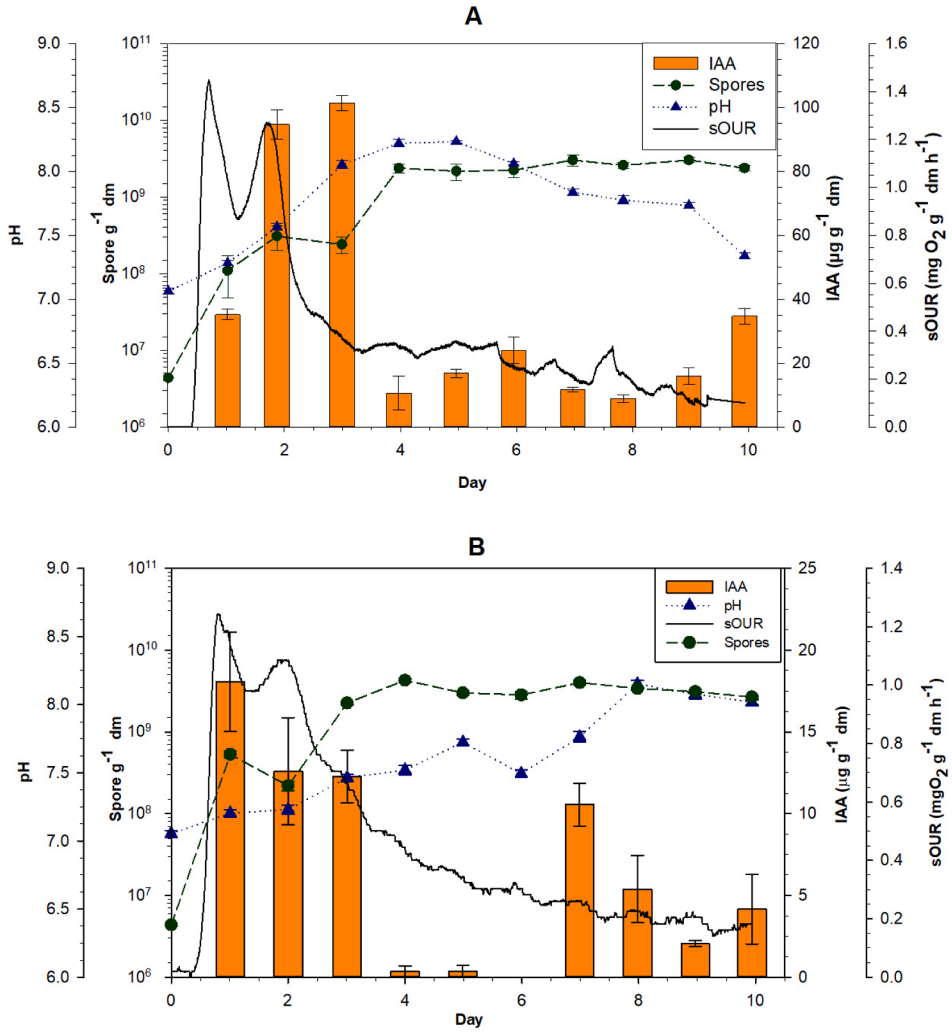

Fig. 5. Results of a 10-day time course experiment. A) *T. harzianum* B) *T.viride.*

more detail.

The pH profile for both strains was not similar, but the pH range was the same (approximately 7–8.3) and agrees with that reported in other studies. It was mentioned previously that the optimum pH range for *T. harzianum* to produce spores was reported to be between 6 and 7 (Sala et al., 2020; Zhang and Yang, 2015). For IAA the optimum pH range is also neutral (5–8) in most microorganisms including *T.harzianum* (Bharucha et al., 2013; Napitupulu et al., 2019).

Overall, these results suggest that for both strains, the fermentation time to obtain the maximum amount of IAA and the highest number of spores is different. In the conditions studied, for TH, the maximum IAA was produced at days 2–3 of fermentation while the highest number of spores was obtained at days 7–9. However, for TV, IAA production peaked on day 1 whereas the number of spores did so on day 4 of fermentation.

4. Conclusion

Throughout this article, it is found that despite the limitations due to the heterogenicity of green waste (GW), it can be an advantageous substrate to produce biostimulant (IAA) and biopesticide (conidial spore) through solid state fermentation. Two strains of *Trichoderma* were able to grow on GW and produced the marketable products. Optimized conditions for *T. harzianum* showed that the highest amount of IAA was obtained after 2–3 days of fermentation when the grass content of substrate and tryptophan (Trp) concentration were higher. The number of conidia peaked after 7 days of fermentation on a substrate with lower grass and lower Trp. Also, when *T. viride* was used as inoculum, the maximum IAA and spores were obtained at different fermentation times. Therefore, determination of the best fermentation time for the maximum IAA and conidia together seems complicated and needs more research. Further studies should be conducted to understand more about the relationship between IAA production and sporulation in TH. The contribution of more factors like carbon and nitrogen source in the production of spore and IAA can also be analyzed, and thus provide the basis to scale up production.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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