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Increase of *Trichoderma harzianum* Production Using Mixed-Level Fractional Factorial Design

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Abstract: This research presents the increase of the *Trichoderma harzianum* production process in a biotechnology company. The NOBA (Near-Orthogonal Balanced arrays) method was used to fractionate a mixed-level factorial design to minimize costs and experimentation times. Our objective is to determine the significant factors to maximize the production process of this fungus. The proposed $2^1 3^2 4^2$ mixed-level design involved five factors, including aeration, humidity, temperature, potential hydrogen (pH), and substrate; the response variable was spore production. The results of the statistical analysis showed that the type of substrate, the air supply, and the interaction of these two factors were significant. The maximization of spore production was achieved by using the breadfruit seed substrate and aeration, while it was shown that variations in pH, humidity, and temperature have no significant impact on the production levels of the fungus.

Keywords: mixed-level fractional factorial design; NOBA method; *Trichoderma harzianum*

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1. Introduction

Economic development based on the mass production of goods and services has brought with it severe damage to the environment due to population, industrial growth, and environmental deterioration. This situation has led to facing an important challenge: to convert industrialized economies into industrial systems of clean, sustainable processes, demanding the integration of human activities with the physical, chemical, and biological systems of the planet [1]. The use of biofungicides has emerged as an alternative to treating diseases in the field. The genus Trichoderma species is a cosmopolitan fungus, a natural inhabitant of the soil with abundant organic matter and high root densities, which can also be found associated with the surface of plants and decomposed wood bark [2]. The species of this genus are of great agricultural interest due to the antagonistic characteristics that they present against phytopathogenic fungi, for which they execute three biocontrol mechanisms: competition for nutrients or space, antibiosis, and mycoparasitism, the latter being its main mechanism of action [3]. To produce this type of fungus in laboratories can be a great challenge since it requires a lot of resources and time. Therefore, the maximization of spore production and the reduction of associated costs has become a priority for the industry.

Recent investigations have been carried out to maximize the production of spores [4–9]; these studies have worked with designs at two levels. The authors were able to obtain a maximum spore production of 3.4×10^8 CFU.g^{-1} [4], 5.5×10^8 CFU.g^{-1} [5], 3.5×10^8 CFU.g^{-1} [6].

A maximum spore concentration of 4.8×10^8 CFU.g^{-1} was obtained in a fermentation parameter study [7]. Another research involving the degradation of biopolymers determined that it was possible to obtain 4.98×10^8 CFU.g^{-1} spore concentration [8], a higher value of 5.9×10^8 CFU.g^{-1} was reached in a single-factor method [9].

This investigation proposes the use of a fractional mixed-level design generated by the NOBA method; the objective is to achieve a higher spore production level. Until today, studies focused on maximizing the production of this fungus have only considered factors at two levels. This research aims to innovate and deepen the production process of this fungus by including qualitative and quantitative factors. Additionally, the use of the NOBA method is proposed as an innovative tool to reduce the number of runs.

In industrial manufacturing processes, it is very common to find both qualitative and quantitative factors; mixed-level designs are very useful in this type of experimentation [10]. Mixed-level designs have the characteristic of being made up of factors from multiple levels. As the levels increase, so does the number of runs; this makes them difficult to apply for economic and time reasons.

Some important works concerning mixed-level designs are mentioned below. A model was developed to obtain designs with high levels of orthogonality based on difference matrices [11]. An algorithm was designed to form mixed-level orthogonal designs using two-level orthogonal designs [12]. Using the concept of J_2-optimality, an algorithm was created to obtain orthogonal and nearly orthogonal designs [13]. The balance coefficient form I was made known [14], in addition to the J_2-optimality published, standardized, and used in an objective function to generate efficient designs. The search for an orthogonal matrix using a methodology based on the polynomial counting function and strata that represent an orthogonal matrix as the positive integer solutions of a system of linear equations where the cost is minimized was exposed [15]. A study was carried out for the maximization of the life of a turbine by means of an orthogonal series of three factors and seven rows [16]. A method using polynomial counting, based on complex level counting for quadratic optimization, was shown [17]. An algorithm to create matrices of mixed levels of generalized minimum aberration using mixed integer optimization with quadratic conic constraints was presented [18]. These methods require great computational skills and statistical knowledge, which overly complicates their application in industry [11,13].

An alternative to these complex algorithms is provided with the NOBA and NONBPA methods [19,20]. The NOBA method was elaborated to generate orthogonal and near-orthogonal balanced mixed-level fractional factorial designs. The NOBA method allows us to optimize the process using fewer runs while ensuring that balance and orthogonality properties are maintained as much as possible in the resulting fraction [19]. The authors developed the NONBPA (Near-Orthogonal Pure array) method for those designs that, due to their nature, once fractionated, present difficulty in possessing the balance property. NOBA and NONBPA methods are less expensive and easier to apply in the industry and can be adapted to any production process [20].

This document shows the maximization of the production process of *Trichoderma harzianum* by making use of the NOBA method. The experimental design involved the following factors:

- Air. Occasional aeration allows good growth and sporulation of the fungus. Concentrations of carbon dioxide in the air higher than 10–15%, a product of cellular respiration, inhibit growth [21];
- Humidity. The amount of water that permeates the environment where the fungus develops is another key characteristic [22]. Trichoderma presents a low level of osmotic tolerance; an excess of humidity lowers the availability of oxygen, limiting the development of the fungus, and compacting the substrate, preventing their full colonization. On the other hand, low humidity inhibits the development of fungus by limiting the mobility of nutrients [23];

- Temperature. This magnitude, referring to the notion of heat, impacts the physiology of fungal growth and is evidenced by the inhibition in the elongation of the hypha, the decrease in the germination of the conidia, and the formation of the germinal tube. For this reason, this factor limits the development of microorganisms [22];
- pH. The measure of acidity or alkalinity is important for Trichoderma species; however, they are not demanding in relation to the pH of the substrate. They can grow in a wide pH range [24];
- Substrate. Any solid material other than soil in situ, natural, synthetic, residual, mineral, or organic, which, placed in a container, in pure form, or in a mixture, allows the anchorage of the root system [25].

It is worth mentioning that in this work, the incubation period and the inoculum concentration are factors that remained fixed at 28 days and 10%, respectively.

Technological and development costs are major challenges for the development of industrial products [22]. Colonies of *Trichoderma harzianum* grow and mature rapidly after five days of incubation on a potato dextrose agar culture medium. In a previous design of 28 runs, the laboratory found an increase in the spore's growth of 50% during the 21 days and 250% during the 28 days of incubation, realizing that in the 29 days onwards, the mass began to decrease.

There are various forms of production of Trichoderma harzianum, solid, liquid, and biphasic. The biphasic is the fastest, because the inoculum is produced by liquid fermentation, which is then used to ferment the solid substrate [26]; in addition, it has been reported that substances that promote plant growth, such as indoleacetic acid, gibberellic acid, cytokinins, and vitamins are produced in a liquid medium [27]. There are reports where up to 5×10^9 CFU.g^{-1} [28] and 1×10^9 [26] CFU.g^{-1} have been obtained using biphasic production media with a shorter incubation time of 8 days and 10 days, respectively, thanks to the use of yeasts such as Saccharomyces cerevisiae and culture media with cellulose, yeast extract, lactose, and lactobionic acid that promote the production of cellulases.

Solid-state fermentation (SSF) was used to produce *Trichoderma harzianum* biomass in this study. SSF is defined as the microbial culture on the surface and interior of a solid matrix with sufficient moisture to permit the growth of microorganisms [29].

Large-scale, cost-effective production is possible through SSF for fungal biomass generation [6]. SSF is a cheaper biomass generation system than liquid or biphasic since it does not require sophisticated formulation procedures, and it is possible to use agro-industrial residues, which makes cost reduction possible [30].

It is a $2^1 3^2 4^2$ mixed-level factorial design that requires 288 runs; when fractioned, it only requires 48 runs. The response variable is spore production (see Table 1).

Table 1. Factors and levels factorial design *Trichoderma harzianum*.

Real Name of the Factors (Uncoded Factors)	Coded Factors	Real Values of the Levels (Uncoded Levels)				Coded Levels
Air	A	Yes	No	---	---	1, 2
Humidity (%)	B	30	50	70	---	1, 2, 3
Temperature (°C)	C	23	25	27	---	1, 2, 3
pH	D	4	5	6	7	1, 2, 3, 4
Substrate	E	Corn cob	Grain of rice	Sesame husk	Breadfruit seed	1, 2, 3, 4

For this study, the fraction was built using the NOBA method that, in combination with the statistical analysis carried out in Design Expert (version 11.0.4.x64) software®, allowed us to determine the factors that have a significant impact on spore production [31]. The results showed that the breadfruit seed substrate and aeration are significant, while it was shown that variations in pH, humidity, and temperature are not significant.

This research was conducted in the industrial sector; the laboratory where the study was carried out uses the Trichoderma fungus in 40% of its products, which represents

35% of the company's net profit. The company is currently in the process of expanding its market, with export being the main objective, which is why they are interested in increasing spore production and having the capacity to meet future demand. We believe this research is important because it constitutes the first real-world application of the NOBA method. In addition, this work can help the industry to understand the utility and ease of use of the NOBA method as well as the benefits and savings that can be achieved.

The paper has been organized into five sections. Section 1 presents an introduction and a literature review focused on mixed-level factorial designs and topics related to the reproduction of *Trichoderma harzianum* spores, Section 2 presents the methodology, Section 3 presents the results for experimentation additionally maximization and confirmatory tests, Section 4 presents conclusions, and finally, Section 5 presents a discussion.

1.1. Mixed-Level Fractional Factorial Designs

Designs of experiments are commonly used to find the optimal configuration of a model to maximize or minimize a process or system. The experimental designs (DOE) allow us to make deliberate changes in the selected variables of the process and thus be able to know the behavior of the response variable due to these changes [10]. These data obtained can be analyzed and thus be able to obtain valid conclusions that will be used to derive an empirical statistical model that unites the inputs with the outputs [14]. The use of experimental design in the initial phases of a product cycle can substantially reduce time and costs, resulting in processes and products with better field performance and greater reliability than those developed using other approaches.

Mixed-level factorial designs are designs in which qualitative and quantitative factors coexist; the characteristic is that at least one of its factors has more than two levels. A design can be symbolized by a matrix containing all level combinations of the $n \times W$ size factors. Where: n is the total number of rows, and W is the total number of factors. Fractional factorial designs are the most popular designs in experimental investigation [32]. Its use is especially popular in screening experiments; these are experiments in which many factors are considered, and the objective is to identify those factors with large effects [10].

Balance and orthogonality are two basic properties of factorial experiments. Balance requires that each possible factor level in each column appears the same number of times [19]. It is due to the orthogonality in the designs that we can obtain the effects of all the factors in an independent way [33].

1.2. Characteristics Factor for the Production of Spores of the Trichoderma harzianum

The most important factors influencing fungal production are temperature, pH, type of substrate, inoculum concentration, incubation period, aeration, and moisture content [34]. For the successful growth of biological control agents during production, it is necessary to know the specific and optimal value of the physiological and environmental parameters [35].

Table 2 shows the operation range as well as the optimal values for the factor levels that have been documented in other investigations.

Table 2. Factor, operation range, and optimal value.

Factor	Operation Range	Optimal Value	References
Air	Yes-No	Yes	[5]
	Yes–No	Yes	[32]
	Yes–No	Yes	[36]
Humidity	50%–80%	50%	[6]
	30%–60%	50%	[37]
	50%-70%	70%	[38]
Temperature	25 °C–35 °C	25 °C	[6]
	25 °C–28 °C	26 °C	[35]
	24 °C–27 °C	25 °C	[37]

Table 2. *Cont.*

Factor	Operation Range	Optimal Value	References
pH	3.8–6.0	6.0	[4]
	5.0–8.0	5.8	[6]
	2.0–6.0	4	[39]
Substrate	Rice husk, sugar cane, coffee husk, corn cob.	Rice husk	[5]
	Rice grains, sorghum, birdseed, broken corn, rice straw.	Rice grains	[40]
	Rice grain, wheat grain, sorghum grain, sesame husk.	Sesame husk	[41]
Incubation period	5–7 days	5 days	[4]
	14–18 days	27.8 days	[6]
	5–15 days	31 days	[24]
Inoculum concentration	0.1%–10%	12%	[6]
	10%-15%	13.9%	[42]
	5%–10%	5%	[43]

A statistical analysis was carried out to determine the influence of ***air*** on the growth of the fungus; the results showed that the presence of air increases the production of the Trichoderma fungus [37]. Several authors [5,36] agree with what was reported by the author [37]; a 30% increase in spore production was reported due to the presence of air in the incubation process.

Humidity is important in the production of the fungus; an optimal level of humidity must be sought since humidity values less than 30% reduce the growth of microorganisms, and the same happens with values greater than 90%; it is recommended to keep humidity at a range of 50%-70% [38]. The authors [6,39] achieved the highest spore production using 50% humidity.

Fungi need a certain temperature to develop and carry out their activities [40]. Through several studies [6,23,41], it has been possible to verify that the optimal range for the development of the fungus is situated at 24 °C–27 °C. Obtaining an optimal value in their studies of 25 °C [6,41] and 26 °C [23].

Hydrogen potential is a critical parameter in the viability of the fungus [42]. The authors examined the effect of pH on the in vitro activities of the extracellular enzymes of Trichoderma. Most of the Trichoderma strains examined were able to grow in a wide pH range from 2.0 to 6.0 with an optimum of 4.0. However, some of the examined pathogenic fungi had optimal pH at alkaline values of 8–10 [22]. A seven-factor design with two levels was used, and it was possible to determine an optimal pH in filamentous fungi. It was found that it develops better in a range of 3.8 to 6, locating the optimum at 6. Additionally, when the pH takes values greater than 8 and less than 3, destabilization is generated, causing a decrease in spore production [4]. A five-factor design with two levels determined that the maximum spore production was achieved using a pH of 5.8 [6].

For fungal spores' growth, it is necessary to provide them with adequate nutrition (carbon, phosphorus, and nitrogen), so the choice of substrate is key for large-scale production. Different types of substrates such as tomato husk, rice husk, garlic husk, cocoa husk, sesame husk, peanut husk, coffee husk, bean husk, soybean and corn husk, corn cob, rice grains, sorghum, birdseed, broken corn, rice straw, wheat straw, agricultural manure, banana pseudo stem, and dry banana leaves, provide this nutrition, the highest spore production of 3.1×10^6 CFU.g^{-1} was obtained using rice grain as a substrate [43]. In another study [44], the substrates rice grain, wheat grain, sorghum grain, and sesame husk were considered, with the highest production of 3.7×10^8 CFU.g^{-1} was obtained with rice grain. Using the substrates wheat straw, broad bean straw, vegetable husks, teff straw, and rice husk in a one-factor-at-a-time design, the rice husk was the one that presented the highest performance with a production of 5.0×10^8 CFU.g^{-1} [5].

Growth phases in Trichoderma beyond 21 days of incubation have been found, which could indicate that as the incubation period increases, spore production will also increase.

However, beyond 28 days, the growth is affected, being 28 days, the period in which the highest production was obtained [6]. The authors [34] found a maximum spore production of 7.3×10^8 CFU.g^{-1} with an incubation period of 5 days.

It is necessary to use an adequate inoculum concentration for good sporulation in the fungus [45]. Using the inoculum size of 5% and 10% in their experiment, they achieved the highest number of spores, 5.9×10^8 CFU.g^{-1} with the 5% inoculum [46]. With values of 0.1% and 10%, the predictive model of the authors determines that the highest point of production would be achieved with an inoculum concentration of 12% [6].

In this investigation, five factors were considered: three quantitative and two qualitative. Originally the "Biotechnological Company" had operated with the factors in the levels without air, humidity at 50%, temperature of 25 °C, pH of 4, and rice grain as substrate. With the aim of increasing spore production, new levels and factors were evaluated, considering the characteristics of the process, the current state of literature, and experience. In the case of the quantitative factors, values were taken both above and below the operation, and in the case of the qualitative factor, according to local availability and the facilities.

1.3. Production Process of Trichoderma harzianum

The reproduction process of *Trichoderma harzianum* using SSF begins with the reactivation of the strains in potato dextrose agar (PDA) for seven days at a temperature of 25 °C, or until the mycelium sporulates. The substrate must undergo a size reduction process until obtaining a particle size of 0.5 mm. The ground substrate is washed to remove impurities and hydrated by immersion in water containing the antibiotic (chloramphenicol) at 500 ppm for 45 min. Then, it is filtered and deposited in the high-density polystyrene bag; it is enriched with molasses, and the pH is manipulated with 10% acetic acid (4 pH) or with NaOH (14 pH) and hydrated for two hours. Then it is then sterilized in an autoclave for 30 min at a pressure of 103,421 Pascals at 120 °C. The sterile substrate is cooled and inoculated with a cube of colonized agar and incubated for 28 days at room temperature (23–27 °C) in a humidity range between 30 and 70%. With air supplied every five days or without air supply. After incubation, the preparation of 500 g of biopreparation is homogenized.

The method used to extract spores from the biopreparation was vibratory screening, which consists of an upper sieve with a pore size of 209 mm and a lower sieve with a size of 35 mm pore. Next, each gram of substrate is resuspended in 10 mL of distilled water plus 0.01% polyoxyethylene sorbitan monooleate at a concentration of the 10^6 sp/mL and stirred for two minutes. Decimal dilutions were made and sown in 9 cm diameter Petri dishes with nutrient agar culture medium for counting conidia and in potato dextrose agar for enumeration of total spores. Conidia were counted in a hemocytometer (Neubauer chamber) with a phase contrast microscope (Zeiss, Mexico City, Mexico), at 800×. The *Trichoderma harzianum* fungus produces three types of propagules: hyphae, chlamydospores, and conidiospores, the latter being the most stable thanks to their thick three-layer wall that allows them to survive adverse conditions until they find the right ones to germinate. Because of this, viability is measured as the germination of spores (conidia) in the substrate [47].

2. Methodology

The experimental strategy for the maximization of *Trichoderma harzianum* spore production consists of five steps.

Step 1. Design structure.

The proposed experiment is a $2^1 3^2 4^2$ mixed-level factorial design, which contains 288 runs. The experiment includes three numerical factors (% humidity, temperature, and pH), two categorical factors (air and substrate), and the response variable spore production. The size of the full factorial makes this design economically infeasible. Therefore, running a fraction is a more suitable approach.

Step 2. Application of NOBA method.

Many alternatives to fractionate mixed-level factorial designs have been proposed; however, these require complex programming techniques and a lot of computational time [11–18]. The NOBA method offers the use of four simple steps to obtain the fraction using basic mathematics, allowing the experimenter to reduce the number of runs significantly. This method is flexible, and the experimenter can decide the size of the fraction to be constructed. The authors recommend choosing a size fraction with a high number of runs because this fraction will have better levels of balance and orthogonality, but of course, financial restrictions will play an important role in this decision [19].

Procedure to apply the NOBA method:

(i) Evaluate the divisor factors and the sizes of the fractions that they produce from the full factorial. The divisor factor is a column of a factor that forms segments into its column and is used to divide the design into s segments and so to create a size s fraction. Therefore, s = number of runs in the full factorial n/size of each segment. Then, for the design ($2^1 3^2 4^2$) if we compute and analyze the divisor factor B, $s = 288/2 = 144$, for factor C, $s = 288/6 = 48$, and for the factor D, $s = 288/18 = 16$, and the factors A and E are discarded as possible divisor factors. To obtain as much information as possible while maintaining a budget, we select C as the divisor factor to produce a fraction with 48 runs.

Note that it is unrecommended to use the first and last columns as divisor factors because they produce a fraction that is too big or too small [19].

(ii) Determine if the fraction will be balanced. To know this, it is necessary to calculate the least common multiple (LCM) for the factor's levels. The least common multiple for level 2,3,3,4,4 is 12, and since $s \geq LCM$ ($48 \geq 12$), the fraction will be balanced.

(iii) Assign positions. A position is a number assigned to each run within a segment, given that, in this case, each segment contains six runs. The positions take values from 1 to 6 within each segment (see Table 3). In this way, every run in the factorial design is labeled as segment i, position j, the number of positions in a segment can be denoted as $p = n/s$, and the number of times that each position appears in the fraction is $m = s/p$. Therefore, $n = 288$, $s = 48$ thus $p = n/s = 288/48 = 6$ and $m = s/p = 48/6 = 8$.

(iv) Use of permuted vectors. Permuted vectors are used to assign the positions in the segments. According to the NOBA method for $s = 48$, $p = 6$, and $m = 8$, we need 8 vectors of size 6 to fill the 48 positions see Table 4.

Table 3. Segments and positions for (288, $2^1 3^2 4^2$) using factor C as divisor factor.

Run	Divisor Factor C	Segment	Position
1	1	1	1
2	1		2
3	1		3
4	1		4
5	1		5
6	1		6
7	2	2	1
8	2		2
9	2		3
10	2		4
11	2		5
12	2		6

Table 3. *Cont.*

Run	Divisor Factor C	Segment	Position
13	3	3	1
14	3		2
15	3		3
16	3		4
17	3		5
18	3		6
.		.	.
.		.	.
.		.	.
271	1	4	1
272	1		2
273	1		3
274	1		4
275	1		5
276	1		6
277	2	5	1
278	2		2
279	2		3
280	2		4
281	2		5
282	2		6
283	3	6	1
284	3		2
285	3		3
286	3		4
287	3		5
288	3		6

Table 4. Permuted vectors for $p = 6$ and $m = 8$.

Permuted Vector 1	Permuted Vector 2	Permuted Vector 3	Permuted Vector 4	Permuted Vector 5	Permuted Vector 6	Permuted Vector 7	Permuted Vector 8
6	6	6	6	6	6	6	6
5	5	5	5	5	5	5	5
4	4	4	4	4	4	3	3
3	3	2	2	1	1	4	4
2	1	3	1	2	3	2	1
1	2	1	3	3	2	1	2

Next, we build the fraction by selecting one run for each segment, making sure that each position appears m times see Table 5.

Table 5. Some runs selected of the ($2^1 3^2 4^2$) for the NOBA fraction. Background color in the indicates the number of every selected run to construct the fraction.

		2	**3**	**3**	**4**	**4**				**2**	**3**	**3**	**4**	**4**				**2**	**3**	**3**	**4**	**4**	
	Run	**A**	**B**	**C**	**D**	**E**	**Position**		**Run**	**A**	**B**	**C**	**D**	**E**	**Position**		**Run**	**A**	**B**	**C**	**D**	**E**	**Position**
Segment 1	1	1	1	1	1	1	1	Segment 2	7	1	1	2	1	1	1	Segment 3	13	1	1	3	1	1	1
	2	2	1	1	1	1	2		8	2	1	2	1	1	2		14	2	1	3	1	1	2
	3	1	2	1	1	1	3		9	1	2	2	1	1	3		15	1	2	3	1	1	3
	4	2	2	1	1	1	4		10	2	2	2	1	1	4		16	2	2	3	1	1	4
	5	1	3	1	1	1	5		11	1	3	2	1	1	5		17	1	3	3	1	1	5
	6	2	3	1	1	1	6		12	2	3	2	1	1	6		18	2	3	3	1	1	6
		2	3	3	4	4				2	3	3	4	4				2	3	3	4	4	
	Run	A	B	C	D	E	Position		Run	A	B	C	D	E	Position		Run	A	B	C	D	E	Position
Segment 4	19	1	1	1	2	1	1	Segment 5	25	1	1	2	2	1	1	Segment 6	31	1	1	3	2	1	1
	20	2	1	1	2	1	2		26	2	1	2	2	1	2		32	2	1	3	2	1	2
	21	1	2	1	2	1	3		27	1	2	2	2	1	3		33	1	2	3	2	1	3
	22	2	2	1	2	1	4		28	2	2	2	2	1	4		34	2	2	3	2	1	4
	23	1	3	1	2	1	5		29	1	3	2	2	1	5		35	1	3	3	2	1	5
	24	2	3	1	2	1	6		30	2	3	2	2	1	6		36	2	3	3	2	1	6
		2	3	3	4	4				2	3	3	4	4				2	3	3	4	4	
	Run	A	B	C	D	E	Position		Run	A	B	C	D	E	Position		Run	A	B	C	D	E	Position
Segment 7	37	1	1	1	3	1	1	Segment 8	43	1	1	2	3	1	1	Segment 9	49	1	1	3	3	1	1
	38	2	1	1	3	1	2		44	2	1	2	3	1	2		50	2	1	3	3	1	2
	39	1	2	1	3	1	3		45	1	2	2	3	1	3		51	1	2	3	3	1	3
	40	2	2	1	3	1	4		46	2	2	2	3	1	4		52	2	2	3	3	1	4
	41	1	3	1	3	1	5		47	1	3	2	3	1	5		53	1	3	3	3	1	5
	42	2	3	1	3	1	6		48	2	3	2	3	1	6		54	2	3	3	3	1	6
	…		….	…	….	…	…		…			…			…		…						…
		2	3	3	4	4				2	3	3	4	4				2	3	3	4	4	
	Run	A	B	C	D	E	Position		Run	A	B	C	D	E	Position		Run	A	B	C	D	E	Position
Segment 40	1	1	1	1	2	4	1	Segment 41	7	1	1	1	2	4	1	Segment 42	13	1	1	1	2	4	1
	2	2	1	1	2	4	2		8	2	1	1	2	4	2		14	2	1	1	2	4	2
	3	1	2	1	2	4	3		9	1	2	1	2	4	3		15	1	2	1	2	4	3
	4	2	2	1	2	4	4		10	2	2	1	2	4	4		16	2	2	1	2	4	4
	5	1	3	1	2	4	5		11	1	3	1	2	4	5		17	1	3	1	2	4	5
	6	2	3	1	2	4	6		12	2	3	1	2	4	6		18	2	3	1	2	4	6
		2	3	3	4	4				2	3	3	4	4				2	3	3	4	4	
	Run	A	B	C	D	E	Position		Run	A	B	C	D	E	Position		Run	A	B	C	D	E	Position
Segment 43	1	1	1	1	3	4	1	Segment 44	7	1	1	2	3	4	1	Segment 45	13	1	1	3	3	4	1
	2	2	1	1	3	4	2		8	2	1	2	3	4	2		14	2	1	3	3	4	2
	3	1	2	1	3	4	3		9	1	2	2	3	4	3		15	1	2	3	3	4	3
	4	2	2	1	3	4	4		10	2	2	2	3	4	4		16	2	2	3	3	4	4
	5	1	3	1	3	4	5		11	1	3	2	3	4	5		17	1	3	3	3	4	5
	6	2	3	1	3	4	6		12	2	3	2	3	4	6		18	2	3	3	3	4	6
		2	3	3	4	4				2	3	3	4	4				2	3	3	4	4	
	Run	A	B	C	D	E	Position		Run	A	B	C	D	E	Position		Run	A	B	C	D	E	Position
Segment 46	1	1	1	1	4	4	1	Segment 47	7	1	1	2	4	4	1	Segment 48	13	1	1	3	4	4	1
	2	2	1	1	4	4	2		8	2	1	2	4	4	2		14	2	1	3	4	4	2
	3	1	2	1	4	4	3		9	1	2	2	4	4	3		15	1	2	3	4	4	3
	4	2	2	1	4	4	4		10	2	2	2	4	4	4		16	2	2	3	4	4	4
	5	1	3	1	4	4	5		11	1	3	2	4	4	5		17	1	3	3	4	4	5
	6	2	3	1	4	4	6		12	2	3	2	4	4	6		18	2	3	3	4	4	6

(v) To build the fractions, these runs are extracted from the full factorial; the fraction generated is shown in Table 6. The notation NOBA (C, 48, $2^1 3^2 4^2$) indicates that this is a 48-run near-orthogonal balanced array for a $2^1 3^2 4^2$ mixed-level design in which factor C was used as the divisor factor.

Table 6. NOBA (*C*, 48,$2^1 3^2 4^2$) using a coded level.

Run	A	B	C	D	E
1	2	3	1	1	1
2	1	3	2	1	1
3	2	2	3	1	1
4	1	2	1	2	1
5	2	1	2	2	1
6	1	1	3	2	1
7	2	3	1	3	1
8	1	3	2	3	1
9	2	2	3	3	1
10	1	2	1	4	1
11	1	1	2	4	1
12	2	1	3	4	1
13	2	3	1	1	2
14	1	3	2	1	2
15	2	2	3	1	2
16	2	1	1	2	2
17	1	2	2	2	2
18	1	1	3	2	2
19	2	3	1	3	2
20	1	3	2	3	2
21	2	2	3	3	2
22	2	1	1	4	2
23	1	1	2	4	2
24	1	2	3	4	2
25	2	3	1	1	3
26	1	3	2	1	3
27	2	2	3	1	3
28	1	1	1	2	3
29	2	1	2	2	3
30	1	2	3	2	3
31	2	3	1	3	3
32	1	3	2	3	3
33	2	2	3	3	3
34	1	1	1	4	3
35	1	2	2	4	3
36	2	1	3	4	3
37	2	3	1	1	4
38	1	3	2	1	4
39	1	2	3	1	4
40	2	2	1	2	4
41	2	1	2	2	4
42	1	1	3	3	4
43	2	3	1	3	4
44	1	3	2	3	4
45	1	2	3	3	4
46	2	2	1	4	4
47	2	1	2	4	4
48	2	1	3	4	4

Step 3. Production process of Trichoderma harzianum.

Experimental runs were performed in the laboratory; this process lasted a total of 28 days from the time the substrate was washed to the end of incubation (see Section 1.3). Spore concentration is the total number of spores in the culture (germinated and non-

germinated). This is used to calculate the percentage viability. Therefore, percentage viability is quantified as the division of germinated spores' number over spore concentration. And spore production is computed as the product of the spore concentration and the percentage viability.

Step 4. Statistical data analysis.

In this step, the Design Expert (version 11.0.4.x64) software® was used, and an ANOVA was generated for statistical analysis of the data; this was conducted to examine the magnitude and direction of the factor's effects and to determine the variables that are significant.

Data obtained experimentally very often lacks important properties, such as normality. Normality is a key assumption in using statistical tests and creating a model with an appropriate level of confidence. This section also includes Fit statistics obtained by Design expert (version 11.0.4.x64) software® to assess the quality of the model.

The Box–Cox transformation was obtained in Design expert (version 11.0.4.x64) software ®; it was useful to determine if it is necessary to apply a potential transformation to the dependent variables to correct the asymmetry of the data, and thus ensure that normality exists.

An optimization was performed using the desirability function to determine the factors levels that maximize spore production.

Step 5. Confirmatory tests of the factorial fractional design.

It is advisable to compare model prediction against real laboratory conditions. Therefore, more than one test should be performed under the optimal conditions recommended by Design Expert (version 11.0.4.x64) software®. In this case, 10 confirmatory tests are also included in the results of this document. This allowed us to corroborate that the selection of levels in the significant factors really shows a behavior like the results of the optimization.

3. Results

The mixed-level fractional factorial design is shown in Table 7. A total of 48 experiments with the corresponding values for spore production are shown.

Table 7. Results of the experimental runs.

Run	Air	% Humidity	Temperature (°C)	pH	Substratum	Spore Concentration $\times 10^8$ CFU.g^{-1}	% Viability	Spore Production $\times 10^8$ CFU.g^{-1}
1	No	70	23	4	Corn Cob	3.8	98	3.724
2	Yes	70	25	4	Corn Cob	4.05	98	3.969
3	No	50	27	4	Corn Cob	3.75	98	3.675
4	Yes	50	23	5	Corn Cob	4.15	98	4.067
5	No	30	25	5	Corn Cob	3.7	97	3.589
6	Yes	30	27	5	Corn Cob	4	97	3.880
7	No	70	23	6	Corn Cob	3.6	98	3.528
8	Yes	70	25	6	Corn Cob	4.45	98	4.361
9	No	50	27	6	Corn Cob	3.6	97	3.492
10	Yes	50	23	7	Corn Cob	4.2	98	4.120
11	Yes	30	25	7	Corn Cob	4.1	99	4.060
12	No	30	27	7	Corn Cob	3.5	97	3.400
13	No	70	23	4	Grain of rice	2.22	95	2.110
14	Yes	70	25	4	Grain of rice	3.03	97	2.940
15	No	50	27	4	Grain of rice	2.42	95	2.300
16	No	30	23	5	Grain of rice	2.01	94	1.890
17	Yes	50	25	5	Grain of rice	3.13	97	3.040
18	Yes	30	27	5	Grain of rice	3.09	97	3.000
19	No	70	23	6	Grain of rice	2.52	96	2.420
20	Yes	70	25	6	Grain of rice	3.22	97	3.123
21	No	50	27	6	Grain of rice	2.13	96	2.044
22	No	30	23	6	Grain of rice	2.32	96	2.230
23	Yes	30	25	7	Grain of rice	3.18	97	3.084
24	Yes	50	27	7	Grain of rice	3.33	97	3.230
25	No	70	23	4	Sesame husk	0.9	92	0.830
26	Yes	70	25	4	Sesame husk	1.3	94	1.222
27	No	50	27	4	Sesame husk	0.85	92	0.782
28	Yes	30	23	5	Sesame husk	1.25	93	1.162

Table 7. *Cont.*

Run	Air	% Humidity	Temperature (°C)	pH	Substratum	Spore Concentration $\times 10^8$ CFU.g^{-1}	% Viability	Spore Production $\times 10^8$ CFU.g^{-1}
29	No	30	25	5	Sesame husk	0.8	92	0.740
30	Yes	50	27	5	Sesame husk	1.41	94	1.330
31	No	70	23	6	Sesame husk	0.95	92	0.874
32	Yes	70	25	6	Sesame husk	1.35	94	1.270
33	No	50	27	6	Sesame husk	0.92	94	0.864
34	Yes	30	23	7	Sesame husk	1.28	94	1.203
35	Yes	50	25	7	Sesame husk	1.3	94	1.222
36	No	30	27	6	Sesame husk	0.79	93	0.734
37	No	70	23	4	Breadfruit seed	7.34	96	7.050
38	Yes	70	25	4	Breadfruit seed	8.12	97	7.880
39	Yes	50	27	4	Breadfruit seed	7.98	97	7.740
40	No	50	23	5	Breadfruit seed	7.23	98	7.090
41	No	30	25	5	Breadfruit seed	6.89	95	6.550
42	Yes	30	27	5	Breadfruit seed	8.23	97	7.983
43	No	70	23	6	Breadfruit seed	6.93	97	6.722
44	Yes	70	25	6	Breadfruit seed	8.52	99	8.434
45	Yes	50	27	6	Breadfruit seed	8.31	98	8.143
46	No	50	23	7	Breadfruit seed	7.52	98	7.370
47	Yes	30	25	7	Breadfruit seed	7.84	98	7.683
48	No	30	27	7	Breadfruit seed	7.76	97	7.530

Table 8 shows the ANOVA; we can see that air and substrate factors, as well as their interaction, are statistically significant. On the other hand, it can be noticed that any change in levels of pH, temperature, and humidity has no significant impact on spore production.

Table 8. ANOVA for spore production maximization. Background color in the table is to indicates the significant factors in the ANOVA.

Source	Sum of Squares	Df	Mean Square	F-Value	*p*-Value	
Model	281.59	10	40.19	823.7	<0.0001	Significant
Air (A)	5.76	1	5.76	144.15	<0.0001	
Humidity (B)	0.2140	1	0.2140	6.26	0.0769	
Temperature (C)	0.0305	1	0.0305	0.8912	0.3513	
Ph (D)	0.1792	1	0.1792	5.24	0.0678	
Substratum (E)	274.94	3	91.40	2673.55	<0.0001	
Air—Substratum (A–E)	0.4758	3	0.1586	4.64	0.004	
Residual	1.26	37	0.0342			
Cor Total	282.85	47				

Table 9 shows a standard deviation of 0.1973 which indicates that our data tends to be distributed very close to the mean. A mean of 3.7424 for spore production was obtained. The coefficient of variation is 5.27, which means that the arithmetic mean is representative of the data set. R^2 = 0.9945 and R^2adj = 0.9935 indicate that a high percentage of the variability in the response can be explained by the regressors. R^2 *pred* = 0.9921 indicates that the model has a high predictive capacity. An *Adeq Precision* = 89.0719 indicates a good signal; based on this information, we conclude that this model can be used to predict and optimize spore production.

Table 9. Fit Statistics.

Std. Dev	0.1973	R^2	0.9945
Mean	3.7424	R^2 *adj*	0.9935
C.V.%	5.27	R^2 *pred*	0.9921
		Adeq Precision	89.0719

Figure 1 shows the normal probability plot for residuals and the Box–Cox method. The normal probability plot indicates that the residuals tend to be normal since the data in

the left tail are very similar to those in the right tail. The *Box–Cox* method indicates that the distribution would be more normal with a square root transformation. Since no outliers are found, and there is no skew in the distribution, it is not necessary to carry out the transformation, the distribution is not perfectly normal, but it is normal enough to perform the analysis. In addition, we must remember that the least squares method is robust to the assumption of normality.

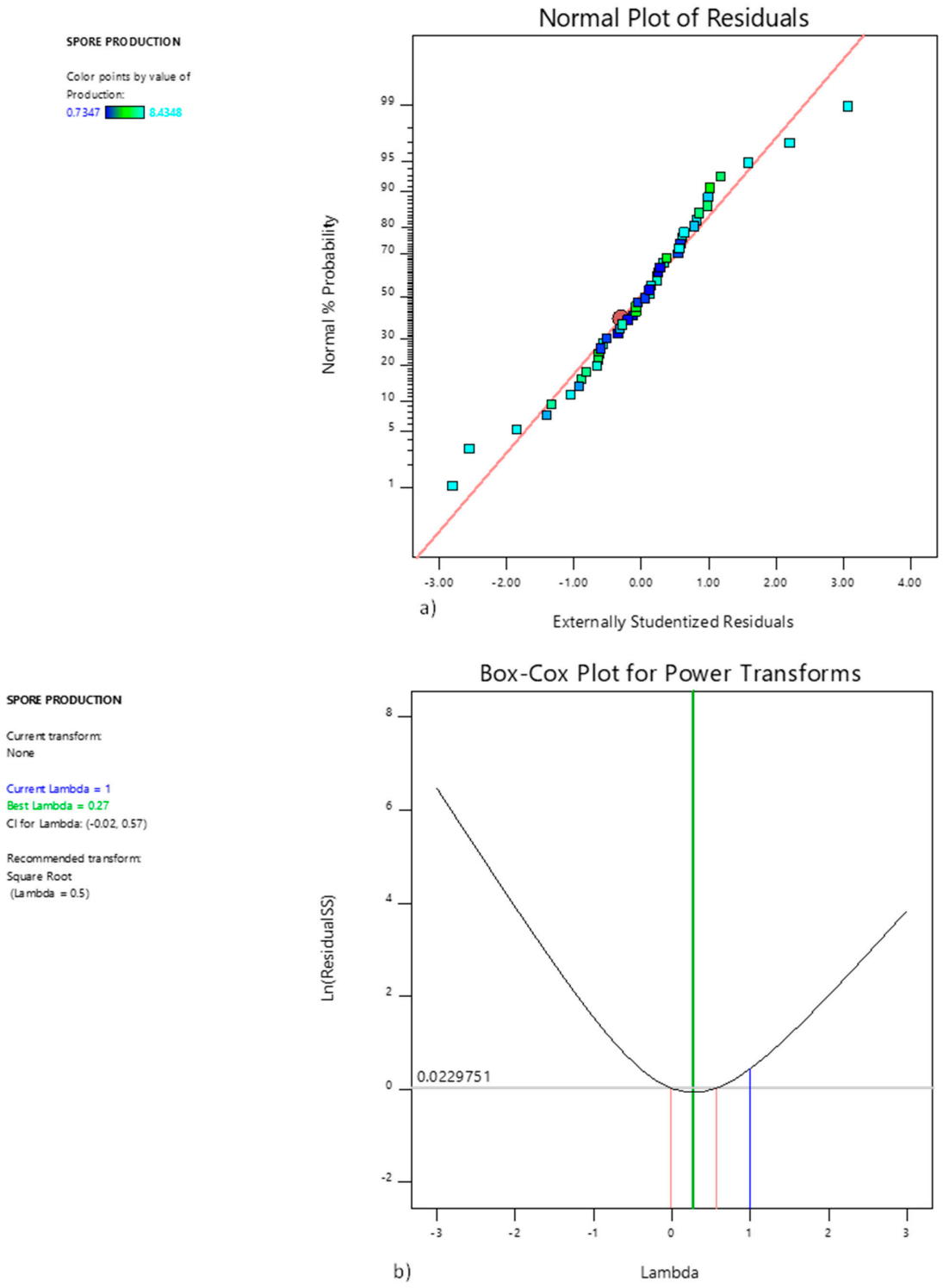


Figure 1. Normal probability plots of the residuals (**a**) and Box−Cox plot (**b**).

Figure 2a shows that humidity, temperature, and pH remain constant as horizontal lines, indicative that it is indistinct which levels of these factors we use. The air-substrate interaction (A–E) is significant; the graphic for this interaction is shown in Figure 2b.

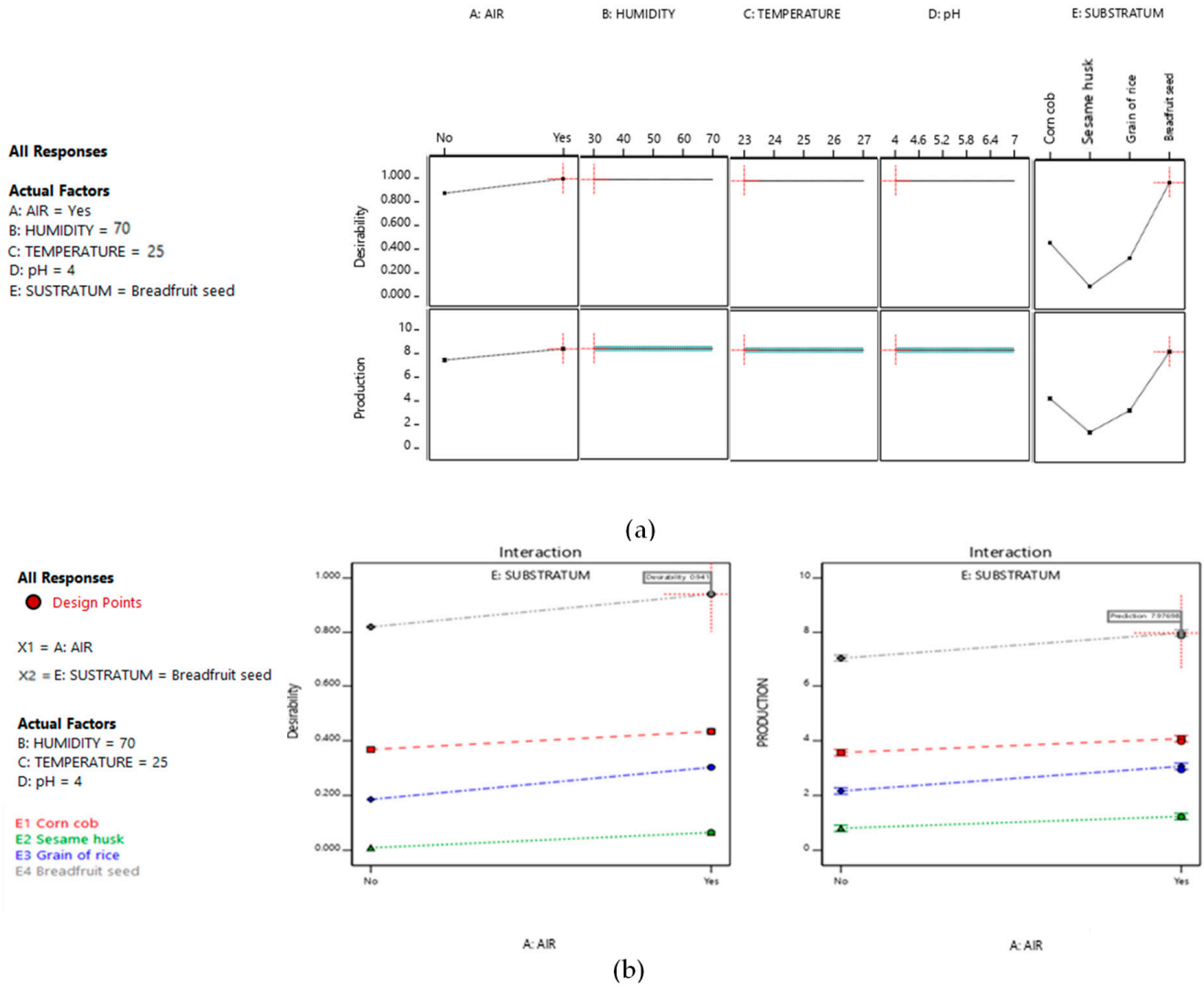


Figure 2. Interaction plots (**a**) all factors; (**b**) interactions.

Figure 3 shows the optimal factors levels. These include air supply, the humidity of 70%, the temperature of 25 °C, pH of 4, and breadfruit seed; this selection of levels maximizes spore production to 7.97698 × 10^8 CFU.g^{-1} with desirability of 0.955.

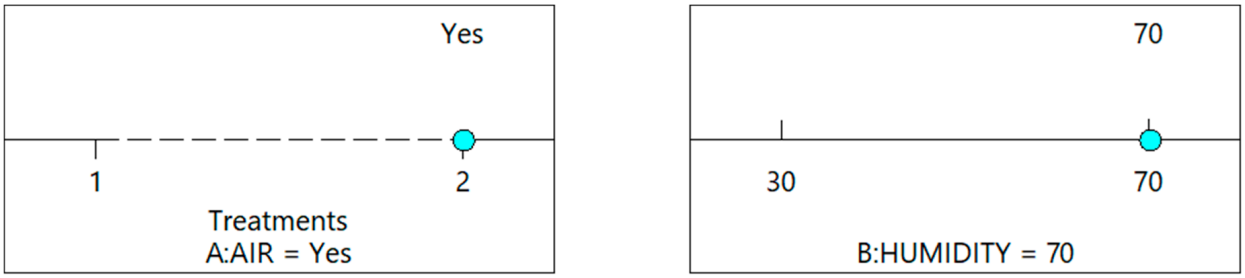


Figure 3. *Cont.*

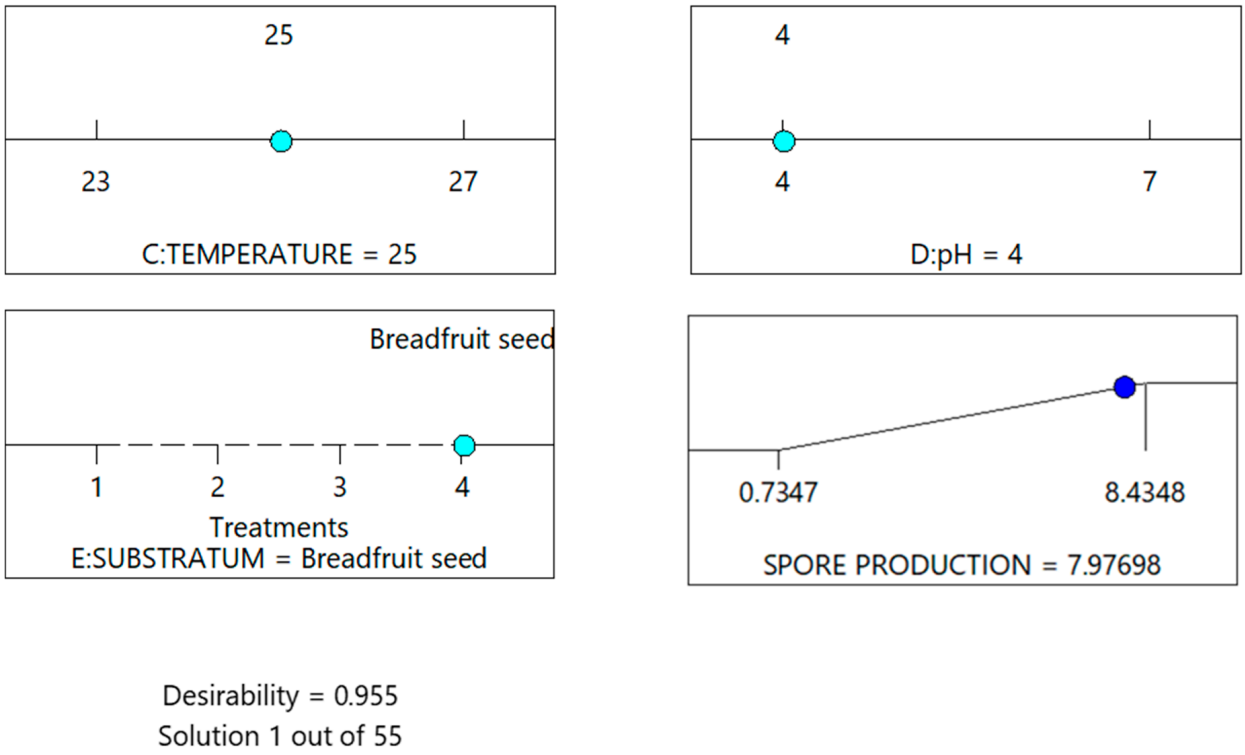


Figure 3. Maximum response configuration.

Figure 4 shows the optimization of spore production. This production is maximized to 7.97698 × 10^8 CFU.g^{-1} when the breadfruit seed and aeration are selected.

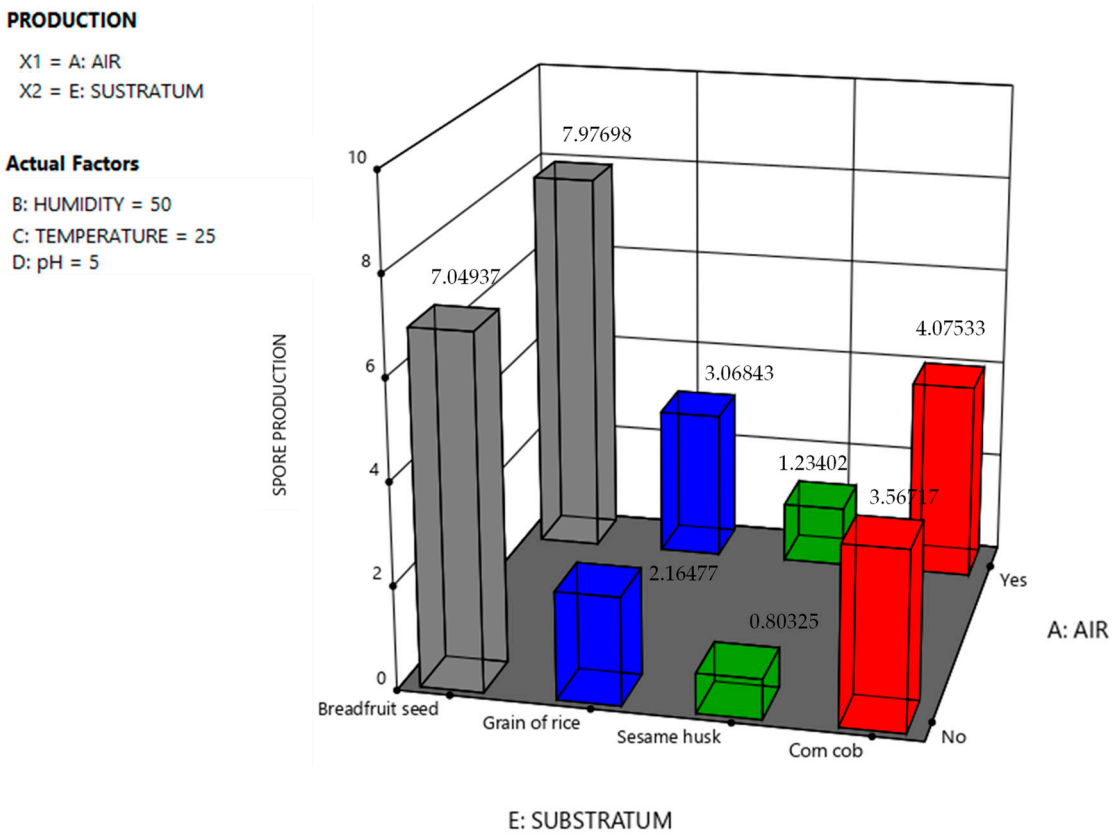


Figure 4. Graph of maximization spore production.

It should be noted that 10 confirmatory runs were carried out to further string the validity of the model. The air and substrate factors remained fixed at optimum conditions, while pH, humidity, and temperature levels were randomly selected in the range in which their respective levels varied. The results are shown in Table 10. When carrying out the experiments, the average spore production was 7.9724 $\times 10^8$ CFU.g^{-1}, demonstrating coincidence with the prediction provided by Design Expert (ver. 11.0.4.x64) software®.

Table 10. Confirmatory runs.

Run	Air	Humidity (%)	Temperature °C	pH	Substratum	Spore Concentration $\times$ 10^8 CFU.g^{-1}	% Viability	Spore Production $\times$ 10^8 CFU.g^{-1}
1	Yes	30	25	4	Breadfruit seed	8.1329	98	7.9703
2	Yes	30	27	4	Breadfruit seed	8.1327	98	7.9701
3	Yes	30	23	5	Breadfruit seed	8.1328	98	7.9702
4	Yes	70	25	5	Breadfruit seed	8.1431	98	7.9803
5	Yes	70	27	5	Breadfruit seed	8.1331	98	7.9705
6	Yes	50	25	6	Breadfruit seed	8.1334	98	7.9708
7	Yes	50	27	6	Breadfruit seed	8.1435	98	7.9807
8	Yes	30	23	7	Breadfruit seed	8.1332	98	7.9706
9	Yes	50	25	7	Breadfruit seed	8.1328	98	7.9702
10	Yes	70	27	7	Breadfruit seed	8.1334	98	7.9708

The "Biotechnological Company" where the research was conducted had an average production of 3.974 $\times$ 10^8 CFU.g^{-1} spores, after the study was carried out, its average production could be doubled, reaching a value of 7.9724 $\times$ 10^8 CFU.g$^{-1,}$ as shown in Table 11.

Table 11. Previous and actual dataset.

Operating Values in the Study Case	Factor					Spore Production CFU.g^{-1}	% Increase
	Air	Humidity	Temperature	pH	Substrate		
Before being improved.	No	40%	24 °C	5	Grain of rice	3.974 $\times$ 10^8	---
After being improved.	Yes	70%	27 °C	7	Breadfruit seed	7.9724 $\times$ 10^8	100.7%

4. Conclusions

In this study, the viability of the NOBA method to maximize the production of Trichoderma spores was demonstrated through an application to a real dataset. The experimental design involved the following factors: air (yes, no), humidity (30%, 50%, 70%), temperature (23 °C, 25 °C, 27 °C), pH (4, 5, 6, 7), substrate (corn cob, rice grain, sesame husk, breadfruit seed). In this scenario, the levels that maximize spore production are (air = yes) and (substratum = breadfruit seed), with a predicted value for spore production of 7.97698 CFU.g^{-1} and a desirability equal to 0.955.

All the factors were selected according to the literature, previous experience in the process, and the laboratory conditions themselves. Only the air and the type of substrate were significant in this study; the use of molasses as a crop enricher could create disturbances in the effects of the remaining factors, due to the humidity and pH of the product itself. Other sources of carbon, nitrogen, or sporulation inducers were not considered in the experiment because the other goal was to reduce the production costs; the raw material selected was the only one available in the region as industrial waste, getting others from outside would increase costs due to transportation and logistics, given that the raw materials used as an organic substrate for biomass production account for 35–40% of production costs [48].

The results of the investigation show that the species of fungus used is capable of reproducing on the different substrates examined and sporulates abundantly on them, but the level of colonization and biomass production differs among the different growth media, most likely reflecting the dietary preference of the Trichoderma species.

This research represents the first real-world application of the NOBA method.

This method is a valuable tool for researchers looking for new optimization options across various industries, and it can be applied to a wide range of populations and real data sets.

As a final comment, we would like to mention that the experience we had when carrying out this project was enriching from a personal and professional point of view, since it allowed us to contribute to the solution of a real problem in the industry, which is the optimization of resources. This was achieved using a technique that we ourselves had previously developed. The practical application of this technique allowed us to demonstrate that it really works in the industrial field and that it will be very useful for companies that want to implement it. This methodology can be used in any real dataset in research and industry; it can be useful in any process that needs to be improved, or even in the process of designing new products.

5. Discussion

In this study, a group of different organic materials available in the region was investigated to determine its viability for the growth and multiplication of *Trichoderma harzianum* and to determine the most suitable organic materials for generating a high amount of conidial biomass with prolonged viability.

Trichoderma harzianum production generates added value and has been proven to be a good approach through which multiple low-cost growth media can be used to formulate bioproducts.

The environment can be the main beneficiary, given that environmental pollution can be reduced by promoting the reduction of chemical products used for treating plant diseases [49]. Finally, many more in-depth studies like the present are required to select substrates that provide large, stable, and efficient microbial populations.

The NOBA method has proven effective in dealing with mixed levels designs that require a large number of experiments. It is easy to apply, does not require complex programming, and significantly reduces the number of runs.

One can use the current real data set as a basis and select the appropriate levels based on the existing literature and then apply the NOBA method to obtain two benefits, optimization of the response variable and minimization of costs.

Our results demonstrated the effectiveness of the NOBA method as an optimization tool; it was possible to double the production of spores, a result above (10^6) conidia/g was obtained, which is the minimum requirement for the application of biopesticides in agriculture.

Different optimization models and methodologies are available like one factor at a time (OFAT) until complex statistical designs such as two-level fractional factorial designs (FFD), the Box–Behnken design (BBD), Taguchi Design (TD) or Placket-Burmann design (PB).

OFAT is a traditional screening method. It consists of selecting a starting point, or baseline of the levels, for each factor, and then successively varying each factor in its range [10].

FFD are experiments where many factors are considered with two levels; the objective is to identify those factors that have large effects through a fraction of the factorial design [10].

BBD is a second-order multivariate technique based on a partial three-level design; it allows the estimation of parameters in a quadratic model and the evaluation of the lack of fit of the model [50].

TD emphasizes the appropriate selection of control factor levels to minimize factor-borne variability of noise and, in this way, generate a robust product or process [10].

PB is one of the most applied screening methods for the recognition of the most significant factors among a large number of variables [51].

The NOBA method presents several advantages in relation to the mentioned methodologies. Considerably reduces the number of runs, therefore, resources and time. The fraction obtained comes from a mixed-level factorial design; you can choose as many factors

or levels as needed and still obtain a balanced orthogonal/semi-orthogonal economic size fraction. Orthogonality makes the effects of the factors independent; therefore, each column provides different information to the design. The balance allows a uniform distribution of information for each level and causes the intersection column to become orthogonal to the main effects. The NOBA method considers the entire factorial design to obtain the fraction, whereas BBD does not. BBD is a spherical response surface, which includes a central point and midpoints between the corners; therefore, the extreme values of the factors are not included in the runs. The NOBA method does consider the extreme values of the variables, and therefore the effects can be estimated more efficiently since it considers the entire region.

TD is a methodology that also offers orthogonal and economic size designs; for the present case study, we could use an arrangement with 16 runs L16 (4^5), 16 runs with five factors and a maximum level in the factors of 4; with the NOBA method, we could also obtain a fraction of only 16 runs using factor D. Factor C was used, which offers us 48 runs, since there were sufficient resources for this number of runs. The main disadvantage of TD in relation to the NOBA Method is that it has more of a focus on main effects and not on interactions. Furthermore, the NOBA method is not limited to a small number of arrays.

The NOBA method has been shown to have many characteristics that some available optimization methods lack, which makes it an invaluable tool for its future application in different fields of knowledge.

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