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Optimization of Esterase Production in Solid-State Fermentation of Agricultural Digestate

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Abstract: The continuous increase in biogas production poses the need for innovative applications for its by-products. Solid-state fermentation (SSF) has regained attention in the development of several products because of the possibility to use low-cost and easily available substrates, such as organic wastes. SSF represents a valuable process for agricultural digestate valorization in terms of enzyme production. In the present study, cellulase and esterase were produced by *Trichoderma asperellum* R on a digestate-based substrate in SSF, with esterase as the highest obtained activity. After assessing the effect of light on it, the esterase production in SSF was optimized using response surface methodology. The influence of substrate composition, temperature and humidity on the enzyme production was evaluated on two sets of data generated based on digestate concentration (50% and 70% *w*/*w*). The statistical analyses revealed that these parameters affected esterase production only when *Trichoderma asperellum* grew on substrate containing 50% *w*/*w* of digestate. The best esterase activity (264.6 mU/mg total protein) was achieved with the following optimized SSF parameters: 50% digestate, 50% fruits, 10% sawdust, 30 °C. The current finding of esterase production on digestate-based substrates makes the SSF method presented here a sustainable and completely circular technology.

Keywords: fungal biorefinery; response surface methodology; *Trichoderma*; cellulase; food waste

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1. Introduction

Esterases are a class of enzymes, also referred to as carboxyl ester hydrolases (EC 3.1.1.), that catalyze the hydrolysis and synthesis of ester bonds [1]. They comprise both lipolytic enzymes, active on lipids, also called lipases, and non-lipolytic esterases, active on water-soluble ester substrates. In recent years, esterases have become relevant in industrial applications due to their high enantioselectivity, wide-spectrum substrate specificity and high stability in organic suspension [2]. All these features make esterases highly valuable for applications such as chiral drug synthesis, the production of flavorings and biomass degradation. Among esterases, carbohydrate esterases and feruloyl esterases have gained an increasing relevance in lignocellulosic biorefineries [3] as accessory enzymes for hemicellulose and lignin-ferulate-arabinoxylan ester bond deconstruction, which is a crucial step for biofuel production and is essential for the conversion of plant biomass into value-added products or energy [4]. Nowadays, biorefineries have achieved enough degrees of

development to produce biofuels and chemical building-blocks as an alternative to refinery products [5]. The development of two-platform biorefineries [6] where integrated biorefinery processes take place to produce both biofuels (e.g., biogas) and value added-products (enzymes, biofertilizers) is the most efficient strategy to valorize biomass in the future bio-based economy. Moreover, sustainable production of these enzymes can contribute to the bioeconomy, supporting several industries. Esterases can be isolated from mammalians, plants or produced by microorganisms, and can be successfully employed as biocatalysts in biorefineries. Fungal biorefinery produces a broad spectrum of products such as organic acids, vitamins, proteins and a cocktail of enzymes. Over the last twenty years, the interest in solid-state fermentation (SSF) processes has markedly increased due to the relative simplicity, low input (energy and water) and inexpensive nutrient requirements. SSF is a promising bioprocess enabling microorganisms to produce bioproducts growing on solid substrates used both as a physical matrix and as a source of nutrients in the absence or near absence of free water. Cereal wastes and residues are the most investigated substrates for enzyme production in SSF, while the most common enzymes reported are xylanases, followed by cellulase, β-glucosidase, laccase and β-xylosidase [7]. Few studies have been carried out on esterase/lipase production in SSF; usually, different waste-based substrates such as digestate and wheat bran, with supplements, have been used with fungi belonging to the genera *Trichoderma, Aspergillus, Cryphonectria* and *Rhizophus* [8–11].

To obtain high enzyme activity, different SSF parameters should be optimized to promote the maximum growth of the microorganism and to increase the production of the enzymes. While nutrient content, aeration, oxygen level and substrate depth affect fungal growth in SSF, moisture content and substrate composition are important factors for both cell growth and enzyme production.

Response surface methodology (RSM) is a group of statistical techniques used to plan experiments, create models, assess the impacts of variables and examine the ideal circumstances under which variables can produce the desired answers. RSM has been successfully applied to optimize the SSF parameters. Due to the reduced time and expense compared with conventional single variable experiments, it has been recognized as a practical method for analyzing optimal circumstances [12]. The concept of using *Trichoderma* sp. in fungal biorefinery is appealing not only for its enzyme production, but also for the obtained fungal biomass that can be used in biofertilizer or biostimulant formulation. Fungi belonging to the genus *Trichoderma* can promote plant growth and protect plants from phytopathogens [13]

In this study, the optimization of the nutritional and environmental conditions for the maximum esterase/lipase production by *Trichoderma asperellum* R under the SSF of digestate was conducted for the first time. Firstly, whole digestate, the by-product of anaerobic digestion, mixed with agro-industrial waste was used as an SSF substrate for fungal growth and screened for cellulase and esterase production. Moreover, substrate composition, temperature and moisture content effect on esterase/lipase production was estimated and optimized with the aid of RSM.

2. Materials and Methods

2.1. Fungal Species

Trichoderma asperellum R comes from the mycotheque of Saida Messgo-Moumene (University of Blida 1, Blida, Algeria) within the framework of scientific collaboration. The strain was maintained as agar plugs of mycelium in sterile distilled water at 4 °C and stored in the collection of the Agri-food and Environmental Microbiology Platform (PiMiAA), University of Brescia, Italy. *T. asperellum* R was routinely grown on Potato Dextrose Agar (PDA, Merck, Darmstadt, Germany) at 26 °C for 7 days and used for solid-state fermentation (SSF) substrate inoculation.

2.2. Organic Wastes

Agricultural digestate, fruits and scraps from yard trimming were the wastes used as substrates in the solid-state fermentation process.

The agricultural digestate was collected from a full-scale biomethane plant operating in the Lombardy Region (northern Italy). The input for the anaerobic digestion (AD) was a mix of manure and vegetables as reported in Table S1. Whole digestate (liquid and solid phase) was collected at the end of the AD and stored at −20 °C until use. Apple, banana and grape fruits no longer suitable for consumption and scraps from yard trimming of *Prunus laurocerasus* were kindly provided by a local non-profit association (Cauto).

2.3. Production of Enzymes in Solid-State Fermentation

2.3.1. Substrate Composition

The ability of *T. asperellum* R to produce enzymes in SSF was verified using a previously set up protocol [10,14]. Briefly, the SSF substrate was composed of the whole digestate (70% *w*/*w*) mixed with apple, banana and grape (30% *w*/*w*). The substrate humidity was modified by adding carpentry sawdust (20% *w*/*w* of digestate mix with fruits). Seventy grams of substrate were transferred in a plant micropropagation box (Micropoili, Cesano Boscone, Italy) and sterilized in autoclave at 121 °C for 15 min for two consecutive cycles. One hundred µL of *T. asperellum* R conidia suspension (10^6 conidia in sterile distilled water) was inoculated in each box. Control samples consisted of SSF substrates not inoculated with the fungus. All the substrates were incubated at 26 °C under illumination of 12 h light/12 h dark cycles, using daylight tubes 24 W/m^2, 9000 lx in a climatic chamber (model 720, Binder) for 6 days.

2.3.2. Crude Extract Collection

Seventy milliliters of sterilized deionized water were added to the different substrates after six days of fermentation. Each obtained mixture was agitated on an orbital shaker for 30 min. The resulting suspension was filtered using Miracloth (Merck, Millipore, Darmstadt, Germany) and centrifuged at 10,000 rpm for 20 min. The supernatants were filtered with 0.45 µm filter, aliquoted and stored at −20 °C until enzyme activity assays.

2.3.3. Cellulase Activity

The endoglucanase (EG) activity (EC 3.2.1.4) was assessed as reported by Bulgari and colleagues [10]. The release of reducing sugars was measured in a reaction mixture containing the crude extract and carboxymethyl cellulose (0.5% *w*/*v*) as a substrate in 50 mM Na acetate buffer (pH 5) at 50 °C for 60 min. One unit (U) of endoglucanase activity was defined as the amount of enzymes that released 1 µmol of glucose equimolar per minute [15].

2.3.4. Esterase Activity

The carboxylesterase activity (EC 3.1.1.1) was determined spectrophotometrically by measuring the hydrolysis of 4-nitrophenyl butyrate (pNPB, Merck, Sigma-Aldrich, Darmstadt, Germany) as a substrate, as previously reported [9]. Ten µL of crude extract was used as an enzyme source. The enzymatic reaction was carried out at 25 °C for 15 min, and the release of pNPB was measured at 405 nm. Enzyme activity was calculated using the extinction coefficient of pNPB, corresponding to 18.5 mM^{-1} cm^{-1}. One unit was defined as the amount of the enzyme that catalyzes the conversion of one micromole of substrate per minute and is normalized by grams of fermented substrate(U/g) or by total protein present in the crude extract (U/mg total proteins). Total protein content in the crude extract was determined using a Bradford assay [16].

2.4. Optimization of Esterase Production in Solid-State Fermentation

To optimize the esterase production by *T. asperellum* R, the interactive effects of substrate composition, temperature and substrate humidity were assessed using Response Surface Methodology (RSM).

As the substrate is one of the main factors affecting enzyme production, various organic wastes were combined in different percentages. The composition of the substrates

and their organic wastes' relative percentages are reported in Table 1. Substrate humidity was adjusted by adding carpentry sawdust at 10% *w*/*w* or 20% *w*/*w* to each substrate. All the substrates at different levels of humidity were incubated at 26 °C and 30 °C under illumination of 12 h light/12 h dark cycles, using daylight tubes 24 W/m^2, 9000 lx in a climatic chamber (model 720, Binder) for 6 days.

Table 1. Solid-state fermentation (SSF) substrate composition.

SSF Substrate ID	% Digestate	% Fruit	% Scraps
SSF-1	100	0	0
SSF-2	70	30	0
SSF-3	70	0	30
SSF-4	70	15	15
SSF-5	50	50	0
SSF-6	50	0	50
SSF-7	50	25	25

2.5. Response Surface Methodology

Two separate analyses were conducted based on the digestate concentration, 70% or 50% *w*/*w*, as they represent the waste that we want to valorize. Fruits and scraps were summarized in a single variable X1. Since % *P. laurocerasus* can be derived as a linear combination of the % Fruit (% Fruit = (30%—% *P. laurocerasus*) at 70% of digestate and % Fruit = (50%—% *P. laurocerasus*) at 50% of digestate), we used the levels of fruits to define the variable X1. We were then able to extend the results associated with X1 to the % *P. laurocerasus* since, at increasing values of X1, we had increasing levels of % Fruit and decreasing values of % *P. laurocerasus*.

For each digestate concentration, we had three levels for the elements Fruits and *P. laurocerasus*. At a digestate concentration of 70%: 30% Fruit/0% *P. laurocerasus* (SSF-2); 0% Fruit/30% *P. laurocerasus* (SSF-3), 15% Fruit/15% *P. laurocerasus* (SSF-4). At a digestate concentration of 50%: 50% Fruits/0% *P. laurocerasus* (SSF-5), 0% Fruit/50% *P. laurocerasus* (SSF-6), 25% Fruit/25% *P. laurocerasus* (SSF-7) (Table 2). Variables X2 and X3 were defined as the level of substrate humidity and temperature, respectively.

Table 2. Values of coded levels of the 3 tested variables.

Digestate 70% (*w*/*w*)				
ID	**Variable**	**Level**		
		−1	0	1
X1	Fruits (*P. laurocerasus*)	0% (30%)	15% (15%)	30% (0%)
X2	Substrate humidity	10%		20%
X3	Temperature	26 °C		30 °C
Digestate 50% (*w*/*w*)				
ID	**Variable**	**Level**		
		−1	0	1
X1	Fruits (*P. laurocerasus*)	0% (50%)	25% (25%)	50% (0%)
X2	Substrate humidity	10%		20%
X3	Temperature	26 °C		30 °C

Humidity and temperature were included in the analysis beside the substrate characterization, considering a total of 3 factors. An experimental D-optimal design (DOD) was applied considering the levels of each factor, as shown in Table 2. In total, 12 factor combinations were considered and all experiments were repeated twice. In total, 12 factor combinations (equal to all the possible permutations of the levels of fruits (*P. laurocera-*

sus), substrate humidity and temperature at each value of digestate: 3 × 2 × 2 = 12; all combinations are displayed in Table 3) were considered.

Table 3. Runs obtained from D-optimal experimental design.

	Factor		
N Run	**X1**	**X2**	**X3**
1	−1	−1	−1
2	−1	−1	1
3	−1	1	−1
4	−1	1	1
5	0	−1	−1
6	0	−1	1
7	0	1	−1
8	0	1	1
9	1	−1	−1
10	1	−1	1
11	1	1	−1
12	1	1	1

The SSF process and the esterase assay were carried out as described above.

The following polynomial regression model was fitted through ordinary least square method for both levels of digestate:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

where β_0 is the intercept; β_1, β_2 and β_3 are the linear coefficients related to the variables X_1, X_2 and X_3, respectively; the parameter β_{11} is associated with the quadratic term of X_1; and the coefficients β_{12}, β_{13} and β_{23} allowed us to test for an interaction effect of the pair of variables X_1X_2, X_1X_3 and X_2X_3.

Student's *t*-test was used to test for the statistical significance of the regression parameters. The validity of the obtained model was assessed through the analysis of variance (ANOVA) and the lack of fit was tested through the F-test. Three-dimensional response surface graphs were also evaluated to represent the variable's effect on esterase activity. All tests were two-sided and the level of significance was set at 5%. Statistical analyses and the validity of the regression models were performed in R (version 4.2.2) [17]: the DOD was conducted through the AlgDesign package (version 1.2.1) [18], while the RSM models were fitted using the rsm package (version 2.10.3) [19].

3. Results and Discussion

Biorefineries were defined for the first time by the United States Department of Energy as processing plants where biomass feedstock is converted and/or extracted into a spectrum of valuable products to produce fuels and high-value chemicals [20]. Nowadays, different kinds of biorefineries have been developed, starting from different materials such as raw crops (whole-crop biorefinery), lignocellulosic biomass (lignocellulosic biorefinery) or cereal and grass biomass natural-wet (green biorefinery) to obtain value-added products such as chemical building blocks, pharmaceutical products, textiles, plastics, lubricants and biofuels [5]. An example of a successful biorefinery is the anaerobic conversion (AD) of biowaste into biogas and digestate. Digestate, the by-product of AD, is mainly and poorly valorized as biofertilizer and, recently, it has been investigated as a substrate in fungal biorefinery to produce organic acids, plant hormone-like molecules and enzymes [10]. This study investigates the potential application of *T. asperellum* R in a fungal biorefinery using digestate mixed with agro-industrial wastes as a substrate for enzyme production. At first, enzyme production obtained through *Trichoderma asperellum* R—digestate SSF was analyzed; then, the enzyme showing the highest activity was chosen and its production was optimized using response surface methodology.

3.1. Cellulase and Esterase Activity in Solid-State Fermentation

In this study, the aqueous crude extract obtained from *T. asperellum* R SSF was analyzed to check enzyme activities after 6 days of fermentation. As previously reported in other *Trichoderma* sp. [10,13], *T. asperellum* R is able to secrete cellulase and esterase growing on digestate-based substrates. In detail, the enzyme activities were 82.3 ± 43.86 mU/g and 259.7 ± 36.43 mU/g for cellulase and lipase/esterase, respectively. The cellulase amount obtained by *T. asperellum* R was comparable with that reported for *T. reesei* RUT-C30 (113.9 ± 11.9 mU/g), a well- known cellulase producer strain, on the same substrate [10]. More interestingly, *T. asperellum* R was able to produce a higher amount of esterase compared with *T. atroviride* Ta13, a strain reported as the best esterase producer (163.1 ± 7.3 mU/g) in the SSF of digestate-based substrates [10]. Based on these results, the esterase production by *T. asperellum* R growing on a digestate-based substrate was optimized using response surface methodology

3.2. Light Does Not Influence Esterase Activity

Light in fungi has a significant impact on numerous signaling pathways that combine light response with metabolism, stress response and development, and is particularly relevant to a variety of physiological control processes [21]. Despite the fact that many of the fungi being researched in academia and industry never experience natural day and night cycles, physiological processes nonetheless adhere to circadian rhythms, which also affect metabolism. For example, in *T. reesei*, the model fungi for industrial cellulase production, light strongly reduces the enzyme activity [22]. Since light can influence enzyme production, we evaluated its effect on esterase. Esterase activity was significantly lower (181.8 ± 29.15 mU/mL, $p < 0.01$) when SSF was carried out in 24 h exposure to light. Conversely, *T. asperellum* R secreted the highest amount of esterase in complete darkness or under illumination with 12 h light/dark cycles (Figure 1). No statistically significant difference in enzyme activity was observed among complete darkness (24 h dark) and 12 h light/dark.

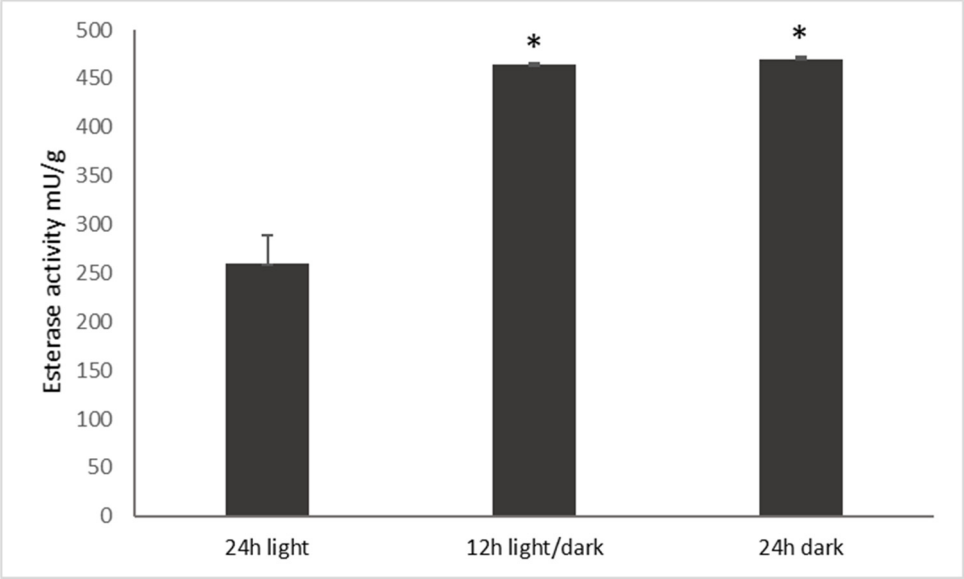

Figure 1. Esterase activity measured in the crude extract obtained from *Trichoderma asperellum* R solid-state fermentation at different light conditions. * $p < 0.01$: statistically significant compared with 24 h light according to Student's *t*-test.

As the esterase activity was comparable among these conditions, the optimization of the SSF process for enzyme production was carried out under 12 h light/dark cycles. This parameter is similar to the natural growth condition of fungi and may preserve *T. asperellum*

R conidiation and spore germination. The fungal biomass entangled with the substrate may be used as a biostimulant. As previously demonstrated, a *Trichoderma*-enriched bio-organic fertilizer better supported plant growth than the sole *Trichoderma* suspension [23]. As one of the goals of this research is the development of a fungal biorefinery with zero waste, the preservation of fungal vitality is an aspect to take into account for the application of the SSF substrate as a biostimulant.

3.3. Esterase Production Optimization

Solid-state fermentation has demonstrated to be an intriguing alternative to submerged fermentation in biorefinery (among other applications [24]). The production of high enzyme quantities using SSF requires optimal environmental conditions to promote maximum growth of the microorganism. In particular, substrate composition determines the success of SSF in terms of fungal growth and enzymes activities [25]. Microbial (fungi, yeast and bacteria) esterase/lipase production in SSF is generally induced by oils and fats principally and produced as secondary metabolites at very high levels [26–28]. Moreover, esterase/lipase activity in *Aspergillus ibericus* is also negatively influenced by high levels of phosphorus, while nitrogen induces an increase in lipase production in SSF [11]. In this study, digestate was chosen as the main substrate components not only for the growing need for its valorization, but also for its chemical composition. At the end of the anaerobic digestion process, the digestate still contained organics, nitrogen components, phosphorous, fatty acid and metabolites that require further treatment prior to disposal and/or reuse [13,29].

3.3.1. Fungal Growth

As previously demonstrated for different *Trichoderma* spp. [14], *T. asperellum* R was not able to grow on 100% digestate (SSF-1); on the other hand, the addition of fruits, *P. laurocerasus* and sawdust allowed fungal growth. The presence and relative abundance of these ingredients influenced the fungal colonization of the substrate. As shown in Figure 2, *T. asperellum* R completely colonized the substrate and grew abundantly on the digestate-based substrate containing 20% sawdust. As the amount of fungal growth can influence enzyme production, the esterase activity was normalized on total proteins present in the crude extract (Table S2). The esterase activity, expressed as mU, on total proteins present in the crude extract (mU/mg) allowed us to reduce the bias associated with fungal biomass. The Bradford assay did not detect proteins in the substrate not inoculated with *T. asperellum* R or not conducive to fungal growth (SSF-1) (Table S2).

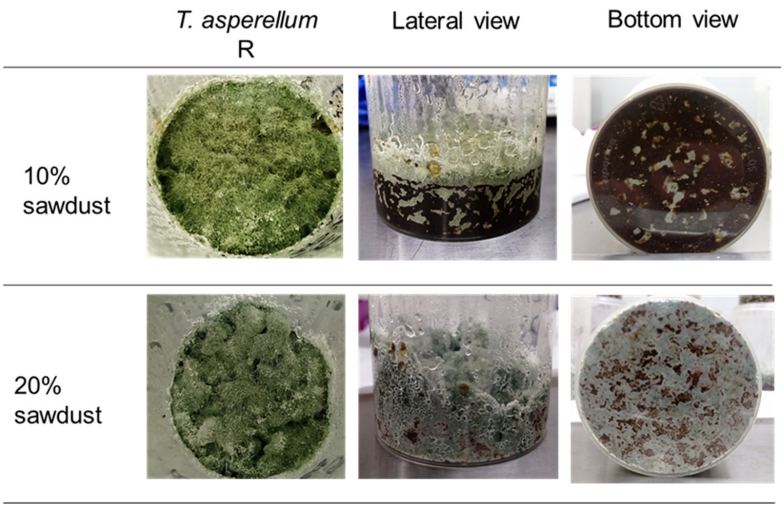

Figure 2. SSF of *Trichoderma asperellum* R after six days of fermentation on a substrate composed of 70% digestate and 30% fruits and different amounts of sawdust that regulate substrate humidity.

3.3.2. The Model Fitness

The successful scale-up of an SSF enzymatic production system is difficult due to the robust control of media composition and cultural conditions. This problem can be overcome through the optimization of processes and nutrient parameters. Interrelating the parameters to establish the best possible conditions for maximum enzyme production required numerous experiments to be performed. Usually, one-factor-at-a-time is used to optimize the parameters, but is time consuming and laborious in cases with many variables. Statistical methods such as response surface methodology (RSM) are helpful to define the interaction and effects of various influencing parameters to obtain enhanced production of enzymes and metabolites. In this study, substrate composition, temperature and moisture were selected as independent variables for the optimization of esterase production in SSF. These three factors were optimized with D-optimal experimental design in RSM. This statistical technique has been successfully applied for antibiotics [30] and enzyme [31] optimization in SSF, reducing the number of experiments carried out. Even if RSM can be combined with different experimental designs, DOD is one of the most accurate [32].

Twelve runs obtained through the DOD (Table 3) allowed us to study how the factors influence esterase production in SSF.

The experimental data (Table S3) were stratified based on digestate concentration in the SSF substrate and analyzed through a linear regression model. The equation of the polynomial regression models whose parameters β were estimated through the ordinary least square method are:

Digestate 70%: $Esterase\ activity = 103.0 - 8.2 * X_1 + 2.4 * X_2 + 16.8 * X_3 + 8.3 * X_1^2 - 1.9 * X_1X_2 - 20.6 * X_1X_3 - 6.1 * X_2X_3$

Digestate 50%: $Esterase\ activity = 119.4 + 59.7 * X_1 - 23.2 * X_2 + 22.3 * X_3 + 7.6 * X_1^2 + 15.5 * X_1X_2 + 19.2 * X_1X_3 - 13.0 * X_2X_3$

ANOVA was applied to investigate the significance of the design to better understand the sources of variation. ANOVA is the most exploited statistical tool to evaluate the impact of variables over a process [33]. The lack of fit *p*-values was 0.085 and 0.338 for the first and second model, respectively, and were not statistically significant, demonstrating that the model developed in this study was significant and may be used to explain esterase production by SSF. However, we only obtained a significant model for digestate = 50% (Table 4).

Table 4. ANOVA results of the RSM on 70% and 50% Digestate scenarios.

	Digestate = 70%					**Digestate = 50%**				
Test	**Df**	**Sum Sq**	**Mean Sq**	**F Value**	**Pr (>F)**	**Df**	**Sum Sq**	**Mean Sq**	**F Value**	**Pr (>F)**
FO (X1, X2, X3)	3	8411.8	2803.9	1.0	0.422	3	106,487.8	35,495.9	8.5	0.001
TWI (X1, X2, X3)	3	10,735.6	3578.5	1.3	0.320	3	17,986.6	5995.5	1.4	0.272
PQ (X1)	1	760.3	760.3	0.3	0.611	1	624.6	624.6	0.1	0.705
Residuals	16	45,255.5	2828.5			16	67,208.5	4200.5		
Lack of fit	4	23,310.6	5827.7	3.2	0.053	4	20,898.5	5224.6	1.4	0.307
Pure error	12	21,944.9	1828.7			12	46,310.0	3859.2		

X1 = % Fruits (100-% Digestate—% *P. laurocerasus*), X2 = % Substrate humidity, X3 = Temperature. FO: first order; TWI: two-way interaction; PQ: pure quadratic term.

The first model (digestate = 70%) revealed that all the terms in the model (the three variables (% Fruits/*P. laurocerasus*, temperature and substrate humidity) (p = 0.422), the two-way interactions (p = 0.320) and the quadratic term of X1 (p = 0.611)) did not influence the esterase production in SSF by *T. asperellum* R.

On the other hand, one variable affected the esterase activity when the SSF substrate was composed of 50% of digestate ($p < 0.001$); in detail, a significant association was observed between % Fruits (50%—*P. laurocerasus*) and esterase activity (Table 5), meaning

that an increased percentage of fruits and a decreased percentage of *P. laurocerasus* are related to an increased esterase activity.

Table 5. Ordinary least squares regression method results of the RSM on 70% and 50% Digestate scenarios.

	Digestate = 70%				Digestate = 50%			
Variable	**Estimate**	**Std. Error**	**t Value**	**Pr (>|t|)**	**Estimate**	**Std. Error**	**t Value**	**Pr (>|t|)**
X1	−8.2	11.1	−0.7	0.472	59.7	13.5	4.4	0.000
X2	2.4	10.9	0.2	0.826	−23.2	13.2	−1.8	0.098
X3	16.8	10.9	1.5	0.142	22.3	13.2	1.7	0.112
X1*X2	−1.9	11.1	−0.2	0.867	15.5	13.5	1.1	0.270
X1*X3	−20.6	11.1	−1.9	0.082	19.2	13.5	1.4	0.175
X2*X3	−6.1	10.9	−0.6	0.581	−13.0	13.2	−1.0	0.342
$X1^2$	8.3	16.0	0.5	0.611	7.6	19.6	0.4	0.705

X1 = % Fruits (100-% Digestate—% *P. laurocerasus*), X2 = % Substrate humidity, X3 = Temperature.

3.3.3. Esterase Production

The effect of the different combinations of three independent variables was interpreted using 3D response surface graphs in the data set with 70% *w*/*w* digestate and 50% *w*/*w* digestate, as it represents the waste that we want to valorize. The analysis provided significant information regarding the role played by each factor and the effect of their interactions on the response under SSF.

The maximum esterase activity in the model digestate = 70% was achieved with Fruit = 0% (*P. laurocerasus* = 30%), humidity = 10% and temperature = 30 °C (Figure 3). The maximum production value estimated by the model in these conditions is equal to 167.6 mU/mg of total proteins.

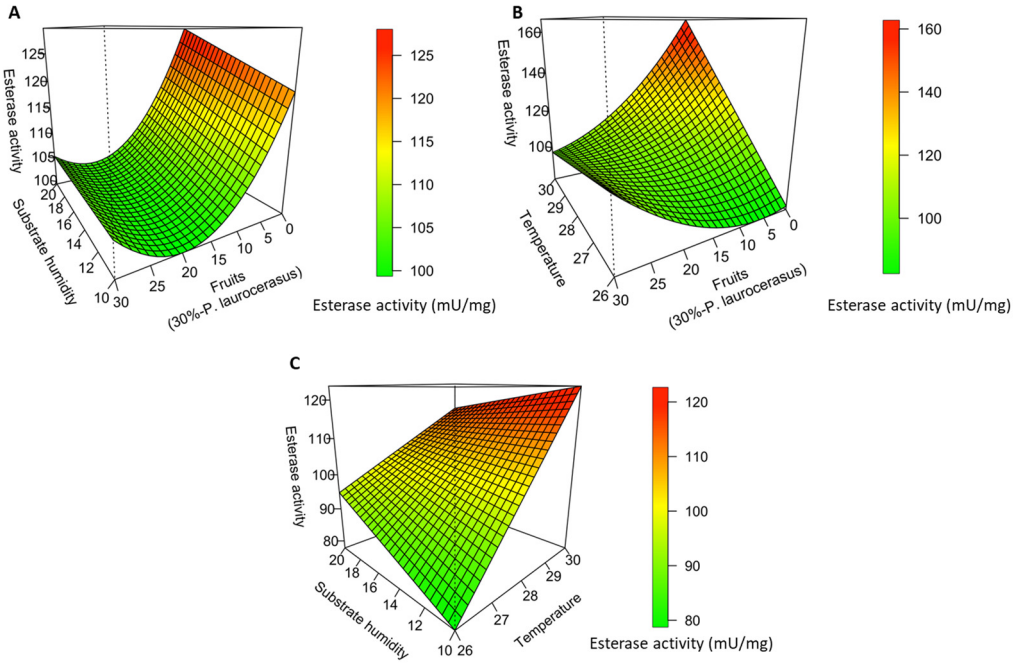

Figure 3. Three-dimensional surface plots of esterase optimization on data set with 70% *w*/*w* of digestate. When the effect of two parameters was plotted, the remaining one was set at central level (**A**) temperature (°C), (**B**) substrate humidity (% *w*/*w* of sawdust added to the substrate), (**C**) % Fruits/*Prunus laurocerasus*.

Interestingly, the highest esterase production (264.6 mU/mg of total proteins) was achieved with a substrate composed exclusively of 50% digestate and 50% fruits in the

condition of higher humidity (X2 = 10% of sawdust) and higher temperature (X3 = 30 °C) (Figure 4). This result is in agreement with a previous work on *Aspergillus niger* lipase production in solid-state fermentation of organic wastes; the highest lipase production was achieved at the highest tested humidity and temperatures [34].

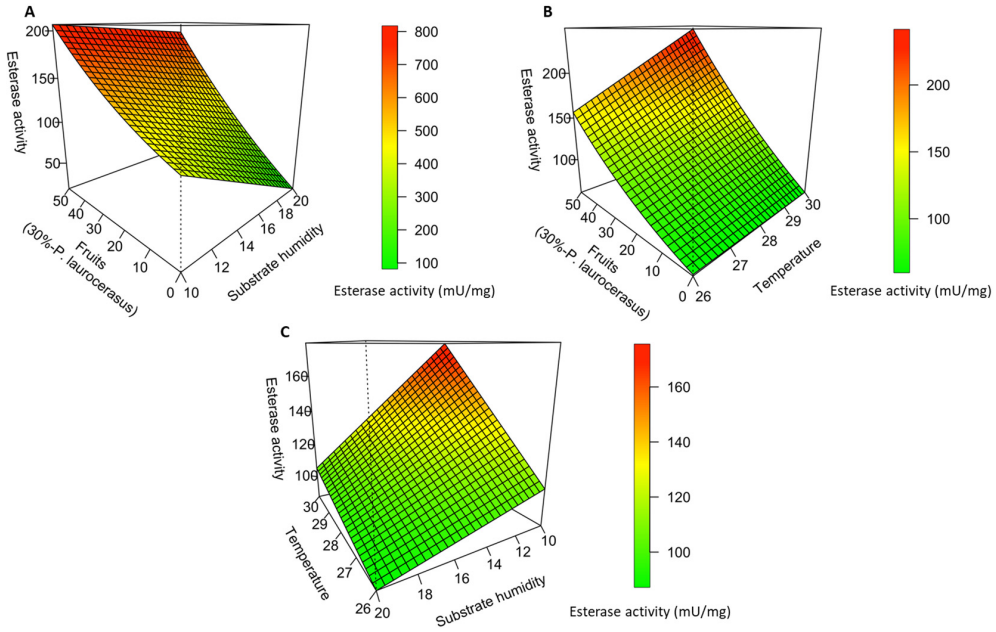

Figure 4. Three-dimensional surface plots of esterase optimization on data set with 50% (*w*/*w*) of digestate. When the effect of two parameters was plotted, the remaining one was set at central level (**A**) temperature (°C), (**B**) substrate humidity (% *w*/*w* of sawdust added to the substrate), (**C**) % Fruits/*Prunus laurocerasus*.

4. Conclusions

Due to its high-water content and alkaline pH, digestate has been considered unsuitable for fungal growth in SSF and, therefore, for fungal biorefinery. This study presents an optimized protocol of SSF to produce esterase using the whole digestate (liquid and solid fraction) and an easily available and cheap substrate.

DOD and RSM highlighted that esterase production differed in the two data sets; the variables (temperature, humidity and substrate composition) did not significantly affect esterase production at the highest digestate concentration (digestate = 70%). Conversely, the ratio between fruit and *P. laurocerasus* significantly influenced esterase activity. The optimum esterase production was obtained with a substrate composed of digestate and spoiled fruits (50:50 ratio *w*/*w*) inoculated with *T. asperellum* R and incubated for six days at 30 °C. These findings open up prospects for the creation of a two-platform biorefinery to produce value-added compounds and support the concrete possibility of developing a two-platform biorefinery that can fulfilling the unmet needs of a bioeconomy to produce value-added compounds. Digestate enriched with *Trichoderma* sp. has been previously demonstrated to induce seed germination and root elongation. Thus, the here-presented process could be combined with a biogas plant where the produced digestate, opportunely amended, could be used for esterase production and as biofertilizer.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/fermentation9060524/s1. Table S1: biomethane plant AD feedstock materials, Table S2: total protein content in the crude extract, Table S3: matrix used for Response Surface Methodology analyses.

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References

1. Bornscheuer, U.T. Microbial Carboxyl Esterases: Classification, Properties and Application in Biocatalysis. *FEMS Microbiol. Rev.* **2002**, *26*, 73–81. [CrossRef] [PubMed]
2. Rafeeq, H.; Hussain, A.; Shabbir, S.; Ali, S.; Bilal, M.; Sher, F.; Iqbal, H.M.N. Esterases as Emerging Biocatalysts: Mechanistic Insights, Genomic and Metagenomic, Immobilization, and Biotechnological Applications. *Biotechnol. Appl. Biochem.* **2022**, *69*, 2176–2194. [CrossRef] [PubMed]
3. De Bhowmick, G.; Sarmah, A.K.; Sen, R. Lignocellulosic Biorefinery as a Model for Sustainable Development of Biofuels and Value Added Products. *Bioresour. Technol.* **2018**, *247*, 1144–1154. [CrossRef]
4. Contesini, F.J.; Frandsen, R.J.N.; Damasio, A. Editorial: CAZymes in Biorefinery: From Genes to Application. *Front. Bioeng. Biotechnol.* **2021**, *9*, 622817. [CrossRef]
5. Calvo-Flores, F.G.; Martin-Martinez, F.J. Biorefineries: Achievements and Challenges for a Bio-Based Economy. *Front. Chem.* **2022**, *10*, 973417. [CrossRef]
6. De Buck, V.; Polanska, M.; Van Impe, J. Modeling Biowaste Biorefineries: A Review. *Front. Sustain. Food Syst.* **2020**, *4*, 11. [CrossRef]
7. Teigiserova, D.A.; Bourgine, J.; Thomsen, M. Closing the Loop of Cereal Waste and Residues with Sustainable Technologies: An Overview of Enzyme Production via Fungal Solid-State Fermentation. *Sustain. Prod. Consum.* **2021**, *27*, 845–857. [CrossRef]
8. Pitol, L.O.; Finkler, A.T.J.; Dias, G.S.; Machado, A.S.; Zanin, G.M.; Mitchell, D.A.; Krieger, N. Optimization Studies to Develop a Low-Cost Medium for Production of the Lipases of Rhizopus Microsporus by Solid-State Fermentation and Scale-up of the Process to a Pilot Packed-Bed Bioreactor. *Process Biochem.* **2017**, *62*, 37–47. [CrossRef]
9. Savino, S.; Bulgari, D.; Monti, E.; Gobbi, E. Agro-Industrial Wastes: A Substrate for Multi-Enzymes Production by Cryphonectria Parasitica. *Fermentation* **2021**, *7*, 279. [CrossRef]
10. Bulgari, D.; Alias, C.; Peron, G.; Ribaudo, G.; Gianoncelli, A.; Savino, S.; Boureghda, H.; Bouznad, Z.; Monti, E.; Gobbi, E. Solid-State Fermentation of Trichoderma Spp.: A New Way to Valorize the Agricultural Digestate and Produce Value-Added Bioproducts. *J. Agric. Food Chem.* **2023**, *71*, 3994–4004. [CrossRef]
11. Oliveira, F.; Abrunhosa, L.; Venâncio, A.; Belo, I.; Pérez-Rodríguez, N.; Domínguez, J.M. Aspergillus Ibericus Lipase Production by Solid-State Fermentation of Olive Pomace. In *Wastes: Solutions, Treatments and Opportunities*; Taylor & Francis Group: London, UK, 2015; pp. 195–201.
12. Vohra, A.; Satyanarayana, T. Statistical Optimization of the Medium Components by Response Surface Methodology to Enhance Phytase Production by Pichia Anomala. *Process Biochem.* **2002**, *37*, 999–1004. [CrossRef]
13. Woo, S.L.; Hermosa, R.; Lorito, M.; Monte, E. Trichoderma: A Multipurpose, Plant-Beneficial Microorganism for Eco-Sustainable Agriculture. *Nat. Rev. Microbiol.* **2022**, *21*, 312–326. [CrossRef] [PubMed]
14. Alias, C.; Bulgari, D.; Gobbi, E. It Works! Organic-Waste-Assisted Trichoderma Spp. Solid-State Fermentation on Agricultural Digestate. *Microorganisms* **2022**, *10*, 164. [CrossRef] [PubMed]
15. Ghose, T.K. Measurement of Cellulase Activities. *Pure Appl. Chem.* **1987**, *59*, 257–268. [CrossRef]
16. Bradford, M.M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef] [PubMed]
17. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2022.
18. Wheeler, A.B.; Braun, M.J. Package ' AlgDesign ' NeedsCompilation Yes. *R Proj. Stat. Comput.* **2022**, *1*, 1–25.
19. Lenth, R. V Response-Surface Methods in R, Using Rsm. *J. Stat. Softw.* **2009**, *32*, 1–17. [CrossRef]
20. Kamm, B.; Kamm, M. Principles of Biorefineries. *Appl. Microbiol. Biotechnol.* **2004**, *64*, 137–145. [CrossRef]
21. Schmoll, M. Assessing the Relevance of Light for Fungi: Implications and Insights into the Network of Signal Transmission. *Adv. Appl. Microbiol.* **2011**, *76*, 27–78. [CrossRef]
22. Schmoll, M. Regulation of Plant Cell Wall Degradation by Light in Trichoderma. *Fungal Biol. Biotechnol.* **2018**, *5*, 10. [CrossRef]

23. Ye, L.; Zhao, X.; Bao, E.; Li, J.; Zou, Z.; Cao, K. Bio-Organic Fertilizer with Reduced Rates of Chemical Fertilization Improves Soil Fertility and Enhances Tomato Yield and Quality. *Sci. Rep.* **2020**, *10*, 177. [CrossRef] [PubMed]
24. Singhania, R.R.; Patel, A.K.; Soccol, C.R.; Pandey, A. Recent Advances in Solid-State Fermentation. *Biochem. Eng. J.* **2009**, *44*, 13–18. [CrossRef]
25. Yafetto, L. Heliyon Application of Solid-State Fermentation by Microbial Biotechnology for Bioprocessing of Agro-Industrial Wastes from 1970 to 2020: A Review and Bibliometric Analysis. *Heliyon* **2022**, *8*, e09173. [CrossRef]
26. Benjamin, S.; Pandey, A. Isolation and Characterization of Three Distinct Forms of Lipases from Candida Rugosa Produced in Solid State Fermentation. *Braz. Arch. Biol. Technol.* **2001**, *44*, 213–221. [CrossRef]
27. Rodriguez, J.A.; Mateos, J.C.; Nungaray, J.; González, V.; Bhagnagar, T.; Roussos, S.; Cordova, J.; Baratti, J. Improving Lipase Production by Nutrient Source Modification Using Rhizopus Homothallicus Cultured in Solid State Fermentation. *Process Biochem.* **2006**, *41*, 2264–2269. [CrossRef]
28. Shabtai, Y.; Daya-Mishne, N. Production, Purification, and Properties of a Lipase from a Bacterium (Pseudomonas Aeruginosa YS-7) Capable of Growing in Water-Restricted Environments. *Appl. Environ. Microbiol.* **1992**, *58*, 174–180. [CrossRef]
29. Tawfik, A.; Eraky, M.; Alhajeri, N.S.; Osman, A.I.; Rooney, D.W. Cultivation of Microalgae on Liquid Anaerobic Digestate for Depollution, Biofuels and Cosmetics: A Review. *Environ. Chem. Lett.* **2022**, *20*, 3631–3656. [CrossRef]
30. El-housseiny, G.S.; Ibrahim, A.A.; Yassien, M.A.; Aboshanab, K.M. Production and Statistical Optimization of Paromomycin by Streptomyces Rimosus NRRL 2455 in Solid State Fermentation. *BMC Microbiol.* **2021**, *21*, 34. [CrossRef]
31. Singh, R.S.; Chauhan, K.; Jindal, A. Response Surface Optimization of Solid State Fermentation for Inulinase Production from Penicillium Oxalicum Using Corn. *J. Food Sci. Technol.* **2018**, *55*, 2544–2551. [CrossRef]
32. Xu, W.; Wong, W.K.; Tan, K.C.; Xu, J.-X. Finding High-Dimensional D-Optimal Designs for Logistic Models via Differential Evolution. *IEEE Access* **2019**, *7*, 7133–7146. [CrossRef]
33. Mahalaxmi, Y.; Sathish, T.; Subba Rao, C.; Prakasham, R.S. Corn Husk as a Novel Substrate for the Production of Rifamycin B by Isolated Amycolatopsis Sp. RSP 3 under SSF. *Process Biochem.* **2010**, *45*, 47–53. [CrossRef]
34. Nema, A.; Patnala, S.H.; Mandari, V.; Kota, S.; Devarai, S.K. Production and Optimization of Lipase Using Aspergillus Niger MTCC 872 by Solid-State Fermentation. *Bull. Natl. Res. Cent.* **2019**, *43*, 82. [CrossRef]