Challenges of Enzymes, Conidia and 6-Pentyl-alpha-pyrone Production from Solid-State-Fermentation of Agroindustrial Wastes Using Experimental Design and T. asperellum Strains

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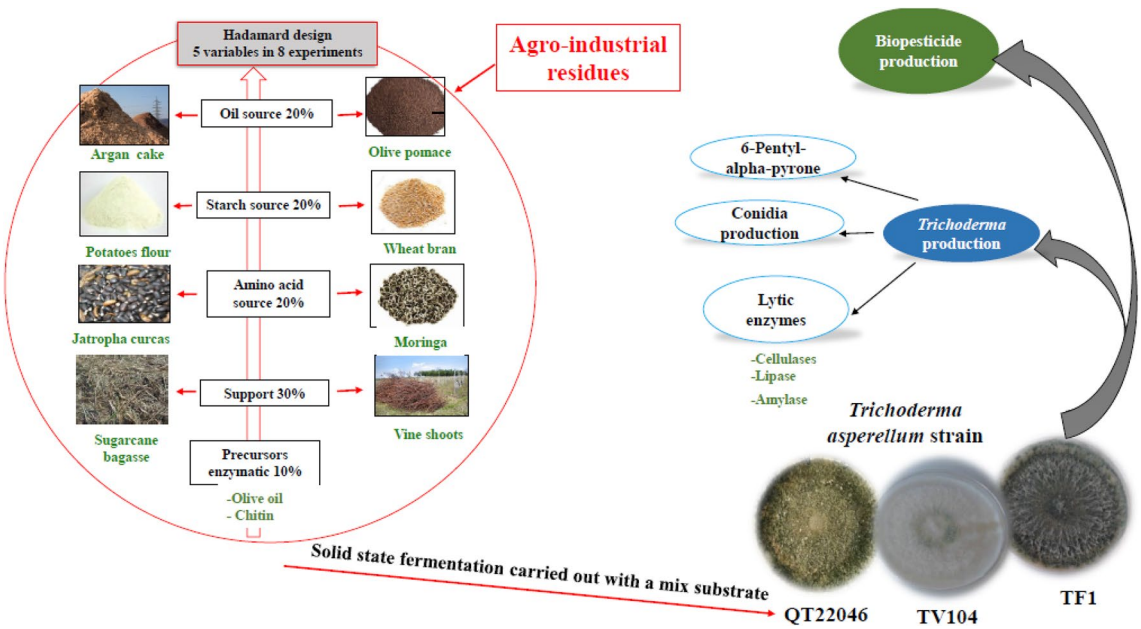
Rayhane Hamrouni[1,2] · Magalie Claeys-Bruno[1] · Josiane Molinet[1] · Ahmed Masmoudi[2] · Sevastianos Roussos[1] · Nathalie Dupuy[1]

Abstract

In a context of growing awareness regarding environmental protection, biomass valorization is gaining a lot of attention. The byproducts volumes generated by agro-industry are massive and, left to decay, can constitute environmental pollutions. Use of agro-industrial wastes and solid-state fermentation (SSF) technology offers advantages to produce value-added products such as antibiotics, pigments, aromas and enzymes of industrial interest like cellulases, chitinases, amylases, etc. Several studies have already demonstrated the advantages of SSF for the production of fungal metabolites, yet the optimal conditions for metabolites production strongly depend on the culture conditions and microbial strain utilized. Therefore, the aim of this study was to improve the conidia, lytic enzymes (cellulase, lipase and amylase), and antifungal—6-pentyl-alpha-pyrones (6-PP)—production by three *Trichoderma asperellum* strains cultivated using SSF. Designs of experiments have been achieved in order to identify influential factors on 6-PP, conidia and enzymes (cellulase, lipase, and amylase) production by the fungal culture. A significantly enzymes activities, conidiation and 6-PP production were observed on mix of substrates: vine shoots, potatoes flour, jatropha, olive pomace and olive oil on high carbon/nitrogen ratio 37 which was used by *T. asperellum* TV104 as a source of nutrients and also as a matrix.

Graphic Abstract



Keywords Solid-state-fermentation · *Trichoderma asperellum* · Optimization · Agroindustrial wastes · Experimental design

Statement of Novelty

The development of sustainable solutions to exploit byproduct is necessary to solve the environmental problems. They are rich in nutrients can serve as a substrate for the production of interested compounds as antifungal ones. In this study various byproduct were valorized to formulate biopesticides by the way of solid state fermentation (SSF) and *Trichoderma asperellum* fungi. For the optimization of antifungal properties, Hadamard design was performed and various byproduct mixture. The final formulation allows to obtain high content in conidia, enzyme and 6-pentyl-alpha-pyrone (6-PP). This work presents two advantages: The valorization of agroindustrials wastes having an impact on the ecology of the world, and the production of interested compounds.

Introduction

Commonly agricultural produce important amount of byproducts whose main part are unused and cause environmental problems [1, 2]. In recent years, numerous studies have been published supporting the use of SSF in valorization of agricultural byproducts in particular for microbial growth [1, 3]. This technology has an economical interest according to agricultural byproducts (which are naturally rich in carbohydrates and other nutrients) as culture medium in SSF is cheaper than using synthetic substrates [3, 4]. Furthermore, one of the main driving forces in the increase of SSF popularity over the last decades is the growing concern about sustainability in bioprocess development linked to the possibility to use agro-industrial wastes having an impact on the worldwide ecology [5, 6]. Generally, SSF can be used for several applications such as enzyme production (pectinases, cellulases amylases, lipases) primary metabolites production (citric acid, lactic acid) secondary metabolites production (aromatic compounds, mycotoxins and organic volatile) and biomass production (mycelium, spores) [7, 8]. The most used agroindustrials wastes are: sorghum pulp, sugarcane bagasse, wheat, rice, corn, coconut fiber, coffee wastes, oil palm wastes, coconut oil mash, mustard oil mash, wheat flour [9].

In addition, there are several reports with the aim of filamentous fungi conidia production using sugarcane bagasse as support/substrate in SSF. Previously sugarcane bagasse is particularly suitable for SSF because of its porosity allowing good water absorption, indispensable to carried out the microbial metabolism [10]. Moreover, in term of volume, it is the major solid agro-industrial byproduct generated in Morocco, Brazil and Mexico [11]. Sugarcane bagasse is an inexpensive material rich in lignin (11%) molecules, cellulose (38%) and around 34% of hemicelluloses components (Table 1) [11, 12]. Vine shoots is another agro-industrial

Table 1 Composition of different substrates tested

Substrates	Origin	Characteristics, composition (g 100 g^{-1} dry matter)	Carbon–nitrogen ratio (C/N)	Reference
Argan press cake	Morocco	Humidity (26.3%), ashes (3.6), lipids (18.9), glucosides (26.6), cellulose (17.6)	7	[45]
Olive oil	France	Fatty acids (%): palmitic acid (13.6), *cis* vaccenic acid (2.45), oleic acid (68.1), linoleic acid (10.2), linolenic acid (0.6)	94	[46]
Olive pomace	France	Humidity (28%), ashes (3.5), lignin (19.5), hemicellulose (16.8), cellulose (11.5), lipid (7.2), protein (6.5) phenols (1.2)	82	[47]
Jatropha cake	Burkina Faso	Humidity (4.1%), protein (28.4), lipid (12), ashes (6.1) crude fiber (25.9)	14	[48]
Moringa oleifera leaves	Burkina Faso	Humidity (75%), protein (25.3), lipid (8.1), ash (5.2), crude fiber (15.3)	10	[49]
Wheat bran	France	Humidity (8.2), holocellulose (58.2), protein (13.8), lignin (5.7), lipid (7.4), crude fibre (2.1), pectin (1.7), soluble sugars (1.4), ash (1.7)	17	[50]
Potato flour	France	Humidity (7.5), carbohydrate (75.2), protein (9.1), lipid (7.4), crude fibre (2.1), ash (3.2)	22	[51]
Vine shoots	France	Humidity (10%), cellulose (51.9), hemicellulose (22.3), lignin (16.6), lipids (0.5), tannins (0.5), total nitrogen (0.8), ashes (2.5)	75	[52]
Sugarcane bagasse	Morocco	Humidity (8%), cellulose (41.6), hemicellulose (25.1), lignin (20.3), ashes (4.8)	151	[53]
Chitin	France	Natural polysaccharide (β-(1–4)-*N*-acetyl-D-glucosamine), the main commercial sources of chitin are crab and shrimp shells	20	[54]

waste that recently attracted a lot of attention in fermentation process for filamentous fungi growth [13]. It is the most important byproduct in vine industry (France, Italy, Spain and Tunisia) generated during the pruning season. In this study, it will be used for the first time for *Trichoderma* metabolites production. Sugarcane bagasse and vine shoots will be compared as support of culture in the medium to possess favorable physical properties having consequence on water availability.

Moreover, the ideal substrate for filamentous fungi growth should offer several factors like mineral salts, starch, carbon, nitrogen, protein sources, etc. Those sources were proportioned with a mix of substrates. In 2017, the feasibility of wheat bran and potato flour as a substrate for SSF in order to produce *Trichoderma harzianum* conidias was demonstrated by De la Cruz-Quiroz et al. [14] showing that potato flour is a profitable starch source for the conidiation of *Trichoderma*. For this reason it is necessary to evaluate the effect of these substrates not only for conidia production but also for enzymes and secondary metabolites. On other hand, Tunisia, France, Italy, Spain and Morocco are the larger producers of olive oil. However, the processing of the high production of olive oil gives rise to a large amount of olive pomace (20 million tons) [15] which accumulation leads to an important problem of environmental pollution. For this reason, the opportunity to use this byproduct waste as the main oleaginous source in SSF is an important economic valorization approach, offering advantages in fermentation process.

Today, moringa and argan are the most commercial products for cosmetic sectors mainly in Africa especially in Cameroon, Burkina Faso, Morocco, and Tunisia [16, 17]. However, at the present time there is no published information about the treatment of the wastes generated during production process. Using moringa and argan cake as protein and lipid source in filamentous fungi growth seems to be a good idea. Generally natural sources (like amino acid) are preferably used than synthetic soluble compounds.

Based on the report published by International Fund for Agricultural Development (IFAD) *Jatropha curcas* represent an important waste generated by Africa country including; Mali, Burkina Faso, Senegal, Cameroon, followed by Asia and Latin America. This waste is toxic to human and environment but rich in protein which induce the production of lactones molecules [18]. Use of SSF and filamentous fungal strains may give an added value to jatropha cake, through bioconversions to produce value-added products such as antibiotics, pigments, lactone (with antifungal activity).

Globally, *Trichoderma* strains are the most important fungus used in SSF process. There are a great number of works reported the SSF process using *Trichoderma* strains for producing enzymes, conidia or secondary metabolites with industrial importance using different culture medium [6, 19, 20]. However, it has not been reported the production of all these components at the same media in SSF by *Trichoderma* strains. In order to optimize fermentation media, statistically efficient tools as experimental designs [21–23] have been used to study the effects of several factors especially in the development of any fermentation process owing to their impact on the economy and to improve product yields. More precisely, screening strategies [24] are intended to identify very quickly active factors among many candidate factors with a few numbers of experiments. Indeed, at the beginning of a study, we generally do not know which factors have an influence on the studied responses. A screening of the factors will thus be performed to detect only the most important factors. This screening stage should involve a minimum number of experiments, and should not take much time of calculation. The most well-known screening designs are the Hadamard or Plackett and Burman design [25].

In this investigation, 6-PP, fungicidal secondary metabolite with coconut aroma, lytic enzymes (cellulase, amylase, lipase) and conidia producing strain of three *T. asperellum*, were subjected to optimization of media and cultivation parameters for metabolites production.

Materials and Methods

Microorganisms and Culture Conditions

The strains of *T. asperellum* TV104, *T. asperellum* TF1 and *T. asperellum* QT22046 from the IRD/IMBE fungi collection were used in present study. These strains were selected after a screening and identification of *Trichoderma* strains for 6-PP and conidia production (Hamrouni et al. [26]). The fungal strains were activated in sterilized potato dextrose agar (PDA) and incubated during 5 days at 30 °C to conserve at 4 °C. Conidia obtained by culturing fungus in Erlenmeyer flasks containing PDA for 5–10 days at 25 °C were resuspended in 100 ml sterile water solution containing Tween 80 [0.01% (v/v)] and quantified by using a traditional hemacytometer counting as described by Roussos et al. [27]. The amount of inoculum used in each fermentation was calculated to achieve an initial concentration of 2×10^7 conidia g^{-1} DM (dry matter) used according to De la Cruz-Quiroz et al. [14].

Solid-State Fermentation

All the results being expressed in by gram of DM, the water content of each sample was measured as followed: after 5 days, 1 g of fermented material was introduced into a lab oven at 105 °C during 24 h, to analyze the relative humidity of the sample [13].

SSF was performed in 250 ml bottles containing 10 g of mixture substrates (DM). Each mixture was sieved to a 3–4 mm particle size. The humidity was set to 50% with distilled water before sterilization. All the culture media were then autoclaved at 121 °C during 1 h. Each mixture was then inoculated with 2×10^7 conidia g^{-1} DM, the volume of this added conidial suspension sets the initial humidity to 66%. The cultures were performed in a laboratory incubator at 27 °C ± 1 °C during 5 days (Fig. 1). Each condition has been performed in triplicate. The characteristics of the different substrate tested are shown in Table 1.

The Carbon to Nitrogen (C/N) Ratio Analysis

Total carbon (% C), total nitrogen (% N) and C/N ratio for all the substrates used and for the 8 medium tested were determined. Samples were firstly lyophilized (Lyophilizator Heto Power Dry LL 1500 Thermo Fischer Scientific, at − 109 °C) and then grounded in order to obtain a fine powder as homogenous as possible with a ball milling (MM 400 Retsch) 0.1 mg of each broyat was used to analyse the % C, total % N and C/N ratio with an elemental analyser Thermo Fisher Scientific© (USA).

Conidia Determination

Counting of conidia was done at the end of culture by mixing 1 g of fermented material and 100 ml of sterile water containing Tween 80 [0.01% (v/v)] in an Erlenmeyer flask, after that the suspension of conidia was counted using Malassez cell.

Enzyme Assays

Amylase activity was measured using soluble starch (1%) in phosphate buffer (0.1 M, pH 7.0) at 50 °C for 10 min [28]. Cellulase activity was determined using carboxymethyl cellulose (1%) in sodium citrate buffer (50 mM, pH 4.8) at 50 °C for 30 min, in according with De la Cruz-Quiroz et al. [14]. An enzyme activity (U) was defined as the amount of enzyme that catalyzes the release of 1 µmol of glucose min^{-1}. Lipase activity was determined using 0.5 ml of *p*-nitrophenyl (25 mM) in phosphate buffer (25 mM, pH 7.0) at 30 °C for 30 min [5, 14]. An enzyme activity (U) was defined as the amount of enzyme required to release 1 µmole of *p*-nitrophenol min^{-1}.

Extraction and Analysis of 6-PP

6-PP was recovered by soxhlet extraction system from fermented material using pure heptane. Samples (10 g of the fermented material) were co-distilled at 60 °C with 100 ml heptane during 45 min. 1 ml of a γ-undecanolactone solution (80 mg l^{-1}) was added as the internal standard before extraction.

6-PP analysis were performed with a gas chromatograph 7890A (GC) (Agilent Technology) equipped with a split/splitless injector (T = 260 °C) and a flame ionization detector (T = 260 °C). Aroma constituents were separated on a Supelcowax capillary column (internal diameter 0.25 mm, length 60 m, film thickness 0.25 µm). The carrier gas was dihydrogen (column flow 1 ml min^{-1}) and the split ratio was 1:2. The oven temperature was set as follows: 30 min at 180 °C, from 180 to 230 °C at 10 °C min^{-1}, 10 min at 230 °C.

Quantitative analysis of 6-PP was carried out using the internal calibration method, with γ-undecanolactone (99%, Aldrich) as internal standard.

Experimental Design

Screening study was performed to quantify the effects of five variables in eight experiments. Culture conditions; incubation temperature, moisture level, inoculum concentration and pH, are fixed according to Sarhy-Bagnon et al. [29]. Responses variables were; 6-PP, conidia production and lytic enzymes (cellulase, lipase and amylase).

Factors and Domain of Interest The influences of five variables (support, starch source, protein source, oleaginous waste and precursors enzymatic) with two levels on the 6-PP, conidia, cellulases, lipases and amylases production were studied.

The same approach was employed for all the strains (*T. asperellum* TV104, TF1 and QT22046). The experimental domain is shown in Table 2.

We can postulate that the result of each experiment is a linear combination of the effect of each five coded variables X_1, X_2,…,X_5. A first order polynomial model for the five variables with two levels is proposed.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + \cdots + b_5X_5 + \varepsilon.$$

In order to estimate the coefficients b_i, a Hadamard design in eight experiments was performed (Table 3). It is valid only in the experimental points, and therefore cannot be used for any interpolation or extrapolation.

Repeatability in order to estimate the variability of each response, the experiment 6 has been triplicate. The generation and the data treatment of the designs were performed using the software Azurad.

Results and Discussion

The experiments of the Hadamard designs were done and the results are given in Table 4.

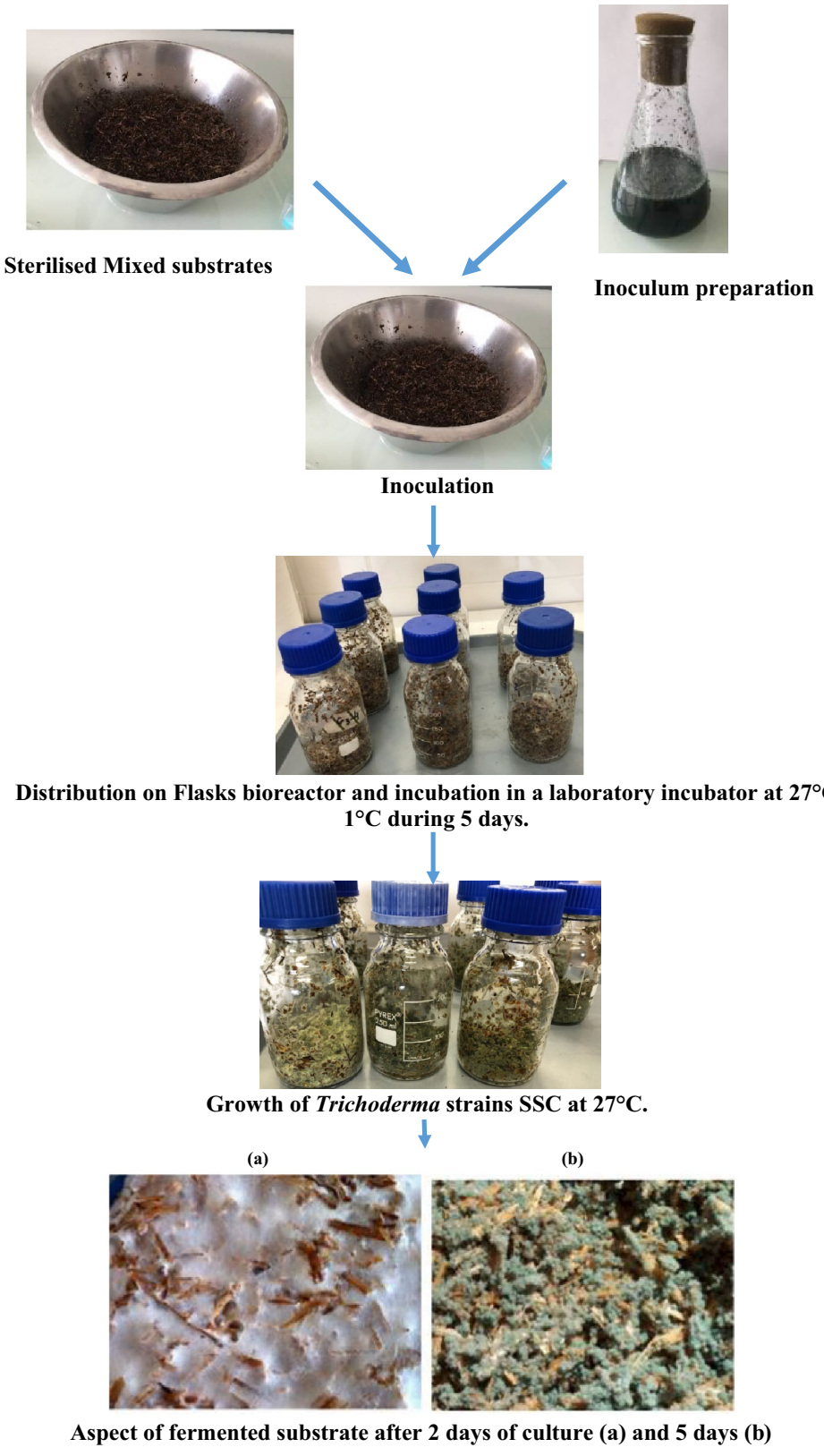


Fig. 1 Representation of the SSF system

Table 2 Variables and levels

Variables	Level (−)	Level (+)
X_1: support	Vine shoots	Sugarcane bagasse
X_2: starch source	Potatoes flour	Wheat bran
X_3: protein source	Jatropha cake	Moringa
X_4: oleaginous waste	Olive pomace	Argan cake
X_5: precursors enzymatic	Olive oil	Chitin

From the results, we can calculate the estimation of the model coefficients using multilinear regression. In order to check the significance of these coefficients, a student test has been performed.

Effects Plot

The coefficient values can be represented by an effect plot (Fig. 2) on which magnitudes and signs of each effect of the variables are shown. The red effects whether they are positive or negative, are the statistically significant ones. A significant limit (example Fig. 2a) could be calculated considering the variability calculated using triplicate which is represented by the vertical dotted line. Any coefficient exceeding this limit is considered active, which means that this variable is influential on the response. All coefficients not exceeding or near this limit are considered as inactive.

Effect of Variables

In order to choose the more favorable level of the variables, the value of the response for each level was calculated. The behaviour of each factor is presented in Fig. 3 (on the right), in orange the level (+) and in blue the level (−).

Determination of the Solid Medium for *T. asperellum* TV104

*Conidia Production

Figure 2 shows the effect, b_i, of the tested variables. All the coefficients are significant. This mean that all the variables have a significant effect on the conidia production.

Thus, though it is a screening study, it can be observed that the presence of vine shoot, wheat bran, jatropha, olive pomace and chitin in the growth medium increases the conidia production by *T. asperellum* TV104. On the other hand, the presence of sugarcane bagasse, moringa, argan cake, olive oil and potatoes flour results in low conidia production.

*Enzymes Activities

Effects plots for cellulase, lipase and amylase activities are presented in the Fig. 3a Results of Hadamard design showed that cellulase activity by *T. asperellum* TV104 is more efficient when vine shoots, wheat bran, jatropha, olive pomace, and chitin are present in the medium. For lipase activity, the nature of the support and the starch source has scarcely significant effects, sugarcane bagasse and potatoes flour give a slightly higher activity than vine shoots and wheat bran. In opposite, protein source, oleaginous waste and enzymatic precursors are very influent variables. Jatropha, olive pomace and olive oil lead to high cellulase activity (Fig. 3b). For amylase activity, factors have no significant influence. Only the factor "oleaginous waste" seems to be important: olive pomace leads to higher activity.

6-PP Production Same statistical tools to identify significant effects on conidia or enzyme activities are used to study 6-PP production. Results obtained showed that all the tested

Table 3 Hadamard experimental design for the evaluation of relative importance of selected substrates for 6-PP, conidia production and enzymes activities produced by *T. asperellum* strains

Experiments	Support 30%	Starch source 20%	Protein source 20%	Oleaginous waste 20%	Enzymatic precursors 10%
1	Vine shoots	Potatoes flour	Jatropha cake	Olive pomace	Olive oil
2	Sugarcane bagasse	Potatoes flour	Jatropha cake	Argan cake	Chitin
3	Sugarcane bagasse	Wheat bran	Jatropha cake	Argan cake	Olive oil
4	Vine shoots	Wheat bran	Moringa	Argan cake	Olive oil
5	Sugarcane bagasse	Potatoes flour	Moringa	Olive pomace	Olive oil
6′	Vine shoots	Wheat bran	Jatropha cake	Olive pomace	Chitin
6″	Vine shoots	Wheat bran	Jatropha cake	Olive pomace	Chitin
6‴	Vine shoots	Wheat bran	Jatropha cake	Olive pomace	Chitin
7	Vine shoots	Potatoes flour	Moringa	Argan cake	Chitin
8	Sugarcane bagasse	Wheat bran	Moringa	Olive pomace	Chitin

Table 4 Values of the responses of the Hadamard design for *T. asperellum* TV104, TF1 and *T. asperellum* QT22046: (6′, 6″, 6‴ are the triplicate)

Runs	*T. asperellum* TV104					*T. asperellum* TF1					*T. asperellum* QT22046				
	Conidia × 10^7 g^{-1} DM	Cellulases ($U g^{-1}$)	Lipases ($U g^{-1}$)	Amylases ($U g^{-1}$)	6-PP [mg (g DM)$^{-1}$]	Conidia × 10^7 g^{-1} DM	Cellulases ($U g^{-1}$)	Lipases ($U g^{-1}$)	Amylases ($U g^{-1}$)	6-PP [mg (g DM)$^{-1}$]	Conidia × 10^7 g^{-1} DM	Cellulases ($U g^{-1}$)	Lipases ($U g^{-1}$)	Amylases ($U g^{-1}$)	6-PP [mg (g DM)$^{-1}$]
1	12.17	4.61	37.30	7.17	1.13	106.44	9.72	43.04	7.91	0.410	14.54	4.28	13.77	2.56	0.034
2	22.99	3.26	10.64	2.37	0.19	18.74	11.27	27.65	3.02	0.005	12.59	1.02	15.78	0.21	0.084
3	22.94	3.21	39.95	4.08	0.004	22.73	6.61	12.60	4.35	0.001	20.87	1.62	14.77	3.02	0.017
4	8.34	4.48	9.85	3.35	0.002	17.25	12.64	25.82	8.46	0.003	10.63	29.69	11.21	4.03	0.015
5	9.34	0.88	39.28	8.94	0.001	175.74	6.54	26.41	10.56	0.001	37.01	2.35	14.08	6.16	0.006
6′	51.01	6.52	26.11	6.27	0.004	101.20	15.31	14.39	12.34	0	26.18	0.44	13.78	1.00	0.002
6″	52.21	6.20	25.75	10.02	0.037	102.11	15.10	13.38	12.32	0	22.88	0.63	14.66	1.4	0.004
6‴	52.1	6.55	26.13	9.85	0.022	101.11	16.19	14.72	11.90	0	25.13	0.54	14.98	1.88	0.003
7	16.34	2.96	9.63	2.22	0.040	10.87	10.07	42.59	12.11	0.001	29.47	37.95	17.35	4.19	0.023
8	23.21	5.48	9.77	3.47	0.005	52.68	7.784	24.82	7.86	0.004	43.00	1.08	26.88	0.62	0.006

variables have a high effect on the 6-PP production. A mix of vine shoots, potatoes flour, jatropha, olive pomace and olive oil are the best conditions in the microbial production of 6-PP (Table 5).

Determination of the Solid Medium for *T. asperellum* TF1 and *T. asperellum* QT22046

Following the same idea, the optimal levels of the variables affecting the production of conidia, enzymes (cellulase, lipase, amylase) and 6-PP have been studied for *T. asperellum* TF1 and *T. asperellum* QT22046. The results of the Hadamard design experiments are presented in Table 4. Optimal conditions are given Table 5 for each response.

The mix of sugarcane bagasse, potatoes, moringa, olive pomace and chitin is the best conditions in the production of 6-PP, conidia, and enzymes activities by *T. asperellum* QT22046. For *T. asperellum* TF1 no significant effect of the entire variable tested on the 6-PP production were founded. Vine shoots have scarcely significant effects on the production of cellulase, lipase and amylase.

Elemental Analysis of C/N Ratio

The C/N ratio is a marker to determine if the mixture substrates tested is a good candidate to fermentation process. Table 6 shows the C/N ratio of the different solid medium tested. Among medium tested, the mixture containing 30% of vine shoots, 20% of potatoes flour, 20% of jatropha, 20% of olive pomace and 10% of olive oil (medium 1) presents the optimal C/N ratio (37). From these results, it is evident that C/N ratio correlates with metabolites production because this mixture is the optimal medium defined for *T. asperellum* TV104 to produce 6-PP, conidia and enzymes.

Discussion

In SSF, cost and availability are the main factors considered to select suitable substrates for SSF [30]. In fact, wastes from the food and agricultural industries produced in large quantities and are rich in nutrients can serve as a substrate for the production of chemical compounds and enzymes by using the technique of SSF [3, 31].

Concerning culture media, substrates for SSF are usually homogeneous agro-industrial byproducts which offer advantages in this fermentation process. The culture medium has to be considered following two aspects: as substrate, it has to efficiently provide microbial nutritional needs, and as support of fungal culture, it has to possess favorable physical properties having consequence on water availability and allowing initial conidial anchorage, mycelial elongation in space and mass and heat transfers to occur over

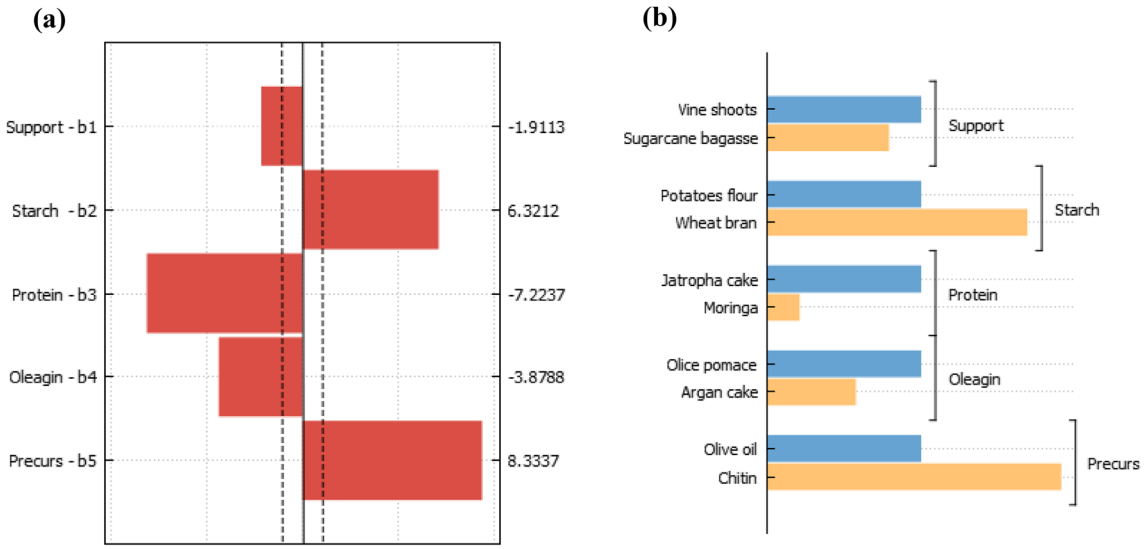


Fig. 2 Effects plot (**a**) and main levels effects (**b**) of the analysis for conidia production

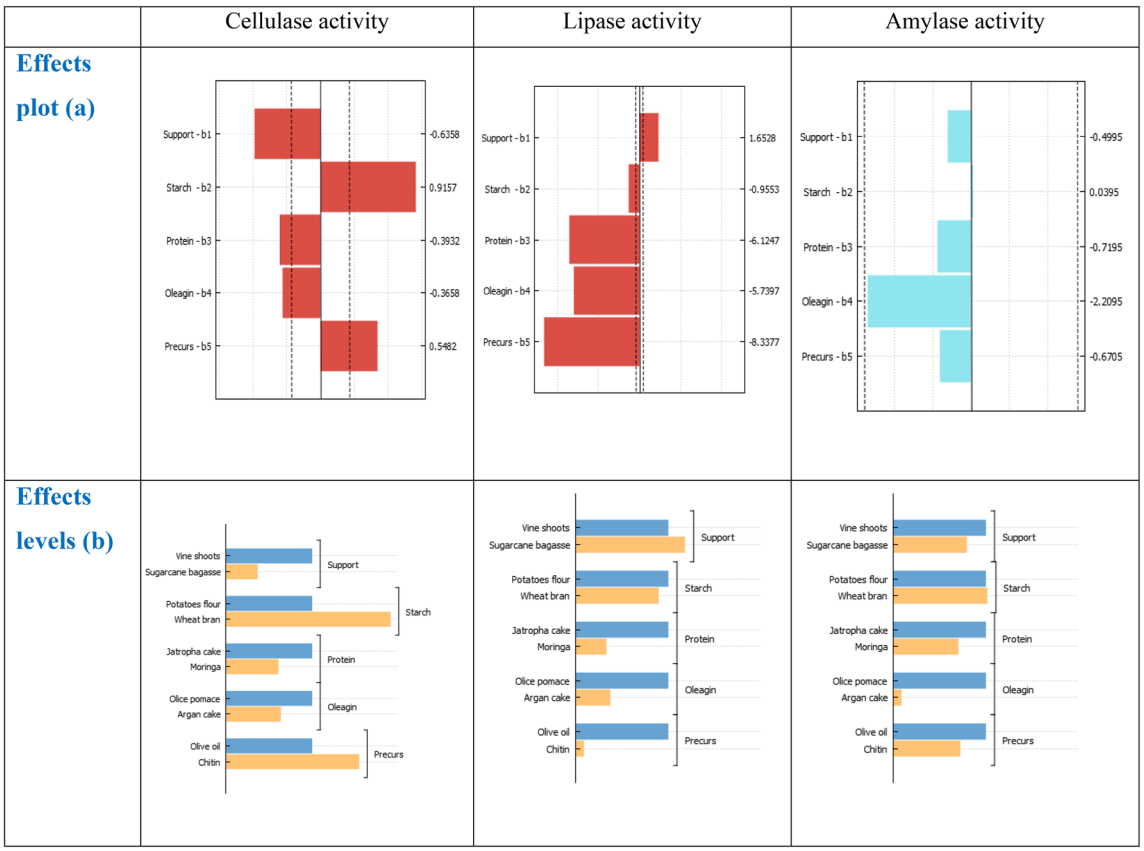


Fig. 3 Main levels effects of the analysis for cellulase, lipase and amylase activities from the Hadamard design for *T. asperellum* TV104

time [9, 11]. Obviously, when using natural byproducts, the distinction between support and substrate may be blurry a solid substrate, because of its physical nature, participates as a support in the general texture of the culture medium. However, in this case, a lignocellulosic byproduct as sugarcane bagasse or vine shoots rich in biopolymers is degraded, due to the production of some enzymes: the fungus preferentially consuming the more easily metabolizable compounds.

Table 5 Effects of variables tested on production of spores, cellulases, lipase, amylase, and 6-PP by *T. asperellum* TF1, *T. asperellum* QT22046 and *T. asperellum* TV104

Variable	*T. asperellum* TV104					*T. asperellum* QT22046					*T. asperellum* TF1				
	Y1 conidia	Y2 cellulases	Y3 lipase	Y4 amylase	Y5 6-PP	Y1 conidia	Y2 cellulases	Y3 lipase	Y4 amylase	Y5 6-PP	Y1 conidia	Y2 cellulases	Y3 lipase	Y4 amylase	Y5 6-PP
Support X1	**Vine shoots**	**Vine shoots**	**Sugarcane bagasse**	**Vine shoots**	**Vine shoots**	**Sugarcane bagasse**	**Vine shoots**	**Sugarcane bagasse**	**Vine shoots**	**Sugarcane bagasse**	No significant	**Vine shoots**	**Vine shoots**	**Vine shoots**	No significant
Starch source X2	**Wheat bran**	**Wheat bran**	**Potatoes flour**	No significant	**Potatoes flour**	**Wheat bran**	**Potatoes flour**	**Wheat bran**	**Potatoes flour**	**Potatoes flour**	**Potatoes flour**	**Wheat bran**	**Potatoes flour**	No significant	No significant
Protein source X3	**Jatropha cake**	**Jatropha cake**	**Jatropha cake**	**Jatropha cake**	**Jatropha Cake**	**Moringa**	**Moringa**	**Moringa**	**Moringa**	**Jatropha cake**	**Jatropha cake**	**Jatropha cake**	**Moringa**	**Moringa**	No significant
Oleaginous waste X4	**Olive pomace**	**Olive pomace**	**Olive pomace**	**Olive pomace**	**Olive pomace**	**Olive pomace**	**Olive pomace**	**Olive pomace**	**Olive pomace**	**Argan cake**	**Olive pomace**	**Olive pomace**	No significant	**Olive pomace**	No significant
Enzymatic precursors X5	**Chitin**	**Chitin**	**Olive oil**	**Olive oil**	**Olive oil**	**Chitin**	**Olive oil**	**Chitin**	**Olive oil**	**chitin**	**Olive oil**	**Chitin**	**Olive oil**	**Chitin**	No significant

Table 6 C/N ratio of the eight solid medium tested

Experience	The carbon–nitrogen ratio (C/N)
Medium 1	37
Medium 2	14
Medium 3	18
Medium 4	16
Medium 5	30
Medium 6	20
Medium 6	20
Medium 6	20
Medium 7	13
Medium 8	24

Following the same idea, substrates were selected, for their nutritional quality more than for their participation in the general texture of the medium. The vine shoot is considered as the best substrate for this SSF and for the secondary metabolites production, because it provides the necessary nutrients for physiology and fungi's growth, also constituting a good source of carbon and nitrogen (Table 1) [13, 32]. It is the first time that vine shoots have been used for the culture of *T. asperellum* strains to produce secondary metabolites. In addition, sugarcane bagasse was selected as substrate for further experimentation, not only because of its commercial potential as an agroindustrial byproduct and its suitability for enzymes, conidia and 6-PP formation but also because it has received much attention as a substrate for SSF [33–35]. For this reason, the opportunity for agro-industrial byproducts of low commercial value to be used as substrates in SSF is an important economic valorization, approach offering advantages in this fermentation process. Medium optimization is generally a time-consuming and labour-intensive process. The Hadamard design proved to be a valuable tool for the rapid evaluation of the effects of the various medium components with significant positive effects on the output. Since this design is a preliminary optimization technique, which tests only two levels of each variable, it cannot provide the optimal quantity of each factors required in the medium. However, this technique provides indications of how each component tends to affect the fungal conidiation and metabolites production. In this study a lower-cost medium to replace such medium described in literature to produce 6-PP, enzymes and conidia [20, 36–39] has been achieved.

Concerning substrate effects, enzymes activities, conidia and 6-PP production by *T. asperellum* TV104 are maximized with the presence of vine shoots, potatoes flour, jatropha cake, olive pomace, and olive oil. However, argan cake and moringa will be omitted because they decreased all the responses tested. Chitin and wheat bran will not be included in the medium because they inhibit 6-PP production and lipase activity. For *T. asperellum* QT22046, argan cake and jatropha cake have a positive effect on the production of 6-PP but they inhibit the production of conidia and enzymes activities. On the other hand, vine shoots and olive pomace maximize cellulases and amylase activities. The mixture of sugarcane bagasse, potatoes flour, moringa, olive pomace and chitin appears to be the adequate medium to maximize the production of all the responses by this strain.

On the other hand, it has been demonstrated a relationship between the high enzymes activities, conidia and 6-PP yields with the carbon nitrogen source supplied. Those sources were proportioned with a mix of substrates mentioned before, which contain amounts of nutrients like sugars, amino acids, lipid, fiber, and minerals. The balance of these nutrients may influence the 6-PP conidia production.

Comparing the C/N ratio of the different medium tested, the maximum metabolites production by *T. asperellum* TV104 were achieved in C/N ratio of 37 (medium 1) showing that *Trichoderma* strains use different carbon and nitrogen sources for metabolites production in SSF.

Considering that the mixture; vine shoots, potatoes flour, jatropha cake, olive pomace, olive oil are richer in protein, lipid and fatty acids, it appears that the biosynthesis of 6-PP are carried out from the metabolism of fatty acids which a common character of lactone production [14]. The cost of the new base medium is one-third of the cost of other medium indicated in literature on the production *Trichoderma* metabolites [11, 29, 40, 41]. For a broad application, the cost of chemical synthesis of 6-PP is one of the main factors limiting the process [29, 40, 42].

Reducing the costs of 6-PP production by optimizing the fermentation medium is the basic research for industrial application. However, using natural substrates as cultural medium, have some disadvantages, such as increasing the experimental variability leading to variations in the process performance, because of the complex nature of the substrate [43, 44]. In order to reduce the heterogeneity that may exist between two stocks of the same byproduct, it is recommended to use byproducts from the same industrial sector that preferentially have the same standardized pretreatments. Of course, the use of a single solid matrix impregnate with pure compounds may improve the repeatability of experiments; it is however possible to use a mixture of solid substrate and to have a good repeatability if the previous recommendations concerning the origin of the medium are respected.

Conclusions

In conclusion, it is important to note that a significant increment in the enzymes, conidia and 6-PP production by *T. asperellum* strains in SSF was achieved by modifying the

culture conditions using an experimental design. The use of a Hadamard design allowed identification of the key variables that significantly affected the response tested. In addition, this strategy of optimizing the best medium leads inevitably to a maximized result. Significantly enzymes activities, conidiation and 6-PP production, were observed on the following mix of substrates: vine shoots, potatoes flour, jatropha cake, olive pomace and olive oil which was used by *T. asperellum* TV104 as a source of nutrients and also as a matrix.

The fact of using agricultural wastes as support or substrate for filamentous fungi growth and conidiation can produce value-added products of metabolites with reduced costs. Also, more variables could also be studied to fully optimize the fermentation process, such as for example, physical parameters like temperature, aeration, water stress and water activity which are of critical importance in SSF.

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