Optimization of Enzyme Production from Streptomyces by Solid-state Fermentation of Agricultural Wastes using the Orthogonal Projections to Latent Structures-Discriminant Analysis (OPLS-DA) Method

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Abstract

This study used Orthogonal Projections to Latent Structures-Discriminant Analysis (OPLS-DA) to identify and optimize the combined effect of the temperature, fermentation time, substrate-type, and fermenting microorganism on solid-state fermentation (SSF) culture of different agricultural wastes, in order to evidence the interactions among variables that allow maximizing the production of each enzyme. Thus, barley and wheat straw were fermented using two *Streptomyces* strains (*S*. MDG 301 and *S*. MDG 147) under SSF conditions. Key degradative enzymes (xylanases, carboxylmethilcellulase (CMCase), mannanase, laccase and peroxidase) and lignin solubilisation were evaluated. Our results highlighted that the highest xylanases and CMCase levels were obtained after treatment of wheat straw for two days with the *S*. MDG 301 strain at 45°C. Similarly, we found that the highest mannanase level was achieved using the *S*. MDG 147 strain on the barley straw at 28°C. Therefore, the integrated approach used based on analytical and bio statistical methods, proved to be a valuable tool for an accurate and rapid determination of the most significant parameters for controlling the bio-transformation process in terms of the enzymatic activity and lignin solubilisation.

Statement Of Novelty

Modern intensive agriculture generates large amounts of lignocellulosic waste that often are being burned or accumulated in landfills. In a sustainable economy, the reuse of these wastes is a biotechnological challenge that aims to obtain value-added products through the biotransformation of these substrates. Our previous experience in this field tells us that the use of statistical methods to identify and optimize the combined effect of multiple variables affecting solid-state fermentation (SSF) process is mandatory.

In this work, we applied for first time Orthogonal Projections to Latent Structures-Discriminant Analysis to optimize SSF processes carried out by Streptomyces and considering all the variables on the production of enzymes and the solubilization of lignin, which will allow us to predict future applications.

1. Introduction

A current challenge for biotechnologists is implementation of commercial products obtained from renewable sources. It is well known that lignocellulosic biomass is the most abundant renewable bioresource and thus, during the last years it has been used for countless industrial applications such as to obtain bioethanol, paper, furniture, construction material, etc [1-3]. However, its full exploitation, although attractive, is still far from being achieved. In fact, residual lignocellulosic biomass from human activities such as pruning and harvesting activities, forestry or forest clearing, agricultural practices, has as principal use to be burned in order to produce electrical energy. Moreover, the disposal of residual lignin from the pulp and paper industries is one of the major environmental pollutants for continental waters encouraging the development of new technologies to prevent this problem [4-7]. Among the different political and social pressures to the different industrial sectors, the introduction of the "Ecolabel"

in the European Union had a considerable impact in order to ensure environmental and sustainable requirements of products and processes. In accordance with all these policies and trends, the development of new biological strategies to provide added value to a variety of residues such as lignocellulosic residues are being investigated [8-9]. These "clean" technologies include the bioconversion of agro-residues through the use of microorganisms capable of excreting enzymes able to attack the main components of lignocellulose. Some of them are hydrolytic enzymes that modify the structure of lignocellulose by breaking the glycosidic bonds that join the different sugar units of cellulose and hemicellulose moieties. Among these microorganisms, *Streptomyces spp*. can also solubilize lignin from lignocellulosic residues through the production of cellulolytic (*i.e.* carboxymethyl cellulase) and hemicellulolytic (xylanases, and mannanases) as well as oxidative (laccases and peroxidases) enzymes that render lignin-carbohydrate complexes (Acid Precipitable Polymeric Lignin, APPL) [10-11,2]. Enzyme technology has entered a phase in whichhe increased understanding of the fundamental biology and bioinformatics, are beginning to shape the discovery, development, purification, and application of biocatalysts to a much greater extent. This development is leading to new enzyme applications and increased impact of enzyme technology in the industry [12]. Currently, enzymes are being employed in industrial biotechnology for numerous purposes to produce novel and sustainable products in various areas such as food processing and preservation, detergents, textile, leather and paper industries, medicine, water and industrial effluent treatment, cosmetics and pharmaceuticals and agriculture [13-17]. Several authors have compared the efficiency of solid and liquid fermentation processes, establishing a series of advantages and disadvantages for solid state fermentation (SSF) [18-21]. Nowadays, SSF is an economically viable, practically acceptable technology for large-scale bioconversion processes. The SSF is a system similar to natural conditions, using less volume of water reduces the volume of the fermenter and production costs, and the amount of contaminant effluents [22]. However, growth and kinetics studies can be complicated as far as most studies were carried out empirically analysing mainly, the production of biomass and enzymes. Thus, the use of predictive mathematical models for effective prediction and optimization of data can be a very useful strategy [23]. In this context, the chemometrics and multivariate data analysis (MVDA) are valuable tools for modelling and explaining the behaviour of a wide range of chemical and biological processes and their data. Multivariate statistical tools are increasingly used for optimizing methods because they help fine-tuning the conditions in the experiments execution. This, in turn, reduces the number and time of the analyses, which directly results in saving time, reagents, and waste disposal. Additionally, such tools may reveal interaction effects among the variables, not observed when using univariate models [24]. In the field of analytical sciences, these multivariate approaches have been successfully used primarily in two scenarios related to the extraction of information of interest from experimental data. These scenarios are the determination of analytical results (qualitative and quantitative approaches), and the optimization of the operational conditions for the suitable design of a certain measuring device, or for the reliable application of a certain analytical process [25]. Particularly, OPLS and OPLS-DA have been widely used for this purpose [26-29]. The OPLS and OPLS-DA are based on the partial least squares to latent structures (PLS). OPLS-DA goes further because it rotates the model which allows detecting the correlated variation that is related to the discrimination (class separation), in the first predictive component. The uncorrelated variation, which is

not related to the class separation, appears in the orthogonal components [30]. To the best of our knowledge this predictive model approach has not been applied for optimizing the solid-state fermentation (SSF) process by *Streptomyces.*

This study aims to apply the OPLS-DA method to identify and optimize the effect of several parameters (i.e., temperature, fermentation time, substrate-type, and fermenting microorganism), in the enzyme production and substrate solubilization during the SSF of different agricultural wastes. Thus, the main goal of this study is to determine the different interactions among the variables that allow maximizing the production of each enzyme. This would, in turn, provide valuable knowledge on the optimal fermentation conditions that would render the desired characteristics for both to the product to be obtained and/or the enzyme production.

2. Materials And Methods

2.1. Raw materials, chemicals, and microbial strains

The lignocellulosic residues used as fermentation substrates, *i.e.*, wheat (*Triticum aestivum* var. maestro) and barley (*Hordeum vulgare*) straws, were both furnished by local Guadalajara farmers (Central Spain). The microorganisms used belonged to the MICRODEG Collection from the Department of Biomedicine and Biotechnology of the University of Alcalá (Universidad de Alcalá de Henares, Madrid, Spain). They were classified as *Streptomyces* sp. based on their morphology and cell wall chemotype [31]. Those strains were *Streptomyces* sp. MDG 301 and *S.* MDG 147, isolated from Nicaraguan volcanic soils and from lignocellulosic residues, respectively. Their spores suspensions were kept at −20 °C in 20 % (w/v) glycerol until used. For the actual experiments, the microorganisms were routinely cultured up to their sporulation in International Streptomyces Project (ISP) medium 2 agar dishes, according to previous protocols [32].

2.2. Solid-state fermentation of the lignocellulosic residues

Prior to the SSF process, both wheat and barley straws were ground and sieved through a 40-mesh screen coupled to a Janke and Kunkel A-10 equipment (Germany), and then air-dried for 24 h at 50 °C. At the same time, the preinoculum was prepared by growing standardized strain spore suspensions (*S.* MDG 301 and *S.* MDG 147) (10^7 cfu mL^{-1}) in 2 L Erlenmeyer flasks containing saline basal medium (MBS) [33] supplemented with 0.6 % (w/v) yeast extract. The SSF was performed in two five-tray bioreactors containing a total of 1 kg of each residue, to which 5.6 L of preinoculum was added. The substrates were aseptically distributed onto the trays (200 g per tray), then incubated at 28 °C and 45 °C for 7 days whilst keeping them at 90−95 % relative humidity by blowing humidified sterile air filtered through a 0.22 μm nominal cut-off membrane every 12 h. The uninoculated controls were incubated under the same conditions. In all samples, the enzymatic activities, and the APPL and alkali lignin yields were determined [34].

2.3. Enzymatic analysis

Both fermented wheat and barley straws (10 g) were extracted with 15 mL of distilled water after 2, 4, and 7 days of incubation at the two tested temperatures (28 °C and 45 °C). The mixtures were sonicated for 15 min and then filtered through Whatman No. 1 filter paper to obtain the crude enzyme extract [35] where the following enzymatic activities were measured on the crude enzyme extract:

- Peroxidase activity (EC 1.11.1.7) was assayed using 2,6-dimethoxyphenol (2,6-DMP) as substrate, according to the protocol described by [36].

- Laccase activity (EC 1.10.3.2) was determined by the oxidation of 2,2-azino-bis-(3 ethylbenzthiazoline-6-sulphonic acid) (ABTS), according to the protocol described by [37]

- Xylanase (EC 3.2.1.8), mannanase (EC 3.2.1.78) and carboxymethylcellulase (EC 3.2.1.4) activities were determined by the release of reducing sugars using the Somogyi Nelson reagent [38]. For the reaction, 25 μL enzyme extract was mixed with 75 μL phosphate buffer 50 mM (pH 7.0) and with the specific substrate for each enzyme, *i.e.*, xylan, mannan and carboxymethyl-cellulose (CMC), respectively. The absorbance was determined at 540 nm, considering that one unit of xylanase, mannanase or CMCase activity is defined as the amount of enzyme required to release 1 μmol of xylose, mannose or glucose per minute, respectively. All enzyme assays were made in triplicate.

2.4. APPL and alkali-lignin extraction

The content for APPL and alkali-lignin was determined according to the protocol described by [32]. After 2, 4, and 7 days of incubation under the different SSF conditions, the APPL was extracted from the fermented wheat and barley straws. To obtain the APPL, 20 mL of distilled water/g was added to the transformed straw and steamed at 100 °C for 1 h, then filtered through Whatman No. 54 filter paper, and washed again with 100 mL of water at 80 °C. The APPL was precipitated from the supernatants by acidification to pH 1−2 with 12 M HCl. The precipitates were collected by centrifugation (12,439 *g*, 20 min), washed twice with deionized acidic water, freeze-dried in a Christ Alpha 1-4 freeze-dryer with LDC-1 M controller (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), and the dry weight of APPL was gravimetrically estimated. For the alkali-lignin obtention, the same procedure was followed but the lignin extraction was carried out with 0.1 M NaOH.

2.5. Statistical analysis

The statistical analysis for the optimization of the fermentation conditions for each enzyme production was carried out using SIMCA (Sartorius Stedim Bio-tech, Aubagne, France). The OPLS-DA method was used to try to discriminate among the different levels of the studied variables. The software was configured to calculate the confidence intervals on the parameters using a 95 % confidence level, whilst

the significance level used when computing the critical limits for the models' distance and the Hotelling's T2 ellipse was set as 0.05 (95 % confidence). The data was previously centred and scaled (unit variance) for eliminating any weight due to the variables or observations magnitude. The analysis was performed using a non-linear iterative partial least squares (NIPALS) algorithm, fully cross validation (CV) which included an uncertainty test, and 1/SDev as weighing. Because OPLS-DA is responsive to the model complexity [39], to avoid overfitting, a repeated 10-fold CV was set to estimate the relevant number of components in the OPLS models [40]. Nonetheless, to avoid overfitting and still have the optimal number of proper factors for generating predictive models, three latent variables were use in all cases.

3. Results And Discussion

3.1. Multivariate Analysis of the enzymatic activities, APPL and alkali-lignin yields under different SFF conditions.

The degradation of lignocellulose was studied by measuring the activity of three hydrolytic enzyme (xylanase EC 3.2.1.8, mannanase EC 3.2.1.78, and CMCase EC 3.2.1.203) and two oxidative enzyme (laccase EC 1.10.3.2 and peroxidase EC 1.11.1.7). This was done to determine the effect of the fermentation time, type of substrate, temperature and microbial strain on the SSF process in terms of enzymatic activity. Additionally, the content of APPL and alkali-lignin in the extracts obtained from SSF cultures was determined to evaluate the degradation capacity of the two strains on the residues.

Table 1 shows the results of the enzymes analysis on the substrates under the different experimental conditions Also, the values obtained for APPL and alkali-lignin contents in the extracts obtained from fermented residues are summarized in Table 2.

Table 1

Extracellular enzymatic activities obtained from wheat and barley straw after SSF process under the different operational conditions tested. All values are given as mean ± standard deviation (n=3).

Substrate	T (°C)	Strain	Days	Xylanase (U/mL)	CMCase (U/mL)	Mannanase (U/mL)
Wheat	28	301	2	0.00±0.00	0.00±0.00	0.00±0.00
Wheat	28	301	4	0.63±0.06	0.01±0.01	0.01±0.02
Wheat	28	301	7	0.26±0.10	0.03±0.01	0.00±0.0
Wheat	28	147	2	1.35±0.29	0.90±0.10	1.23±0.65
Wheat	28	147	4	8.16±0.20	1.76±0.01	6.31±0.13
Wheat	28	147	7	6.66±0.34	0.88±0.02	3.68±0.14
Wheat	45	301	2	31.11±1.21	10.19±1.29	0.00±0.00
Wheat	45	301	4	32.78±0.16	8.76±0.10	0.00±0.00
Wheat	45	301	7	33.91±0.52	7.43±0.31	0.00±0.00
Wheat	45	147	2	0.52±0.05	0.15±0.01	0.08±0.00
Wheat	45	147	4	4.25±1.75	1.18±0.02	4.30±0.13
Wheat	45	147	7	1.10±0.51	0.00±0.00	0.00±0.00
Barley	28	301	2	3.73±1.34	0.48±0.05	0.72±0.10
Barley	28	301	4	7.15±1.46	0.61±0.13	0.28±0.23
Barley	28	301	7	7.47±1.52	0.53±0.12	0.04±0.04
Barley	28	147	2	1.50±0.56	0.59±0.23	0.00±0.00
Barley	28	147	4	1.07±0.08	0.00±0.00	0.00±0.00
Barley	28	147	7	0.87±0.01	0.34±0.16	1.16±0.10
Barley	45	301	2	5.35±0.40	1.25±0.05	1.22±0.11
Barley	45	301	4	22.85±1.88	1.16±0.09	0.77±0.12
Barley	45	301	7	27.95±1.58	1.55±0.2	0.69±0.19
Barley	45	147	2	1.13±0.63	0.46±0.070	0.00±0.00
Barley	45	147	4	2.433±0.61	0.17±0.00	0.37±0.14
Barley	45	147	7	5.71±1.35	0.30±0.04	1.15±0.01

Table 2

APPL and alkali-lignin yields obtained from the wheat and barley straws after the SSF process under the different tested operational conditions. All values are given as mean ± standard deviation (n=3).

Substrate	T (ºC)	Strain	Days	APPL (g)	Alkali-lignin (g)
Wheat	28	301	2	0.10±0.01	0.21±0.01
Wheat	28	301	4	0.14±0.03	0.24±0.01
Wheat	28	301	7	0.17±0.00	0.61±0.00
Wheat	28	147	2	0.03±0.01	0.55±0.03
Wheat	28	147	4	0.10±0.00	1.31±0.02
Wheat	28	147	7	0.06±0.01	1.46±0.02
Wheat	45	301	2	0.17±0.00	0.54±0.01
Wheat	45	301	4	0.11±0.00	0.72±0.01
Wheat	45	301	7	0.17±0.00	1.38±0.01
Wheat	45	147	2	0.07±0.03	0.49±0.01
Wheat	45	147	4	0.10±0.00	0.43±0.02
Wheat	45	147	7	0.11±0.00	1.35±0.12
Barley	28	301	2	0.09±0.01	0.62±0.19
Barley	28	301	4	0.06±0.00	0.57±0.14
Barley	28	301	7	0.09±0.00	0.49±0.01
Barley	28	147	2	0.05±0.00	0.54±0.00
Barley	28	147	4	0.03±0.01	0.50±0.02
Barley	28	147	7	0.04±0.00	0.50±0.00
Barley	45	301	2	0.07±0.02	0.35±0.03
Barley	45	301	4	0.08±0.01	0.43±0.06
Barley	45	301	7	0.13±0.01	0.46±0.05
Barley	45	147	2	0.07±0.02	0.53±0.01
Barley	45	147	4	0.07±0.02	0.47±0.02
Barley	45	147	7	0.09±0.01	0.45±0.02

In all the extracts obtained from both fermented substrates, neither laccase nor peroxidase activities were detected, probably due to the adsorption of these enzymes to the substrate, which makes their recovery difficult [11].

These data were analysed using OPLS-DA, evaluating the individual effect and the interaction of the variables studied in terms of the enzymatic response.

The OPLS-DA model indicated that the discrimination among the *Streptomyces* strains and substrates-type levels was already clear after calculating three predictive t(1-3) components. In other words, the fermentation using wheat and barley straws, combined with any of these strains showed a clearly differentiated pattern. The R2X(1) parameter indicated that about 24.1 % of the X main-data matrix variation was modelled by the t [1] predictive component. Likewise, R2X(2) and R2X(3) showed that 20.5 % and 11.5 % of the X main-data matrix variation was modelled by the t(2) and t(3) predictive components, respectively. Moreover, a total 25 % of the X variation was modelled by three to(1-3) orthogonal components. Thus, a total 81.1 % of the X variation was modelled by all the (predictive and orthogonal) components, which renders a rather good explanation of the separation. The outliers outside the 95 % confidence Hotelling's T2 ellipse were not excluded from the analysis because, in perspective, they did not affect the overall separation results.

Figure 1 shows the 3D Scores Scatter plot from the OPLS-DA model used to test the effect of the substrate type and the strains employed in the SSF process for the enzymatic production. In both cases, it can be observed that it is possible to clearly discriminate the effect of each studied variable.

On the other hand, despite that the incubation temperature showed a less homogeneous groups distribution, it allowed differentiating two distinct effects. Additionally, the incubation time did not show a homogeneous behaviour, possibly because this parameter is a determining factor of the studied variables (Figure 2).

3.2. Optimized enzyme activities in the bio-treated substrates

According to the OPLS-DA model, the level of xylanase production during the incubation time showed clear differences between the two evaluated *Streptomyces* strains. Thus, the combined observation of the model showed in Figure 1, 2 and 3a, indicates that the maximum xylanase activities were obtained in fermented wheat straw with *S.* MDG 301 strain, after 7 days of incubation at 45 °C (dot 1). It is important to highlight that no apparent differences were observed between the maximum xylanase production in wheat straw at the above-mentioned conditions and the xylanase production after 2 and 4 days (Figure 3a, coloured region). Therefore, the obtained data suggest that high production of this enzyme can be reached in shorter fermentation periods, which implies an important advantage for industrial applications due to the consequent reduction in production time and cost. Similarly, in fermented barley straw, despite obtaining a general lower enzyme production in comparison to wheat, the results showed that a few

samples had a significant xylanase production, in the same conditions (Figure 1, 2 and 3a, dot 2). This aspect was not evidenced by ANOVA analysis which revealed large significant differences (Pr > F: 0.0006) between the wheat (Figure 1, 2 and 3a, dot 1) and the barley (Figure 1, 2 and 3a, dot 2) samples.

The high levels of xylanase obtained in both fermented substrates are a consequence of their chemical composition. Agricultural residues, including wheat and barley straw, contain high amounts (approximately 25 % w/w) of hemicellulosic sugars, mainly xylose, which induces the production of this enzyme [41].

Regarding the CMCase activity, the OPLS-DA analysis showed a higher production rate of this enzyme in the wheat straw after 2 days of incubation with the S. MDG 301 strain at 45 °C (Figure 1, 2 and 3b, dot 3). In the barley straw, the differences found throughout the incubation time were not significant and the enzyme production was significantly lower than those obtained in wheat straw. However, low concentrations of mannanase enzyme activity were detected in these conditions. This result could be attributed to the practically absence of inducers of this activity in the residues, corresponding the activity detected to the enzyme basal or constitutive level. On the contrary to the conditions obtained for the maximum production of xylanase and CMCase activities, the highest activity of the mannanase, was detected using wheat straw treated with the S. MDG 147 strain incubated at 28 °C during 4 days (Figure 1, 2 and 3c, dot 4).

3.3. Optimized APPL and alkali-lignin yields from fermented substrates

According to the OPLS-DA model the amount of solubilized APPL was significantly higher using wheat straw as substrate, and when it was inoculated with the S. MDG 301 strain. The maximum solubilisation of APPL was reached on the day 7 (Figure 1, 2 and 4a, dot 1). In order to increase the extraction yield of solubilized lignin from the fermented substrates, an alkaline extraction with 0.1 M NaOH was carried out. As expected, the amount of lignin solubilized from the wheat and barley straws using alkali was higher than using water. Thus, as estimated, these results coincided with the highest enzymatic activities which could be attributed to the fact that the enzymatic cleavage of the structure bonds is bound to the consequent substrate solubilisation [34]. It is important to take into consideration that the reported arabinoxylan/glucomannane ratio in non-aqueous angiosperms is 20:1 [11]. For this reason, the main role of the APPL solubilisation should be mostly ascribable to the production of xylanase activity for the chemical composition of the substrate [42].

The highest lignin solubilization (APPL) and highest alkali-lignin extractions were obtained from wheat straw fermented with the S. MDG 147 strain, after 7 days of fermentation at a temperature of 28 °C (Figure 1, 2 and 4b, dot 5). The synergistic action of xylanases, mannanases and CMCase activities produced by this strain might facilitate the extraction of lignin by the alkali. Similar results were obtained

from wheat straw fermented with *S.* MDG 301 strain after 7 days of incubation likely due to the enhanced solubilization caused by enzymatic action of xylanase (Figure 4a, dot 1).

Undoubtedly, lignocellulosic biomass is considered a low cost and easy to acquire residue that can be revalorized and used as fermentable substrate to produce inputs/raw materials of industrial interest. The use of products generated by SSF of lignocellulosic residues have increased significantly in the pharmaceutical, food, lubricants, paper, and textile industries, acknowledging the potential of depolymerizing enzymes to generate favourable chemical changes in substrates [43-45]. Hence, among the parameters influencing the fermentation process, the enzymes production is the most relevant aspect. However, it is important to emphasize that the economy, efficiency and success of such biotechnological processes largely depend on the control of other crucial aspects, including the substrate nature, the microorganisms employed, and the process operational conditions [46]. These parameters will in turn determine the enzymatic activity reached, the extent of the chemical changes in the substrates, and thus, the production of bio-products with specific characteristics.

For instance, we previously reported that lignocellulosic residues, microbiologically treated and subsequently functionalized, were successfully used as thickeners to elaborate lubricant greases. The rheological and tribological characteristics of the new greases largely depended on the substrate employed and the activity of the enzymes involved in the lignin depolymerization process. A clear increase in the rheological and viscoelastic properties were directly related to the reduction of the soluble lignin. This is due to the β-O-4 bonds disruption, together with an increase in the C, H, and N content due to the influence of the xylanase enzyme activity [34]. However, in specific cases, the desired substrate changes occur with a minimal enzyme production. For instance, Rajeev *et al*., (2009) [47] studied the ethanol production by saccharificating three different feedstocks (rice straw, sugarcane bagasse and water hyacinth biomass). They showed that the highest reaction yield occurred in SSF with rice straw, whose saccharification process occurred with low cellulase activity. In these studies, the production of the desired metabolite was clearly governed by the paramount enzymatic action, which in turn was dependent on the optimization of the specific process control variables.

Multivariate analysis can concurrently optimize the experimental variables in order to find the best operational conditions for biotechnological processes and discriminate systems regarding one or more interesting features. At the same time, it helps determining the value of multiple physical-chemical properties directly or indirectly related to the substrate or product chemical composition. Therefore, the use of conventional biochemical analyses and powerful biostatistical tools (OPLS-DA model) represents a natural marriage. It allows to determine the most suitable conditions for maximising the production of a metabolite of industrial interest (*e.g.,* specific enzymatic production), considering the overall interaction of the operating variables that affect such processes. This integrated approach provides valuable information to set up the appropriate fermentation conditions to produce the desired chemical changes in the substrates, and thus, to get a particular product of industrial interest.

4. Conclusions

This study revealed that the biotransformation process of lignocellulosic biomass is largely affected by several parameters involved in the SSF. While both tested agro-residues (wheat and barley straws) were successfully processed by *Streptomyces*, differences in the enzyme production were detected depending on several factors, including the substrate characteristics, the fermentation temperature and time, and the employed microbial strains. The OPLS-DA analysis revealed the combined effect of the operative conditions on the bioconversion process. It highlighted that the highest xylanases and CMCase levels were obtained after fermenting the wheat straw for two days with the *S*. MDG 301 strain at 45 °C. Likewise, it showed that the highest mannanese level was achieved using the *S*. MDG 147 strain on the barley straw at 28 °C. Therefore, the multivariate statistical analysis proved to be a valuable tool for an accurate and rapid determination of the most significant parameters for controlling the bio-transformation process in terms of enzymatic activity. This control is vital because it directly results in reducing the time and cost of the industrial application of such processes.

An integrated approach based on both analytical and biostatistical methods will improve the control of the enzymatic synthesis of new biobased products and allow the optimization of the enzyme technology application for industrial processes.

Declarations

Funding sources

Conflicts of interest/Competing interests

The first author of the referenced manuscript, on behalf of him/herself and all authors, declares that there is no potential conflict of interest related to the article. The authors have no relevant financial. The authors have no conflicts of interest to declare that are relevant to the content of this article. All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript. The authors have no financial or proprietary interests in any material discussed in this article.

Availability of data and material

All data generated or analysed during this study are included in this published article and there are not supplementary information files.

Author contribution

All authors contributed to the study conception and design. Conceptualization: Fernando Ortega-Ojeda, Gabriela Domínguez and Manuel Hernández. Data curation: Gabriela Domínguez and Juana Rodríguez. Formal analysis: Carmen Fajardo, Gabriela Domínguez and Fernando Ortega-Ojeda. Funding acquisition: Manuel Hernández. Methodology: Gabriela Domínguez, Carmen Fajardo, Juana Rodríguez, José M. Molina-Guijarro and Fernando Ortega-Ojeda. Project administration: Manuel Hernández and María E. Arias. Resources: Manuel Hernández and María E. Arias. Supervision: Manuel Hernández, María E. Arias, and María E. Eugenio. Validation: Manuel Hernández, María E. Arias, María E. Eugenio and Fernando Ortega-Ojeda. Writing review & editing: Gabriela Domínguez, Manuel Hernández M. Enriqueta Arias and Fernando Ortega-Ojeda. The first draft of the manuscript was written by Gabriela Domínguez, Carmen Fajardo, Manuel Hernández, and M. Enriqueta Arias and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Consent to participate

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Consent for publication

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References

1. Tribot, A; Amer, G; Maarouf, A; Hélène de Baynast; Delattre, C; Pons, A; Jean-Denis, M; Callois, J; Vial, C and Michaud, P. Wood-Lignin: Supply, Extraction Processes and use as Bio-Based Material. Eur.

Polym. J.112:228-240. (2019).

2. Blánquez, A; Ball, A; González-Pérez, J; Jiménez-Morillo, N; González-Vila, F; Arias, ME and Hernández, M. Laccase SilA from *Streptomyces Ipomoeae* CECT 3341, a Key Enzyme for the Degradation of Lignin from Agricultural Residues?. PloS One. 12(11): (2017). DOI: 10.1371/journal.pone.0187649.

3. Pinales-Márquez, C; Rodríguez-Jasso, R; Araújo, R; Loredo-Treviño, A; Nabarlatz, D; Gullón, B and Ruiz, H. Circular Bioeconomy and Integrated Biorefinery in the Production of Xylooligosaccharides from Lignocellulosic Biomass: A Review. IND CROP PROD. (2021). 162:113274. DOI: 10.1016/j.indcrop.2021.113274.

4. Ashrafi, O; Laleh, Y and Fariborz, H. Wastewater Treatment in the Pulp-and-Paper Industry: A Review of Treatment Processes and the Associated Greenhouse Gas Emission. J. Environ. Manage. 158:146-157. (2015). DOI: 10.1016/j.jenvman.2015.05.010.

5. Collett, J; Billing, J; Meyer, P; Schmidt, A; Remington, B; Hawley, E; Hofstad, B; Panisko, E; Dai, Z; Hart, T; Santos, D; Magnuson, J; Hallen, R; Jones, S. Renewable Diesel Via Hydrothermal Liquefaction of Oleaginous Yeast and Residual Lignin from Bioconversion of Corn Stover. Appl. Energy. 233-234(C):840-853. (2019). DOI: 10.1016/j.apenergy.2018.09.115.

6. Singh, G and Arya, S. Utility of Laccase in Pulp and Paper Industry: A Progressive Step Towards the Green Technology. Int. J. Biol. Macromol. 134:1070-1084. (2019). DOI: 10.1016/j.ijbiomac.2019.05.168.

7. Torres, L; Adenise, Z; Tanobe, W; Oliveira, V; Karp, S; Lorenci, L; Faulds, C and Soccol, C. Lignin as a Potential Source of High-Added Value Compounds: A Review. J. Clean. Prod. 263:121499. (2020). DOI: 10.1016/j.jclepro.2020.121499.

8. Gouveia, S., Otero, L.A., Fernández-Costas, C., Filgueira, D., Sanromán, Á, Moldes, D. Green Binder Based on Enzymatically Polymerized Eucalypt Kraft Lignin for Fiberboard Manufacturing: A Preliminary Study. (2018). Polym. J. 10(6):642. DOI: 10.3390/polym10060642

9. Kamali, M; Khodaparast, Z. Review on Recent Developments on Pulp and Paper Mill Wastewater Treatment. Ecotoxicol Environ Saf. 114:326-342. (2015). DOI: 10.1016/j.ecoenv.2014.05.005.

10. Arias, M.E., Blánquez, A., Hernández, M., Rodríguez, J., Ball, A.S., Jiménez-Morillo, N.T., González-Vila, F.J., González-Pérez, J.A. Role of a thermostable laccase produced by *Streptomyces ipomoeae* in the degradation of wheat straw lignin in solid state fermentation. J. Anal. Appl. 122:202-208. (2016). DOI: 10.1016/j.jaap.2016.09.023.

11. Borrero-López, A.M., Blánquez, A., Valencia, C., Hernández, M., Arias, M.E., Franco, J.M. Influence of Solid-State Fermentation with *Streptomyces* on the Ability of Wheat and Barley Straws to Thicken Castor Oil for Lubricating Purposes. IND CROP PROD. 140:111625. (2019). DOI: 10.1016/j.indcrop.2019.111625

12. van Beilen, J and Li, Z. Enzyme Technology: An Overview. Curr Opin Biotech. 13(4):338-344. (2002). DOI: 10.1016/S0958-1669(02)00334-8.

13. Arbige, M; Shetty, J and Chotani, G. Industrial Enzymology: The Next Chapter. Trends Biotechnol. 37(12): 1355-1366. (2019).

14. Fasim, A., More, V and More, S. Large-scale production of enzymes for biotechnology uses. Curr Opin Biotech. 69, 68-76. (2021). DOI: 10.1016/j.copbio.2020.12.002.

15. Cavaco-Paulo, A and Gubitz, G. Textile Processing with Enzymes. pp. 240 in Woodhead Publishing, ISBN 9781855736108. (2003).

16. Mojsov, K. D; Andronikov, D; Janevski, A; Kuzelov, A; Gaber, S. The application of enzymes for the removal of dyes from textile effluents. Adv. Mater. Technol. 5(1), 81-86. (2016). DOI: 10.5937/SAVTEH1601081M.

17. Khan, N. Enzyme Technology - an Emerging Trend in Biotechnology. Enzyme Engineering. 7(1). (2018). DOI: 10.4172/2329-6674.1000163.

18. Hesseltine, C. Biotechnology Report: Solid State Fermentations. Biotechnol. Bioeng. 14(4):517-532. (1972). DOI: 10.1002/bit.260140402

19. Ibarruri, J; Cebrián, M; Hernández, I. Valorisation of Fruit and Vegetable Discards by Fungal Submerged and Solid-State Fermentation for Alternative Feed Ingredients Production. J. Environ. Manage. 281:111901. (2021). DOI: 10.1016/j.jenvman.2020.111901

20. Vyas, A; Vyas, D. Production of fungal cellulases by solid state bioprocessing of groundnut shell wastes. scient. ind. Res. 64:767-770. (2005).

21. Nigam, P; and Singh, D. Solid-state (Substrate) Fermentation Systems and their Applications in Biotechnology. Basic Microbiol. 34(6):405-423. (1994). DOI: doi.org/10.1002/jobm.3620340607.

22. Krishna C. Solid-state fermentation systems-an overview. Crit. Rev. Biotechnol. 25(1-2):1-30. (2005). DOI: 10.1080/07388550590925383.

23. Webb, C. and Manan, M. Design aspects of solid-state fermentation as applied to microbial bioprocessing. J. Appl. Biotechnol. 4(1):511-532. (2017). DOI: 10.15406/jabb.2017.04.00094.

24. Govarthanan, M; Selvankumar, T; Selvam, K; Sudhakar, C; Aroulmoji, V; Kamala-Kannan, S. Response surface methodology based optimization of keratinase production from alkali-treated feather waste and horn waste using bacillus sp. MG-MASC-BT. Ind. Eng. Chem. Res. 27, 25–30. (2015). DOI: 10.1016/j.jiec.2014.12.022

25. Cuadros, L; Jiménez-Carvelo, A; Fernández-Ramos, M. Multivariate Thinking for Optical Microfluidic Analytical devices–A Tutorial Review. Microchem J. 164:105959. (2021). DOI: 10.1016/j.microc.2021.105959

26. O'Leary, J; Hiscox, J; Eastwood, D; Savoury, M; Langley, A; McDowell, S; Rogers, H; Boddy, L; Müller, C. The whiff of decay: Linking volatile production and extracellular enzymes to outcomes of fungal interactions at different temperatures. Fungal Ecol. 39:336-348. (2019). DOI: 10.1016/j.funeco.2019.03.006.

27. Wei, M; Xiong, S; Chen, F; Geladi, P; Eilertsen, L; Myronycheva, O; Lestander, T; Thyrel, M. Energy smart hot-air pasteurisation as effective as energy intense autoclaving for fungal preprocessing of

lignocellulose feedstock for bioethanol fuel production. Energy. 155: 237-247. (2020). DOI: 10.1016/j.renene.2020.03.154

28. Yan, Ma, Ling, T; Su, X; Jiang, B; Nian, B; Chen, L; Liu, M; Zhang, Z; Wang, D; Mu, Y; Jiao, W; Liu, Q, Pan, Y; Zhao, M. Integrated proteomics and metabolomics analysis of tea leaves fermented by *Aspergillus niger, Aspergillus tamarii* and *Aspergillus fumigatus*. Food Chem.. 334:127560. (2021). DOI: 10.1016/j.foodchem.2020.127560

29. Gromski, P; Muhamadali, H; Ellis, D; Xu, Y; Correa, E; Turner, M; Goodacre, R. A tutorial review: Metabolomics and partial least squares-discriminant analysis – a marriage of convenience or a shotgun wedding. Chim. Acta. 879:10-23. (2015). DOI: 10.1016/j.aca.2015.02.012

30. Kvalheim, O; Karstang, T. Interpretation of latent-variable regression models. Chemom. Intell. Lab. Syst. 7: 39–51. (1989). DOI: 10.1002/cem.1217

31. Anderson, A; Wellington, E. The taxonomy of *Streptomyces* and related genera. Int. J. Syst. Evol. Microbiol. 51:797–814. (2001).

32. Hernández-Coronado, M; Hernández, M; Centenera, F; Pérez, MI; Ball, A; Arias, ME. Chemical characterization and spectroscopic analysis of the solubilization products from wheat straw produced by *Streptomyces* strains grown in solid-state fermentation. J. Microbiol. 143: 1359–1367. (1997). DOI: 10.1099/00221287-143-4-1359.

33. Crawford, D. L. Lignocellulose decomposition by selected *Streptomyces* Appl. Environ. Microbiol. 35, 1041–1045. (1978). DOI: 10.1128/AEM.35.6.1041-1045.1978

34. Borrero-López, A.M., Blánquez, A., Valencia, C., Hernández, M., Arias, M.E., Eugenio, M.E., Fillat, U., Franco, J.M. Valorization of Soda Lignin from Wheat Straw Solid-State Fermentation: Production of Oleogel. ACS Sustain. Chem. Eng. 6(4):5198-5205. (2018). DOI: 10.1021/acssuschemeng.7b04846

35. Berrocal, M.á, Rodriguez, J., Ball, A.S., Perez-Leblic, M.I., Arias, M.E. Solubilisation and Mineralisation of [14 C] Lignocellulose from Wheat Straw by *Streptomyces Cyaneus* CECT 3335 during Growth in Solid-State Fermentation. Appl. Microbiol. Biotechnol.. 48(3):379-384. (1997). DOI: 10.1007/s002530051066.

36. Hernández, M; Rodríguez, J; Soliveri, J; Copa, J; Pérez, MI; Arias, ME. Paper mill effluent decolorization by fifty *Streptomyce*s strains. Appl. Environ. Microbiol. 60: 3909–3913. (1994).

37. Moya, R; Hernández, M; García-Martín, A; Ball, A; Arias, ME. Contributions to a Better Comprehension of Redox-Mediated Decolouration and Detoxification of Azo Dyes by a Laccase Produced by *Streptomyces Cyaneus* CECT 3335. Technol. 101(7):2224-2229. (2010). DOI: 10.1016/j.biortech.2009.11.061

38. Somogyi, M. A New Reagent for the Determination of Sugars. Biol. Chem. 160:61-68. (1945).

39. Bylesjö, M; Rantalainen, M; Cloarec, O; Nicholson, J; Holmes, E; Trygg, J. OPLS discriminant analysis: Combining the strengths of PLS-DA and SIMCA classification. J. Chemom. 20: (8-10):341-351. (2006). DOI: 10.1002/cem.1006

40. Shenk J.S. y Westerhaus M.O. Analysis of Agriculture and Food Products by Near Infrared Reflectance Spectroscopy. Monograph, NIR Systems, Silver Spring, MD.(1995).

41. Panagiotou, G; Olsson, L. Effect of Compounds Released during Pretreatment of Wheat Straw on Microbial Growth and Enzymatic Hydrolysis Rates. Bioeng. 96(2):250-258. (2007). DOI: 10.1002/bit.21100.

42. Puls, J. Chemistry and Biochemistry of Hemicelluloses: Relationship between Hemicellulose Structure and Enzymes Required for Hydrolysis. Macromolecular symposia. 120(1):183-196. (1997). DOI: 10.1002/masy.19971200119.

43. Roopesh, K., Ramachandran, S., Nampoothiri, K.M., Szakacs, G., Pandey, A. Comparison of Phytase Production on Wheat Bran and Oilcakes in Solid-State Fermentation by Mucor *Racemosus*. Bioresour. Technol. 97(3):506-511. (2006). DOI: 10.1016/j.biortech.2005.02.046.

44. Sindhu, R., Binod, P, Pandey, A. Biological Pretreatment of Lignocellulosic biomass–An Overview. Bioresour. Technol. 99:76-82. (2016). DOI: 10.1016/j.biortech.2015.08.030.

45. Thomas, L; Abhilash, J; Arumugam, M; Pandey, A. Production, Purification, Characterization and Over-Expression of Xylanases from Actinomycetes. Indian J. Exp. Biol. 51(11):875-84. (2013).

46. Lizardi-Jiménez, M; Hernández-Martínez; R. Solid state fermentation (SSF): diversity of applications to valorize waste and biomass. 3 Biotech. 7: 44. (2017). DOI 10.1007/s13205-017-0692-y.

47. Rajeev, K, Reeta, S; Singhania, R; Gincy, M; Pandey, A. Cellulase production using biomass feed stock and its application in lignocellulose saccharification for bio-ethanol production. Energy. 34:421-424. (2009). DOI: 10.1016/j.renene.2008.05.0

Figures

R2X[1] = 0.241; R2X[2] = 0.205; R2X[3] = 0.115; Ellipse: Hotelling's T2 (95%)

R2X[1] = 0.241; R2X[2] = 0.205; R2X[3] = 0.115; Ellipse: Hotelling's T2 (95%)
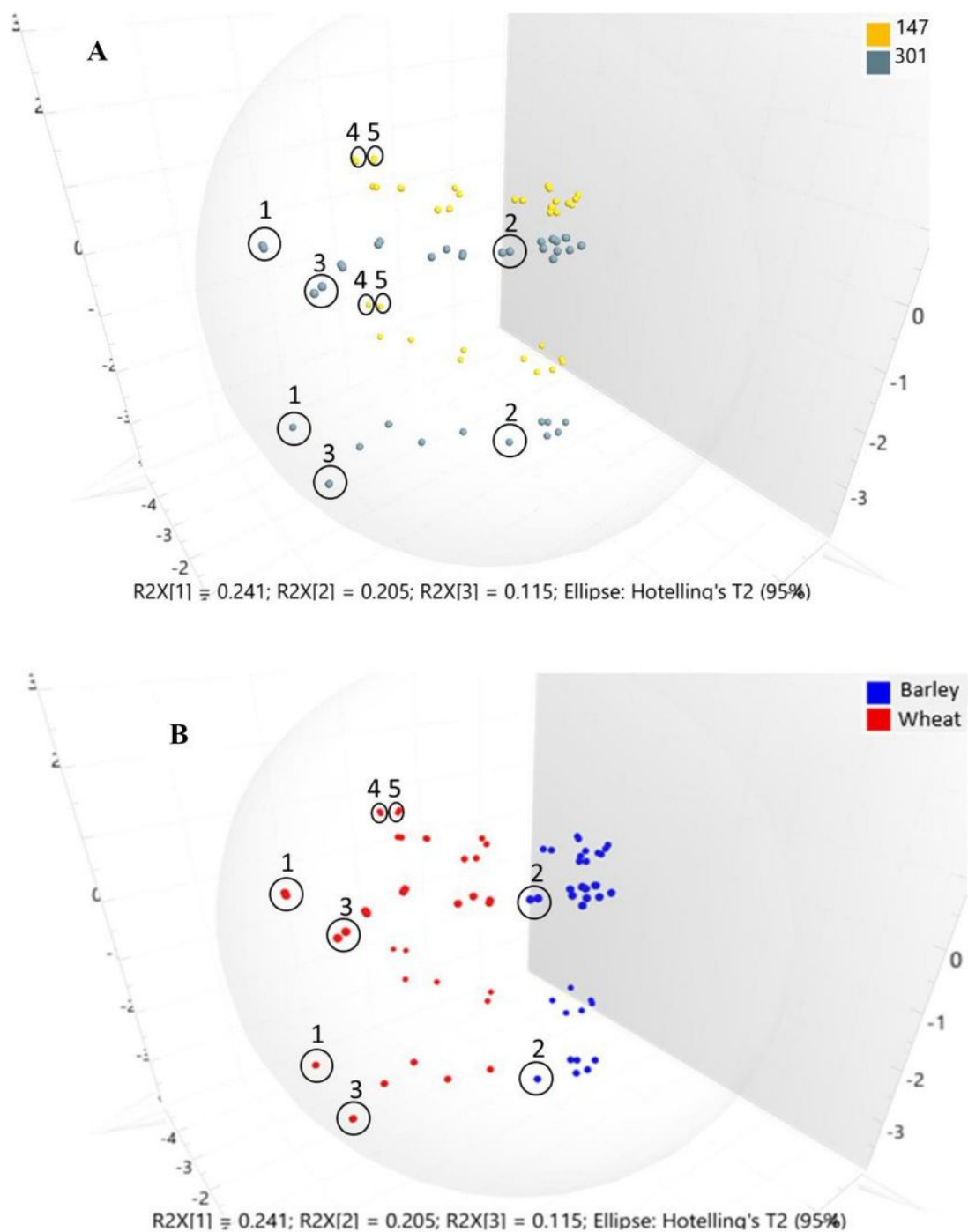
Figure 1

3D Scores Scatter plots from the OPLS-DA models used to test the effect of temperature, substrate-type, and the strain employed in the SSF process for the enzymatic production. Each view is coloured according to the strain (A) and substrate-type (B) variables, respectively (n=3).

R2X[1] = 0.241; R2X[2] = 0.205; R2X[3] = 0.115; Ellipse: Hotelling's T2 (95%)

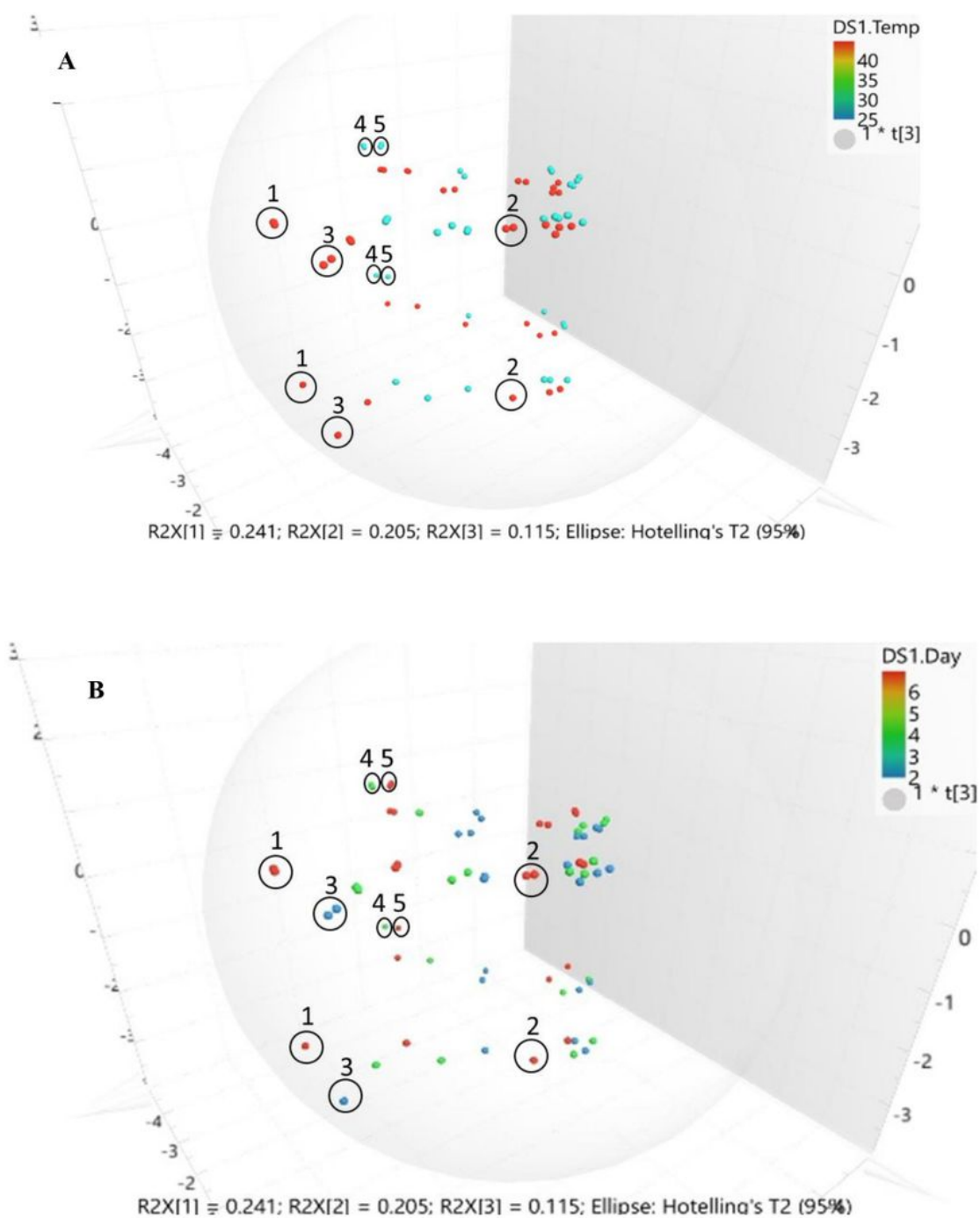R2X[1] = 0.241; R2X[2] = 0.205; R2X[3] = 0.115; Ellipse: Hotelling's T2 (95%)

Figure 2

3D Scores Scatter plots from the OPLS-DA models shown in Figure 1. This view is coloured according to the incubation temperature (A) and days variables (B), respectively (n=3).
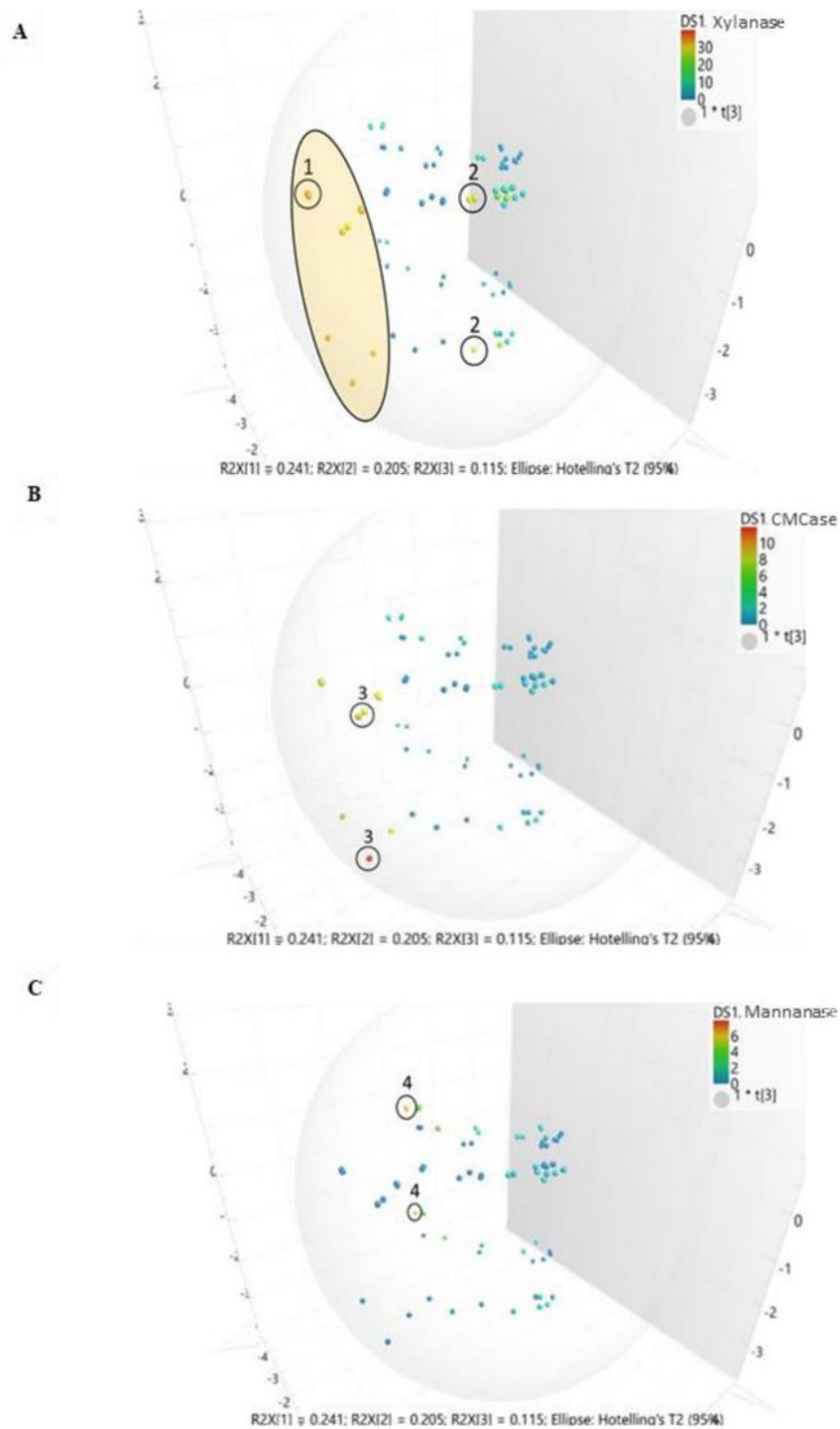
Figure 3

3D Scores Scatter plots from the OPLS-DA models shown in Figure 1 and 2. The views are coloured according to the following variables: xylanase (A), CMCase (B) and mannanase (C) activities, respectively (n=3).
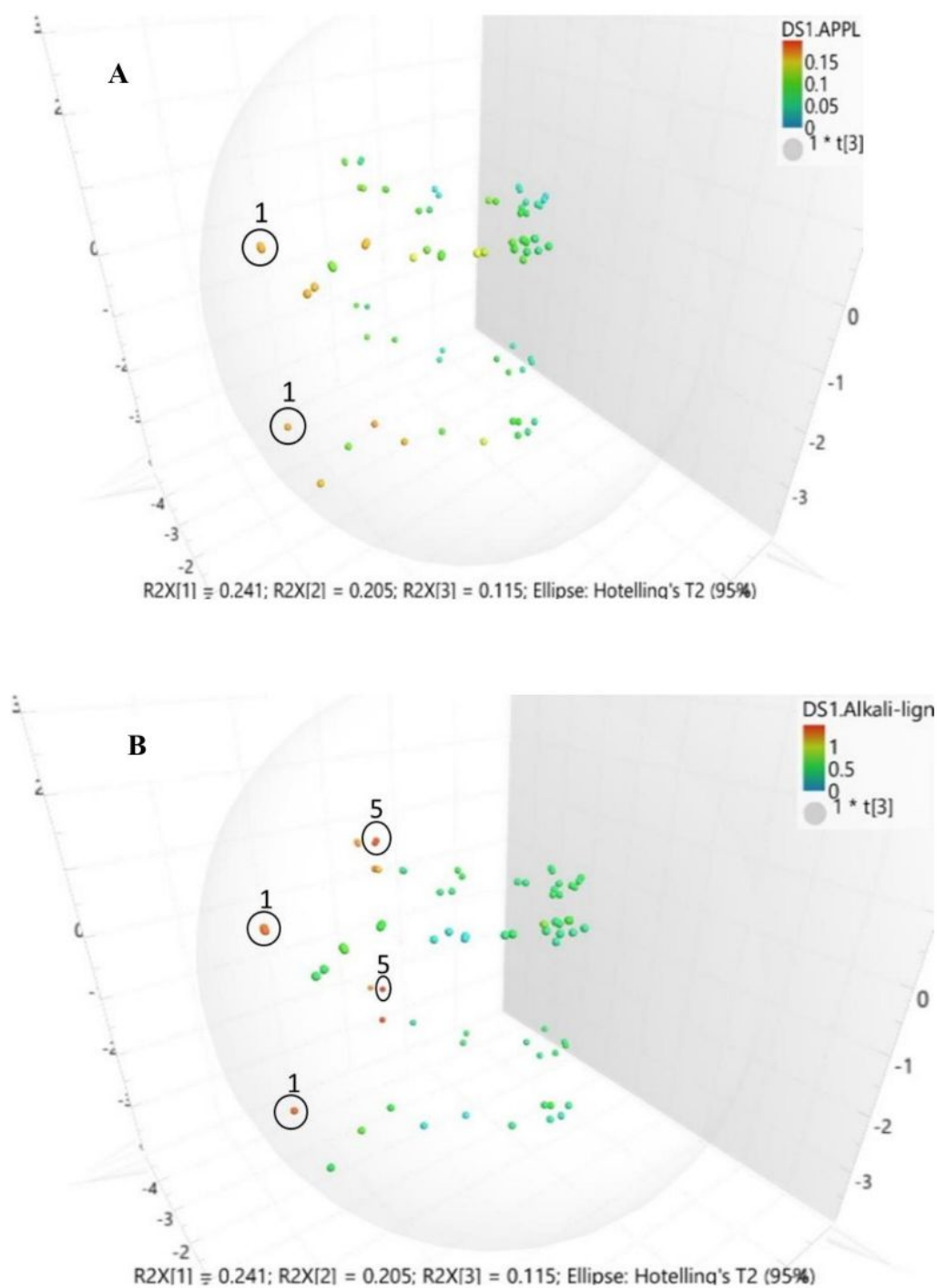
Figure 4

3D Scores Scatter plots from the OPLS-DA models shown in Figure 1 and 2. The views are coloured according to the APPL (A) and alkali-lignin (B) variables, respectively (n=3).

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