

Shaping the Extracellular Pectinolytic Activity Profile of *Trametes Hirsuta* Ibk 1569 Through Multi-Response Cultivation Optimization

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Abstract

Background.

Pectinolytic enzyme systems comprise functionally distinct hydrolases, lyases and esterases whose coordinated action is required for efficient pectin degradation. However, optimization of fungal pectinase production is commonly focused on a single enzyme activity, whereas the cultivation conditions required for the simultaneous formation of different components of a pectinolytic complex may not coincide. This study aimed to optimize the nutrient medium composition for the balanced production of a multicomponent pectinolytic system by the basidiomycete *Trametes hirsuta* IBK 1569 under submerged cultivation.

Results.

Response surface methodology based on a Box–Behnken design was used to evaluate the effects of sugar beet pulp concentration, ammonium sulfate concentration and initial pH on endopolygalacturonase, exopolygalacturonase, pectin lyase and pectin esterase activities. The four responses showed distinct factor dependencies and substantially different individual optima, demonstrating that maximization of a single activity did not provide a balanced pectinolytic activity profile. Multi-response optimization using a max–min criterion identified compromise conditions under which the minimum predicted relative response reached 73.3% of the corresponding individual maxima. Experimental validation yielded endopolygalacturonase, exopolygalacturonase, pectin lyase and pectin esterase activities of 64.11 ± 7.18 U/mL, 798.26 ± 72.15 U/L, 3.82 ± 0.49 U/mL and 205.56 ± 17.78 U/L, respectively, with relative prediction errors of 2.81–17.32%. Time-course analysis revealed asynchronous accumulation of the individual activities, and 144 h was identified as a rational cultivation time for obtaining the enzyme complex.

Conclusions.

The results demonstrate that cultivation conditions can be used to shape the functional profile of the pectinolytic system of *T. hirsuta* IBK 1569 and that multi-response optimization is more appropriate than single-response maximization when several complementary enzyme activities are targeted simultaneously. The study also supports the use of sugar beet pulp as a renewable substrate for producing multicomponent fungal pectinase systems.

Background

Pectinases are a functionally heterogeneous group of enzymes responsible for the modification and depolymerization of pectic substances in the plant cell wall. The main components of the pectinolytic complex include polygalacturonases, pectin and pectate lyases, and pectin esterases, which differ in

their mechanisms of action and substrate specificity [1]. Endopolygalacturonases cleave the internal bonds of the polygalacturonic acid chain, exopolygalacturonases sequentially remove terminal galacturonic acid residues, lyases catalyze the depolymerization of esterified pectin or polygalacturonic acid via a β -elimination mechanism, whereas pectin esterases catalyze the de-esterification of pectin [2]. The complementary action of these enzymes determines the efficiency of pectic substance degradation, which underlies their application in fruit juice clarification and extraction, plant tissue maceration, agro-industrial biomass processing, and other biotechnological processes [3]. Therefore, for the production of a complex enzyme preparation, not only the maximum level of an individual pectinase activity but also the relative proportions of the different components of the pectinolytic complex are of technological importance.

The main microbial producers of pectinases remain bacteria and filamentous fungi, particularly representatives of the genera *Aspergillus* and *Penicillium*, for which the conditions for the synthesis of polygalacturonases, lyases, and esterases have been extensively studied [4, 5]. Considerably less attention has been devoted to the pectinolytic enzymes of basidiomycetous macrofungi [6]. At the same time, wood-decaying representatives of this phylum are characterized by well-developed secretory systems adapted to the degradation of complex polysaccharides of the plant cell wall [7].

The biosynthesis of pectinolytic enzymes strongly depends on the composition of the culture medium and cultivation conditions. At the initial stages of optimization, the one-factor-at-a-time (OFAT) approach is often used to assess the effects of individual factors, whereby one parameter is varied while the others are kept constant. Although OFAT makes it possible to determine working ranges of parameters and identify factors with the most pronounced effects, this approach does not allow interactions between the factors to be assessed [8].

For the simultaneous investigation of multiple factors, response surface methodology (RSM) is widely used, combining experimental design, regression modeling, and numerical optimization [9]. In particular, the Box–Behnken design (BBD) enables the evaluation of linear and quadratic effects as well as pairwise interactions among factors with a relatively small number of experiments [10–14]. Similar approaches have been successfully applied to optimize the production of fungal enzymes [15–18], including studies investigating the effect of medium composition on pectinase production [19–21].

Nevertheless, most studies on the optimization of pectinase biosynthesis have focused on maximizing a single target response, such as polygalacturonase, lyase, or esterase activity [22–24]. For pectinases from basidiomycetous macrofungi, statistical optimization has been applied in only a limited number of studies, including those investigating the production of pectin methylesterase, polygalacturonase, and pectin lyase by *Schizophyllum commune* [25, 26]. In our previous studies, *T. hirsuta* IBK 1569 was selected as a promising producer of pectinolytic enzymes, and the main conditions for its submerged cultivation were determined using the OFAT approach [27, 28]. At the same time, these studies revealed a fundamental feature of the producer: EndoPG and PL responded differently to changes in medium composition and pH. This finding highlighted the need for an approach that would enable the combined

effects of these factors on the enzyme complex to be investigated rather than assessing individual factors separately.

In the presence of multiple potentially conflicting responses, multi-response optimization is appropriate for identifying an optimal combination of factors that simultaneously ensures high levels of all target parameters [29, 30]. This approach is particularly relevant for the formulation of multicomponent enzyme preparations [31, 32].

Despite the available data on the optimization of fungal pectinase production, the simultaneous formation of several functionally distinct components of the pectinolytic complex, particularly in Basidiomycota, remains insufficiently studied [6]. In particular, limited information is available on how culture medium composition affects the simultaneous formation of endopolygalacturonase, exopolygalacturonase, pectin lyase, and pectin esterase activities. Moreover, the optimal conditions for maximizing the production of individual pectinolytic enzymes may differ [25, 27, 28], which limits the applicability of single-response optimization in the development of a complex enzyme preparation. Since the complete degradation of pectin requires the complementary action of enzymes with different mechanisms of action, both high levels of individual activities and their relative proportions are important for obtaining a complex enzyme preparation [32, 33].

Therefore, the aim of this study was to optimize the nutrient medium composition for the balanced pectinolytic activity profile of *T. hirsuta* IBK 1569 under submerged cultivation.

Methods

Fungal strain and inoculum preparation

The object of study was *T. hirsuta* IBK 1569 deposited in the IBK Mushroom Culture Collection of the M.G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine. The strain is stored on agarized malt extract agar [34]. The inoculum was prepared on a glucose-peptone-yeast medium (supplied by Khimlaborreaktiv, Ukraine) according to [35].

Cultivation conditions

Submerged cultivation was performed under conditions established in our previous one-factor-at-a-time experiments [27, 28]: cultivation temperature – (28 ± 1) °C, agitation speed – 120 rpm, cultivation time – 9 days, and inoculum size – 5% (v/v). Dried and ground sugar beet pulp (Sugar factory in Rokytne, Ukraine) with a particle size of 1–3 mm was used as the carbon source, while ammonium sulfate (supplied by SpheraSim, Ukraine) was used as the nitrogen source. The culture medium contained 1.0 g/L KH_2PO_4 (supplied by Khimlaborreaktiv, Ukraine) and 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (supplied by Khimlaborreaktiv, Ukraine) as mineral components. The concentrations of sugar beet pulp and

ammonium sulfate, as well as the initial pH, were varied according to the statistical optimization design. The culture media were sterilized at 121°C for 20 min.

Following experimental validation of the optimized culture medium composition, the dynamics of pectinolytic activities, dry biomass accumulation, protein content, pH, and reducing substances were investigated during cultivation of *T. hirsuta* IBK 1569.

Box–Behnken design and response surface modelling

Response surface methodology (RSM) was applied for the optimization of the culture medium composition using a three-factor Box–Behnken design (BBD) according to [36]. The factor levels used in the design are presented in Table 1.

Table 1
Optimization factors and their values used in Box-Behnken design

Factor	Factor designation	−1	0	+ 1
Beet pulp concentration, %	X_1	0.5	1.0	1.5
Ammonium sulphate concentration, g/L	X_2	0.5	1.0	1.5
pH	X_3	5.0	6.0	7.0
After cultivation, the culture broth was separated from the mycelium and insoluble sugar beet pulp residues by filtration. The resulting culture filtrate was used to determine pectinolytic activities. Endopolygalacturonase (EndoPG), exopolygalacturonase (ExoPG), pectin lyase (PL), and pectin esterase (PE) activities were used as model responses. The experimental data were analyzed using regression analysis and response surface methodology. The experimental responses were fitted to a second-order polynomial model including linear, quadratic, and two-factor interaction effects of the investigated variables. The general form of the regression model was:				

$$y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_{ii}^2 + \sum_{i < j} \beta_{ij} X_i X_j$$

1

where Y is the predicted enzyme activity, β_0 is the intercept, β_i is linear coefficient, β_{ii} is quadratic coefficient, and β_{ij} is interaction coefficients, X_i та X_j are coded values of independent variables [36].

Multi-response optimization and experimental validation

In addition to determining the individual optima for each response, multi-response optimization was performed to establish the optimal culture medium composition that simultaneously ensured high levels of all four pectinolytic activities. Multi-response optimization was carried out using the max-min

approach with response normalization [29, 30, 37] (Bera & Mukherjee, 2012; Derringer & Suich, 1980; Kim & Lin, 2000). For each point within the investigated factor space, the predicted activities of EndoPG, ExoPG, PL, and PE were calculated using the corresponding regression equations. Since all four responses were to be maximized and differed in numerical ranges and units of measurement, the predicted activity of each enzyme was normalized relative to its individual maximum predicted value within the investigated region:

$$R_i = \frac{\hat{Y}_i}{\max \hat{Y}_i} \cdot 100\%$$

2

where R_i is the relative predicted activity of the i -th enzyme; $\hat{Y}_i$ is the predicted enzyme activity at a given combination of factors.

The optimal combination of factors was defined as the one for which the minimum relative activity among all four components of the complex reached the maximum possible value:

$$\max[\min[R_{EndoPG}, R_{ExoPG}, R_{PL}, R_{PE}]]$$

3

This approach was used to determine a rational culture medium composition that ensured balanced activity profile of EndoPG, ExoPG, PL, and PE.

Numerical optimization was performed by dividing the coded range of each factor from -1 to $+1$ into 40 equally spaced levels ($40^3 = 64,000$ combinations evaluated). Thus, the selected conditions represented a rational solution that maintained the highest possible balanced profile of all four pectinolytic activities rather than the absolute maximum of a single component of the complex.

The conditions determined by individual optimization of the responses and by the max-min approach were experimentally validated in an additional series of cultivation experiments. Validation was performed using the predicted optimal concentrations of sugar beet pulp and ammonium sulfate and the corresponding initial pH, while the other cultivation parameters were kept as described above.

Pectinolytic enzyme activity assays

Endopolygalacturonase activity (EC 3.2.1.171) was determined based on the decrease in pectin viscosity according to the method described by [38]. The reaction mixture, containing the culture broth and a 0.5% solution of apple pectin (Apfelpektin, Germany) with pH 5.0, was incubated at 30°C for 10 min in an Ostwald viscometer ($d = 0.73$ mm). One unit of endopolygalacturonase activity (EndoPGA, U) was defined as the amount of enzyme that reduces the viscosity of the pectin solution by 30%.

Exopolygalacturonase activity (EC 3.2.1.15) was determined spectrophotometrically according to a modified method described by Alajlani M. M., Gomes E., et al. [39, 40]. The reaction mixture, containing the culture broth and a 1% solution of apple pectin (Apfelpektin, Germany) with pH 5.0, was incubated at 40°C for 10 min. One unit of exopolygalacturonase activity (ExoPGA, U) was defined as the amount of enzyme that catalyzes the formation of 1 μ mol of reducing sugars per minute, as determined by the DNS method.

Pectin lyase activity (EC EC 4.2.2.10) was determined spectrophotometrically according to a modified method described by Çelik S. Y. et al. [41]. The reaction mixture, containing 0.5 mL of culture broth and 1.5% apple pectin (Apfelpektin, Germany) with pH 7.0, was incubated at 30°C for 10 min. Then, 0.1 mL of 1 N NaOH (supplied by Khimlaborreaktiv, Ukraine) was added, and the mixture was incubated in a water bath at 80°C for 5 min and cooled to room temperature. Subsequently, 1.2 mL of 1 N HCl (supplied by Khimlaborreaktiv, Ukraine) and 1 mL of 0.04 M thiobarbituric acid solution (supplied by Khimlaborreaktiv, Ukraine) were added to the mixture, with mixing after each addition. The mixture was incubated in a water bath at 80°C for 5 min. After cooling, the reaction mixture was centrifuged at 3000 rpm for 15 min. The absorbance was then measured at 550 nm against a control. The control consisted of a mixture containing culture broth inactivated at (100 ± 5) °C. One unit of pectin lyase activity (PLA, U) was defined as the amount of enzyme that catalyzes the formation of 1 μ mol of unsaturated galacturonides per minute.

Pectin esterase activity (EC 3.1.1.11) was determined titrimetrically according to a modified method described by [38]. The reaction mixture, containing the culture broth as the enzyme source and a 1% solution of apple pectin (Apfelpektin, Germany) with pH 5.0, was incubated at 30°C for 60 min. The mixture was then titrated with 0.1 N NaOH (supplied by Khimlaborreaktiv, Ukraine) to pH 7.5. One unit of pectin esterase activity (PEA, U) was defined as the amount of enzyme that catalyzes the formation of 1 μ equivalent of acid per minute [42].

Time-course analysis and analytical measurements

Following experimental validation of the multi-response optimum, the time course of cultivation was investigated under the optimized medium composition. EndoPG, ExoPG, PL, and PE activities were monitored together with dry mass of the insoluble fraction, extracellular protein concentration, pH, and reducing sugar concentration.

The dry biomass mass, comprising the mycelium of *T. hirsuta* IBK 1569 and residual sugar beet pulp particles, was determined gravimetrically after drying to constant weight according to [43].

Protein concentration was determined spectrophotometrically using the Lowry method [44].

The active acidity (pH) was determined potentiometrically using a pH/millivolt meter

Sugar concentration was determined spectrophotometrically using 3,5-dinitrosalicylic acid (Thermo Fisher Scientific, USA) according to [45]. Sugar content was calculated from a calibration curve constructed using standard glucose solutions in the concentration range of 0–1 g/L.

Statistical analysis

Statistical analysis and visualization of the optimization results were performed in the Python 3.13 environment using the pandas, NumPy, statsmodels, SciPy, and Matplotlib libraries. Separate second-order regression models were constructed for endopolygalacturonase, exopolygalacturonase, pectin lyase, and pectin esterase activities. Regression coefficients were estimated using the ordinary least squares method with the OLS() function from the statsmodels library.

The statistical significance and goodness of fit of the regression models to the experimental data were evaluated using the coefficient of determination R^2 , adjusted coefficient of determination R_{adj}^2 , Fisher's F-statistic, model p-value, and lack-of-fit (LOF). A model was considered statistically significant at $p \leq 0.05$. Standard errors, t-statistics, and corresponding p-values were calculated for the regression coefficients. The effects of linear and quadratic terms and pairwise factor interactions were assessed using a Type II analysis of variance (ANOVA) with the anova_lm() function. Effects with $p \leq 0.05$ were considered statistically significant.

The agreement between the models and the experimental data was additionally assessed using diagnostic plots, including residuals versus predicted values, experimental versus predicted responses, and Q–Q plots of the residuals. The effects of individual terms in the regression models were visualized using Pareto charts of standardized effects, while the relationships between enzyme activities and the investigated factors were visualized using three-dimensional response surface plots.

For comparison of mean values in the cultivation dynamics experiments, one-way analysis of variance (ANOVA) followed by multiple comparisons using Duncan's multiple range test was performed. Differences were considered statistically significant at $p < 0.05$. The experiments were conducted in three independent replicates, and the results are presented as the mean $\pm$ standard deviation.

LLM disclosure

During manuscript preparation, ChatGPT (OpenAI, GPT-5.6) was used to assist with language editing, manuscript structuring, and stylistic refinement. All scientific content, data, analyses, interpretations, references, and final wording were reviewed and verified by the authors, who take full responsibility for the manuscript.

Results

The activities of the four pectinolytic components varied markedly across the 15 experimental conditions of the Box–Behnken design (Table 2). EndoPG (Y_1 , U/mL), ExoPG (Y_2 , U/L), PL (Y_3 , U/mL) and

PE (Y_4 , U/L) activities in the culture filtrate were used as the responses.

Table 2
Pectinolytic activities of *T. hirsuta* IBK 1569 under the experimental conditions of the Box–Behnken design

Run	X_1	X_2	X_3	Y_1	Y_2	Y_3	Y_4
1	0.5	0.5	6.0	90.62	291.25	3.84	111.11
2	0.5	1.5	6.0	45.07	638.94	1.54	133.33
3	1.5	0.5	6.0	57.18	1018.23	3.63	200.00
4	1.5	1.5	6.0	25.85	876.00	0.97	266.67
5	0.5	1.0	5.0	43.65	387.30	1.37	125.00
6	0.5	1.0	7.0	69.52	634.85	2.85	160.00
7	1.5	1.0	5.0	48.99	957.57	2.30	231.00
8	1.5	1.0	7.0	41.00	1117.57	2.40	222.22
9	1.0	0.5	5.0	57.63	812.10	2.46	208.00
10	1.0	0.5	7.0	65.35	1087.88	3.00	150.00
11	1.0	1.5	5.0	33.32	547.57	0.70	237.00
12	1.0	1.5	7.0	49.65	678.56	1.60	240.00
13	1.0	1.0	6.0	66.28	756.34	2.23	222.22
14	1.0	1.0	6.0	53.92	810.52	1.97	266.70
15	1.0	1.0	6.0	50.20	765.37	1.33	197.00

Changes in the proportions of the investigated factors had a substantial effect on the production of pectinolytic enzymes. EndoPGA values ranged from 25.85 to 90.62 U/mL, ExoPGA from 291.25 to 1117.57 U/L, PLA from 0.70 to 3.84 U/mL, and PEA from 111.11 to 266.70 U/L.

The fitted response surfaces further illustrated these response-specific patterns within the investigated factor space (Fig. 1). For EndoPG (Fig. 1A), increasing the concentrations of sugar beet pulp and ammonium sulfate was associated with a decrease in the predicted activity, whereas an increase in the initial pH had a less pronounced positive effect. In contrast, for ExoPG (Fig. 1B), the most pronounced trend was an increase in activity with increasing sugar beet pulp concentration; an increase in pH also enhanced the response, whereas increasing the ammonium sulfate concentration showed a negative trend.

Pectin lyase activity (Fig. 1C) decreased most markedly with increasing ammonium sulfate concentration. The effect of sugar beet pulp was nonlinear, with higher PLA values occurring

predominantly at lower ammonium sulfate concentrations and higher pH. For PE (Fig. 1D), a different relationship was observed: increasing the concentrations of both sugar beet pulp and ammonium sulfate promoted an increase in activity, while the effect of sugar beet pulp was nonlinear, with a region of high activity forming near the upper end of the investigated range.

Note

BP – beet pulp; AS – ammonium sulphate.

Overall, the fitted surfaces indicated distinct factor–response relationships for the four pectinolytic activities, providing a basis for their subsequent regression analysis and separate optimization.

Second-order polynomial models were fitted separately to each pectinolytic activity, and their statistical characteristics are summarized in Table 3. The models explained a substantial proportion of the observed variation; however, only the PL model reached overall statistical significance at the predefined $p < 0.05$ threshold, while the PE model was close to this level. Lack-of-fit was not significant for EndoPG, PL, or PE, whereas significant lack-of-fit was observed for ExoPG.

Table 3
Statistical characteristics of regression models for pectinolytic activities of *T. hirsuta* IBK 1569

Enzyme	Parameter				
	R ²	Adjusted R ²	F-statistic	p-value	LOF
EndoPG	0.879	0.661	4.033	0.0695	0.45581
ExoPG	0.876	0.652	3.919	0.0734	0.07533
PL	0.914	0.758	5.870	0.0328	0.55154
PE	0.894	0.704	4.702	0.0515	0.79880

Accordingly, the models were interpreted primarily as local empirical descriptions of factor–response relationships within the investigated experimental domain. They were subsequently used to identify candidate conditions for individual and multi-response optimization, which were evaluated experimentally.

The standardized effects confirmed that the four pectinolytic activities differed in their responses to the investigated cultivation factors (Fig. 2). EndoPG activity was primarily associated with negative effects of ammonium sulfate and sugar beet pulp concentrations, whereas ExoPG showed an opposite response to sugar beet pulp, with higher substrate concentrations favouring this activity. PL was most strongly negatively associated with ammonium sulfate concentration and also showed a nonlinear response to sugar beet pulp. In contrast, PE was favoured by increasing sugar beet pulp and ammonium sulfate concentrations, with a pronounced quadratic contribution of sugar beet pulp.

The relative importance and direction of the investigated factors were response-specific. In particular, conditions associated with higher EndoPG and PL activities differed from those favouring ExoPG and PE, providing a quantitative basis for the divergence of their model-derived optima. The full second-order polynomial models, including all linear, quadratic, and two-factor interaction terms, were used for all predictions and optimization procedures. For clarity, only statistically significant terms ($p < 0.05$) are shown below; the omitted terms were retained in the fitted models.

$$EndoPGA = 56,7973 - 9,4814 \cdot X_1 - 14,6097 \cdot X_2 + 5,2431 \cdot X_3 - 8,4652 \cdot X_1 \cdot X_3$$

$$ExoPGA = 777,4106 + 252,2030 \cdot X_1 - 58,5809 \cdot X_2 + 101,6844 \cdot X_3$$

$$PLA = 2,3600 - 1,3012 \cdot X_2 + 0,4840 \cdot X_3 + 0,6030 \cdot X_1^2 - 0,4445 \cdot X_1 \cdot X_3$$

$$PEA = 228,6407 + 48,8056 \cdot X_1 + 25,9861 \cdot X_2 - 37,5287 \cdot X_1^2$$

Optimization of the individual responses resulted in clearly different factor combinations (Table 4). The model-derived optima for EndoPG and PL were located at the lower limits of both sugar beet pulp and ammonium sulfate concentrations and at the upper limit of initial pH. ExoPG, in contrast, was favoured by the highest investigated sugar beet pulp concentration combined with low ammonium sulfate concentration and high initial pH. The predicted PE optimum was shifted toward high concentrations of both sugar beet pulp and ammonium sulfate and an intermediate initial pH.

Table 4
Predicted optimal conditions for maximizing individual pectinolytic activities of *T. hirsuta* IBK 1569

Target enzyme	Sugar beet pulp		Ammonium sulfate		pH	
	Coded	Actual, %	Coded	Actual, g/L	Coded	Actual
EndoPG	-1.000	0.50	-1.000	0.50	+ 1.000	7.00
ExoPG	+ 1.000	1.50	-1.000	0.50	+ 1.000	7.00
PL	-1.000	0.50	-1.000	0.50	+ 1.000	7.00
PE	+ 0.793	1.397	+ 1.000	1.50	+ 0.241	6.24

The divergence among these conditions was particularly evident for sugar beet pulp and ammonium sulfate concentrations. Consequently, maximizing one component of the pectinolytic profile necessarily moved the cultivation conditions away from those predicted to favour at least one of the other activities. This incompatibility provided the rationale for subsequent multi-response optimization.

The divergence among the individual model-derived optima indicated that simultaneous maximization of all four pectinolytic activities was not achievable within the investigated factor space. Therefore, a multi-response optimization based on the max–min criterion was applied to identify a compromise combination of cultivation factors.

Each predicted response was normalized to its corresponding individual predicted maximum, and the optimization procedure maximized the lowest relative response among EndoPG, ExoPG, PL, and PE. The resulting feasible region is shown in Fig. 3.

Comparison of the relative enzyme activities under conditions optimized separately for each response showed that maximizing one component of the pectinolytic complex did not ensure the simultaneous maximization of the production of the other enzymes (Fig. 4). Thus, under the conditions optimized for EndoPG or PL, the relative activities of ExoPG and PE were substantially lower, whereas optimization for PE was accompanied by a decrease in the relative activities of EndoPG and PL.

The selected combination of cultivation factors maximized the minimum relative predicted response among the four activities. Under these conditions, the lowest normalized response corresponded to 73.3% of its individual model-derived maximum, indicating a compromise solution in which none of the four activities was strongly suppressed relative to its own predicted optimum.

The model-derived conditions corresponding to the individual and multi-response optima were evaluated experimentally (Table 5). The response-specific conditions generally produced high values of their respective target activities; however, the agreement between predicted and experimental values varied considerably for the non-target responses.

Table 5
Experimental verification of individual optimal conditions for
pectinase activity responses

Response	Enzyme activity		Relative error. %
	Predicted	Experimental	
EndoPG and PL optimum			
EndoPGA	94.6	82.67 ± 10.17	14.43
ExoPGA	685.47	712.32 ± 31.95	3.77
PLA	5.19	4.54 ± 0.38	14.38
PEA	116.32	111.55 ± 17.78	4.28
ExoPG optimum			
EndoPGA	58.7	50.90 ± 4.99	15.33
ExoPGA	1189.88	1132.67 ± 106.22	5.05
PLA	4.3	2.98 ± 0.21	44.42
PEA	213.93	144.44 ± 25.56	48.11
PE optimum			
EndoPGA	34.31	38.74 ± 3.88	11.42
ExoPGA	943.33	682.45 ± 91.77	38.23
PLA	1.47	1.68 ± 0.13	12.52
PEA	269.73	233.33 ± 44.44	15.6
Complex of enzymes optimum			
EndoPGA	67.73	64.11 ± 7.18	5.65
ExoPGA	936.53	798.26 ± 72.15	17.32
PLA	3.71	3.82 ± 0.49	2.81
PEA	216.47	205.56 ± 17.78	5.31

Under the multi-response conditions, all four pectinolytic activities were detected simultaneously, and the relative prediction errors ranged from 2.81 to 17.32%. In contrast, under some response-specific conditions, prediction errors for non-target activities exceeded 40%. Thus, the multi-response solution showed a more consistent agreement between predicted and experimental values across the four measured activities.

These results supported the selected factor combination as an experimentally reproducible compromise within the investigated design space. The validation, however, concerned the simultaneous levels of the four pectinolytic activities and did not assess functional synergy or superiority of the resulting enzyme profile in pectin conversion.

The medium optimized for EndoPG and PL provided the highest EndoPGA and PLA values but was accompanied by the lowest PE activity. The medium optimized for ExoPG resulted in the maximum ExoPGA, whereas the activities of the other enzymes were lower. The highest PEA was observed under the conditions optimized for PE, however, this composition resulted in decreased EndoPG, ExoPG, and PL activities. In contrast, the medium determined using the max-min criterion for the production of the pectinolytic enzyme complex did not provide the maximum activity of any individual enzyme but was characterized by the most balanced pectinolytic activity profile.

Following the determination of the culture medium composition using the max-min criterion, the growth dynamics of *T. hirsuta* 1569 and the production of pectinolytic enzymes during cultivation were investigated (Fig. 5). ExoPG activity increased rapidly up to 144 h of cultivation, when its maximum value of 823.68 ± 26.97 U/L was recorded (Fig. 5). PLA also increased substantially during this period, reaching 2.84 ± 0.37 U/mL at 144 h, while its maximum value was observed later, at 216 h of cultivation (Fig. 5). EndoPGA reached its highest values at later stages of cultivation (Fig. 5), whereas PEA was highest during the early stages of cultivation and subsequently gradually decreased (Fig. 5).

During cultivation, the dry biomass gradually increased from 1.19 ± 0.05 g/L at the beginning of the experiment to 3.46 ± 0.17 g/L at 216 h of cultivation (Fig. 6), after which its values remained at a similar level ($p > 0.05$). Thus, biomass accumulation continued considerably longer than the formation of the maximum levels of most enzymatic activities. The protein content in the culture broth increased during the first 96 h of cultivation, reaching its maximum value on day 4, after which it gradually decreased (Fig. 6).

The initial pH of the medium was 6.21. During the first 96 h of cultivation, the pH decreased to 4.31–4.55, after which it remained in the slightly acidic range (Fig. 6). This change in medium pH indicates active metabolic processes during the initial cultivation period and the development of conditions favorable for the production of pectinolytic enzymes. The content of reducing sugars in the medium increased up to 120–144 h (Fig. 6), which may be associated with the enzymatic hydrolysis of polysaccharide components of sugar beet pulp, after which their concentration decreased, presumably as a result of subsequent consumption by the culture.

Discussion

The present study demonstrates that the four pectinolytic activities produced by *T. hirsuta* IBK 1569 responded differently to changes in sugar beet pulp concentration, ammonium sulfate concentration, and initial pH. This divergence was reflected both in the fitted response surfaces and in the model-derived individual optima. EndoPG and PL were favoured by comparatively low concentrations of the two

medium components, whereas ExoPG required a higher sugar beet pulp concentration and PE was favoured by higher concentrations of both sugar beet pulp and ammonium sulfate. Thus, the pectinolytic phenotype of *T. hirsuta* IBK 1569 cannot be represented adequately by a single aggregate pectinase response, because cultivation conditions that favour one activity may simultaneously reduce another.

Such response-specific behaviour is consistent with the complex regulation of fungal systems involved in pectin degradation. In *Aspergillus niger*, genes encoding different pectinolytic enzymes are controlled by several transcriptional regulators and do not necessarily respond uniformly to the same environmental signals [46]. For wood-decaying basidiomycete fungi, the composition of secreted enzymes has likewise been shown to depend on the substrate and the stage of its utilization, indicating the dynamic nature of the extracellular enzymatic response [47]. Thus, the differences observed among EndoPG, ExoPG, PL, and PE in *T. hirsuta* IBK 1569 may reflect differential regulation of individual components of the secretory system, although the specific mechanisms underlying this process require molecular confirmation.

The enzyme-specific nature of the response was particularly evident with respect to sugar beet pulp concentration. Increasing its content promoted higher ExoPGA and PEA activities, whereas maximum EndoPGA and PLA activities were achieved at a lower substrate concentration. This indicates that increasing the amount of a complex plant-derived substrate does not result in a proportional increase in all components of the pectinolytic complex. Sugar beet pulp contains not only pectic substances but also cellulose and hemicellulose components; therefore, changes in its concentration simultaneously alter the availability of several potential substrates and inducers of enzymatic systems. In *A. niger*, the major polysaccharide components of sugar beet pulp have been shown to be utilized sequentially, while changes in pectin availability are accompanied by a reorganization of the regulation of enzymes also involved in cellulose and hemicellulose utilization [48]. Therefore, the different optima of pectinolytic activities in *T. hirsuta* IBK 1569 may be associated not only with the amount of pectin as an inducer but also with changes in the relative availability of substrate components and the corresponding secretory response of the fungus.

The effect of ammonium sulfate was also enzyme-specific: increasing its concentration adversely affected EndoPGA and, particularly, PLA, whereas the opposite trend was observed for PEA. A similarly differential response of individual pectinases to medium composition has been reported for other fungi. For example, in *Schizophyllum commune*, the optimal conditions for pectin methylesterase and polygalacturonase production differed substantially in terms of pH, temperature, cultivation time, and substrate concentration [25]. In other fungal systems, the nitrogen source and its concentration also substantially affected pectinase activity, although the direction of this effect depended on both the producer and the enzyme [49]. Furthermore, the results obtained here refine the conclusions of previous one-factor-at-a-time studies of *T. hirsuta* IBK 1569, in which 1.0 g/L ammonium sulfate was selected as a rational concentration for further cultivation [27, 28]. When three factors were varied simultaneously, the individual optima for EndoPG and PL shifted, clearly demonstrating that the factor value selected at fixed

levels of the other variables does not necessarily correspond to the optimum in a multidimensional factor space.

Differences among the enzymes were also observed with respect to the initial pH. For EndoPG, ExoPG, and PL, the calculated optima shifted toward pH 7.0, whereas the optimal value for PE was approximately 6.24. In this context, the initial pH should be regarded as a factor affecting the physiological state of the culture and the regulation of enzyme secretion rather than as the biochemical pH optimum of the enzymes themselves. In ascomycetes, it has been well established that medium pH can affect the expression of genes encoding plant cell wall-degrading enzymes, including pectinolytic enzymes, through pH-dependent regulatory systems [50]. However, the specific regulatory mechanism in *T. hirsuta* IBK 1569 was not investigated. During cultivation, the pH rapidly decreased from 6.21 to 4.31–4.55; therefore, the calculated optimum primarily characterizes the initial conditions for culture development rather than the actual medium conditions at the time of maximum enzymatic activity. In the RSM-based optimization of pectin lyase production by *Schizophyllum commune*, an initial pH of 6.0 was among the optimal parameters, which is consistent with our findings regarding the importance of the initial medium pH for the formation of pectinolytic activity [26].

Although the second-order models explained a substantial proportion of the observed variation, only the PL model was statistically significant at $p < 0.05$, while the PE model was close to the significance threshold and the EndoPG and ExoPG models were not significant. Therefore, the calculated optima should be interpreted as local model-derived conditions within the investigated factor space rather than as universally precise predictions. Their practical relevance was assessed by subsequent experimental validation. At the same time, the LOF was statistically non-significant for all responses, indicating no systematic deviation of the corresponding models from the experimental data within the investigated factor space.

Experimental validation confirmed the main pattern identified by the models: optimization of a single response did not provide an optimal profile of the enzyme complex. Under the conditions calculated for ExoPG, the highest ExoPGA value of 1132.67 ± 106.22 U/L was obtained experimentally; however, PLA and PEA activities were substantially lower than those achieved under their respective optimal conditions. Similarly, the medium optimized for PE provided a PEA value of 233.33 ± 44.44 U/L but was accompanied by decreased activities of the other enzymes. Importantly, prediction errors for non-target responses under some individually optimized conditions exceeded 40%, whereas under the multi-response conditions the relative errors for all four activities ranged from 2.81 to 17.32%. This indicates that the compromise region provided more consistent agreement between predicted and experimental responses across the complete activity profile.

Multi-response optimization represents the key distinction between this study and most published studies on the statistical optimization of fungal pectinases. For *S. commune*, for example, RSM was applied to maximize pectin lyase production on citrus waste, yielding an optimum for a single target enzyme [25]. However, for a technological enzyme complex, such an approach is insufficient when the

optima of its individual components are conflicting. Classical approaches to multiple-response optimization involve transforming responses with different scales into comparable functions and searching for an optimal solution [29], whereas the maximin approach is specifically aimed at maximizing the lowest of the normalized responses [30]. The minimum predicted relative response of 73.3% indicates that the selected conditions represented a compromise among the four measured activities rather than the absolute optimum of any individual enzyme. This mathematically balanced activity profile should not be interpreted as evidence of functional synergy or as an application-specific optimal enzyme ratio. Experimentally, the resulting complex exhibited EndoPGA, ExoPGA, PLA, and PEA activities of 64.11 ± 7.18 U/mL, 798.26 ± 72.15 U/L, 3.82 ± 0.49 U/mL, and 205.56 ± 17.78 U/L, respectively. Thus, the advantage of the multi-response optimum was not the achievement of the absolute maximum of any individual activity, but the reproducible formation of a complex in which none of the four components was markedly limited.

The asynchronous maxima of individual pectinolytic activities indicate that the extracellular activity profile changed during cultivation. Such temporal restructuring of extracellular enzyme systems has also been reported for wood-decaying basidiomycetes, although direct confirmation of changes in the secretory protein composition of *T. hirsuta* IBK 1569 would require proteomic or transcriptomic analysis. For *T. hirsuta* st. 072, the secretome composition was shown to depend substantially on the nature of the substrate and cultivation conditions [51], while dynamic transcriptomic and proteomic analyses of *Phlebia radiata* revealed substantial changes in the abundance of individual CAZymes during the utilization of a woody substrate; the expressed genes included, among others, polygalacturonases, pectinesterase, and polysaccharide lyases [47]. Thus, the different temporal maxima of pectinase activities in *T. hirsuta* IBK 1569 are consistent with the temporal remodeling of the enzyme profile characteristic of wood-decaying fungi, although direct confirmation of this mechanism in the studied strain would require secretome analysis or analysis of the expression of the corresponding genes.

The dynamics of the analytical parameters additionally indicated that the accumulation of pectinolytic activities was not directly associated with the total protein concentration in the culture filtrate. The maximum protein concentration was observed at 96 h, whereas some enzymatic activities continued to increase at later stages. This may reflect temporal changes in the composition of the extracellular protein pool; however, without proteomic analysis, it is not possible to distinguish the contributions of the synthesis of individual enzymes, their degradation, or other secreted proteins. The increase in the concentration of reducing substances during 120–144 h, followed by a subsequent decrease, may reflect changes in the balance between the release of low-molecular-weight products resulting from enzymatic substrate degradation and their utilization by the fungus. Sequential utilization of different components of sugar beet pulp has been demonstrated for *A. niger*, supporting the possibility of a similar dynamic during fungal growth on this substrate [48]. However, the DNS method determines the total content of reducing compounds and does not allow their individual composition to be established; therefore, this interpretation remains hypothetical.

Considering the simultaneous levels of the four pectinolytic activities and the duration of the process, 144 h was selected as a rational cultivation endpoint. At this time, ExoPGA reached its maximum value, while the other components of the pectinolytic complex retained substantial levels of activity. Further cultivation promoted an increase in some late-stage activities but simultaneously altered the enzyme profile and extended the production cycle. In the previous OFAT study of *T. hirsuta* IBK 1569, a cultivation period of 9 days was selected for obtaining the enzyme complex, despite the earlier maximum of PLA activity [28]. Following multi-response optimization, this period can be reduced to 6 days, indicating that optimization affected not only the medium composition but also the rational duration of the process.

The results obtained demonstrate that optimization of pectinase production by *T. hirsuta* IBK 1569 cannot be reduced to maximizing a single enzymatic activity, as the individual components of the complex exhibited different factor-dependent and temporal optima. Application of the max-min criterion enabled the identification of rational conditions that simultaneously provided high levels of all four pectinolytic activities and were experimentally validated. At the same time, the optimum identified is local to the investigated factor space and cultivation conditions, while equal weighting of the responses corresponds to the objective of obtaining a balanced rather than specialized enzyme complex. For specific technological applications, the relative contribution of the target activities can be further optimized by assigning them differential weights. Further validation in a laboratory-scale bioreactor is required to assess whether the established activity profile is maintained during process scale-up.

Conclusions

The study demonstrated that optimization of pectinase production by *Trametes hirsuta* IBK 1569 based on a single target response does not ensure a balanced pectinolytic activity profile. The concentrations of sugar beet pulp and ammonium sulfate, as well as the initial pH, affected EndoPG, ExoPG, PL, and PE differently, resulting in distinct individual optima. Application of multi-response optimization using the max–min criterion enabled the identification of compromise conditions under which the minimum predicted level of each of the four activities was no lower than 73.3% of its individual model-derived maximum.

Experimental validation supported the selected multi-response conditions: the obtained activities were 64.11 ± 7.18 U/mL for EndoPG, 798.26 ± 72.15 U/L for ExoPG, 3.82 ± 0.49 U/mL for PL, and 205.56 ± 17.78 U/L for PE, while the relative prediction errors ranged from 2.81 to 17.32%. Analysis of the temporal profiles additionally revealed asynchronous accumulation of the individual pectinolytic activities and allowed 144 h of cultivation to be identified as a rational process duration.

The results demonstrate the feasibility of shifting from the maximization of a single enzymatic response toward the simultaneous consideration of several components of the pectinolytic activity profile. The identified cultivation conditions provide a basis for further evaluation and scale-up of pectinase production by *T. hirsuta* using sugar beet pulp-containing media.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author Contribution

P.Z.: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Visualization, Writing – original draft. I.K.: Conceptualization, Methodology, Supervision, Project administration, Writing – review & editing. All authors reviewed and approved the final manuscript.

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Availability of data and materials

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

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Figures

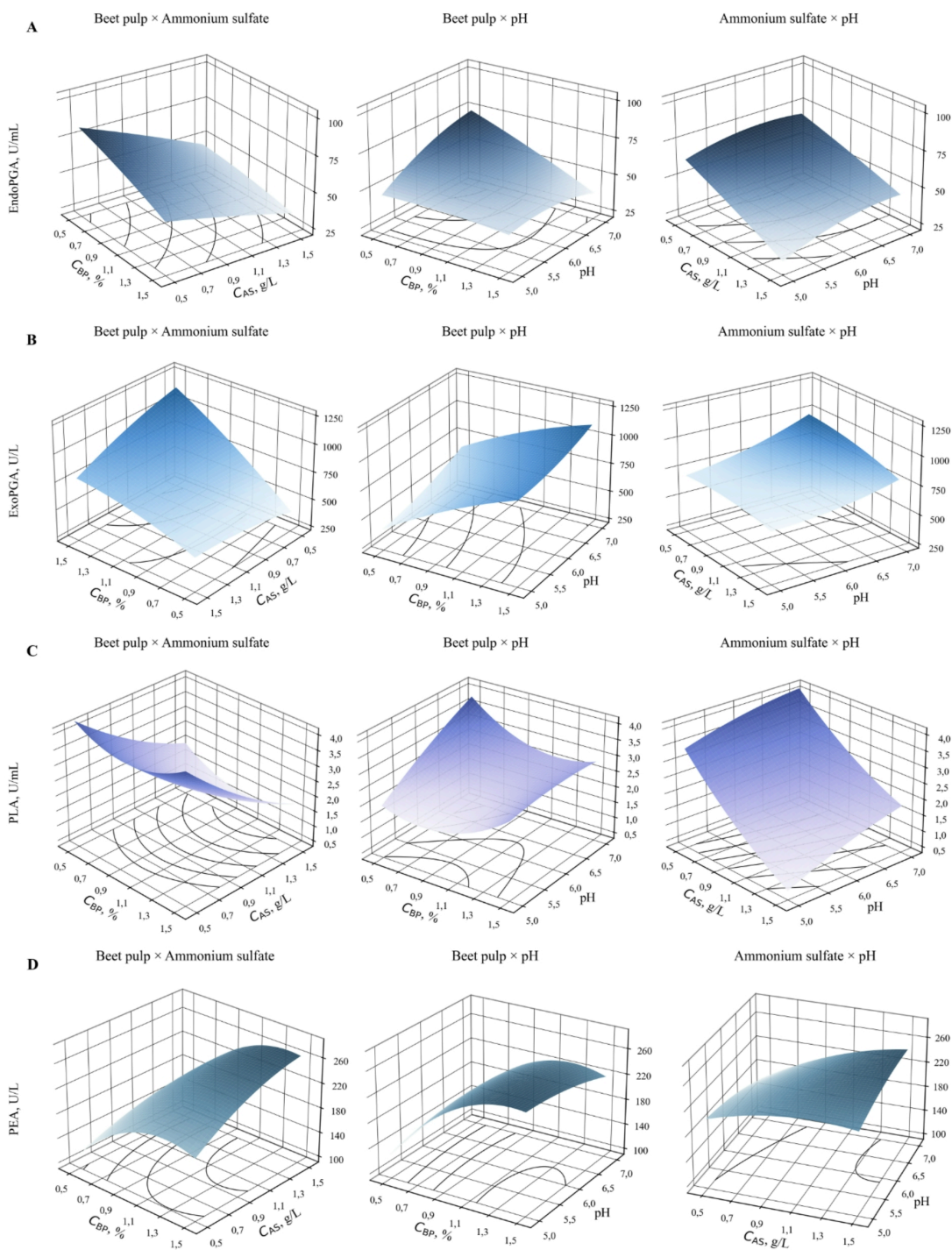


Figure 1

Response surface plots for pectinase activity of *T. hirsuta* IBK 1569 obtained using the Box–Behnken design: A – EndoPGA; B – ExoPGA; C – PLA; D – PEA

Note:BP – beet pulp; AS – ammonium sulphate.

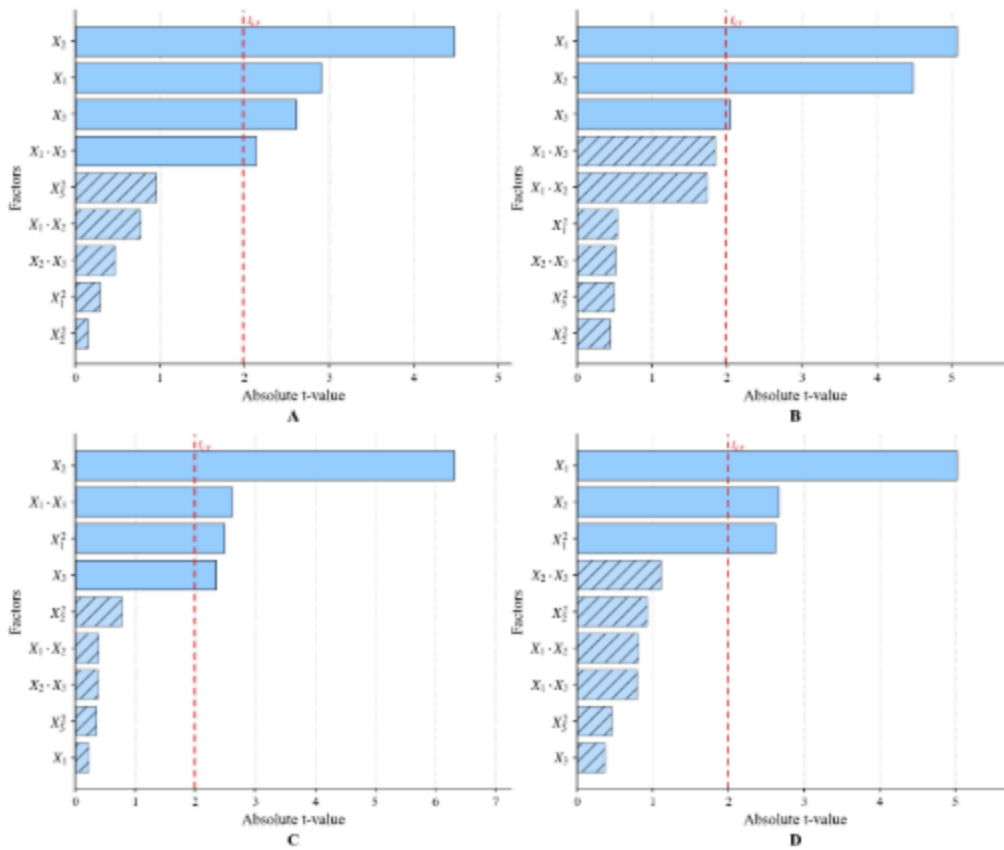


Figure 2

Pareto charts of standardized effects of sugar beet pulp concentration, ammonium sulfate concentration, and initial pH on the pectinolytic activities of *T. hirsuta* IBK 1569: A – EndoPGA; B – ExoPGA; C – PLA; D – PEA

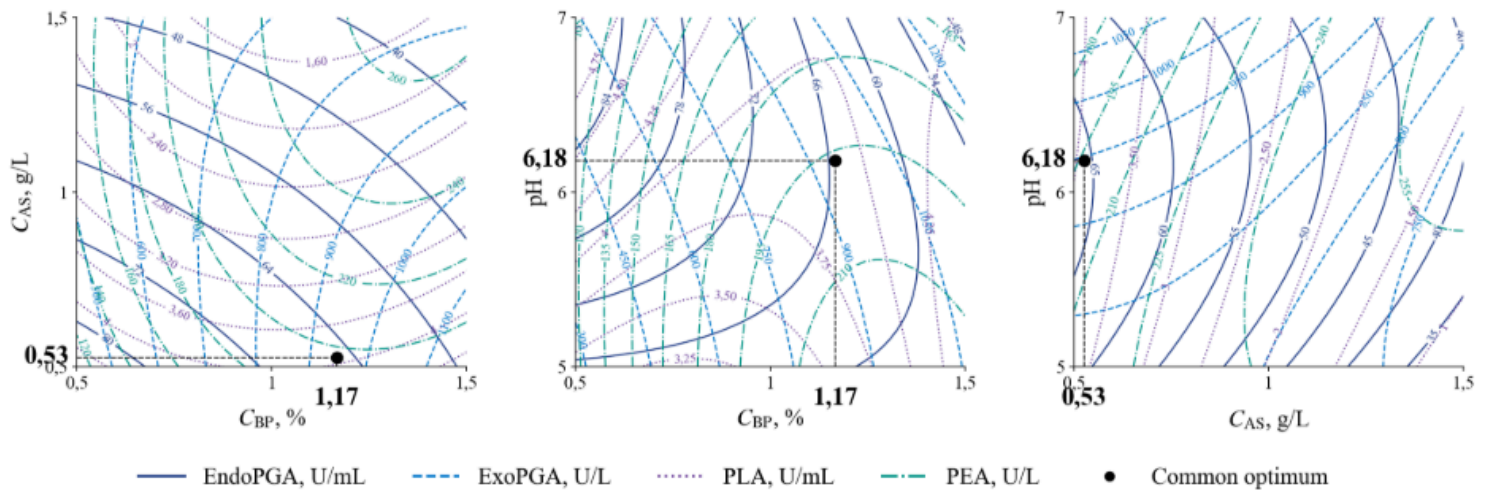


Figure 3

Overlay contour plots for simultaneous optimization of pectinolytic enzyme activities

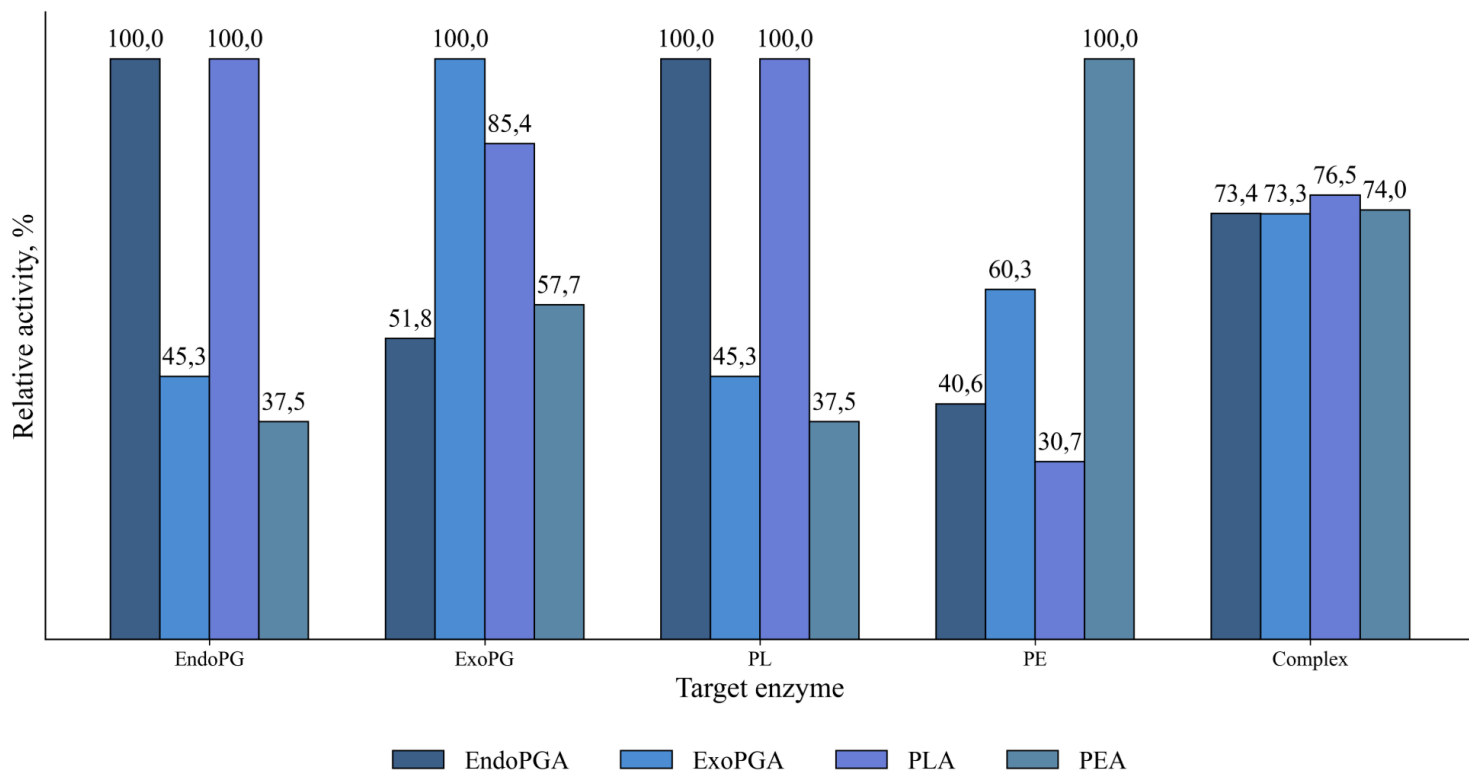


Figure 4

Relative predicted pectinolytic activities of *T. hirsuta* IBK 1569 under individual and multi-response optimized conditions

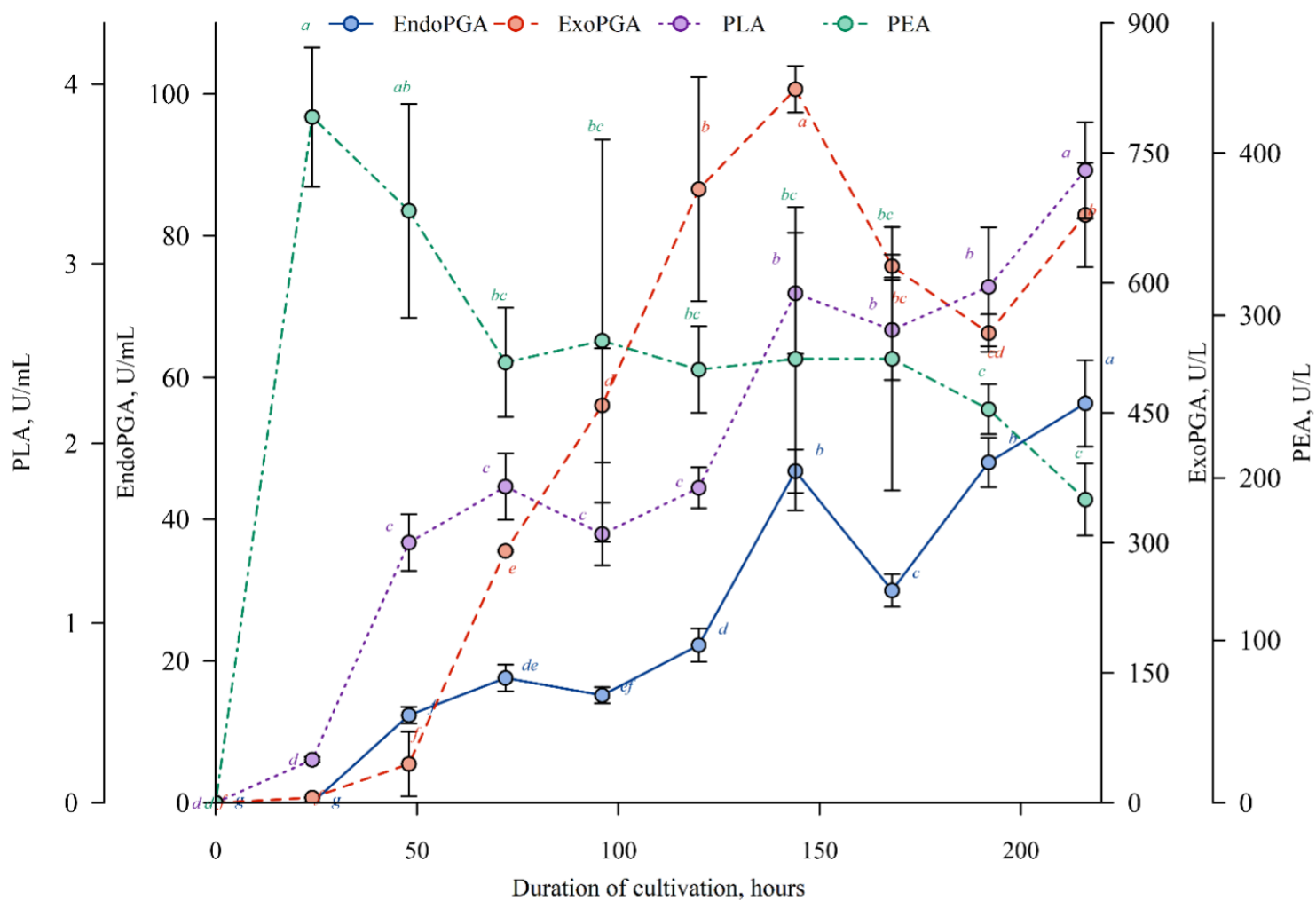


Figure 5

Time course of pectinolytic activities of *T. hirsuta* IBK 1569 under optimized cultivation conditions

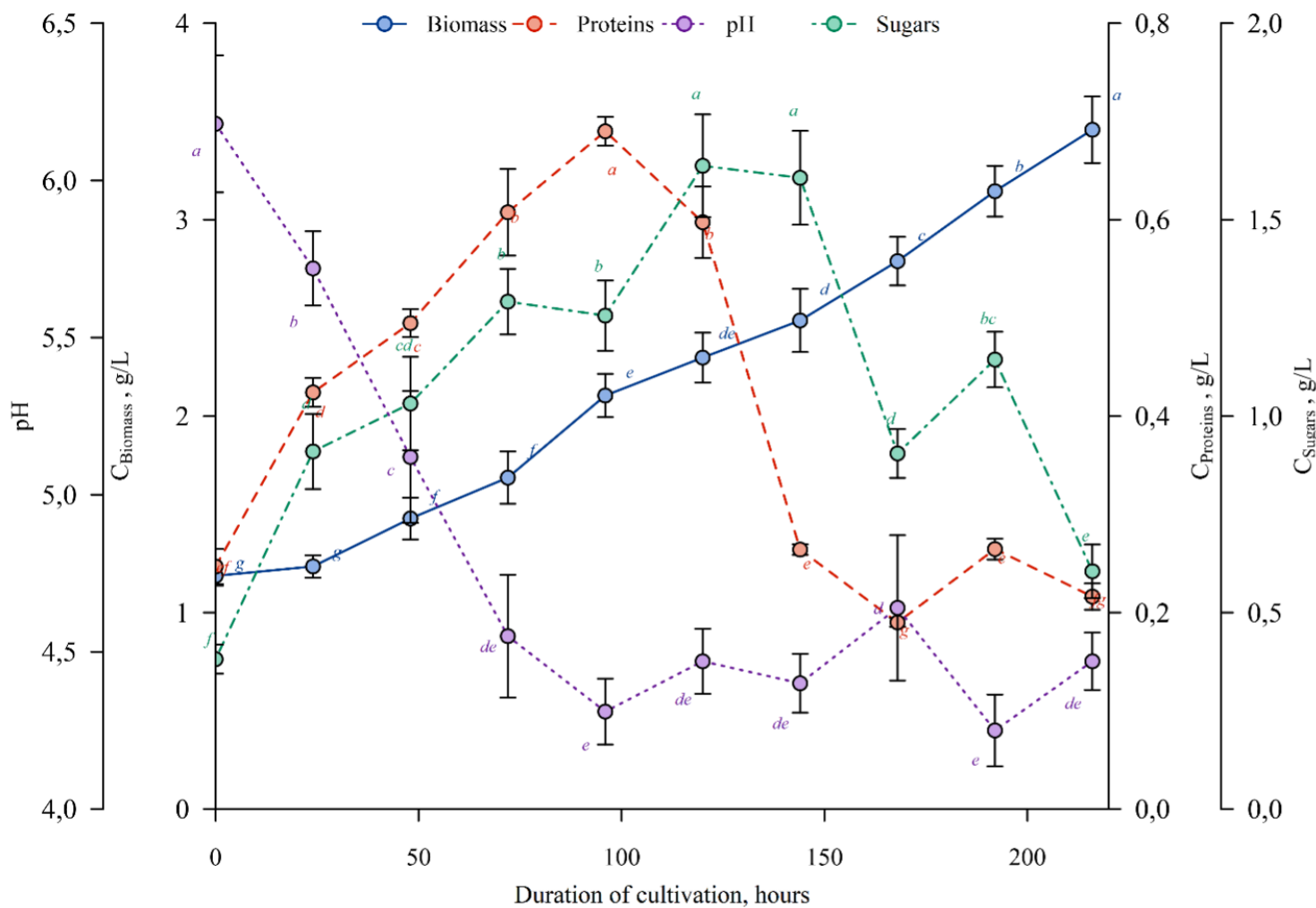


Figure 6

Time course of culture parameters during cultivation of *T. hirsuta* IBK 1569 on the optimized medium