A CCRD STUDY ON CHITINASE AND PROTEASE PRODUCTION BY THE ACARICIDAL FUNGUS *HIRSUTELLA THOMPSONII*

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ABSTRACT

This study investigated the impact of varying carbon, nitrogen, and initial pH levels on chitinase and protease production by an acaricidal filamentous fungus Hirsutella thompsonii using a central composite rotatable design (CCRD) during liquid fermentation. Carbon concentrations ranged from 5 to 30 g/L, nitrogen from 1 to 15 g/L, and initial pH from 4 to 8. Responses measured included chitinase production (U/mL) and protease production (U/mL) over 30 days. Chitinase production peaked under nuetral pH pH and high carbon conditions, reaching up to 41,79 U/mL, while protease production was optimized at neutral pH and high nitrogen levels, resulting in a production of 0,19 U/mL. ANOVA confirmed model significance, with determination coefficients (R^2) of 77,51 for chitinase production, and 72,04 for protease production. These findings underscore the importance of optimizing environmental conditions to enhance enzyme production by filamentous fungi, offering valuable insights for industrial biotechnology and efficient bioinput production processes.

Keywords: Central composite design. Filamentous acaricidal fungus. Chitinase. Protease. Liquid fermentation.

1 INTRODUCTION

The production of enzymes, particularly chitinase and protease, by filamentous fungi such as Hirsutella thompsonii, assumes a critical role not only in the fungal life cycle but also in its interaction with target organisms, such as mites. Chitinase, an enzyme that catalyzes the hydrolysis of chitin, a major component of the exoskeleton of arthropods including mites, is pivotal for fungal infection. By degrading the chitinous barriers of the mite's exoskeleton, chitinase facilitates fungal penetration and colonization of the host. Proteases, on the other hand, play a crucial role in the breakdown of host tissues, aiding in nutrient acquisition by the fungus during colonization.

Understanding the intricate mechanisms underlying enzyme production by *Hirsutella thompsonii* is essential for harnessing its potential as a biocontrol agent against mite infestations. By exploiting the enzymatic capabilities of this fungus, researchers aim to develop sustainable alternatives to chemical pesticides for managing mite populations in agricultural and urban environments. Unlike traditional chemical agents, biocontrol agents such as H. thompsonii offer environmentally friendly solutions that pose minimal risk to non-target organisms and ecosystems.

The utilization of *H. thompsonii* as a biocontrol agent holds considerable promise due to its specificity, efficacy, and environmentally benign nature. By leveraging the enzymatic arsenal of this fungus, biocontrol strategies can be developed to target specific pests while minimizing ecological disruptions. Moreover, the development of formulations incorporating H. thompsonii and its enzymes offers innovative approaches for integrated pest management, providing growers with effective tools for sustainable crop protection.

In this context, the present study delves into elucidating the effects of carbon, nitrogen, and initial pH levels on the fungal concentration and enzyme production of an acaricidal filamentous fungus. Utilizing a central composite rotatable design (CCRD), which enables the exploration of complex interactions between variables, we aimed to model these effects and identify optimal conditions for liquid fermentation. By systematically varying these key parameters within predetermined ranges, we aimed to uncover insights into the intricate relationship between environmental factors and enzyme production. Ultimately, our findings could contribute to advancing the field of biotechnology and facilitating the development of sustainable solutions for various applications, including biocontrol agents and biofertilizers.

2 MATERIAL & METHODS

The variables and their levels were systematically varied according to the CCRD. Sucrose served as the carbon source, while soybean extract was used as the nitrogen source, with initial pH being adjusted as per the experimental design. The design incorporated a total of 16 experimental runs, including two central points for replication. Liquid fermentation was carried out in Erlenmeyer flasks containing the formulated basal (with KCl, $MgSO_4$, K_2HPO_4), and the fermentation process was monitored over a period of 30 days.

Samples were collected on days 3, 4, 5, 6, 7, 10, 15, and 30 to assess chitinase activity and protease activity. Chitinase activity was measured using the DNS (3,5-Dinitrosalicylic Acid) method (Miller,1959)[1], where the culture supernatant was reacted with chitin substrate, and the reducing sugars released were quantified using DNS reagent, with absorbance measured at 540 nm.

Protease activity was assessed using the Folin-Ciocalteu reagent method (Cupp-Enyard,2008)[2], where the culture supernatant was incubated with a casein substrate, and the tyrosine released was reacted with Folin-Ciocalteu reagent, with absorbance measured at 660 nm.

The experimental data were analyzed using analysis of variance (ANOVA) to determine the significance of the effects of the independent variables on the response variables. Response surface methodology (RSM) was employed to develop models and generate response surface plots for visualization of the effects. Optimal conditions for maximum chitinase, and protease activities were identified through desirability function optimization.

3 RESULTS & DISCUSSION

The highest chitinase production was observed in treatment T10, which used 38.5 g/L of sugar, 8 g/L of soybean extract, and pH 6, reaching 6,351.35 U/mL on day 30. These results indicate that chitinase production is favored by high carbon concentrations and neutral pH. The activity peak was observed at 15 days, as expected for this type of fermentation. Other treatments, such as T12 (17.5 g/L of sugar, 20 g/L of soybean extract, pH 6), also showed high activity, reaching 9,594.59 U/mL on day 15, highlighting the influence of carbon and nitrogen concentration.

Treatments with extreme pH (T02 and T04 with pH 8) showed smaller variations, suggesting that a higher pH is not ideal for chitinase production. This corroborates the need for a more neutral environment for maximum enzymatic activity.

Protease production peaked under neutral to slightly alkaline pH conditions and high nitrogen concentrations. In treatment T12, with 17.5 g/L of sugar, 20 g/L of soybean extract, and pH 6, the maximum protease production was 0.195 U/mL on day 7. This suggests that the fungus favors a nitrogen-rich environment for protease production. The production time was optimized at 7 days, with treatments such as T04 (5 g/L of sugar, 15 g/L of soybean extract, pH 8) and T08 (30 g/L of sugar, 15 g/L of soybean extract, pH 8) showing high levels of enzymatic activity.

Other treatments, such as T06 (30 g/L of sugar, 1 g/L of soybean extract, pH 8), also achieved significant protease production but showed consistent production over time. In contrast, treatments like T13 (17.5 g/L of sugar, 8 g/L of soybean extract, pH 2.5) resulted in no protease production over the 30 days, highlighting the importance of pH and nitrogen concentration.

The tables below show the analysis of variance (ANOVA) for chitinase and protease production data, confirming the significance of the models. Table 1 summarizes the ANOVA results for chitinase production, while Table 2 does so for protease production:

Table 1: analysis of variance for chitinase production in 15 days.

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	Fcal	p-value
Regression	4753,2	9	528,1	6,8	0,00012
Residual	1376,8	20	68,8		
Lack of fit	1368,5	5	273,7	467,7	0,0
Pure Error	8,3	15	0,6		
Total	6130,0	29			
R^2 = 77,51%					

Table 2: analysis of variance for proteases production in 7 days.

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	Fcal	p-value
Regression	3777,8	9	419,8	5,7	0,00057
Residual	1466,1	20	73,3		
Lack of fit	1458,0	5	291,6	537,9	0,0
Pure Error	8,1	15	0,5		
Total	5243,9	29			
R^2 = 72,04%					

ANOVA showed that the sum of squares for regression was 4753.2 for chitinase and 3777.8 for protease, with statistical significance in both cases (p-value < 0.001). The coefficient of determination (R^2) was 77.51% for chitinase and 72.04% for protease, suggesting that the models explain the variability in enzyme production well.

The results indicate that chitinase production by *H. thompsonii* is favored by high carbon concentrations and neutral pH, while protease production is optimized in nitrogen-rich environments and neutral to slightly alkaline pH. These environmental conditions are crucial for maximizing the production of these enzymes.

The statistical analysis, despite confirming the significance of the models, revealed a notable lack of fit, suggesting that other factors beyond those considered (carbon, nitrogen, pH) may influence enzyme production. Additional factors such as aeration, temperature, and medium composition could improve the accuracy of the models.

4 CONCLUSION

In conclusion, optimizing environmental conditions is crucial to maximize chitinase and protease production by *H. thompsonii*, providing fundamental insights for industrial biotechnology and efficient bioinput development. These results highlight the importance of precisely adjusting factors such as pH, carbon and nitrogen concentration to achieve maximum levels of enzyme activity, promoting more effective applications in the biotechnology industry.

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