JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

OPTIMIZATION OF ENZYMATIC PRODUCTION OF RHIZOPUS OLIGOSPORUS USING RESPONSE SURFACE METHODOLOGY

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https://doi.org/10.55251/jmbfs.10790

ARTICLE INFO

Received 12. 4. 2023
Revised 22. 4. 2025
Accepted 19. 5. 2025
Published 1. 8. 2025

Regular article

OPEN ACCESS

ABSTRACT

In the present study, we aimed to optimize the levels of abiotic factors involved in maximizing the production of extracellular fermentative *Rhizopus oligosporus* (*R. oligosporus*) enzymes using substrates such as soy and dextrose. In the first trial, we determined the effect of environmental factors on *R. oligosporus* growth. The redundancy analysis showed that three levels, namely temperature (X_1), carbon dioxide concentration (CO_2; X_2), and light intensity (X_3), which accounted for 34.3% of the variation observed in the organoleptic characteristics of micelles, including color and spore quantity. In the second trial, a three-level and three-factor Box–Behnken design with one replicate was used to study the interaction of the significant abiotic factors (X_1, X_2, and X_3) with alkaline protease (AlkP; Y_1), acid protease (AcP; Y_2), lipase (LiP; Y_3), and carbohydrase (Cbh; Y_4). Three levels, namely temperature (28, 30, and 32 °C), CO_2 concentration (385, 436, and 494 ppm), and light intensity (0, 53, and 100 lux), were optimized using the response surface methodology. Analysis of variance showed that X_1 and X_3 were significant factors for the yield of AlkP, AcP, and Cbh. X_1 and X_2 significantly affected the LiP yield. The desirability surface plot indicated that the optimal yields of AlkP, AcP, and Cbh (0.557, 0.387, and 0.098 U/mg, respectively) were recorded at 25 °C and 0 lux. A LiP yield of 0.0752 U/mg was obtained at 25 °C of temperature and 494 ppm of CO_2 concentration. Taken together, the present study provides conditions for the optimal production of *R. oligosporus* enzymes using soy and dextrose as substrates. The present data can be used to improve soy fermentation in the search for protein-rich foods with excellent digestibility.

Keywords: *Rhizopus oligosporus;* response surface methodology; alkaline and acid proteases; lipase; carbohydrase

INTRODUCTION

In recent years, interest in high-quality plant foods as substitutes or supplements for animal protein sources is growing, and studies have shown that consuming plant-based proteins can prevent cardiovascular diseases and regulatory changes in the intestinal microbiota (**Tong *et al*., 2021; Qiao *et al*., 2022**). Soy is an abundant and valuable source of plant protein that adequately contains essential amino acids, and its consumption may reduce blood cholesterol by means of peptides released during digestion that intervene in cholesterol binding to bile acids, inhibit cholesterol solubilization in micelles, and stimulate low-density lipoprotein receptor transcription (**Yamatani *et al*., 2009; Boachie *et al*., 2018; Zhu *et al*., 2023**). This multitude of studies has emphasized the health benefits of soy; nevertheless, some contrasting results have been reported for soy as a protein source. Compared with other proteins, the soy protein exhibits lower digestibility because peptides released during digestion hydrophobically interact with each other and for aggregates (**Tan *et al*., 2023**). Furthermore, a soy-based diet can be disadvantageous as it may contain anti-nutritional factors (**Becker-Ritt *et al*., 2004**). Microbial enzymes can attenuate these anti-nutritional factors in soybeans during fermentation.

Various microorganisms, including lactic acid-producing bacteria and fungi, have been used in fermentation to improve the low digestibility of soy to increase its acceptability (**Chen *et al*., 2023; Drabo *et al*., 2023; Xie and Gänzle, 2023).** Some species of fungi from the Mucoraceae family, such as *Rhizopus oryzae* and *Rhizopus oligosporus* (*R. oligosporus*), have been used as starters in solid-state fermentation to increase the nutritional and bioactive value of the soy protein in order to expand its possible applications in the food industry (**Feng *et al*., 2005; Drabo *et al*., 2023**). *R. oligosporus* produces soybean tempeh (**Rusmin and Ko, 1974**). Biochemical changes during fermentation crucially affect the nutritional quality of the final product. These fungi form a metabolic complex and produce several enzymes that allow them to utilize diverse nutrients. During processing, the activity of these enzymes modifies the main macronutrients and anti-nutritive components of soy, resulting in food products with greater digestibility. Their mycelia penetrate several layers in the soybean grain, colonizing and solubilizing the interlaminar material via the action of extracellular enzymes such as lipases (LiPs), proteases, phytases, and carbohydrases (Cbhs). Chemical changes occurred due to the fungal-enzymatic action improve the digestibility and availability of the protein and fatty acids in the food, respectively, thereby increasing the nutritional value and shelf life while modifying the sensory properties and resulting in better acceptability (**Drabo *et al*., 2023; Liu *et al*., 2023**).

Fungal enzymes, including amylases, LiPs, cellulases, proteases, and phytases, play a unique role of biocatalysts to accelerate biochemical reactions during fermentation (**Lennartsson *et al*., 2014**), and microorganisms are the preferred source of these enzymes because of their rapid growth rate. Moreover, these enzymes can be extracted from cells and then used to catalyze diverse commercially significant processes (**Raveendram *et al*., 2018; Gullian-Klanian *et al*., 2022**) and can be genetically modified to develop new enzymes with desirable properties or simply for overproduction (**Zhang *et al*., 2019**). Microorganisms used during fermentation and the enzymes they produce should exhibit high plasticity and withstand changing environmental and nutritional conditions. The concentration of such enzymes produced by microorganisms depends mainly on abiotic factors present in a culture medium (**Sharma *et al*., 2017; López-Trujilla *et al*., 2023**). Data on the optimal conditions for enzyme production are varied. Some researchers have reported that the highest protease and LiP activity of *R. oligosporus* occurs after 48 h of incubation at 35 °C and 95%–97% humidity, whereas under the same temperature and humidity conditions, the maximum amylase activity occurs after 36 h of incubation (**Han and Nout, 2000**). Other researchers have reported that temperatures between 35–40 °C improve *R. oligosporus* growth; however, enzyme production in the fungus is optimized at lower temperatures (25 °C), which is possibly related to less enzyme denaturation (**Nahas, 1988**).

A model should be established to optimize parameters that are most relevant to achieving the maximum fungal-enzymatic production. Response surface methodology (RSM) is a widely used statistical method to determine optimal conditions for enzyme production, specifically when several parameters and their interactions can affect the response (**Gullian-Klanian and Terrats-Preciat, 2017; Kayaroganam, 2023**). The RSM approach substantially reduces the number of experiments to study the effect of various parameters and their combination that can result in optimal responses. Thus, herein, we aimed to identify abiotic conditions necessary for optimal *R. oligosporus* biomass production. Subsequently, RSM was used to optimize growth variables associated with *R. oligosporus* to obtain the maximum production of proteases, Cbhs, and LiPs.

MATERIAL AND METHODS

Microbial strain and culture condition

R. oligosporus spores were purchased commercially and activated by spreading the spores on the surface of Petri dishes containing Sabouraud dextrose agar (MCD Lab, #7031) acidified to pH 3.5 with tartaric acid (10%). The Petri dishes were incubated for 6 days at 26 ±1 °C (Thermo Fisher, Massachusetts, USA).

Effect of environmental conditions on *R. oligosporus* growth

The first experiment was conducted to determine the effect of environmental conditions on *R. oligosporus* growth. Five environmental conditions, namely temperature, carbon dioxide concentration (CO_2), formaldehyde concentration (HCOH), light intensity, and humidity, were considered to determine the optimal conditions for the exponential growth of *R. oligosporus*. For each treatment, eight Petri dishes were seeded and incubated for five days under the experimental conditions presented in Table 1.

Table 1. Levels of abiotic factors considered for *Rhizopus oligosporus* growth (n = 8)

Experimental condition	T (ºC)	CO_2 (ppm)	HCOH (mg/m^3)	Light intensity (Lx)	Humidity (%)
1	23.1	394	0.001	100	56
2	27.0	385	0.000	186	52
3	24.0	494	0.024	132	53
4	23.6	436	0.009	22	57
5	37.0	571	0.034	0	54
6	25.0	200	1215	0	54

T = Temperature; CO_2 = Carbon dioxide; HCOH = Formaldehyde.

At the end of the incubation period, the percentage of growth, the number of spores, and the coloration of micelles were determined. The growth percentage was determined according to the percentage of colonies spread on the surface of the agar. The micellar color was described based on three color attributes, namely lightness (L*), hue (h*), and color purity (C*), using a colorimeter (LS171, Linshang, Haishu District Ningbo, China). The spores were counted using a Newbauer chamber under a phase contrast microscope at 40x. The results were expressed in cells/g.Temperature, CO_2 concentration, HCOH, and humidity were recorded using an air quality monitor (DVMZ07235, Sangkee, Shenzhen, China). The light intensity was measured using a luxmeter (HER-408, Steren, México).

Optimization of *R. oligosporus* enzyme production using RSM

The Box–Behnken design (BBD) with one replicate was used to optimize environmental conditions and study the interaction effects on the production of acid protease (AcP; Y_1), alkaline protease (AlkP; Y_2), LiP (Y_3), and amylase (Y_4) produced by *R. oligosporus*. A three-level and three-factor BBD with one replicate was used to study the interaction of significant abiotic factors on enzyme production. Significant variables from the first trial, namely temperature (X_1), CO_2 concentration (X_2), and light intensity (X_3), were optimized using the RSM. A total of 15 experiments were performed in duplicates based on the BBD, and three levels of temperature (28, 30, and 32 °C), CO_2 concentration (385, 436, and 494 ppm), and light intensity (0, 53, and 100 lux) were used as predictors. Further, the production of AlkP (Y_1), AcP (Y_2), LiP (Y_3), and Cbh (Y_4) from *R. oligorsporus* was analyzed. *R. oligosporus* micelles were inoculated in flasks containing 180 mL of dextrose broth and 1.5% soybean and incubated for five days under different experimental conditions. After incubation, enzymes released into the culture broth were collected by filtration using a 0.45-µm membrane filter to remove the cells. The fungal biomass was estimated by weight difference using a control flask and expressed as dry-weight biomass concentration per liter of the medium. The data were used to standardize the milligrams of the fungal protein per milligram of dry weight. The biuret-reaction-based Lowry protein assay was performed to estimate the protein amount in the samples (**Lowry *et al*., 1951**).

Determination of enzymatic activity

Protease activity: AcP activity was determined by mixing 60 µL of the enzyme extract with 1 mL of 0.5% hemoglobin in glycine-HCl (100 mM, pH 3.5) (**Yoshida & Nagasawa, 1956**). After 15 min at 25 °C, 500 µL of 20% trichloroacetic acid (TCA) was added. The extracts were incubated at 5 °C for 15 min and centrifuged at 12,000 × *g* for 5 min. AlkP activity was determined by the caseinolytic method (**Kunitz, 1947**), in which 250 µL of the enzyme extract was mixed with 180 µL of 5% Tris-HCl casein solution (100 mM, pH 7.6) and incubated at 37 °C for 1 h. Subsequently, 280 µL of 10% TCA solution was added and kept at 27 °C for 10 min. The samples were centrifuged at 12,000 × *g* for 3 min at 5 °C, and 80 µL of the supernatant was mixed with 60 µL of NaOH (100 mM). Absorbance was measured at 405 nm by transferring 140 µL of the mixture to a 96-well microplate. AlkP and AcP units were calculated using the equation $\varepsilon = 8800\ M^{-1}cm^{-1}$. One unit (U) of activity corresponded to the amount of enzyme that catalyzes the formation of 1 µg of tyrosine per minute. Specific enzyme activity was expressed as enzyme units per mL divided by the protein concentration.

LiP activity: The common p-nitrophenyl phosphate (pNPP) hydrolysis method was used to evaluate LiP production following the procedure of **Oliveira *et al.* (2014).** An aliquot (100 µL) of each extract was used in which 800 µL of 0.25% polyvinyl alcohol solution at pH 6.5 and 100 µL of pNPP solution (3 mM) in isopropanol were added. The reaction mixture was incubated for 15 min at 30 °C. The reaction was stopped by adding 300 µL of HCl (3 mM). The sample was then centrifuged at 10,000 *xg*, and 500 µL of the supernatant was added to a microtube containing 1 mL of NaOH (2 mM). The absorbance was measured using a visible light spectrophotometer at 410 nm. LiP units were calculated using the equation $\varepsilon = 17500\ M^{-1}cm^{-1}$. One unit (U) of activity corresponded to the amount of enzyme that catalyzes the formation of 1 µmol of pNPP per minute. Specific enzyme activity was expressed as enzyme units per mL divided by the protein concentration.

Cbh activity: The qualitative determination of Cbh activity was performed according to **Vega-Villasante *et al.* (1999)** using a starch solution (1%) as a substrate. The reaction was performed using 10 µL of the extract, 500 µL of Tris-HCL (50 mM, pH 8), and 500 µL of soluble starch (1% in Trs-HCL). The mixture was shaken and incubated for 10 min at room temperature. Subsequently, 200 µL of sodium carbonate (2N) and 1.5 µL of dinitro salicylic acid reagent was added. The tubes were covered and boiled in a water bath for 15 min. Once the boiling time was over, 7.3 mL of distilled water was added (adjusted to 10 mL) and stirred and read at 550 nm. Cbh units were calculated using the equation $\varepsilon = 4950\ M^{-1}cm^{-1}$. One unit (U) of activity corresponded to the amount of the enzyme that catalyzes the formation of 1 µmol of glucose per minute. Specific enzyme activity was expressed as enzyme units per mL divided by the protein concentration.

Data analysis

The software Statistica (StatSoft v.8.0) was used for the BBD and statistical analysis of the experimental data. The functional relationships between the response (Y_n) and the set of predictors (X_1, X_2, and X_3) were inferred by fitting a second-order polynomial quadratic model based on experimental data. In the first trial, redundancy analysis (RDA) was performed to evaluate the statistical effect of each abiotic factor on the characteristics of the micelles. The predictors were accepted with an estimated probability of type I error less than a/Nc (Bonferroni correction), where Nc is the number of independent variables used in the analysis. In the second trial, the significance of each coefficient was evaluated using the F test of the analysis of variance (ANOVA) at a confidence level of 95%. The main effect of the predictors and their interactions were visualized using the Pareto diagram. The quality of fit of the polynomial model equation was determined by calculating the coefficient of determination (R^2) and the local outlier factor (LOF). The desirability analysis was performed to identify the optimization point for each enzyme. Values were optimized on a scale ranging from 0 (undesirable) to 1.0 (highly desirable). Three-dimensional response surface plots were generated by plotting the enzyme concentration against two significant predictors.

RESULTS AND DISCUSSION

Significant predictors of *R. oligosporus* growth

The environmental conditions significantly affected the number of spores. The luminosity and color saturation of the micelles are presented in Table 2. Experimental conditions 1–4 (Table 1) showed favorable sensory characteristics such as low concentration of spores, lighter color (L*), uniform tone (h*), and lower saturation (C*) for defined soy-based fermented foods. The highest concentration of spores occurred at 37 °C, 571 ppm CO_2 concentration, and 0 light intensity (Table 2). The spore concentration decreased significantly at a temperature of 25 °C and CO_2 concentration of 200 ppm (1.64E+07), which were associated with lower vegetative growth. Experimental conditions #1, 2, 3, and 4 presented similar spore production (log 7.0), luminosity, and hue of the micelles.

Table 2 Significant variation of the response variables depending on different experimental growth conditions of *Rhizopus oligosporus* (n = 8)

Experimental condition	5 day-growth (%)	Spores (cell/g)	L*	h*	C*
1	100	1.25E+07a	53.9a	72.7	2.1b
2	100	1.44E+07a	62.3a	78.9	2.9b
3	100	3.47E+07a	62.4a	76.2	1.6a
4	100	6.08E+07a	60.8a	84.7	1.2a
5	100	1.57E+08b	46.8b	75.9	3.6b
6	10	1.64E+07a	64.2a	79.1	3.9c
p-value	*0.0001*	*0.0001*	*0.0001*	*0.686*	*0.005*

The one-way ANOVA test with a significance value of $\alpha = 0.05$, followed by the Tukey test, was used to compare the differences in the response variables. Means presented in the same column with different letters are significantly different. L* = Lightness; h* = Tonality; C*= Chroma, saturation.

L* is the measure of luminosity, and its value varies from 0 for black to 100 for white. C* is the measure of the purity or saturation of a color, which is measured from the center point of the color space where a* = b* = 0 and along a straight line that extends outward from the said point. A color with a high C* value is a highly saturated color. The h* value is the angle that measures the hue, indicating the orientation of the color concerning the 0° origin (**Otles and Ozyurt, 2015**). Generally, micelles do not exhibit specific values of L*, h*, and C*. These values become important when the objective is to achieve sensory characteristics appropriate to a standard. In the presence of more spores, the lightness decreases and the darkness increases, which is why the L* value increases. Herein, no significant differences were observed in tone (h*), with yellow tones dominating. Quality tempeh, without modifications, is white. As tempeh continues to ripen, it turns slightly whitish. Any other color is considered a detriment to quality (**Owens, 2014**). Conditions that increase spore production (#5), decrease luminosity (L*) and increase saturation (C*) are not appropriate to maintain the target sensory characteristics of the micelle.

The contribution of each environmental variable to the characteristics of the micelles is presented in Table 3. The RDA results showed that the environmental variables accounted for 34.3% of the variance in the organoleptic characteristics of the micelles. Temperature, CO_2 concentration, and light intensity together accounted for 26.5% of the variance, where temperature accounted for 17%, CO_2 concentration for 7.4%, and light intensity for 6.5% of the variance. As temperature and CO_2 concentration increased, the spore number increased significantly; consequently, the micelle luminosity decreased (Fig. 1). Increasing light intensity decreased spore production by 23%.

The present results are consistent with previous results showing that fungi are severely affected by physicochemical factors that affect their growth rate (**Mustafa *et al.*, 2023**), and temperature plays a critical role in it. For each fungus, there is a specific temperature range (minimum–maximum); the fungus cannot grow beyond this range, and the optimal temperature lies between these two extremes (**Madan and Thind, 1998**).

Table 3 Forward selection of environmental variables from the redundancy analysis with a significant effect on *Rhizopus oligosporus* growth.

Predictors	Explains (%)	Contribution (%)	F-value	*p*-value
Temperature	17.0	49.5	9.4	0.002
CO_2	7.4	21.6	4.7	0.006
Light intensity	6.5	19.0	3.8	0.024
Humidity	1.9	5.5	1.2	0.336
HCOH	1.6	4.6	1.0	0.408

Stepwise selection was performed to identify statistically significant predictor variables related to response variables.
Response variables: Spores, Lightness; h* = Tonality; C*= Chroma, saturation.

Additionally, CO_2 exerts a series of indirect effects on fungal growth and reproduction (**Stiling and Cornelissen, 2007**). An increase in CO_2 concentration by 3% significantly affects the growth of *Penicillium* sp., whereas the growth of other fungi such as *P. rugulosum*, *Gliomastix convoluta*, and *Mucor rammanianus* is not inhibited even at an increase in CO_2 concentration by 10% (**Taniwaki *et al.*, 2010**). *Rhizopus* spp. microorganisms grow under anaerobic conditions (**Soccol *et al.*, 1994**); nonetheless, increased amounts of CO_2 affect their biomass density (**De Reu *et al.*, 1995**). A study has shown the effect of light intensity on the production of secondary metabolites in some fungi (**Shu *et al.*, 2010**). **Madan and Thind (1998)** reported that the effect of light intensity was divided into two effects as follows: morphogenetic effects, in which light inhibited or induced structure formation, and non-morphogenetic effects, in which light affected structural growth or compound synthesis.

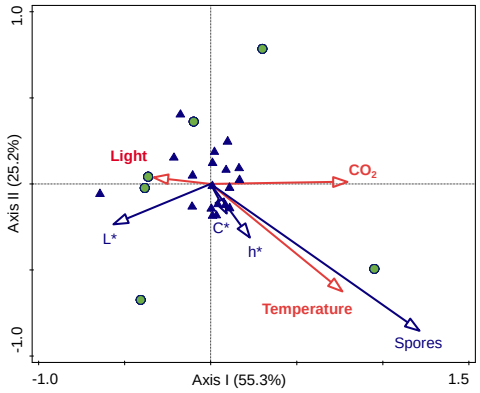

Figure 1 Redundancy analysis shows the association between the predictors and the response variables of *Rhizopus oligosporus* sensorial characteristics. Explained variance for axes I & II are presented in parentheses (F = 4.8; p = 0.001, Monte Carlo test with 900 permutations). Green circles = Centroids of experimental conditions; Blue triangles = Response variable scores; Red arrows = Predictors; and blue arrows = Response variables. The direction of the arrow indicates an increase in the variable values. The angle between the arrows indicates the correlation between the variables.

Enzymatic production of *R. oligosporus* from Box Behnken design

The observed experimental data of enzymatic production in *R. oligosporus* are presented in Table 4. The average values of enzyme production in *R. oligorsporus* based on the environmental variables are presented in Figure 2. The results showed that AlkP concentration was higher, followed by AcP. LiP was the lowest-produced enzyme.

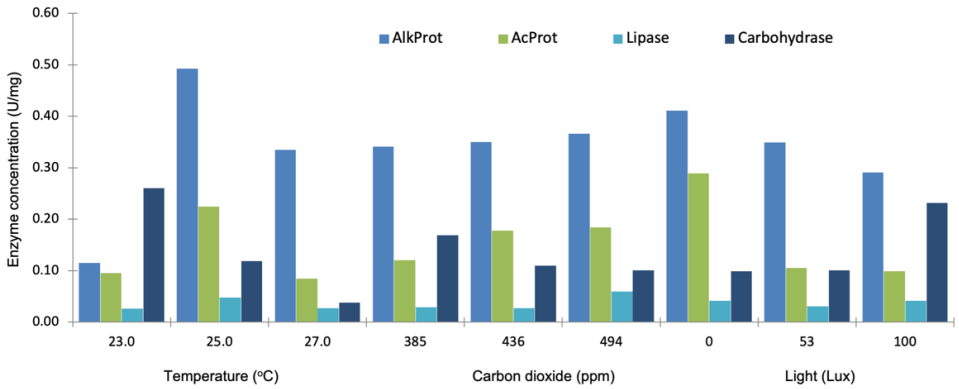

Figure 2 Enzyme production in *Rhizopus oligosporus* according to the level of the environmental variables. Data are obtained using the Box–Behnken design.

Fungal proteases play a fundamental role in several physiological functions and commercial applications (**Christensen *et al.*, 2022**). The present results showed that in the presence of soybeans, *R. oligosporus* produced protease, LiP, and Cbh; however, their concentrations varied depending on environmental conditions. AlkP and AcP were predominantly present in soy protein-based extracts, which agreed with previous studies performed on *R. oryzae* (**Banerjee and Bhattacharyya, 1993**), *R. chinensis* (**Ohtsuru *et al.*, 1982**), and *R. oligosporus* (**Ikasari and Mitchell, 1996**). Proteolysis occurs in *Rhizopus* spp. fungi so that the fungi can survive and grow, and the resulting peptides and amino acids increase the nutritional value of foods, which is helpful for human nutrition. After proteases, high Cbh concentrations were observed, mainly at lower temperatures and higher light intensities. In addition to releasing free peptides and amino acids, the activity of carbohydrate-cleaving enzymes produced by *R. oligosporus* during fermentation is enhanced because of proteolysis. This combination of the microbe and the corresponding enzymes improves the nutritional and flavor properties of fermented soybeans (**Vong *et al.*, 2018**). LiP concentration was the lowest compared with those of other extracellular enzymes; LiP concentration is a function of the fat content of the food. Extracellular LiP production cleaves ester bonds in triglycerides at the interface between the aqueous layer and the oil layer (**Treichel *et al.*, 2010**). The lipolytic activity of the fungus in tempeh contributes to imparting desirable flavors to the fermented food (**Nur *et al.*, 2020).**

Table 4 The Box–Behnken design (response surface methodology; three-level, three-factor, and two-replicates) and observed experimental data for alkaline protease, acid protease, amylase, and lipase obtained from *Rhizopus oligosporus*.

Run	Block	X_1 T (°C)	X_2 CO_2 (ppm)	X_3 Light (mg/m^3)	Y_1 Alk Protease (U/mg)	Y_2 Ac Protease (U/mg)	Y_3 Carbohydrase (U/mg)	Y_4 Lipase (U/mg)
1	1	23.0	436	0	0.154	0.130	0.010	0.114
2	1	23.0	385	53	0.108	0.118	0.011	0.112
3	1	23.0	494	53	0.103	0.025	0.067	0.124
4	1	23.0	436	100	0.100	0.103	0.024	0.289
5	1	25.0	494	100	0.421	0.100	0.098	0.100
6	1	25.0	385	103	0.435	0.099	0.012	0.116
7	1	25.0	436	53	0.511	0.133	0.036	0.114
8	1	25.0	436	53	0.455	0.114	0.034	0.112
9	1	25.0	494	0	0.550	0.528	0.082	0.132
10	1	25.0	385	0	0.532	0.456	0.035	0.137
11	1	25.0	436	53	0.511	0.133	0.036	0.114
12	1	27.0	436	100	0.212	0.070	0.032	0.016
13	1	27.0	436	0	0.433	0.088	0.037	0.013
14	1	27.0	494	53	0.321	0.000	0.012	0.078
15	1	27.0	385	53	0.333	0.132	0.010	0.079
16	2	23.0	436	0	0.134	0.135	0.013	0.116
17	2	23.0	385	53	0.113	0.028	0.065	0.122
18	2	23.0	494	53	0.118	0.113	0.009	0.120
19	2	23.0	436	100	0.089	0.112	0.012	0.286
20	2	25.0	494	100	0.431	0.112	0.088	0.112
21	2	25.0	385	100	0.425	0.111	0.022	0.117
22	2	25.0	436	53	0.512	0.124	0.039	0.120
23	2	25.0	436	53	0.516	0.123	0.037	0.113
24	2	25.0	494	0	0.553	0.518	0.083	0.122
25	2	25.0	385	0	0.522	0.356	0.045	0.134
26	2	25.0	436	53	0.522	0.232	0.025	0.118
27	2	27.0	436	100	0.211	0.086	0.043	0.021
28	2	27.0	436	0	0.412	0.100	0.030	0.020
29	2	27.0	494	53	0.430	0.078	0.035	0.020
30	2	27.0	385	53	0.330	0.122	0.019	0.059

Response surface analysis Alkaline protease (AlkP)

Table 5 presents the ANOVA statistics for AlkP. The regression model showed significant results (p = 0.0006; Table 5). The interaction between two independent variables (X_1 and X_3) was significant for the AlkP yield ($p < 0.05$). Additionally, the linear and quadratic effects of the temperature (X_1) and the linear effect of the light intensity (X_3) were significant ($p < 0.001$). These predictors were extended beyond the reference line of the Pareto chart at $\alpha = 0.05$ (Fig. 3a). The p-value of 0.4759 for the LOF, according to the RSM analysis, implied that the model was insignificant relative to the pure error. The regression coefficient (R^2 = 0.9856) was reasonably in line with the experimental results, indicating that 98.6% of the variability could be measured by the model (Table 5). The overall effect of the temperature and light intensity (X_1 and X_3) on AlkP is shown in the three-dimensional (3D) response surface plot as a representation of the polynomial equation (Eq. 1; Fig. 4a).

$$AlkP = -43.3286 + 3.4345(x) + 0.0093(y) - 0.0672(x^2) - 0.0004(xy) - 7.1186E\text{-}6(y^2) \quad (1)$$

An increase in AlkP was recorded at a low light intensity and medium temperature. The optimal point determined by the desirability surface contour plot indicated that a high AlkP of 0.557 U/mg was obtained when *R. oligosporus* grew at 25 °C, 494 ppm, and 0 lux (Fig. 4a).

Response surface analysis of AcP

Table 5 presents the ANOVA statistics for AcP. The RSM model showed significant results (p = 0.0079), indicating that temperature (X_1) and the light intensity (X_3) were the main factors affecting AcP production (Fig. 3b). The interactive effects between the variables did not change significantly. The p-value of 0.0728 for the LOF implied that the model was not significantly relative to pure error and fitted the data well. According to R^2, 70.78% of the variability was explained by the model. The equation, without considering the non-significant variables, was expressed as follows:

$$AcP = -19.6295 + 1.6027(x) - 0.0055(y) - 0.0321(x^2) + 2.5086E\text{-}5(xy) + 2.8943E\text{-}5(y^2) \quad (2)$$

The effect of temperature and light intensity on AcP production is shown in the 3D response surface plot as a representation of the polynomial equation (Eq. 2). An increase in AcP production was recorded at a medium temperature and low light intensity (Fig. 4b). Based on the desirability score of 1.0, the AcP yield was optimized to 0.387 U/mg under the optimized conditions of 25 °C, 494 ppm, and 0 lux (Fig. 5b).

Table 5 Analysis of variance from the response surface model of *Rhizopus oligosporus* enzyme extract

	Alk Protease (U/mg)			Ac Protease (U/mg)			Lipase (U/mg)			Carbohydrase (U/mg)		
	MS	P-value		MS	P-value		MS	P-value		MS	P-value	
Model		0.0006			0.0079			0.0004			0.0015	
Linear + Quadratic												
X_1 Temperature	0.3657	0.0000	**	0.0571	0.0046	**	0.0014	0.0489	**	0.0306	0.0000	**
X_2 CO_2	0.0005	0.4106		0.0125	0.2319		0.0026	0.0103	**	0.0003	0.8135	
X_3 Lux	0.0304	0.0000	**	0.0995	0.0003	**	0.0003	0.4512		0.0028	0.1846	
Interaction												
X_1X_2	0.0011	0.1973		0.0032	0.5276		0.0000	0.8331		0.0001	0.7648	
X_1X_3	0.0128	0.0002	**	0.0000	0.9349		0.0000	0.9046		0.0139	0.0074	**
X_2X_3	0.0003	0.4539		0.0098	0.2778		0.0004	0.3153		0.0000	0.8759	
Lack of Fit	0.0006	0.4759		0.0094	0.0728		0.0005	0.0055		0.0019	0.0000	
Pure error	0.0005			0.0020			0.0000			0.0000		
R^2 (RSU)	0.9856			0.7078			0.5340			0.7381		

Temperature is a critical parameter for fungal metabolism, and the optimum temperature range at which the maximum cell growth and enzyme production are achieved varies among organisms. The optimization assay showed that protease production significantly depended on temperature and light. For AlkP, 98.5% of the production was dependent on the interaction between the two variables. For AcP, the interaction between the two variables was less significant; however, the independent effect of temperature and light accounted for 70.8% of the variation. Both enzyme groups reached their optimal point under dark conditions and at 25 °C, showing yields of 0.557 U/mg for AlkP and 0.387 U/mg for AcP. Other fungi, such as *Penicillium* sp. and *Aspergillus oryzae* (**Sandhya *et al.*, 2005; Murthy and Naidu, 2010**), required higher temperatures than 25 °C and reached their maximum protease production at 28 and 30 °C. The optimal temperature of 25 °C required for *R. oligosporus* is consistent with the requirements of the fungus *Engyodontium album*, which yields its maximum protease production at this temperature (**Chellappan *et al.*, 2011**).

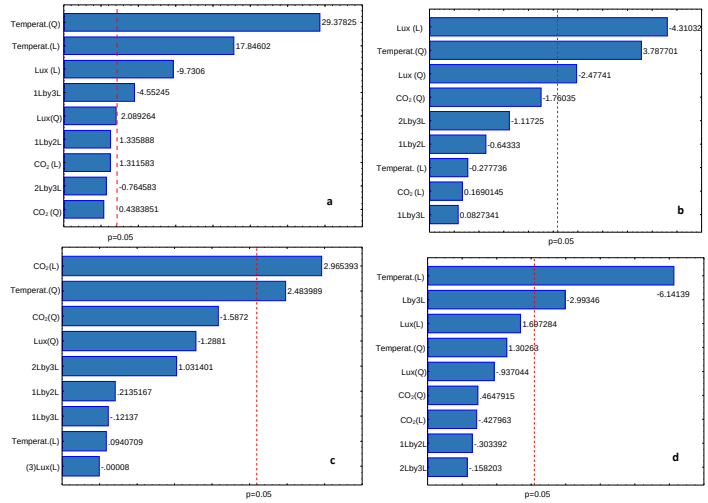

Figure 3 Standardized effects of predictor variables on enzyme production in *Rhizopus oligosporus* represented as a Pareto chart. **(a)** Alkaline Protease; **(b)** Acid Protease; **(c)** Lipase; **(d)** Carbohydrase. The vertical red line indicates the significance level at $p = 0.05$. Horizontal columns show the t-test value for each effect.

Light intensity affected fungal growth and protease production. At low light intensity, AlkP concentration reached 0.411 U/mg, which was 15.1% higher than that of AlkP at 53 lux and 29.2% higher than that at 100 lux. AcP yield reached 0.289 U/mg in dark conditions, which was 64%–66% higher than that at 53 lux or 100 lux. Filamentous fungi use light signals as an indicator of the exposure of hyphae to air and adapt their physiology to this situation to activate morphogenetic pathways (**Rodriguez-Romero *et al.*, 2010**). Flavin chromophore is a low-molecular-weight organic light receptor that detects blue, green, or red-light emissions (**Griffin *et al.*, 1997**). Light intensity affects several functions, including the growth of sporangiophores and the expression of metabolic pathway-related genes (**Idnurm *et al.*, 2006**). Exposure to light delays the vegetative phase of *A. oryzae* as well as the production of AcP (**Murthy *et al.*, 2015**). In Ascomycota fungi, particularly *Aspergillus nidulans*, the exposure of fungal colonies to white and fluorescent light in a liquid medium affected gene expression. The expression of some genes was upregulated under the lighting regime, and these genes were required to regulate asexual development. Similarly, genes with downregulated expression were related to transport, oxidoreductase functions, and nuclear components with regulatory functions of sexual and asexual stages (**Tisch and Schmoll, 2009; Ruger-Herreros *et al.*, 2011**). Probably, light intensity affects the activation of genes that regulate the overexpression of enzymatic pathways necessary for microbial nutrition.

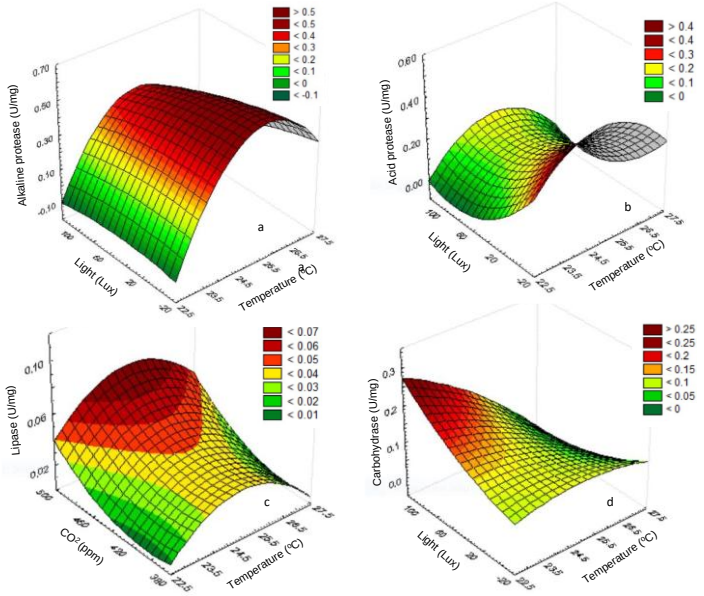

Figure 4 Response surface plot shows the mutual effect of light intensity, dioxide carbon, and temperature on the concentrations of (a) Alkaline Protease; (b) Acid Protease; (c) Lipase; (d) Carbohydrase in *Rhizopus oligosporus.*

Response surface analysis of Lipase (Lip)

The ANOVA results for LiP are presented in Table 5. The RSM model showed significant results ($p = 0.0004$), indicating that temperature (X_1) and the linear effect of CO_2 concentration (X_2) were the factors affecting LiP yield (Fig. 3c). The interaction between these two variables did not significantly alter LiP production. The regression coefficient was lower than those observed for previous enzymes ($R^2 = 0.53$) but was reasonably consistent with the experimental data. The LOF test ($p = 0.0055$) showed that the predicted model was suitable for data analysis. The final predictive equation was as follows:

$$Lip = -2.3623 + 0.2562(x) - 0.0031(y) - 0.0051(x^2) + 1.4757E\text{-}5(xy) + 3.9546E\text{-}6(y^2) \quad (3)$$

The effect of temperature and CO_2 concentration is presented in 3D response surface contour plots (Eq. 3; Fig. 4c). Based on the desirability score of 1.0, the optimum LiP yield was obtained at 0.0752 U/mg at 25 °C and 494 ppm of CO_2 concentration (Fig. 5c).

Microbial LiPs are industrially attractive enzymes because of their broad substrate-specificity and optimal alkaline pH values. Many *Rhizopus* intracellular LiPs with potential activity have been purified and characterized, such as those from *Rhizopus niveus* (**Kohno *et al.*, 1994**), *Rhizopus arrhizus* (**Gancet, 1991**), *Rhizopus javanicus* (**Uyttenbroeck *et al.*, 1993**), and an extracellular LiP from *R. oryzae* (**Salah *et al.*, 1994**). The extracellular LiP or triacylglycerol hydrolases transform fatty acid molecules present in soybeans into free fatty acids by hydrolyzing ester bonds present in triacylglycerols (**Kohno *et al.*, 1994**). The action of LiP contributes to the 30% loss of the crude lipids present in soybeans (**Ruiz-Terán and Owens, 1996**). Of the enzymes investigated, LiP was the only enzyme that showed significant variations in the concentration depending on CO_2 concentration and temperature. Alterations in the modified CO_2 atmosphere significantly affect many enzymatic activities (**Couvert *et al.*, 2023**). According to Henry's law, the stability of LiPs at alkaline pH indicates that they require higher CO_2 concentrations to be active. Herein, the highest LiP concentration was achieved at 494 ppm of CO_2 concentration, reaching values of 0.059 U/mg of the protein. When the CO_2 concentration was lower, LiP production decreased by 53.7%. Importantly, high CO_2 concentrations of 5%–10% inhibit *Rhizopus* spp. growth. Although *Rhizopus microsporus* and *R. oligosporus* manage to grow at high CO_2 concentrations, they exhibit a low micelle density (**Han and Nout, 2000).** A similar result was obtained for temperature; LiPs act in a narrow temperature range and exhibit low thermostability (**Kumar *et al.*, 2019**). Herein, the maximum LiP concentration was achieved at 25 °C (0.048 U/mg), and when the temperature was increased by 2 °C, LiP concentration decreased to 0.027 U/mg.

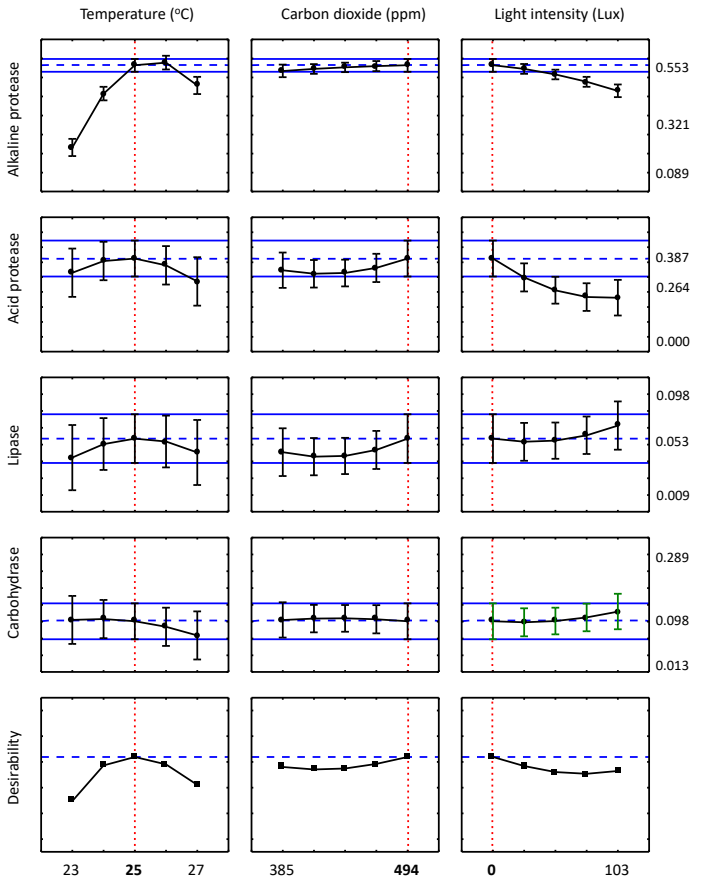

Figure 5 Profiles for predicted values and desirability function for the average recovery of the enzyme concentration of *Rhizopus oligosporus*. Dashed lines indicate the optimization values.

Response surface analysis of Carbohydrase (Cbh)

The linear effect of temperature (X_1) and the interaction between the variables X_1 and X_3 affected Cbh concentration (Fig. 3d). The LOF value (p = 0.0578) and R^2 = 73.81% showed that the predicted model fitted the data well (Table 5). The final predictive equation obtained using the significant predictors is given below:

$$Cbh = -2.5261 + 0.2201(x) + 0.0099(y) - 0.0046(x^2) - 0.0004(xy) + 5.3336E\text{-}6(y^2) \quad (4)$$

The effect of temperature (X_1) and light intensity (X_3) is shown in 3D response surface contour plots (Eq. 4; Fig. 4d). According to the desirability analysis, Cbh concentration was optimized to 0.098 U/mg under conditions of 25 °C and 0 light intensity (Fig. 5d).

Cbhs are enzymes that hydrolyze complex carbohydrates and transform them into simple sugars. Enzymes such as amylase, pectinase, and glucoamylase obtained from *R. oryzae* have been extensively used in various industrial procedures, offering multifaceted benefits. Herein, the concentration of Cbhs, such as proteases, was significantly affected by the interaction between temperature and light, reaching an optimal concentration of 0.098 U/mg at 25 °C and in darkness. This concentration was lower than Cbhs produced in *R. oryzae* after 20 days at 30 °C (1.02 U/mg) when yeast extract and starch were provided as substrates (**Amadioha, 1998**). The authors observed that amylase production was not related to micelle biomass but was directly related to the carbon source type in the medium. Compared with the present study, in which we used dextrose as a carbon source, the maximum amylase concentration reached after five days of incubation was ≤0.69 U/mg. Cbh production probably increases with an increase in sources of polymers other than glucose, such as amylose, fructose, and amylopectin (**Hellín *et al.*, 2003**).

CONCLUSION

Three predictors, namely temperature, CO_2 concentration, and light intensity, were optimized using the RSM to maximize enzyme production in *R. oligosporus*. Increasing temperature and light intensity significantly decreased enzyme production, particularly that of AlkP, AcP, and Cbh. The optimal conditions for enzyme production using dextrose and soy as substrates were set at 25 °C, 494 ppm, and 0 lux. Under these conditions, the enzymes reached their optimal concentrations of 0.557 U/mg (AlkP), 0.387 U/mg (AcP), 0.098 U/mg (*Cbh*), and 0.0752 U/mg (LiP). Exploring fungal metabolic capacity to improve enzyme production using statistical modeling is a research hotspot with enormous potential for developing new fermented products.

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