fermentation

MDPI

Article

Response-Surface Statistical Optimization of Submerged Fermentation for Pectinase and Cellulase Production by *Mucor circinelloides* and *M. hiemalis*

Amal A. Al Mousa [1,*], Abdallah M. A. Hassane [2,*], Abd El-Rahman F. Gomaa [2], Jana A. Aljuriss [3], Noura D. Dahmash [1] and Nageh F. Abo-Dahab [2]

[1] Department of Botany and Microbiology, College of Science, King Saud University, P.O. Box 145111, Riyadh 11362, Saudi Arabia; 438204570@student.ksu

[2] Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Assiut 71524, Egypt; abdfathy96@azhar.edu.eg (A.E.-R.F.G.); abodahabn@azhar.edu.eg (N.F.A.-D.)

[3] Research Assistant Internship Program, Vice Rectorate for Graduate Studies and Scientific Research, Deanship of Scientific Research, King Saud University, P.O. Box 145111, Riyadh 11362, Saudi Arabia; jana120120@gmail.com

* Correspondence: aalmosa@ksu.edu.sa (A.A.A.M.); abdallahhassane@azhar.edu.eg (A.M.A.H.); Tel.: +966-505-389-419 (A.A.A.M.); +20-882-148-095 (A.M.A.H.)

Abstract: Cellulase and pectinase are degrading cellulosic and pectic substances that form plant cell walls and, thereby, they have a wide range of applications in the agro-industrial by-products recycling and food industries. In the current research, *Mucor circinelloides* and *M. hiemalis* strains were tested for their ability to produce cellulase and pectinase from tangerine peel by submerged fermentation. Experiments on five variables: temperature, pH, incubation period, inoculum size, and substrate concentration, were designed with a Box–Behnken design, as well as response surface methodology (RSM), and analysis of variance was performed. In addition, cellulase and pectinase were partially purified and characterized. At their optimum parameters, *M. circinelloides* and *M. hiemalis* afforded high cellulase production (37.20 U/mL and 33.82 U/mL, respectively) and pectinase (38.02 U/mL and 39.76 U/mL, respectively). The partial purification of *M. circinelloides* and *M. hiemalis* cellulase produced 1.73- and 2.03-fold purification with 31.12 and 32.02% recovery, respectively; meanwhile, 1.74- and 1.99-fold purification with 31.26 and 31.51% recovery, respectively, were obtained for pectinase. Partially purified cellulase and pectinase from *M. circinelloides* and *M. hiemalis* demonstrated the highest activity at neutral pH, and 70 and 50 °C, for cellulase and 50 and 60 °C, for pectinase, respectively. Moreover, 10 mM of K+ increased M. circinelloides enzymatic activity. The production of cellulase and pectinase from *M. circinelloides* and *M. hiemalis* utilizing RSM is deemed profitable for the decomposition of agro-industrial wastes.

Keywords: cellulase; pectinase; *Mucor circinelloides*; *Mucor hiemalis*; submerged fermentation; optimization; response-surface methodology; Box–Behnken design

Citation: Al Mousa, A.A.; Hassane, A.M.A.; Gomaa, A.E.-R.F.; Aljuriss, J.A.; Dahmash, N.D.; Abo-Dahab, N.F. Response-Surface Statistical Optimization of Submerged Fermentation for Pectinase and Cellulase Production by *Mucor circinelloides* and *M. hiemalis*. *Fermentation* **2022**, *8*, 205. https://doi.org/10.3390/fermentation8050205

Academic Editor: Diomi Mamma

Received: 7 April 2022
Accepted: 27 April 2022
Published: 30 April 2022

1. Introduction

Filamentous fungi biotechnological processes have enabled the industrial exploitation of their capability to produce profitable enzymes, due to their easy propagation and high production of extracellular enzymes with particular properties such as stability in wide ranges of temperature and pH [1–3]. The genus *Mucor* belonging, to the class Zygomycetes, shows a variety of bioactivities such as multi-metal remediation by *M. hiemalis* [4]; production of biosurfactants [5], fungal chitosan [6] and bio-oil by *M. circinelloides* [7,8]; the yielding of ethanol by *M. indicus* [9] along with the potential to produce extracellular hydrolytic enzymes on various agro-industrial wastes that can be harnessed in diverse applications in industry [10]. These enzymes include milk-clotting proteases [11], malic enzyme [12] and polygalacturonase by *M. circinelloides* [13], ferulic acid esterase from *M. hiemalis* [14],

lipase by *M. geophillus* [15], amylase [16], xylanase by *M. indicus* and *M. hiemalis* [17], and endoglucanase by *M. racemosus* [18].

The increasing expansion of agricultural-waste activity has led to the accumulation of a large quantity of lignocellulosic residues across the world [19]. Lignocellulosic plant biomass is mainly constituted of hemicellulose, cellulose, and lignin [20]. Cellulases are a group of synergetic enzymes that catalyze the degradation of cellulose into fermentable sugars and can be divided into three major components, namely, endoglucanase, exoglucanase and β-glucosidase. Furthermore, endoglucanase is considered the most economical type to create free end groups on the cellulose, thereby producing starting points for the other cellulase synergetic components [21,22]. Fungi have the capability to produce higher quantities of cellulases as compared to other organisms. Cellulase has been included in a wide range of industrial applications for alcohol fermentation, biofuel and starch production, juices extraction, animal-feed processing, and textile and paper manufacturing [23].

Pectinase is involved in the hydrolysis of pectin present in the middle lamella and primary cell wall of vegetables and fruits into D-galacturonic acid by breaking down α-1-4 chains [24]. Pectic compounds are copious in the plant biomass composition; their levels are between 4 and 30% in the pulp of beet and the peel of citrus fruits [25]. Pectinase has a broad domain of implementations and plays critical roles in the food industry, such as the clarification of fruit juice, oil squeezing, the beverage industry, waste management, tea fermentation, the paper and pulp industry, and softening plant-based fibers [26]. Genera of Zygomycetes and Ascomycetes could be a preferred source of pectinase, as 50% of total pectinases are obtained from fungi, because of their easy growth, high productivity rate, being cost effective and having a short life span [18].

The amount of fungal-enzyme manufacturing relies on the conditions of the fermentation process, along with the necessity to optimize these conditions in order to reduce enzyme-production cost [27]. Fungal-enzyme manufacturing is prevalently implemented by submerged or solid-state fermentations [28]. Submerged fermentation, reported in 90% of the industrial enzymes production, occurs in the presence of excess water, thus offering easy handling and better monitoring [29,30]. Concerning the accumulation of vast bulks of agro-industrial by-products around the world, this study aimed to evaluate the production of high-value, partially purified pectinase and cellulase by *Mucor circinelloides* and *M. hiemalis* strains, using agro-industrial by-products as a cheap substrate in submerged-fermentation conditions. The optimization of cellulase and pectinase production and characterization of partially purified enzymes were investigated.

2. Materials and Methods

2.1. Tested Fungi

Mucor circinelloides AUMC 6696.A (Accession no. MT509983) and *M. hiemalis* AUMC 6031 (Accession no. MT365791) [31] were utilized in the in the current search for pectinase and cellulase production. Pure cultures were kept in potato-dextrose-agar (PDA) tubes and preserved at 4 °C for further use.

2.2. Enzymes Preliminary Screening

2.2.1. Screening on Agar Plates

Czapek's agar medium (g/L: KH_2PO_4, 1; $NaNO_3$, 2; $MgSO_4.7H_2O$, 0.5 and $CaCl_2.2H_2O$, 0.5) was supplemented with 10 g/L of pectin and carboxymethylcellulose as carbon source for pectinase and cellulase production. The pH was set to 7 and the inoculated plates were incubated at 28 ± 2 °C for 5 days, and then screened for enzyme production [32]. After flooding the cultured agar plates in iodine solution for 15 min, they were checked for the appearance of a clear zone.

2.2.2. Screening by Submerged Fermentation (SmF)

Wheat straw, pomegranate, and tangerine peels were desiccated at 65 °C for 24 h, then grounded to fine powders and passed through a sieve of mesh size 600 µm and

used, subsequently, as a carbon source (10 g/L) in Czapek's mineral-salts broth. Before sterilization, the broth-containing flasks were adjusted to pH 7.0 using potassium phosphate buffer (pH 7.0, 0.05 M) [33], and then autoclaved at 121 °C, 1.5 bar for 20 min. The strains' spores were removed from the colony surface into suspensions of 10 mL sterile distilled water containing 0.1% Tween-80. The spores' concentration was prepared by measuring and calculating with a haemocytometer and a binocular microscope, then dilution was performed to give a final concentration of approximately 1×10^7 spores/mL which was then utilized to inoculate 250 mL flasks holding 100 mL liquid culture medium. The inoculated flasks were incubated at 28 ± 2 °C for seven days on both shaking at 120 rpm and static conditions. Subsequently, broth media were centrifuged at 10,000 rpm under cooling, and supernatants were maintained at 4 °C for further enzymatic analysis.

2.2.3. Quantitative Screening of Cellulase and Pectinase

Cellulase and pectinase activities were measured according to Miller [34]. In addition, 0.5 mL culture supernatant was added to 0.5 mL pectin or carboxymethylcellulose (1% w/v) in acetate buffer (pH 4.8, 0.05 M) [33], and the mixture was incubated at 50 °C for 30 min. Afterward, the interaction was intercepted by appending 1 mL of 3,5-dinitrosalicylic acid reagent and incubated at 100 °C for 10 min. After cold dishing, the absorbance was measured at 570 nm using a spectrophotometer (Jenway 7315, UK). The amount of reducing sugars was determined using glucose as a standard for plotting the calibration curve (Figure 1). All the tests were carried out three times, and the production was expressed as an average value. A unit of the enzyme was acquainted as the quantity of the enzyme per one mL required to release one µmol of reducing sugar from a substrate per 60 s under the optimum trial conditions, including pH, temperature, and incubation time [35].

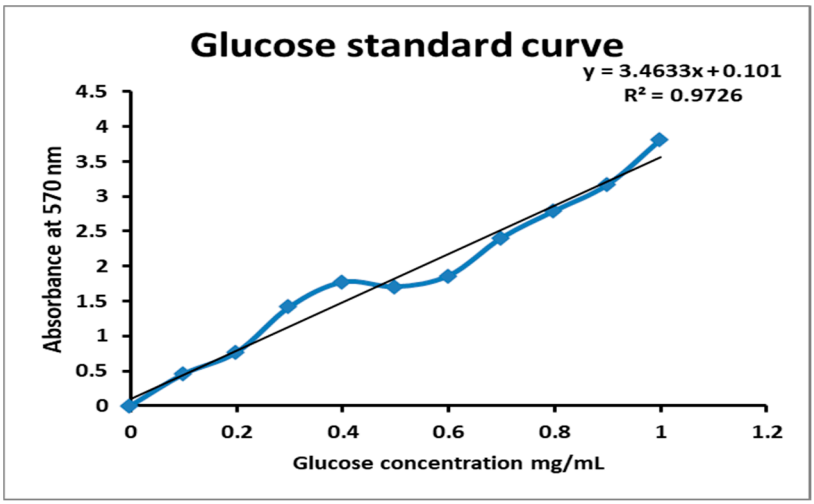


Figure 1. Glucose standard curve.

2.3. Optimization of Enzymatic Productivity under Submerged Fermentation (SmF)

Response-surface methodology (RSM) tactic using Box–Behnken design (BBD) was used to determine the optimum factors for boosted cellulase and pectinase production including A, temperature; B, pH; C, incubation period; D, inoculum size and E, substrate concentration (Table 1). Different pH values were adjusted using buffer system including: sodium acetate buffer (pH 5.0, 0.05 M); potassium phosphate buffer (pH 7.0, 0.05 M); glycine-NaOH buffer (pH 9.0, 0.05 M) [33]. The strains' spore suspensions, of approximately 1×10^7 spores/mL of varied sizes, were utilized to inoculate 250 mL flasks holding 100 mL liquid culture medium with different tangerine-powder concentrations and incubated under static conditions at different tested temperatures for diverse incubation periods. Forty-six experiments with the central points were employed to satisfy the polynomial pattern which is established on a Box–Behnken design (BBD, 5 variables) attained by

Minitab 18® software (Version18.1.1.0. LLC., Pennsylvania State University, State College, PA, USA). A three-level and five-factors experimental BBD was examined, and the number of the tests (N) was determined corresponding to the subsidiary equation:

$$N = 2k \times (k - 1) + C0 \quad (1)$$

where k is the digit of factors and C0 is the digit of central points, which equal to 6.

Table 1. Box–Behnken design levels of independent factors.

No.	Factor	Variables	Units	Range		
				Minimum	Maximum	Mean
1	A	Temperature	°C	20	40	30
2	B	pH	-	5	9	7
3	C	Incubation period	day	5	9	7
4	D	Inoculum size	mL	1	5	3
5	E	Substrate concentration	g	1	5	3

The impact of variables on the simulation (Y) was construed by employing a second-order polynomial equation that was utilized to foretell the quixotic states of the cellulase and pectinase biosynthesis.

$$Y = \beta_0 + \beta_A A + \beta_B B + \beta_C C + \beta_D D + \beta_E E + \beta_{AA} A^2 + \beta_{BB} B^2 + \beta_{CC} C^2 + \beta_{DD} D^2 + \beta_{EE} E^2 + \beta_{AB} AB + \beta_{AC} AC + \beta_{AD} AD + \beta_{AE} AE + \beta_{BC} BC + \beta_{BD} BD + \beta_{BE} BE + \beta_{CD} CD + \beta_{CE} CE + \beta_{DE} DE \quad (2)$$

where Y is response variable; β_0 intercept; β_A, β_B, β_C, β_D and β_E are linear coefficients; β_{AA}, β_{BB}, β_{CC}, β_{DD}, and β_{EE} are square coefficients; β_{AB}, β_{AC}, β_{AD}, β_{AE}, β_{BC}, β_{BD}, β_{BE}, β_{CD}, β_{CE}, and β_{DE} are interaction coefficients; and A, B, C, D, E, A^2, B^2, C^2, D^2, E^2, AB, AC, AD, AE, BC, BD, BE, CD, CE and DE are levels of independent variables. The corresponding coefficients of variables, interaction variables, and contour graphs were obtained by Minitab 18® software. By analyzing the regression equation and constructing the response plots, the ideal values of the tested variables were secured. The coefficient of limitation R^2 was used to express the fineness of profit of the polynomial equation, and the F test was used to determine its statistical significance level.

2.4. Partial Purification of Cellulase and Pectinase from Mucor Strains

After incubation period under optimum conditions obtained from runs no. 20 and 36 for cellulase from *M. circinelloides* and *M. hiemalis*, respectively, and runs no. 18 and 40 for pectinase from *M. circinelloides* and *M. hiemalis*, respectively, the contents of the broth culture were centrifuged at 10,000 rpm under cooling, and the supernatant was utilized for enzymes assay.

Crude enzymes solutions were partially purified by precipitation using cold acetone. Pre-cooled acetone (−20 °C) was subjoined to the enzyme solution until the volume ratio between enzyme solution and acetone reached 1:1; 1:2; 1:3; 1:4, and 1:5 (*v*/*v*). The solution was left at −20 °C overnight to allow protein precipitation. The precipitates were gathered by centrifugation at 10,000 rpm for 15 min and resuspended in a small volume of sodium-citrate buffer (pH 4.8, 0.05 M) [33]. Cellulase and pectinase activities and protein concentration were measured in the supernatant according to Miller [34] and Lowry et al. [36], respectively, utilizing standard of bovine serum albumin to generate the calibration curve spectrophotometerically at 750 nm (Figure 2). These samples were used for determining the activities of cellulase and pectinase, purification fold, and enzyme-recovery yield [37]. Protein was estimated and suitable precipitants (crude:acetone, 1:4)

for characterization were selected. The following equations were used to calculate specific activity, yield, and purification fold of the partially purified enzymes.

$$\text{Specific activity}(\text{U/mg}) = \frac{Total\ activity}{Total\ protein} \quad (3)$$

$$\text{Yield } (\%) = \frac{Total\ units\ in\ partially\ purified\ enzyme\ \times 100\%}{Total\ units\ in\ crude\ enzyme} \quad (4)$$

$$\text{Purification fold } = \frac{Specific\ activity\ of\ partially\ purified\ enzyme}{Specific\ activity\ of\ crude\ enzyme} \quad (5)$$

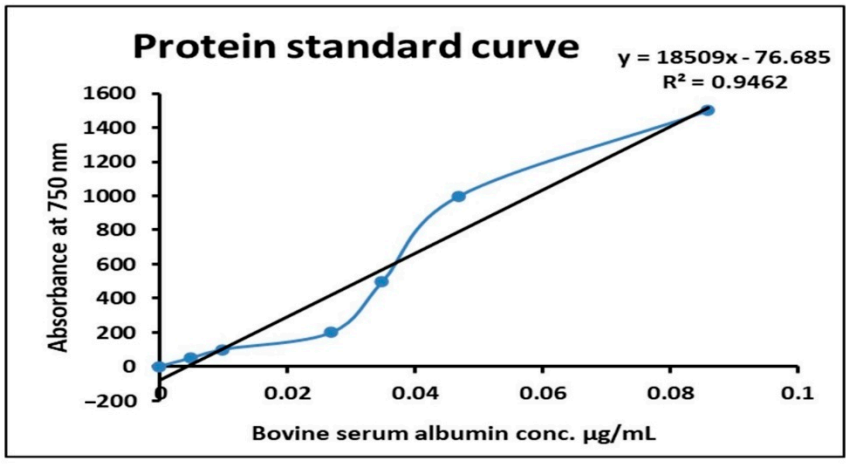


Figure 2. Protein standard curve.

2.5. Characterization of Partially Purified Cellulase and Pectinase

The optimum temperature for partially purified cellulase and pectinase activities was determined in the range of 30–90 °C, and the thermal stability was determined after the pre-maintaining of enzyme at each temperature degree for one hour before screening. Ideal pH estimation was carried out at optimum temperature utilizing various buffers with values 3–11, including: glycine-HCl (pH 3.0, 0.05 M); sodium acetate buffer (pH 5.0, 0.05 M); potassium phosphate buffer (pH 7.0, 0.05 M); glycine-NaOH buffer (pH 9.0 and 11.0, 0.05 M) [33], and the pH stability was assessed after preserving with these pH values for 1 h before screening. In addition, cellulase and pectinase activities were valued after maintaining the enzyme with different metal ions (10 mM of K^+, Mg^{2+}, Ba^{2+} and Ni^{2+}) for 1 h at optimum temperature and pH, including 70 and 50 °C for cellulase and 50 and 60 °C for pectinase from *M. circinelloides* and *M. hiemalis*, respectively, and pH 7.0. Cellulase and pectinase activities were assessed after processing with diverse detergents comprising tween 80 and 20 at concentrations of 1 and 5% v/v, urea (1 and 5% w/v), and Na_2CO_3 (50 and 75 mM), in comparison to control (100% activity) [38,39].

2.6. Data Analysis

All tests and measurements were repeated three times and the values were expressed as the mean ± SD. Significant differences were detected with one-way ANOVA, differences between means were considered using Duncan's new multiple range test (DMRT) at the 0.05 significance level, using the SPSS software program (version No. 16).

3. Results

3.1. Preliminary and Quantitative Screening of Cellulase and Pectinase Production

The preliminary screening for extracellular fungal cellulase and pectinase revealed that both *M. circinelloides* and *M. hiemalis* had a high ability to produce cellulase and pectinase qualitatively on a solid assay medium. They were then quantitatively assayed under SmF

and showed high cellulase production (15.70 and 13.85 U/mL, respectively) and pectinase activity (18.21 and 11.98 U/mL, respectively) on tangerine peel as a substrate under static conditions (Table 2).

Table 2. Cellulase and pectinase production on different substrates under shaking and static conditions.

Substrate	Enzymes	Enzyme Activity (U/mL)			
		M. circinelloides		*M. hiemalis*	
		Shaking	Static	Shaking	Static
Pomegranate peel	Cellulase	3.54 ± 0.47^{b}	3.65 ± 0.34^{b}	4.53 ± 0.27^{b}	6.18 ± 0.27^{b}
	Pectinase	6.01 ± 0.58^{B}	7.22 ± 0.54^{B}	7.66 ± 0.41^{B}	7.06 ± 0.27^{B}
Tangerine peel	Cellulase	14.67 ± 0.71^{a}	15.70 ± 0.63^{a}	13.17 ± 0.36^{a}	13.85 ± 0.18^{a}
	Pectinase	12.45 ± 0.65^{A}	18.21 ± 0.59^{A}	11.20 ± 0.29^{A}	11.98 ± 0.24^{A}
Wheat straw	Cellulase	0.78 ± 0.02^{c}	0.37 ± 0.04^{c}	2.66 ± 0.16^{c}	3.79 ± 0.18^{c}
	Pectinase	0.99 ± 0.08^{C}	0.60 ± 0.06^{C}	1.90 ± 0.20^{C}	3.51 ± 0.02^{C}

The data were given as averages of three replicates (mean ± SD). Values followed by the different letters are significantly different at $p < 0.05$. Small superscripted letters i.e., "^{a}" were affiliated for cellulase activities on different substrates under shaking and static conditions, while capital superscripted letters i.e., "^{A}" were affiliated for pectinase activities on different substrates under shaking and static conditions.

3.2. Response-Surface Methodology for Optimization of Cellulase and Pectinase Production

Table 1 shows the independent factors with their competent levels employed in the optimization of cellulase and pectinase production, while the BBD of the independent factors along with the predicted as well as experimental values are depicted in Table 3. The production of cellulase by *M. circinelloides* was predicted by the following equation:

$$\begin{aligned} Y\ (U/mL) = 147.0 - 2.858A - 7.08B - 20.72C - 0.38D + 5.22E + 0.01299A^2 + 0.450\ B^2 + 1.121C^2 + 0.124D^2 \\ + 0.395E^2 - 0.0021AB + 0.3042AC + 0.0144AD - 0.1083AE - 0.294BC - 0.015BD + 1.088BE - 0.021CD \\ - 0.975CE - 0.340DE \end{aligned} \quad (6)$$

While the production of cellulase by *M. hiemalis* was fitted by the following equation:

$$\begin{aligned} Y\ (U/mL) = -189.4 + 2.187A + 16.02B + 23.91C + 13.19D + 11.66E - 0.02728A^2 - 0.034B^2 - 0.465C^2 - \\ 0.788D^2 - 0.929E^2 + 0.0691AB - 0.1712AC - 0.0196AD + 0.0650AE - 1.846BC - 1.274BD - 0.134BE + 0.067CD \\ - 0.268CE - 0.124DE \end{aligned} \quad (7)$$

M. circinelloides pectinase biosynthesis was fitted by the following equation:

$$\begin{aligned} Y\ (U/mL) = 168.2 - 0.245A - 8.11B - 31.47C + 2.49D - 9.01E - 0.00215A^2 + 0.589B^2 + 1.689C^2 - 0.071D^2 + \\ 0.718E^2 - 0.0289AB + 0.1165AC + 0.0340AD - 0.2032AE - 0.041BC - 0.284BD - + 0.670BE - 0.196CD + \\ 1.619CE - 0.345DE \end{aligned} \quad (8)$$

Meanwhile, *M. hiemalis* pectinase activity was predicted by the following equation:

$$\begin{aligned} Y\ (U/mL) = -228.9 + 3.868A + 2.40B + 38.68C + 12.78D + 20.38E - 0.03621A^2 + 0.979B^2 - 1.754C^2 - 0.447D^2 - \\ 1.175E^2 - 0.1039AB - 0.1039AC - 0.0536AD - 0.0650AE - 1.361BC - 0.315BD - 0.526BE - 0.526CD \\ - 0.009CE - 0.866DE \end{aligned} \quad (9)$$

The highest cellulase activities of both *M. circinelloides* (37.20 U/mL) and *M. hiemalis* (33.82 U/mL) were obtained from runs no. 20 and 36, respectively. Run no. 20, for *M. circinelloides*, consisted of incubation temperature 30 °C, pH value 7, incubation period 5 days, inoculum size 3 mL, and substrate concentration 5 g/100 mL, while run no. 36 for *M. hiemalis* consisted of incubation temperature 30 °C, pH value 9, incubation period 5 days, inoculum size 3 mL, and substrate concentration 3 g/100 mL. Meanwhile, the highest pectinase production of both *M. circinelloides* (38.02 U/mL) and *M. hiemalis* (39.76 U/mL) were obtained from runs no. 18 and 40, respectively. Run no. 18 for *M. circinelloides*

consisted of incubation temperature 30 °C, pH value 7, incubation period 9 days, inoculum size 3 mL, and substrate concentration 5 g/100 mL. In comparison, run no. 40 for *M. hiemalis* consisted of incubation temperature 30 °C, pH value 5, incubation period 7 days, inoculum size 3 mL, and substrate concentration 5 g/100 mL.

Table 3. Box–Behnken design of optimization variables with experimental and predicted cellulase and pectinase activities of both *M. circinelloides* and *M. hiemalis*.

Run Order	Variables					Cellulase Activity (U/mL)				Pectinase Activity (U/mL)			
						M. circinelloides		*M. hiemalis*		*M. circinelloides*		*M. hiemalis*	
	A	**B**	**C**	**D**	**E**	**Exp.**	**Pred.**	**Exp.**	**Pred.**	**Exp.**	**Pred.**	**Exp.**	**Pred.**
1	30	7	5	1	3	20.20	22.28	21.19	22.76	23.67	24.82	18.14	19.80
2	30	9	7	1	3	17.15	18.74	29.86	27.72	19.96	21.94	36.62	35.36
3	30	7	5	5	3	19.30	20.51	18.64	18.46	20.20	21.14	24.74	24.04
4	20	7	7	3	5	27.46	29.96	26.64	25.32	32.99	33.60	35.63	34.70
5	30	7	9	3	1	11.54	14.36	5.77	6.36	8.82	9.90	8.90	7.79
6	20	7	5	3	3	32.66	30.44	17.89	17.89	28.87	28.05	19.05	19.42
7	30	5	7	3	1	14.02	13.13	8.24	8.07	12.94	15.05	14.10	15.32
8	30	7	7	1	5	26.56	27.16	28.87	28.29	31.75	30.80	37.94	37.16
9	30	9	7	5	3	16.08	16.69	14.02	13.76	13.77	14.43	32.58	32.87
10	30	7	7	3	3	13.60	14.53	22.27	23.09	16.08	15.82	29.28	30.13
11	30	7	7	5	1	9.15	8.76	5.52	5.14	8.65	8.78	15.67	17.06
12	40	7	7	3	5	19.05	21.32	29.36	27.35	18.14	20.12	29.61	29.05
13	40	7	7	3	1	8.82	9.18	6.59	5.37	11.21	11.48	11.54	11.52
14	30	7	7	3	3	14.18	14.53	21.85	23.09	16.82	15.82	29.86	30.13
15	20	7	7	3	1	8.57	9.16	9.07	8.54	9.81	8.70	12.37	11.97
16	30	9	7	3	1	7.99	6.21	10.72	11.02	10.22	10.27	21.94	23.24
17	30	7	7	3	3	15.34	14.53	23.92	23.09	15.83	15.82	30.52	30.13
18	30	7	9	3	5	22.84	23.03	22.93	23.60	38.02	39.62	27.55	27.86
19	20	9	7	3	3	20.04	20.63	18.80	20.07	21.03	21.51	36.29	35.89
20	30	7	5	3	5	37.20	34.61	29.44	30.80	29.69	28.04	28.87	29.11
21	20	7	7	1	3	20.95	19.73	18.97	18.98	21.85	21.30	23.92	25.16
22	30	7	7	5	5	21.52	22.51	24.58	23.53	23.26	22.78	28.45	30.26
23	20	7	7	5	3	16.99	17.22	15.67	16.00	14.18	14.69	27.88	27.34
24	30	5	5	3	3	18.97	20.63	16.66	15.30	24.25	25.16	21.03	20.32
25	20	7	9	3	3	16.57	14.49	18.97	19.68	22.43	22.02	22.76	22.62
26	30	7	7	1	1	8.74	7.97	7.83	7.92	11.62	11.26	11.29	10.10
27	40	7	9	3	3	23.50	22.34	12.37	12.26	20.62	21.33	15.25	15.19
28	40	7	7	1	3	15.50	14.84	18.47	19.20	14.84	14.59	23.50	24.26
29	30	9	9	3	3	17.65	18.63	13.19	12.12	24.99	24.37	22.27	22.85
30	40	9	7	3	3	17.07	16.40	20.20	22.26	17.32	15.00	29.05	28.68
31	40	5	7	3	3	14.18	14.54	17.32	17.62	17.07	15.57	28.29	29.12
32	30	9	7	3	5	33.82	31.39	27.63	29.33	33.98	32.40	39.18	39.17
33	30	7	7	3	3	14.51	14.53	22.68	23.09	15.25	15.82	28.04	30.13
34	30	7	5	3	1	10.30	10.35	7.99	9.28	26.39	24.22	10.07	8.90
35	20	5	7	3	3	17.32	18.93	21.44	20.95	18.47	19.76	27.22	28.01
36	30	9	5	3	3	23.67	24.77	33.82	31.95	24.74	26.07	35.05	34.92
37	40	7	5	3	3	15.25	13.96	24.99	24.17	17.73	18.04	20.29	20.74
38	30	7	9	5	3	18.14	16.56	14.92	13.93	18.97	18.20	19.63	18.65
39	30	5	9	3	3	17.65	19.20	25.57	25.01	25.15	24.12	30.02	30.03
40	30	5	7	3	5	22.43	20.90	27.30	28.52	25.98	26.45	39.76	39.66
41	30	7	7	3	3	14.10	14.53	24.74	23.09	14.43	15.82	31.75	30.13
42	30	7	9	1	3	19.38	18.66	16.41	17.16	25.57	25.01	21.44	22.83
43	30	5	7	5	3	16.90	15.03	20.62	22.08	17.89	16.11	31.92	31.68
44	40	7	7	5	3	12.70	13.49	13.60	14.65	9.89	10.70	23.17	22.14
45	30	7	7	3	3	15.42	14.53	23.09	23.09	16.49	15.82	31.34	30.13
46	30	5	7	1	3	17.73	16.83	16.08	15.65	19.54	19.09	30.93	29.13

Exp.: experimental; Pred.: predicted.

Analysis of variance (ANOVA) for the cellulase and pectinase quadric model of *M. circinelloides* and *M. hiemalis* is shown in Tables 4–7. Model terms with a *p*-value < 0.05 were deemed significant. The model's F values for cellulase produced by *M. circinelloides* and *M. hiemalis*, (25.63 and 56.13, respectively) and the model's F values for pectinase produced by the same fungi (48.34 and 88.77, respectively) indicate that the model is significant. Values of "Prob > F" < 0.05 indicated that the model terms are significant. In cellulase activity, A, C, D, E, A^2, B^2, C^2, E^2, AC, AE, BE, and CE are significant model terms for *M. circinelloides*; while for *M. hiemalis*, B, C, D, E, A^2, C^2, D^2, E^2, AC, BC, and BD are significant model terms. Regarding pectinase biosynthesis, A, D, E, B^2, C^2, E^2, AC, AE, BE, and CE are significant model terms for *M. circinelloides*; while for *M. hiemalis*, A, B, E, A^2, B^2,C^2, D^2, E^2, AB, AC, BC, BE, CD, and DE are significant model terms. The "lack of fit F-value" of 7.93 and 1.94 for *M. circinelloides* and *M. hiemalis* cellulase, and values of 3.46 and 0.85 for their pectinase, respectively, indicated that the lack of fit is insignificant concerning the pure error. A non-significant lack of fit is proper for the model to be convenient. The multiple correlation coefficient R^2 = 0.9535 for *M. circinelloides* and R^2= 0.9782 for *M. hiemalis* cellulase and R^2 = 0.9748 for *M. circinelloides* and R^2 = 0.9861 for *M. hiemalis* pectinase, nigh to one, indicated preferable interconnection between experimental and predicted values and elucidated the model accuracy with an upgrade response. Regression values were in harmony with adjusted and predicted R^2.

Table 4. ANOVA for the experimental results of cellulase biosynthesis by *M. circinelloides*.

Source	Sum of Squares	Degree of Freedom	Mean of Squares	F-Value	*p*-Value	Prob > F
Model	1747.96	20	87.40	25.63	0.000	Significant
Linear	1244.08	5	248.82	72.96	0.000	
A	74.32	1	74.32	21.79	0.000	
B	12.73	1	12.73	3.73	0.065	
C	57.29	1	57.29	16.80	0.000	
D	14.87	1	14.87	4.36	0.047	
E	1084.86	1	1084.86	318.11	0.000	
Square	187.20	5	37.44	10.98	0.000	
A^2	14.73	1	14.73	4.32	0.048	
B^2	28.31	1	28.31	8.30	0.008	
C^2	175.35	1	175.35	51.42	0.000	
D^2	2.14	1	2.14	0.63	0.436	
E^2	21.82	1	21.82	6.40	0.018	
2-Way Interaction	316.68	10	31.67	9.29	0.000	
AB	0.01	1	0.01	0.00	0.965	
AC	148.07	1	148.07	43.42	0.000	
AD	0.33	1	0.33	0.10	0.757	
AE	18.76	1	18.76	5.50	0.027	
BC	5.53	1	5.53	1.62	0.215	
BD	0.02	1	0.02	0.00	0.947	
BE	75.75	1	75.75	22.21	0.000	
CD	0.03	1	0.03	0.01	0.930	
CE	60.78	1	60.78	17.82	0.000	
DE	7.41	1	7.41	25.63	0.153	
Residual	85.26	25	3.41			
Lack of Fit	82.65	20	4.13	7.93	0.150	Not significant
Pure error	2.61	5	0.52			
Total	1833.22	45				

R^2: 0.9535, adjusted R^2: 0.9163, and predicted R^2: 0.8176.

Contour plots explained the relationship between parameters and defined each factor's optimum scale for cellulase (Figures 3a–d and 4a–d) and pectinase (Figures 5a–d and 6a–d) activities by *M. circinelloides* and *M. hiemalis*, respectively. The response surface plot constructed any two variables, while other variables were maintained at their optimal level. Contour plots of cellulase and pectinase activities by *M. circinelloides* revealed significant

interactions derived from the analysis of variance and described as significant model terms (Figures 3b,d,g,i and 5b,d,g,i). On the other hand, contour plots of the interactions obtained by ANOVA illustrated model terms significantly influenced cellulase and pectinase production by *M. hiemalis* (Figures 4b,e,f and 6a,b,e,g,h,j). The remaining interactions insignificantly influenced enzymes production.

Table 5. ANOVA for the experimental results of cellulase biosynthesis by *M. hiemalis*.

Source	Sum of Squares	Degree of Freedom	Mean of Squares	F-Value	*p*-Value	Prob > F
Model	2272.99	20	113.65	56.13	0.000	Significant
Linear	1676.41	5	335.28	165.60	0.000	
A	1.29	1	1.29	0.64	0.433	
B	14.09	1	14.09	6.96	0.014	
C	102.55	1	102.55	50.65	0.000	
D	56.67	1	56.67	27.99	0.000	
E	1501.82	1	1501.82	741.79	0.000	
Square	205.80	5	41.16	20.33	0.000	
A^2	64.93	1	64.93	32.07	0.000	
B^2	0.16	1	0.16	0.08	0.780	
C^2	30.24	1	30.24	14.93	0.001	
D^2	86.81	1	86.81	42.88	0.000	
E^2	120.61	1	120.61	59.57	0.000	
2-Way Interaction	390.78	10	39.08	19.30	0.000	
AB	7.64	1	7.64	3.77	0.063	
AC	46.89	1	46.89	23.16	0.000	
AD	0.61	1	0.61	0.30	0.587	
AE	6.75	1	6.75	3.34	0.080	
BC	218.07	1	218.07	107.71	0.000	
BD	103.80	1	103.80	51.27	0.000	
BE	1.15	1	1.15	0.57	0.458	
CD	0.29	1	0.29	0.14	0.709	
CE	4.60	1	4.60	2.27	0.144	
DE	0.98	1	0.98	0.48	0.493	
Residual	50.61	25	2.02			
Lack of Fit	44.83	20	2.24	1.94	0.239	Not significant
Pure error	5.78	5	1.16			
Total	2323.61	45				

R^2: 0.9782, adjusted R^2: 0.9608, and predicted R^2: 0.9192.

Table 6. ANOVA for the experimental results of pectinase biosynthesis by *M. circinelloides*.

Source	Sum of Squares	Degree of Freedom	Mean of Squares	F-Value	*p*-Value	Prob > F
Model	2169.50	20	108.47	48.34	0.000	Significant
Linear	1358.31	5	271.66	121.07	0.000	
A	114.58	1	114.58	51.06	0.000	
B	1.38	1	1.38	0.62	0.440	
C	7.52	1	7.52	3.35	0.079	
D	110.20	1	110.20	49.11	0.000	
E	1124.62	1	1124.62	501.19	0.000	
Square	508.38	5	101.68	45.31	0.000	
A^2	0.40	1	0.40	0.18	0.675	
B^2	48.46	1	48.46	21.60	0.000	
C^2	398.37	1	398.37	177.54	0.000	
D^2	0.70	1	0.70	0.31	0.581	
E^2	71.98	1	71.98	32.08	0.000	
2-Way Interaction	302.81	10	30.28	13.49	0.000	
AB	1.33	1	1.33	0.59	0.448	
AC	21.73	1	21.73	9.68	0.005	

Table 6. *Cont.*

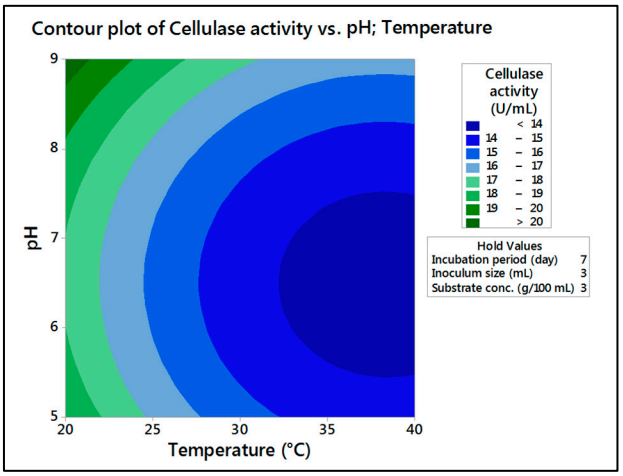
Source	Sum of Squares	Degree of Freedom	Mean of Squares	F-Value	*p*-Value	Prob > F
AD	1.85	1	1.85	0.83	0.372	
AE	66.03	1	66.03	29.43	0.000	
BC	0.11	1	0.11	0.05	0.827	
BD	5.15	1	5.15	2.29	0.142	
BE	28.75	1	28.75	12.81	0.001	
CD	2.46	1	2.46	1.09	0.305	
CE	167.76	1	167.76	74.76	0.000	
DE	7.64	1	7.64	3.40	0.077	
Residual	56.10	25	2.24			
Lack of Fit	52.32	20	2.62	3.46	0.086	Not significant
Pure error	3.78	5	0.76			
Total	2225.59	45				

R^2: 0.9748, adjusted R^2: 0.9546, and predicted R^2: 0.9035.

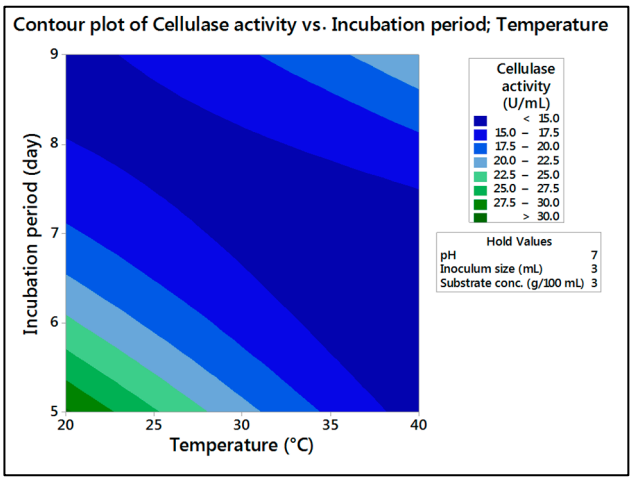
Table 7. ANOVA for the experimental results of pectinase biosynthesis by *M. hiemalis*.

Source	Sum of Squares	Degree of Freedom	Mean of Squares	F-Value	*p*-Value	Prob > F
Model	2938.40	20	146.92	88.77	0.000	Significant
Linear	1720.06	5	344.01	207.85	0.000	
A	37.22	1	37.22	22.49	0.000	
B	55.19	1	55.19	33.34	0.000	
C	5.55	1	5.55	3.35	0.079	
D	0.00	1	0.00	0.00	0.962	
E	1622.11	1	1622.11	980.06	0.000	
Square	962.23	5	192.45	116.27	0.000	
A^2	114.40	1	114.40	69.12	0.000	
B^2	133.71	1	133.71	80.79	0.000	
C^2	429.69	1	429.69	259.61	0.000	
D^2	27.85	1	27.85	16.83	0.000	
E^2	192.78	1	192.78	116.48	0.000	
2-Way Interaction	256.11	10	25.61	15.47	0.000	
AB	17.29	1	17.29	10.45	0.003	
AC	19.12	1	19.12	11.55	0.002	
AD	4.60	1	4.60	2.78	0.108	
AE	6.75	1	6.75	4.08	0.054	
BC	118.59	1	118.59	71.65	0.000	
BD	6.33	1	6.33	3.83	0.062	
BE	17.70	1	17.70	10.70	0.003	
CD	17.70	1	17.70	10.70	0.003	
CE	0.01	1	0.01	0.00	0.954	
DE	48.02	1	48.02	29.01	0.000	
Residual	41.38	25	1.66			
Lack of Fit	31.96	20	1.60	0.85	0.646	Not significant
Pure error	9.41	5	1.88			
Total	2979.78	45				

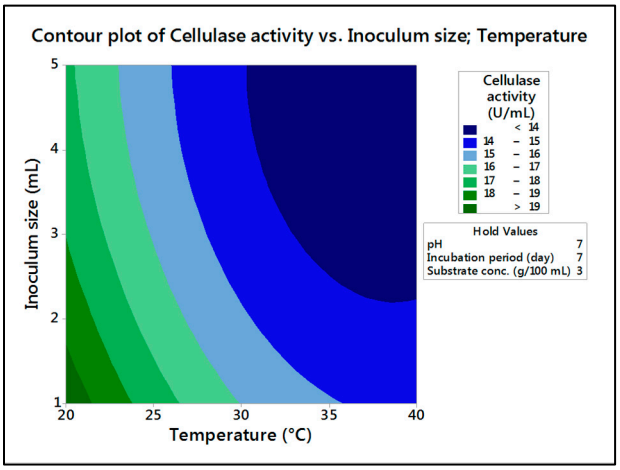
R^2: 0.9861, adjusted R^2: 0.9750, and predicted R^2: 0.9525.



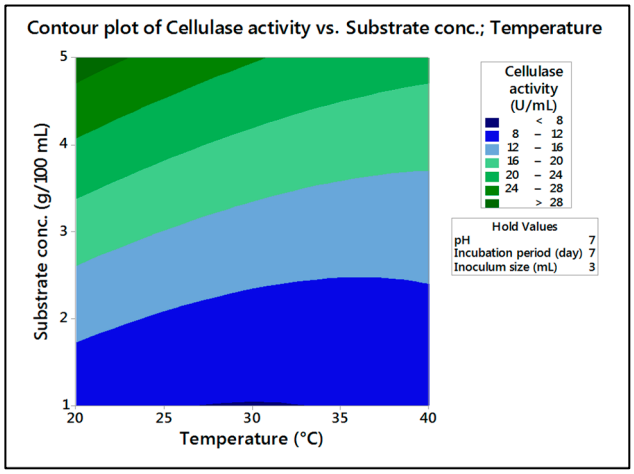
(**a**)



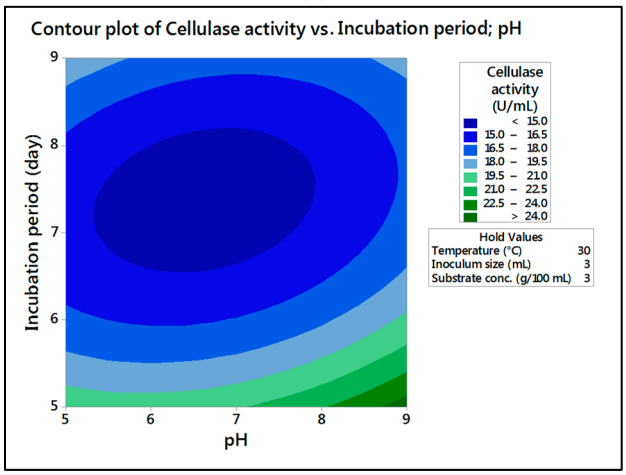
(**b**)



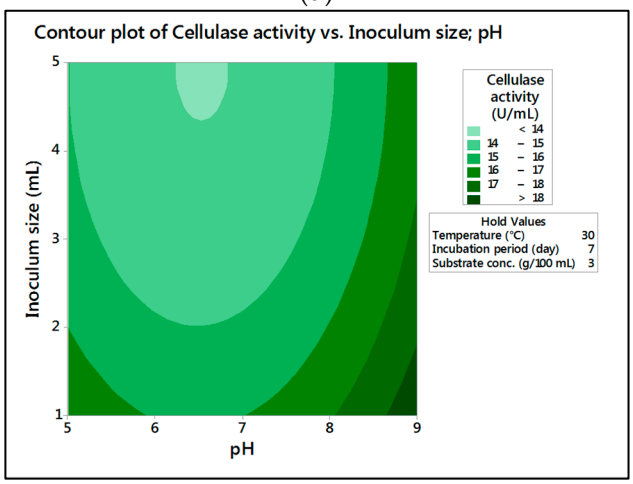
(**c**)



(**d**)



(**e**)



(**f**)

Figure 3. *Cont.*

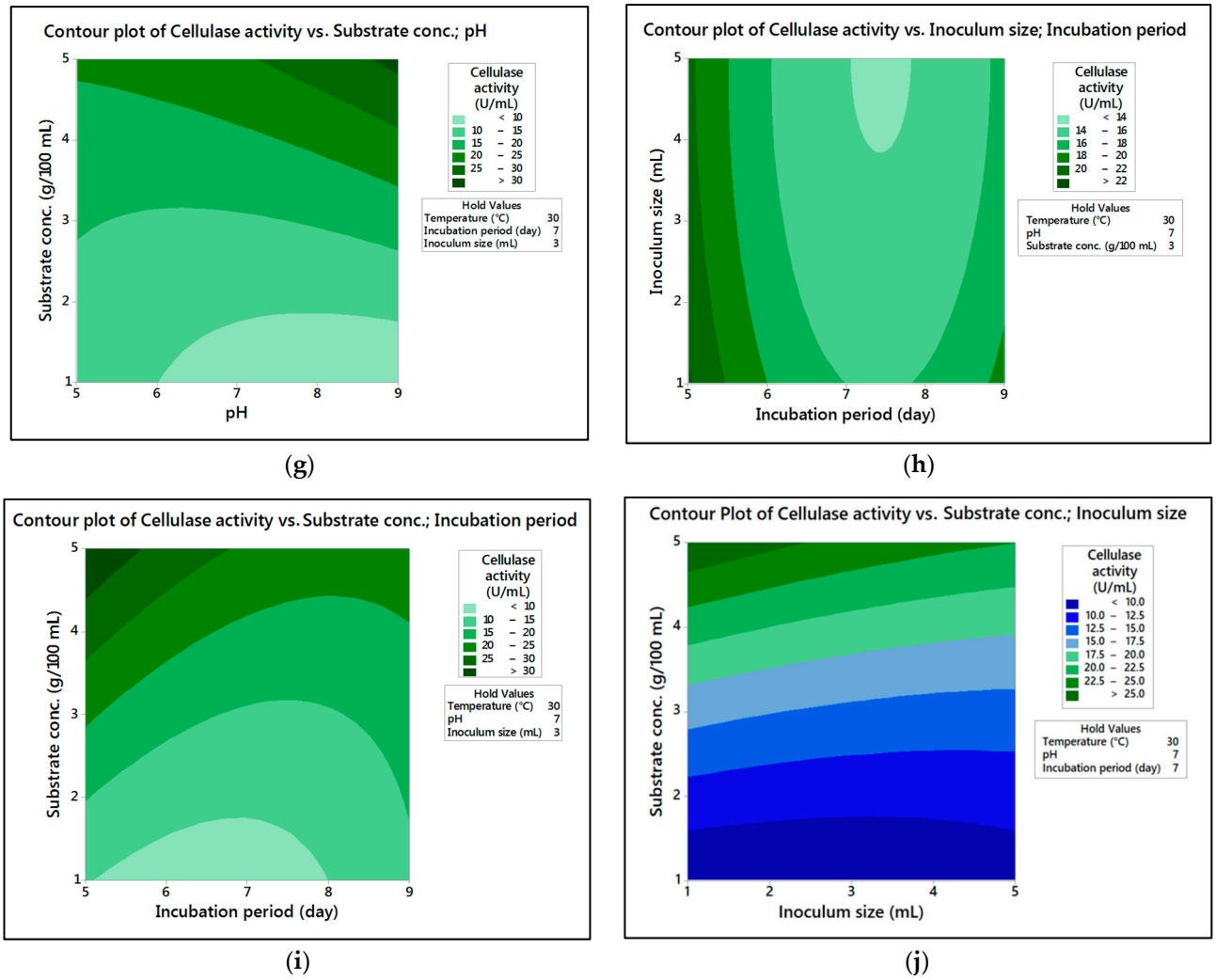
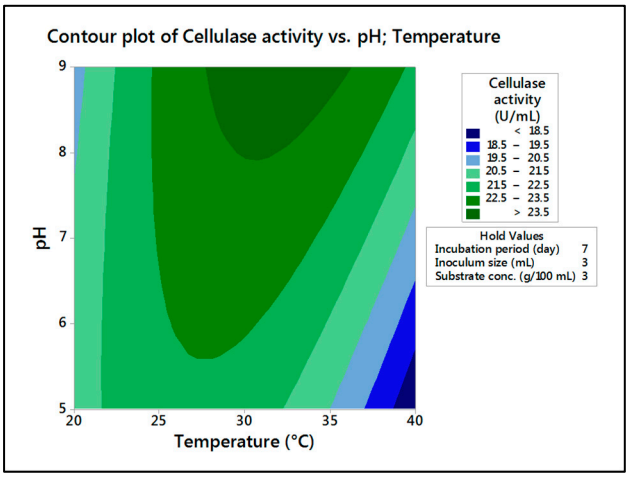


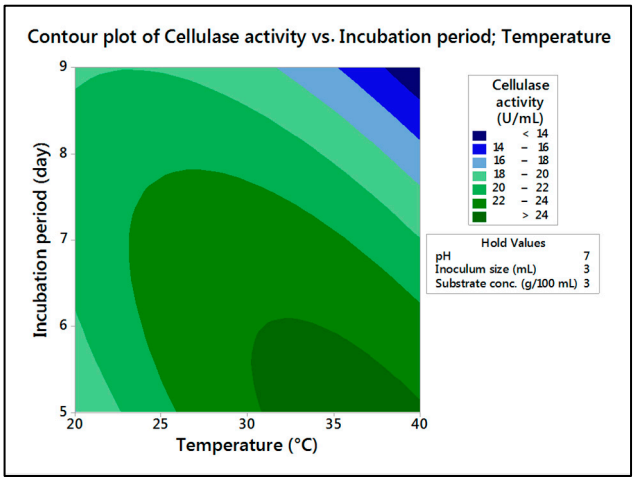
Figure 3. Contour plot showing interactions between independent variables: (**a**) incubation temperature with pH; (**b**) incubation temperature with incubation period; (**c**) incubation temperature with inoculum size; (**d**) incubation temperature with substrate concentration; (**e**) pH with incubation period; (**f**) pH with inoculum size; (**g**) pH with substrate concentration; (**h**) incubation period with inoculum size; (**i**) incubation period with substrate concentration; (**j**) inoculum size with substrate concentration for cellulase activity produced by *M. circinelloides*.

3.3. Partial Purification of Cellulase and Pectinase

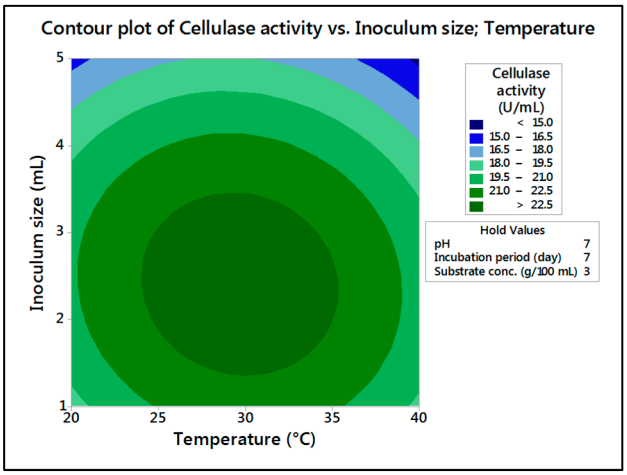
Extracellular cellulase and pectinase from *M. circinelloides* and *M. hiemalis* were partially purified from broth cultures by using different acetone concentrations. The highest cellulase (6.37 and 8.10 U/mL) and pectinase (7.23 and 5.50 U/mL) activities from *M. circinelloides* and *M. hiemalis*, respectively, were obtained via the precipitation of crude filtrate with acetone at the ratio 1:4 (Table 8). Cellulase purification produced 1.73- and 2.03-fold purification, 31.12 and 32.02% cellulase recovery with specific activity of 199.41 and 163.43 U/mg from *M. circinelloides* and *M. hiemalis*, respectively, while 1.74- and 1.99-fold purification, 31.26 and 31.51% recovery with specific activity of 216.83 and 215.36 U/mg were obtained from *M. circinelloides* and *M. hiemalis* pectinase, respectively (Table 9).



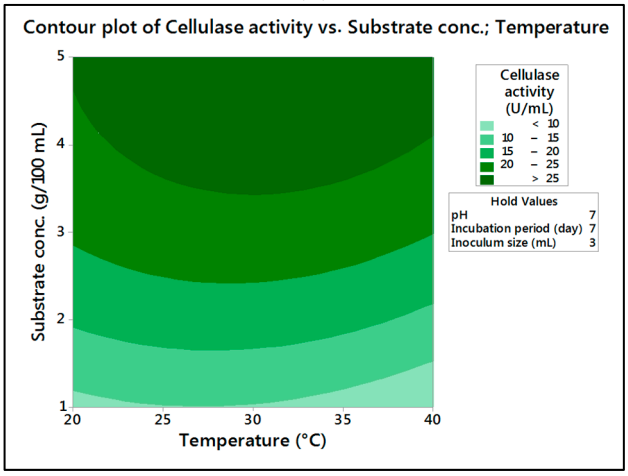
(**a**)



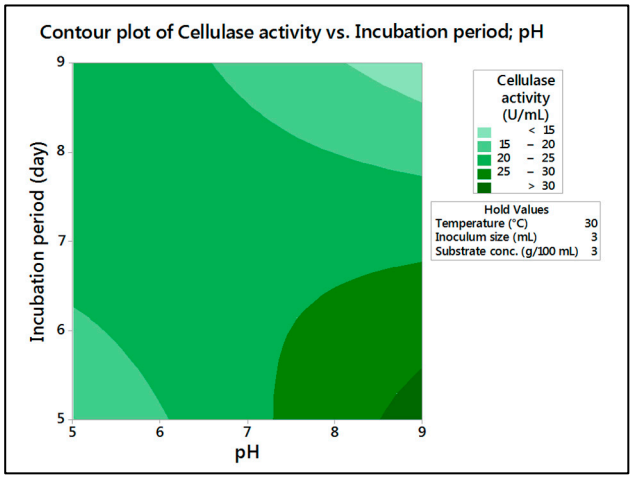
(**b**)



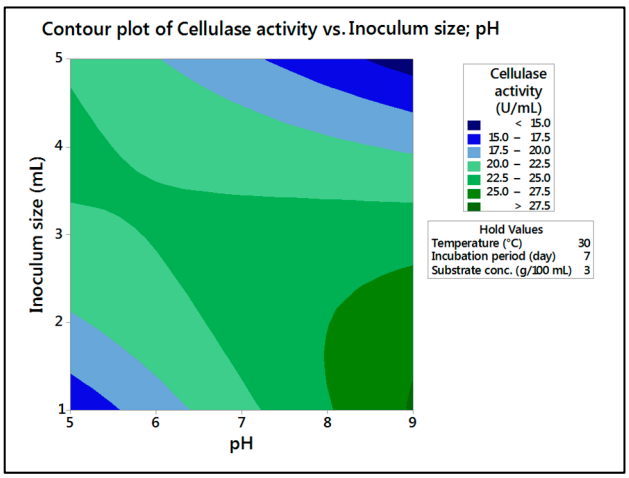
(**c**)



(**d**)

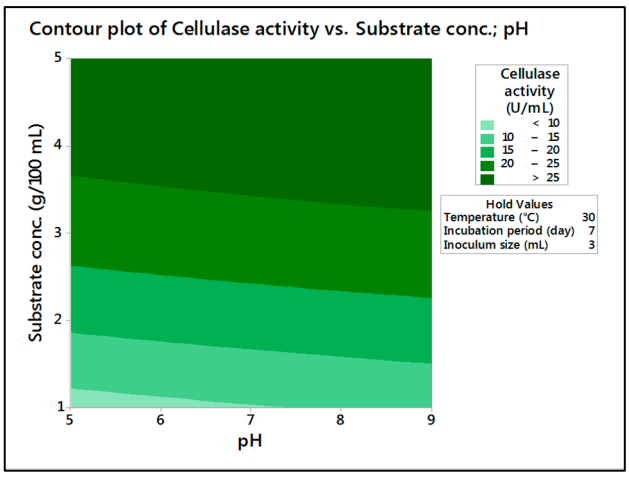


(**e**)

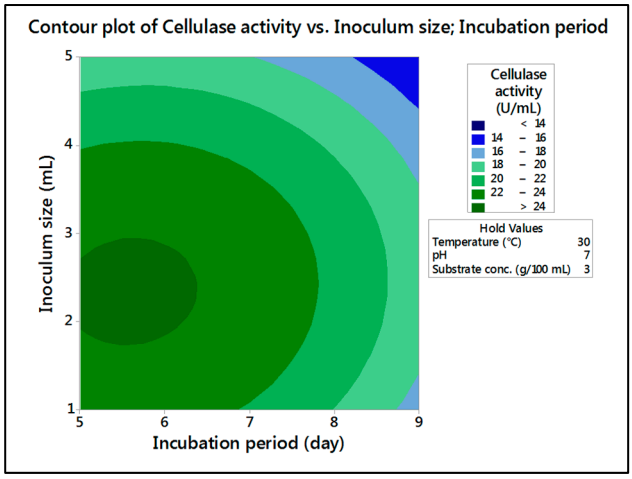


(**f**)

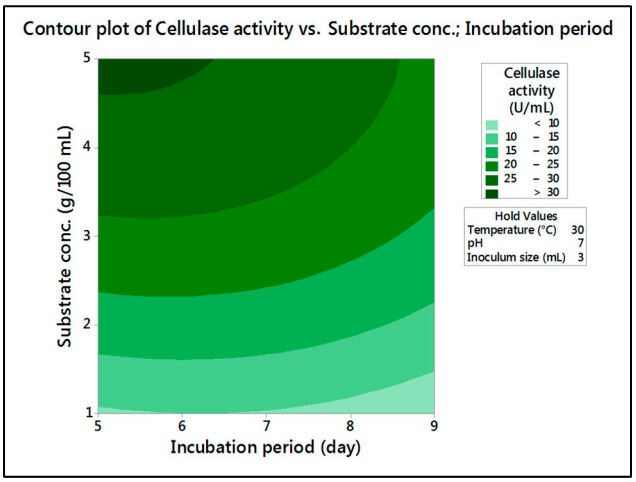
Figure 4. *Cont.*



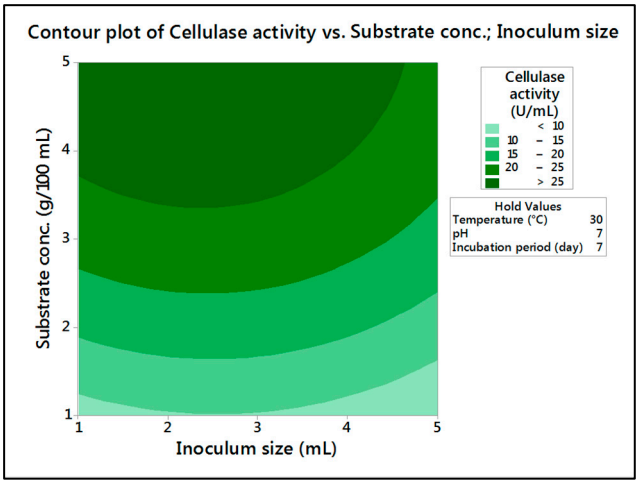
(**g**)



(**h**)

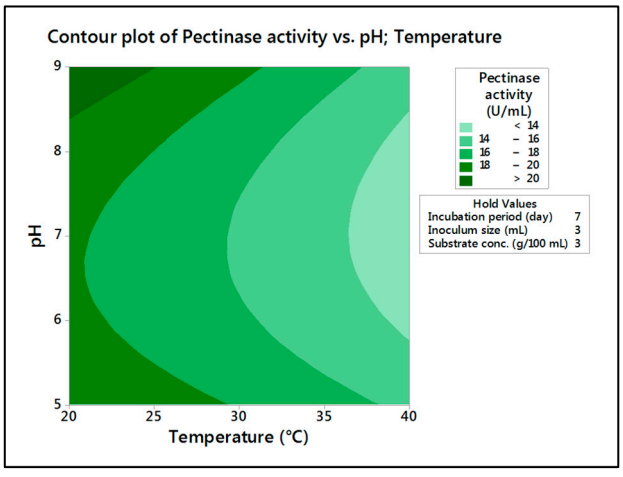


(**i**)

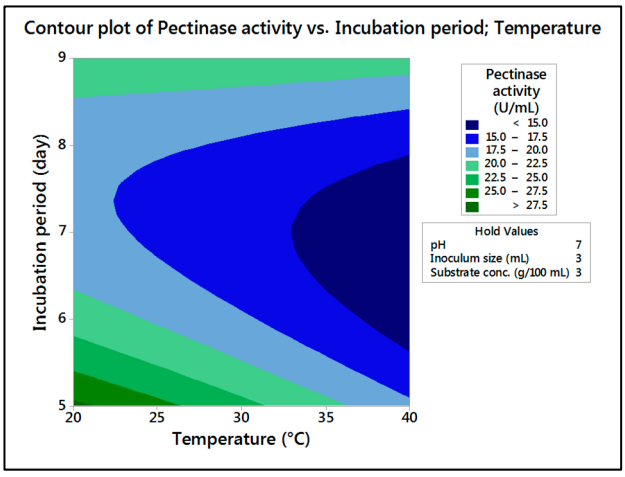


(**j**)

Figure 4. Contour plot showing interactions between independent variables: (**a**) incubation temperature with pH; (**b**) incubation temperature with incubation period; (**c**) incubation temperature with inoculum size; (**d**) incubation temperature with substrate concentration; (**e**) pH with incubation period; (**f**) pH with inoculum size; (**g**) pH with substrate concentration; (**h**) incubation period with inoculum size; (**i**) incubation period with substrate concentration; (**j**) inoculum size with substrate concentration for cellulase activity produced by *M. hiemalis*.

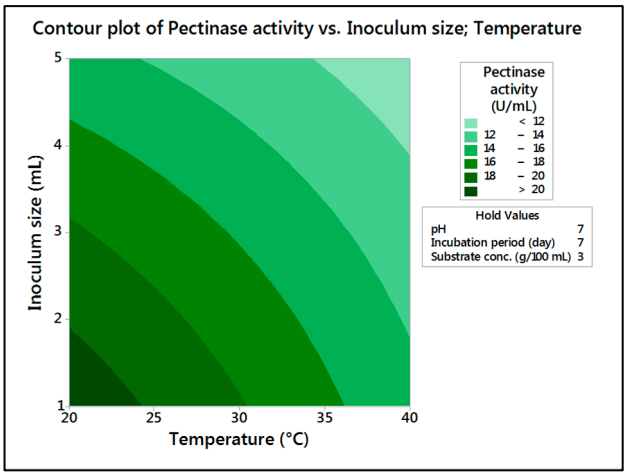


(**a**)

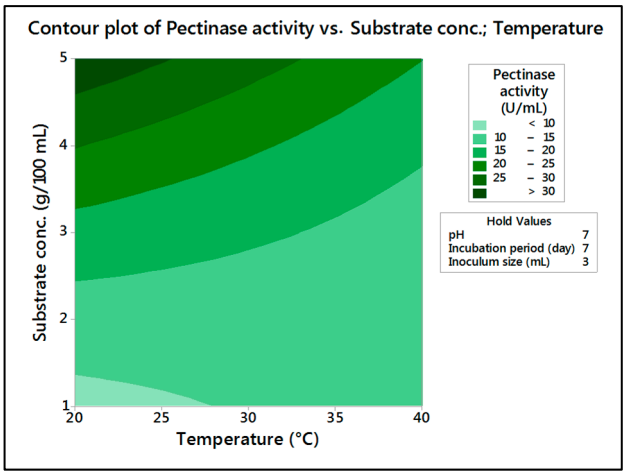


(**b**)

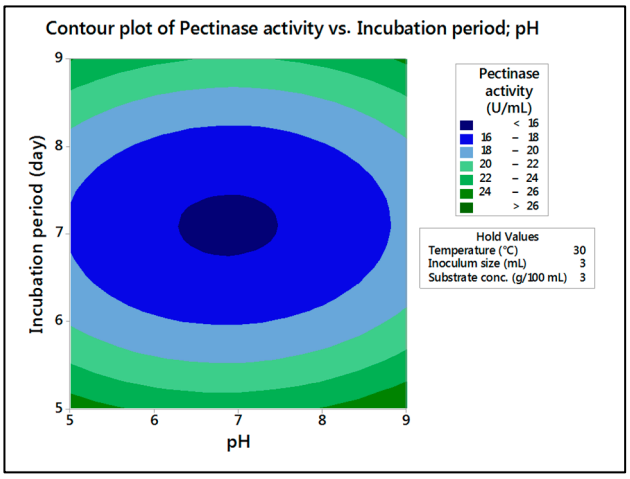
Figure 5. *Cont.*



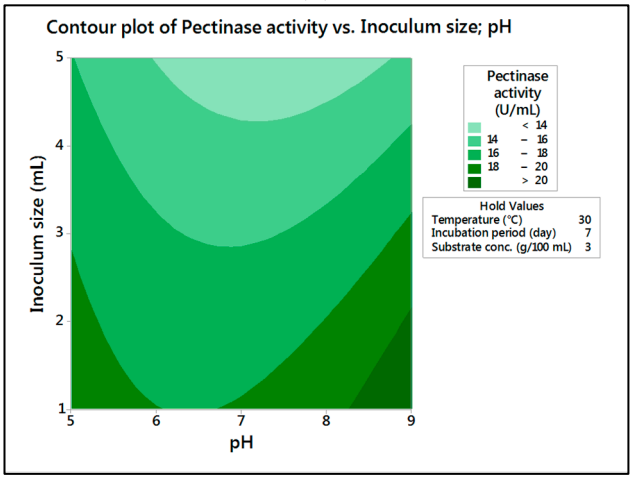
(**c**)



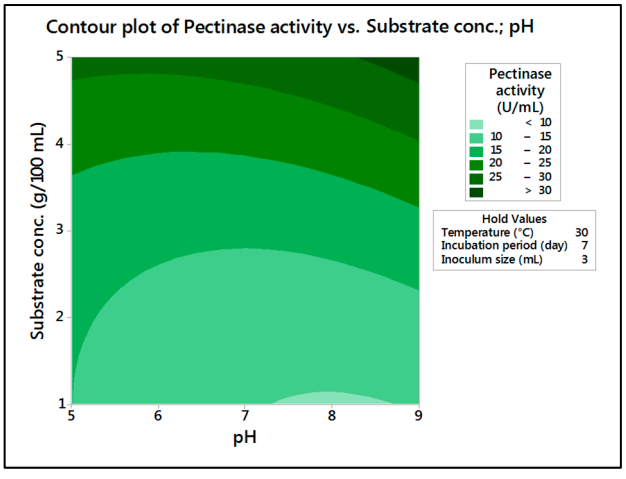
(**d**)



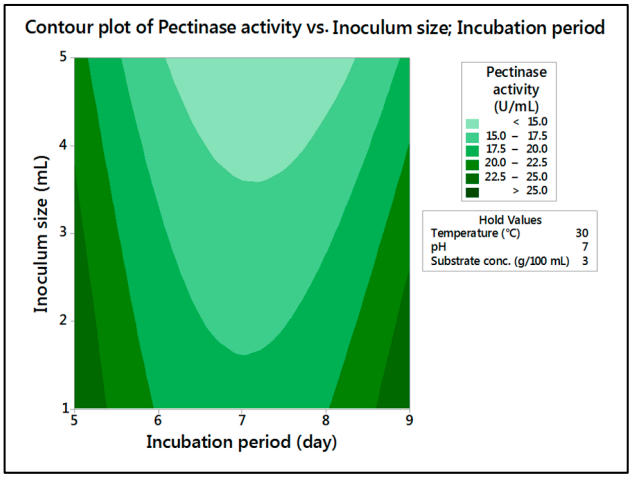
(**e**)



(**f**)

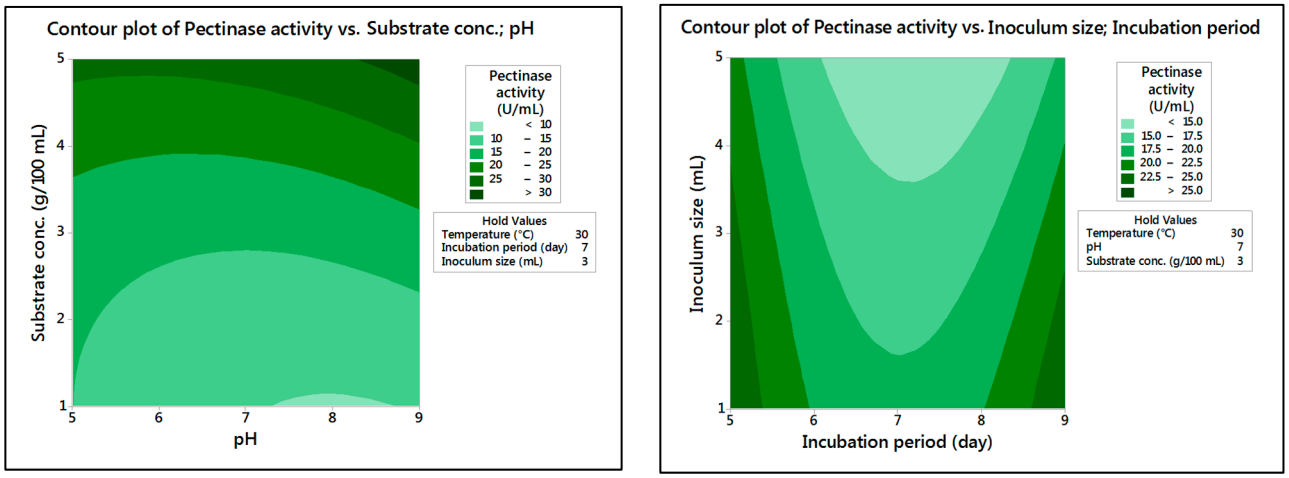


(**g**)



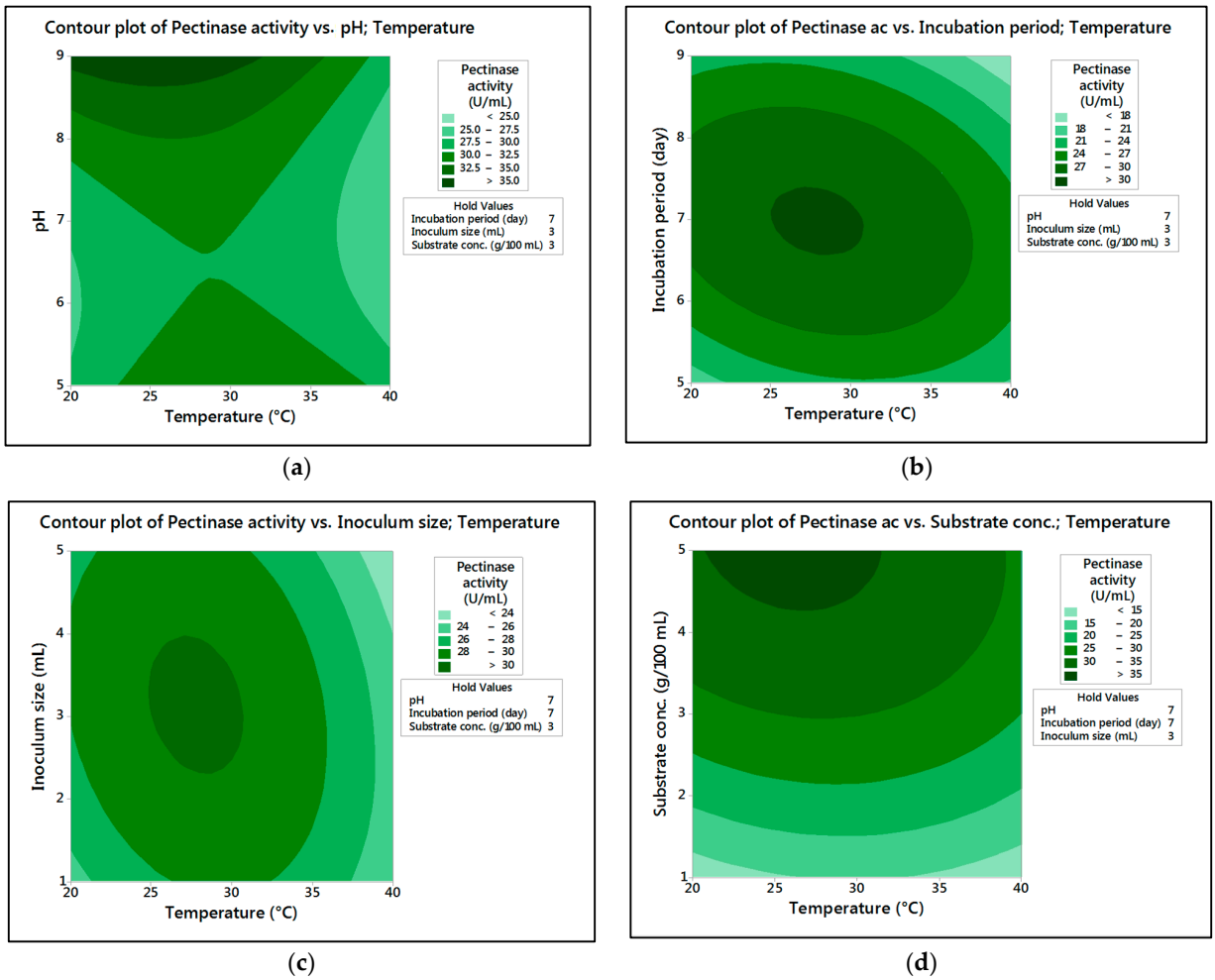
(**h**)

Figure 5. *Cont.*



(**g**) (**h**)

Figure 5. Contour plot showing interactions between independent variables: (**a**) incubation temperature with pH; (**b**) incubation temperature with incubation period; (**c**) incubation temperature with inoculum size; (**d**) incubation temperature with substrate concentration; (**e**) pH with incubation period; (**f**) pH with inoculum size; (**g**) pH with substrate concentration; (**h**) incubation period with inoculum size; (**i**) incubation period with substrate concentration; (**j**) inoculum size with substrate concentration for pectinase activity produced by *M. circinelloides*.



(**a**) (**b**)

(**c**) (**d**)

Figure 6. *Cont.*

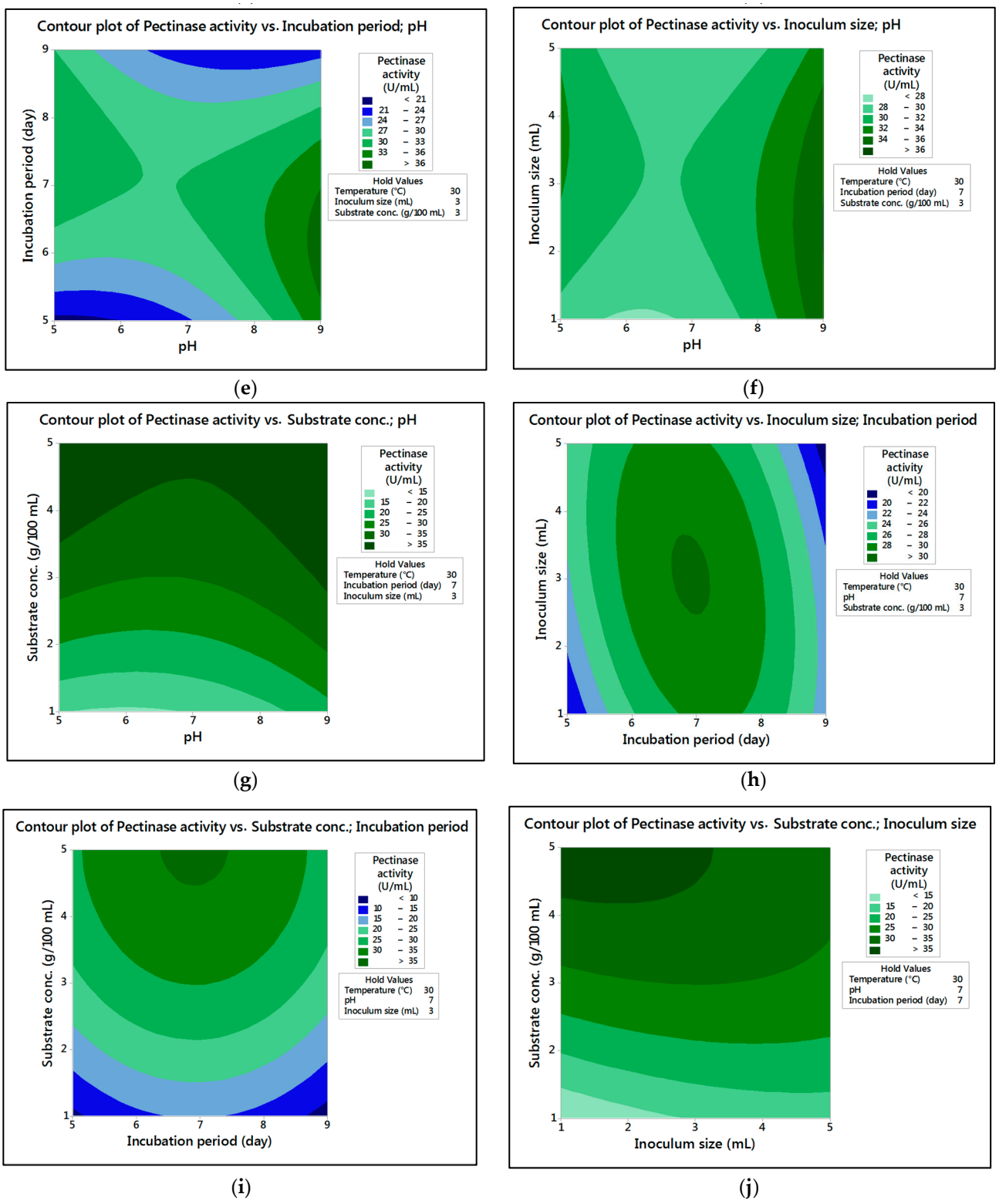


Figure 6. Contour plot showing interactions between independent variables: (**a**) incubation temperature with pH; (**b**) incubation temperature with incubation period; (**c**) incubation temperature with inoculum size; (**d**) incubation temperature with substrate concentration; (**e**) pH with incubation period; (**f**) pH with inoculum size; (**g**) pH with substrate concentration; (**h**) incubation period with inoculum size; (**i**) incubation period with substrate concentration; (**j**) inoculum size with substrate concentration for pectinase activity produced by *M. hiemalis*.

Table 8. Precipitation of cellulase and pectinase produced by *M. circinelloides* and *M. hiemalis* using different concentrations of acetone.

Ratio (Crude:Acetone)	Cellulase Activity (U/mL)		Pectinase Activity (U/mL)	
	M. circinelloides	*M. hiemalis*	*M. circinelloides*	*M. hiemalis*
1:1	3.11 ± 0.08 ^{d}	4.85 ± 0.08 ^{d}	4.60 ± 0.24 ^{e}	2.87 ± 0.24 ^{e}
1:2	3.63 ± 0.10 ^{c}	5.36 ± 0.10 ^{c}	5.06 ± 0.07 ^{d}	3.32 ± 0.07 ^{d}
1:3	5.36 ± 0.11 ^{b}	7.09 ± 0.11 ^{b}	7.08 ± 0.06 ^{b}	5.34 ± 0.06 ^{b}
1:4	6.37 ± 0.04 ^{a}	8.10 ± 0.04 ^{a}	7.23 ± 0.05 ^{a}	5.50 ± 0.05 ^{a}
1:5	5.43 ± 0.10 ^{b}	7.05 ± 0.26 ^{b}	6.89 ± 0.11 ^{c}	5.22 ± 0.07 ^{c}

The data were given as averages of three replicates (mean ± SD). Values followed by the different letters are significantly different at $p < 0.05$.

Table 9. Summary of specific activity, yield and purification fold of cellulase and pectinase produced by *M. circinelloides* and *M. hiemalis*.

Purification Steps	Fungal Strain	Enzyme	Total Activity (U/mL)	Total Protein (mg/mL)	Specific Activity (U/mg)	Yield (%)	Purification Fold
Culture supernatant	*M. circinelloides*	Cellulase	9918.06 ± 63.92	86.05 ± 4.49	115.58 ± 6.33	100.00 ± 0.00	1.00 ± 0.00
		Pectinase	10,740.62 ± 39.60	86.05 ± 4.49	125.17 ± 6.73	100.00 ± 0.00	1.00 ± 0.00
	M. hiemalis	Cellulase	8258.02 ± 91.15	102.39 ± 1.26	80.68 ± 1.86	100.00 ± 0.00	1.00 ± 0.00
		Pectinase	11,063.86 ± 192.37	102.39 ± 1.26	108.07 ± 2.03	100.00 ± 0.00	1.00 ± 0.00
Acetone	*M. circinelloides*	Cellulase	3087.06 ± 67.34	15.48 ± 0.08	199.41 ± 5.27	31.12 ± 0.61	1.73 ± 0.05
		Pectinase	3357.16 ± 17.87	15.48 ± 0.08	216.83 ± 1.09	31.26 ± 0.22	1.74 ± 0.10
	M. hiemalis	Cellulase	2644.05 ± 27.34	16.19 ± 0.30	163.43 ± 4.34	32.02 ± 0.02	2.03 ± 0.04
		Pectinase	3485.80 ± 57.71	16.19 ± 0.30	215.36 ± 0.70	31.51 ± 0.03	1.99 ± 0.04

The data were given as averages of three replicates (mean ± SD).

3.4. Characterization of Partially Purified Cellulase and Pectinase

M. circinelloides partially purified cellulase was highly active at 70 °C (total activity 100%) and decreased gradually at 60–30 °C and 80–90 °C, while the cellulase activity of *M. hiemalis* was highly active at 50 °C (total activity 100%) and decreased gradually at 40–30 °C and 60–90 °C. The relative cellulase stability from both *M. circinelloides* and *M. hiemalis* was high at 30 °C and decreased in the range of 40–90 °C (Figure 7a,b). Partially purified pectinase from *M. circinelloides* showed the best activity at 50 °C (total activity 100%) and decreased gradually at 40–30 °C and 60–90 °C; meanwhile, the pectinase activity of *M. hiemalis* was highly active at 60 °C (total activity 100%) and decreased gradually at 50–30 °C and 70–90 °C. The relative pectinase stability from both *M. circinelloides* and *M. hiemalis* was high at 30 °C and decreased in the range of 40–90 °C (Figure 7c,d). Furthermore, partially purified cellulase and pectinase from both strains had the highest activity and stability (100%) at pH 7.0, and then, at high or low pH values, the activity and stability of enzymes were reduced (Figure 8a–d).

The cellulase activity of both strains after incubation with 10 mM of K^+, Mg^{2+}, Ba^{2+} and Ni^{2+} was decreased except for K^+, which increased *M. circinelloides* partially purified cellulase relative activity and stability by 20.05 and 2.78%, respectively, than control. In contrast, stability decreased except for Ba^{2+} with the *M. circinelloides* enzyme and K^+, Mg^{2+}, and Ni^{2+} with the *M. hiemalis* enzyme. Detergents including tween 80 (1 and 5%), tween 20 (1%), and urea (1%), increased *M. circinelloides* partially purified cellulase relative activity, while a remarkable decrease in relative stability was reported at all concentrations of the tested detergents for enzymes of both strains (Table 10).

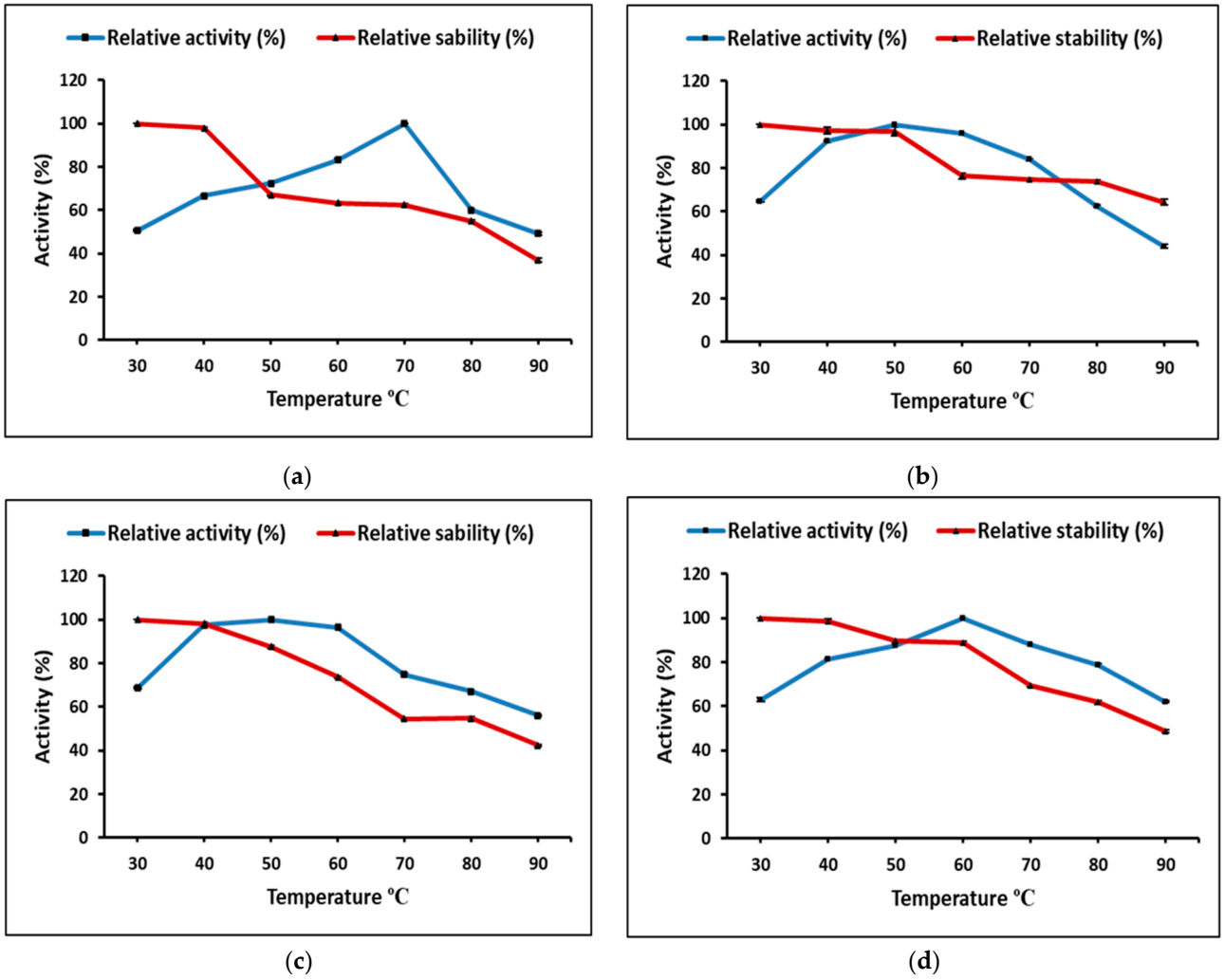


Figure 7. Effects of temperature on activity and stability of partially purified cellulase: (**a**) *M. circinelloides*; (**b**) *M. hiemalis*; and pectinase: (**c**) *M. circinelloides*; and (**d**) *M. hiemalis*.

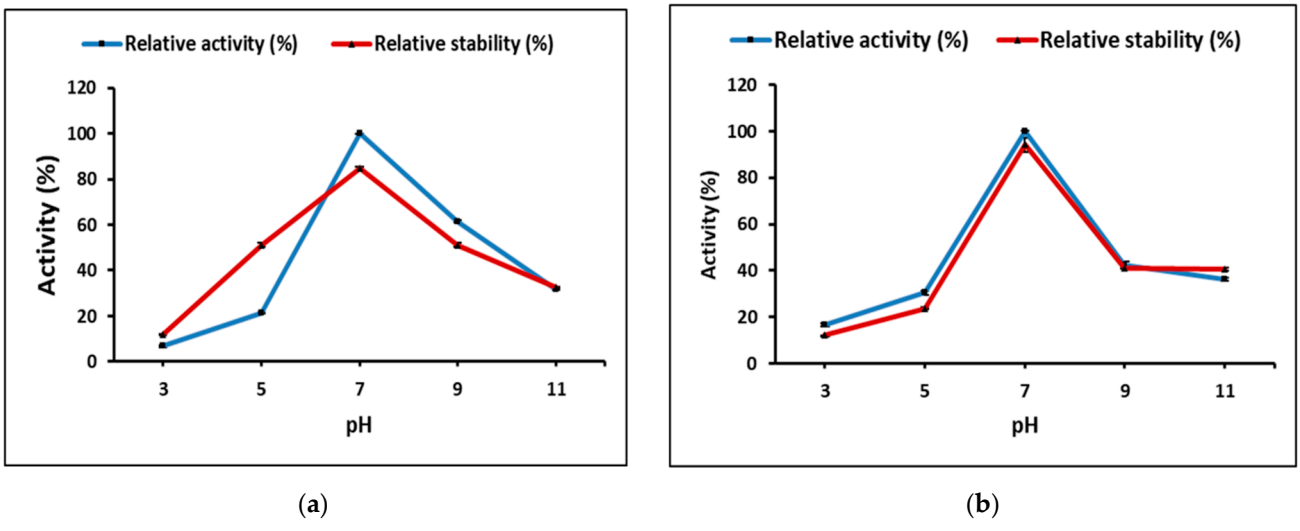


Figure 8. *Cont.*

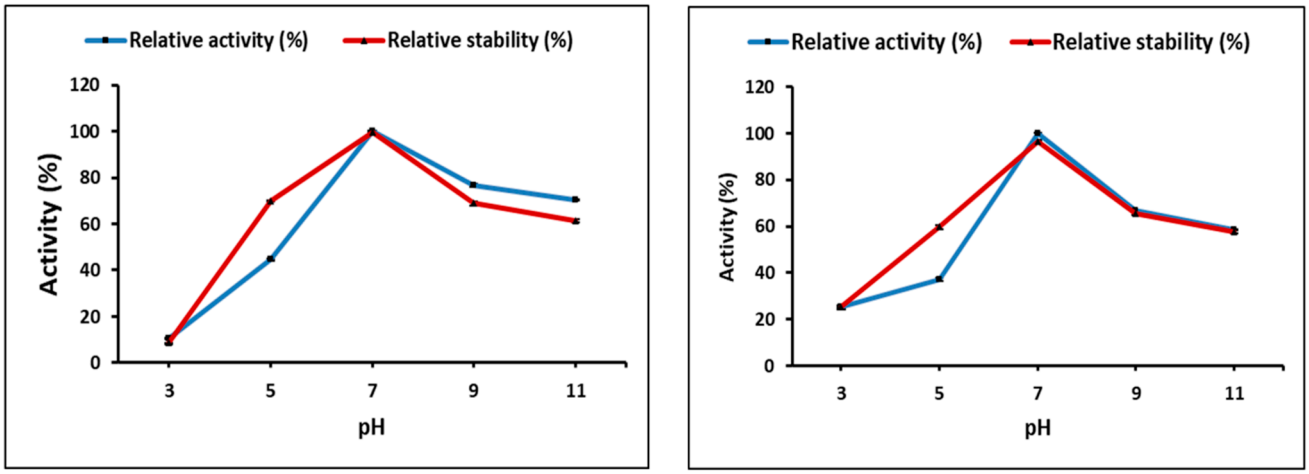


Figure 8. Effects of pH on activity and stability of partially purified cellulase: (**a**) *M. circinelloides*; (**b**) *M. hiemalis*; and pectinase: (**c**) *M. circinelloides*; and (**d**) *M. hiemalis*.

Table 10. Effects of metal ions and detergents on activity and stability of partially purified cellulase from *M. circinelloides* and *M. hiemalis*.

Metal Ions and Detergents	Conc.	*M. circinelloides*		*M. hiemalis*	
		Relative Activity (%)	Relative Stability (%)	Relative Activity (%)	Relative Stability (%)
Control	0	100.00 ± 0.00 ^{e}	-	100.00 ± 0.00 ^{a}	-
K^+	10 mM	120.05 ± 0.69 ^{b}	102.78 ± 0.78 ^{A}	61.18 ± 0.79 ^{g}	94.62 ± 0.76 ^{A}
Mg^{2+}	10 mM	60.36 ± 0.69 ^{i}	42.83 ± 0.57 ^{I}	34.12 ± 0.20 ^{k}	73.11 ± 0.39 ^{B}
Ba^{2+}	10 mM	30.49 ± 0.21 ^{l}	59.83 ± 0.38 ^{H}	43.23 ± 0.59 ^{j}	35.01 ± 0.73 ^{I}
Ni^{2+}	10 mM	41.58 ± 0.24 ^{k}	30.64 ± 0.11 ^{J}	11.12 ± 0.91 ^{l}	50.43 ± 0.92 ^{F}
Tween 80	1% (*v*/*v*)	105.60 ± 0.81 ^{d}	89.41 ± 0.32 ^{C}	69.03 ± 0.21 ^{c}	58.16 ± 0.93 ^{D}
	5% (*v*/*v*)	108.43 ± 0.78 ^{c}	97.00 ± 0.89 ^{B}	68.87 ± 0.25 ^{c,d}	49.95 ± 0.75 ^{F}
Tween 20	1% (*v*/*v*)	106.39 ± 0.24 ^{d}	77.17 ± 0.69 ^{F}	71.01 ± 0.38 ^{b}	42.68 ± 0.83 ^{G}
	5% (*v*/*v*)	87.46 ± 0.29 ^{g}	68.67 ± 0.74 ^{G}	64.08 ± 0.35 ^{e}	64.29 ± 0.96 ^{C}
Urea	1% (*w*/*v*)	121.78 ± 0.43 ^{a}	88.38 ± 0.38 ^{D}	67.97 ± 0.77 ^{d}	58.88 ± 0.42 ^{D}
	5% (*w*/*v*)	91.33 ± 0.35 ^{f}	79.55 ± 0.80 ^{E}	62.44 ± 0.54 ^{f}	56.60 ± 0.99 ^{E}
Na_2CO_3	50 mM	75.25 ± 0.50 ^{h}	69.55 ± 0.61 ^{G}	56.24 ± 0.54 ^{i}	39.66 ± 0.91 ^{H}
	75 mM	53.46 ± 0.78 ^{j}	42.06 ± 0.76 ^{I}	60.14 ± 0.98 ^{h}	39.65 ± 0.95 ^{H}

The data were given as averages of three replicates (mean ± SD). Values followed by the different letters are significantly different at $p < 0.05$.

After incubation with 10 mM K^+, Mg^{2+}, Ba^{2+}, and Ni^{2+}, the partially purified pectinase activity of both strains decreased with the exception of K^+, which increased *M. circinelloides* pectinase activity by 16.38% compared to the control; meanwhile, stability increased except for K^+ and Ba^{2+} with *M. circinelloides* enzyme, and K^+ and Mg^{2+} with *M. hiemalis* enzyme. Detergents including tween 80 (1%) increased *M. circinelloides* pectinase activity by 12.92% compared to the control. In contrast, there was a significant decrease in *M. circinelloides* and *M. hiemalis* partially purified pectinase activity with other tested detergents. A fluctuation in significant increase and decrease in pectinase stability of both strains was reported at low and high concentrations of tween 80, tween 20, urea and Na_2CO_3 (Table 11).

Table 11. Effects of metal ions and detergents on activity and stability of partially purified pectinase from *M. circinelloides* and *M. hiemalis*.

Metal Ions and Detergents	Conc.	*M. circinelloides*		*M. hiemalis*	
		Relative Activity (%)	Relative Stability (%)	Relative Activity (%)	Relative Stability (%)
Control	0	100.00 ± 0.00 ^{c}	-	100.00 ± 0.00 ^{a}	-
K^+	10 mM	116.38 ± 0.68 ^{a}	110.11 ± 0.71 ^{A}	68.73 ± 0.46 ^{c}	68.15 ± 0.75 ^{A}
Mg^{2+}	10 mM	98.56 ± 0.78 ^{d}	100.10 ± 0.41 ^{C}	53.57 ± 0.26 ^{e}	53.51 ± 0.44 ^{C}
Ba^{2+}	10 mM	61.70 ± 0.57 ^{f}	23.28 ± 0.16 ^{I}	61.16 ± 0.49 ^{d}	67.13 ± 0.94 ^{B}
Ni^{2+}	10 mM	26.68 ± 0.35 ^{j}	70.47 ± 0.53 ^{G}	27.00 ± 0.61 ^{l}	46.14 ± 0.72 ^{G}
Tween 80	1% (*v*/*v*)	112.92 ± 0.87 ^{b}	102.08 ± 0.82 ^{B}	35.57 ± 0.67 ^{i}	52.42 ± 0.41 ^{D}
	5% (*v*/*v*)	37.82 ± 0.22 ^{i}	89.22 ± 0.43 ^{E}	51.83 ± 0.24 ^{f}	48.55 ± 0.27 ^{F}
Tween 20	1% (*v*/*v*)	55.80 ± 0.71 ^{g}	90.43 ± 0.48 ^{D}	41.08 ± 0.42 ^{g}	52.07 ± 0.49 ^{D}
	5% (*v*/*v*)	75.14 ± 0.88 ^{e}	85.95 ± 0.59 ^{F}	70.61 ± 0.51 ^{b}	50.20 ± 0.80 ^{E}
Urea	1% (*w*/*v*)	62.62 ± 0.64 ^{f}	70.44 ± 0.88 ^{G}	38.88 ± 0.30 ^{h}	50.85 ± 0.74 ^{E}
	5% (*w*/*v*)	47.27 ± 0.61 ^{h}	85.52 ± 0.89 ^{F}	30.65 ± 0.49 ^{k}	48.51 ± 0.73 ^{F}
Na_2CO_3	50 mM	99.42 ± 0.47 ^{d}	58.21 ± 0.82 ^{H}	33.83 ± 0.52 ^{j}	38.68 ± 0.75 ^{I}
	75 mM	56.26 ± 0.41 ^{g}	58.13 ± 0.93 ^{H}	31.45 ± 0.51 ^{k}	40.97 ± 0.55 ^{H}

The data were given as averages of three replicates (mean ± SD). Values followed by the different letters are significantly different at $p < 0.05$.

4. Discussion

Fungi are an excellent source for the production of various beneficial enzymes. The lignin present in the polymer matrix in plant cell walls form a hydrophobic network which inhibits the access of microbial enzymes to degrade cell-wall components [40,41]. Therefore, the lowest content of lignin in the citrus peels could enhance microbial-enzyme production due to their tissue structure flexibility, allowing the access of the microorganism to the cellulose, pectin and hemicellulose [18]. In this research, *M. circinelloides* and *M. hiemalis* were investigated as potential sources for producing cellulase and pectinase on tangerine peel by submerged fermentation. The type of substrate and the presence of growth factors affect the fungal growth and the biosynthesis of enzymes. Therefore, there is an urgent need to enhance the biosynthesis of the enzymes by optimizing fungal production.

In the current research, *M. circinelloides* and *M. hiemalis* afforded high cellulase production (37.20 U/mL and 33.82 U/mL, respectively) and pectinase (38.02 U/mL and 39.76 U/mL, respectively) at the optimum parameters, which consisted of, respectively, incubation temperature 30 and 30 °C, pH value 7 and 9, incubation period 5 and 5 days, inoculum size 3 and 3 mL, and substrate concentration 5 and 3 g/100 mL; while, for pectinase, optimum conditions included incubation temperature 30 and 30 °C, pH value 7 and 5, incubation period 9 and 7 days, inoculum size 3 and 3 mL, and substrate concentration 5 and 5 g/100 mL, respectively, for *M. circinelloides* and *M. hiemalis*. The multiple correlation coefficient $R^2 = 0.9535$ for *M. circinelloides* and $R^2 = 0.9782$ for *M. hiemalis* cellulase and $R^2 = 0.9748$ for *M. circinelloides* and $R^2 = 0.9861$ for *M. hiemalis* pectinase, nigh to one, indicated that experimental and predicted values are well-correlated and predicted values elucidated the model accuracy with upgrade response. The low *p* values, which are attained by the F test, and high R^2 values indicated that the employed model attained a high significance, and its sufficiency was confirmed [42]. Similarly, *Aspergillus niger*-ATCC 1640 achieved 0.6045 µmol/mL pectinase production using *Citrus macroptera* peel (8.4 g/L) in solid-state fermentation. RSM results indicated that the experimental response for pectinase production was convenient with fitted data ($R^2 = 0.9836$) [43]. The highest cellulase activity (5.60 IU/mL) was obtained after incubation for 4 days and 5% substrate concentration with pH 5.0 at 30 °C using RSM of *Trichoderma viride* in SmF of the seed pods of the silk cotton tree [44]. *Rhizopus delemar* F2 optimal variable values for the maximum production of cellulase (10.40 U/gds) and of pectinase (31.20 U/gds) using solid state fermentation on apple pomace substrate included a moisture ratio of 1:3:5 for 7 days at 30 °C [45].

Conversely, statistical design for the maximum production of pectinase (179.83 U/g in SSF and 1.64 U/mL in SmF) and cellulase (10.81 U/g in SSF and 0.36 U/mL in SmF) by *Aspergillus niger* NCIM 548 was achieved at optimum conditions in SmF consisting of carbon source concentration 65 g/L, pH 4.6, and time 126 h; while in SSF, moisture content was 65% and pH 4.80 for 156 h [46]. On the other hand, the optimum condition for *A. oryzae* producing the maximal pectinase (139.56 U/gds) and cellulase (6.01 U/gds) was 67% of moisture content with pH 5.9 at 33 °C, and for 71.8 h of fermentation on soybean residue [47]. Optimum cellulase activity (124.94 U/g) was attained at 1.5% *w*/*v* rice straw with pH 7 at 30 °C for 8 days by *Aspergillus terreus* RS2 [48]. Ramos-Ibarra et al. [18] utilized RSM for a high production of cellulase (1.0 U/g after 24 h) and pectinase (12.3 U/g after 120 h) using *Mucor racemosus* N9C1 on orange peels by SSF in humidity 70% at 30 °C. They anticipated that increased enzymatic activity reaching its maximum and decreasing at the end of the fermentation period may be attributed to enzyme hydrolysis by proteases.

In the present investigation, *M. circinelloides* and *M. hiemalis* partially purified cellulase and pectinase showed 6.37 and 8.10 U/mL and 7.23 and 5.50 U/mL activities, respectively, and cellulase 1.73- and 2.03-fold purification, 31.12 and 32.02% cellulase recovery with specific activity of 199.41 and 163.43 U/mg; while 1.74- and 1.99-fold purification, 31.26 and 31.51% recovery with specific activity of 216.83 and 215.36 U/mg, respectively, were obtained. Our results were in agreement with Almowallad et al. [25], who utilized *Aspergillus niger* AUMC 4156, *Penicillium oxalicum* AUMC 4153, and *Paecilomyces variotii* AUMC 4149 on orange peel (3% *w*/*v*) by SmF and obtained pectinase activity in static (52.22, 14.06 and 49.26%) and shaken cultures (48.89, 2.94, 50.00%), respectively. Orange peel as a sole carbon source afforded the highest protein content in filtrates with all tested fungal strains in stirred (2.57, 3.75, and 3.40 mg/mL) and static cultures (4.74, 4.45, and 4.98 mg/mL), respectively. Statistical-derived optimum conditions for crude cellulase produced by the SmF of *A. niger* using *A. hypogaea* shells as a carbon source involved 120 h incubation with pH 4 at 40 °C, along with of 13×10^5 CFU/mL inoculum size, while purified cellulase resulted in a 68.12-fold purification with yield 3.87% and specific activity of 484.3 U/mg [23].

Partially purified cellulase and pectinase from *M. circinelloides* and *M. hiemalis* demonstrated the highest activity at neutral pH, and 70 and 50 °C, for cellulase and 50 and 60 °C, for pectinase, respectively. Thakur et al. [13] highlighted that each enzymatic application requires unique properties with respect to specificity, stability, temperature, and pH dependence. High temperature increases the solubility of reactants and products by decreasing viscosities, resulting in faster hydrolysis [49], and longer active life under high temperatures would make enzymes favorable for efficient biomass conversion. Therefore, thermo-stability is the most significant property for the enzyme used under extreme bioprocessing conditions to be efficient [50]. Optimally, purified pectinase from *Rhizomucor pusillus* was active at 55 °C and pH 5.0, and showed stability up to 50 °C and a pH range between 4.0 and 5.0 for 120 min incubation, while the stability decreased rapidly over pH 5.0 and 60 °C [51]. *Aspergillus* sp. Gm showed the highest pectinase production by SmF using 1% pectin at 30 °C for 48 h; meanwhile, the purified pectinase activity optimum temperature was 30 °C, 75.4 U/mL; pH was 5.8, 72.3 U/mL; and substrate concentration 0.5%, 112.0 U/mL, and enzyme thermo-stability decreased 50% within 10 min incubation at 60 °C [52].

Our results revealed a decrease in partially purified pectinase activity of both strains after incubation with 10 mM K^+, Mg^{2+}, Ba^{2+}, and Ni^{2+}, while 10 mM K^+ increased *M. circinelloides* pectinase activity by 16.38%. In contrast, notable pectinase stability increased with Mg^{2+} and Ni^{2+} for *M. circinelloides* enzyme, and with Ba^{2+}, and Ni^{2+} for *M. hiemalis* enzyme. Thakur et al. [13] tested phenolic acids (0.05 mM), metal ions (Mn^{2+}, Co^{2+}, Mg^{2+}, Fe^{3+}, Al^{3+}, Hg^{2+}, and Cu^{2+}), and thiols, and found that they exerted an inhibitory impact on the polygalacturonase from *Mucor circinelloides* ITCC 6025. They suggested that the enzyme did not need any metal ions for its activity expression.

5. Conclusions

The present investigation utilized the response-surface methodology via the Box–Behnken design to improve cellulase and pectinase production by *M. circinelloides* and *M. hiemalis* strains. The experimental results are consistent with predicted responses. The produced enzymes were partially purified and characterized. The optimum parameters for cellulase production by *M. circinelloides* were incubation temperature 30 °C, pH value 7, incubation period 5 days, inoculum size 3 mL, and substrate concentration 5 g/100 mL, and for pectinase production were incubation temperature 30 °C, pH value 7, incubation period 9 days, inoculum size 3 mL, and substrate concentration 5 g/100 mL. For *M. hiemalis*, the optimum parameters for cellulase production were incubation temperature 30 °C, pH value 9, incubation period 5 days, inoculum size 3 mL, and substrate concentration 3 g/100 mL, and for pectinase production were incubation temperature 30 °C, pH value 5, incubation period 7 days, inoculum size 3 mL, and substrate concentration 5 g/100 mL. The influence of single, interaction and quadratic factors on cellulase and pectinase production was investigated using non-linear regression equations with significant R^2 and p values. The partial purification of *M. circinelloides* and *M. hiemalis* cellulase produced 1.73- and 2.03-fold purification with 31.12 and 32.02% recovery, respectively. Meanwhile, 1.74- and 1.99-fold purification with 31.26 and 31.51% recovery were obtained from *M. circinelloides* and *M. hiemalis* pectinase, respectively. A significant increase and decrease in the activity and stability of *M. circinelloides* and *M. hiemalis* partially purified enzymes was reported after incubation with different concentrations of metal ions and detergents. The response-surface methodology was effective and satisfactory, and investigated many factors simultaneously. More research is needed to scale up enzymes production for a wide range of applications.

Author Contributions: Conceptualization, A.M.A.H. and A.A.A.M.; methodology, A.A.A.M., A.M.A.H. and A.E.-R.F.G.; software, A.M.A.H. and J.A.A.; validation, A.M.A.H., A.A.A.M. and N.F.A.-D.; formal analysis, N.D.D.; investigation, A.M.A.H. and J.A.A.; resources, A.M.A.H.; data curation, A.M.A.H.; writing—original draft preparation, A.E.-R.F.G. and N.D.D.; writing—review and editing, A.M.A.H. and A.A.A.M.; visualization, A.M.A.H. and J.A.A.; supervision, A.A.A.M. and N.F.A.-D.; project administration, A.A.A.M. and N.F.A.-D.; funding acquisition, A.A.A.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Deanship of Scientific Research at King Saud University for funding this work through research group No. RG-1441-419 and the Deanship of Scientific Research at King Saud University for the logistic support through the Research Assistant Internship Program, Project no. RAIP-4-21-2.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding this work through research group No RG-1441-419 included in the Research Assistant Internship Program, Project no. RAIP-4-21-2.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Guimaraes, L.H.S.; Peixoto-Nogueira, S.C.; Michelin, M.; Rizzatti, A.C.S.; Sandrim, V.C.; Zanoelo, F.F.; Aquino, A.C.M.M.; Junior, A.B.; Polizeli, M.L.T.M. Screening of filamentous fungi for production of enzymes of biotechnological interest. *Braz. J. Microbiol.* **2006**, *37*, 474–480. [CrossRef]
2. Nigam, P.S. Microbial Enzymes with special characteristics for biotechnological applications. *Biomolecules* **2013**, *3*, 597–611. [CrossRef] [PubMed]
3. Torres-Barajas, R.L.; Aguilar-Osorio, G. Xylanolytic system proteins desorption from Aspergillus flavipes FP-500 cultures with agrowastes. *Rev. Mex. Ing. Quím.* **2013**, *12*, 513–525.
4. Hoque, E.; Fritscher, J. Multimetal bioremediation and biomining by a combination of new aquatic strains of Mucor hiemalis. *Sci. Rep.* **2019**, *9*, 10318. [CrossRef] [PubMed]
5. Zadeh, P.H.; Moghimi, H.; Hamedi, J. Biosurfactant production by Mocur circinelloides on waste frying oil and possible uses in crude oil remediation. *Water Sci. Technol.* **2017**, *76*, 1706–1714. [CrossRef]

6. Tan, Y.N.; Lee, P.P.; Chen, W.N. Dual extraction of crustacean and fungal chitosan from a single Mucor circinelloides fermen-tation. *Fermentation* **2020**, *6*, 40. [CrossRef]
7. Bhanja, A.; Minde, G.; Magdum, S.; Kalyanraman, V. Comparative studies of oleaginous fungal strains (Mucor circinelloides and Trichoderma reesei) for effective wastewater treatment and bio-oil production. *Biotechnol. Res. Int.* **2014**, *2014*, 479370. [CrossRef]
8. Hussain, S.A.; Nazir, Y.; Hameed, A.; Yang, W.; Mustafa, K.; Song, Y. Optimization of diverse carbon sources and cultivation conditions for enhanced growth and lipid and medium-chain fatty acid (MCFA) production by Mucor circinelloides. *Fermentation* **2019**, *5*, 35. [CrossRef]
9. Sharifyazd, S.; Karimi, K. Effects of fermentation conditions on valuable products of ethanolic fungus Mucor indicus. *Electron. J. Biotechnol.* **2017**, *30*, 77–82. [CrossRef]
10. Tako, M.; Kotogan, A.; Krisch, J.; Vagvolgyi, C.; Mondal, K.C.; Papp, T. Enhanced production of industrial enzymes in Mucoromycotina fungi during solid-state fermentation of agricultural wastes/by-products. *Acta Biol. Hung.* **2015**, *66*, 348–360. [CrossRef]
11. Bensmail, S.; Boudjema, K.; Naimi-Fazouane, F. Production of extracellular rennin-like enzyme by a newly isolate Mucor cir-cinelloides (von Tieghem) and its application in camembert cheese making. *J. Appl. Biotechnol. Rep.* **2020**, *7*, 16–24. [CrossRef]
12. Song, Y.; Wynn, J.P.; Li, Y.; Grantham, D.; Ratledge, C. A pre-genetic study of the isoforms of malic enzyme associated with lipid accumulation in Mucor circinelloides. *Microbiology* **2001**, *147*, 1507–1515. [CrossRef] [PubMed]
13. Thakur, A.; Pahwa, R.; Singh, S.; Gupta, R. Production, purification, and characterization of polygalacturonase from Mucor circinelloides ITCC 6025. *Enzym. Res.* **2010**, *2010*, 170549. [CrossRef] [PubMed]
14. Singh, S.; Nigam, V.K.; Sachan, A. Parametric optimization of ferulic acid esterase production from Mucor hiemalis NCIM837. *Int. J. Pharm. Pharm. Sci.* **2015**, *7*, 230–233.
15. Naqvi, S.H.; Dahot, M.U.; Ali, A.; Khan, M.Y.; Rafiq, M. Production and characterization of extracellular lipase secreted by Mucor geophillus. *Afr. J. Biotechnol.* **2011**, *10*, 19598–19606. [CrossRef]
16. Mohapatra, B.R.; Banerjee, U.C.; Bapuji, M. Characterization of a fungal amylase from *Mucor* sp. associated with the marine sponge *Spirastrella* sp. *J. Biotechnol.* **1998**, *60*, 113–117. [CrossRef]
17. Behnam, S.; Karimi, K.; Khanahmadi, M.; Salimian, Z. Optimization of xylanase production by Mucor indicus, Mucor hiemalis, and Rhizopus oryzae through solid state fermentation. *Biol. J. Microorg.* **2016**, *4*, 1–10.
18. Ramos-Ibarra, J.R.; Miramontes, C.; Arias, A.; Arriola, E.; Guatemala, G.; Corona-Gonzalez, R.I. Production of hydrolytic enzyme by solid-state fermentation with new fungal strains using orange by-products. *Rev. Mex. Ing. Quím.* **2017**, *16*, 19–31. [CrossRef]
19. Javed, U.; Ansari, A.; Aman, A.; Ul Qader, S.A. Fermentation and saccharification of agro-industrial wastes: A cost-effective approach for dual use of plant biomass wastes for xylose production. *Biocatal. Agric. Biotechnol.* **2019**, *21*, 101341. [CrossRef]
20. Mamma, D.; Kourtoglou, E.; Christakopoulos, P. Fungal multienzyme production on industrial by-products of the cit-rus-processing industry. *Bioresour. Technol.* **2008**, *99*, 2373–2383. [CrossRef]
21. Henriksson, G.; Nutt, A.; Henriksson, H.; Pettersson, B.; Stahlberg, J.; Johansson, G.; Pettersson, G. Endoglucanase 28 (Cel12A), a new Phanerochaete chrysosporium cellulase. *Eur. J. Biochem.* **1999**, *259*, 88–95. [CrossRef] [PubMed]
22. Imran, M.; Anwar, Z.; Irshad, M.; Asad, M.J.; Ashfaq, H. Cellulase production from species of fungi and bacteria from agri-cultural wastes and its utilization in industry: A review. *Adv. Enzym. Res.* **2016**, *4*, 44–55. [CrossRef]
23. Sulyman, A.O.; Igunnu, A.; Malomo, S.O. Isolation, purification and characterization of cellulase produced by Aspergillus niger cultured on Arachis hypogaea shells. *Heliyon* **2020**, *6*, e05668. [CrossRef] [PubMed]
24. Sethi, B.; Satpathy, A.; Tripathy, S.; Parida, S.; Singdevsachan, S.K.; Behera, B. Production of ethanol and clarification of apple juice by pectinase enzyme produced from Aspergillus terreus NCFT 4269.10. *Int. J. Biol. Res.* **2016**, *4*, 67–73. [CrossRef]
25. Almowallad, S.A.; Aljobair, M.O.; Alkuraieef, A.N.; Aljahani, A.H.; Alsuhaibani, A.M.; Alsayadi, M.M. Utilization of agro-industrial orange peel and sugar beet pulp wastes for fungal endo- polygalacturonase production. *Saudi J. Biol. Sci.* **2022**, *29*, 963–969. [CrossRef]
26. Schwan, R.F.; Wheals, A.E. The microbiology of cocoa fermentation and its role in chocolate quality. *Crit. Rev. Food Sci. Nutr.* **2004**, *44*, 205–221. [CrossRef] [PubMed]
27. Maciel, G.M.; Vandenberghe, L.P.S.; Haminiuk, C.W.I.; Fendrich, R.C.; Bianca, B.E.D.; Brandalize, T.Q.S.; Pandey, A.; Soccol, C.R. Xylanase production by Aspergillus niger LPB 326 in solid-state fermentation using statistical experimental designs. *Food Technol. Biotechnol.* **2008**, *46*, 183–189.
28. Selwal, M.K.; Selwal, K.K. High-level tannase production by Penicillium atramentosum KM using agro residues under submerged fermentation. *Ann. Microbiol.* **2012**, *62*, 139–148. [CrossRef]
29. Joo, H.; Chang, C. Production of an oxidant and SDS-stable alkaline protease from an alkaophilic Bacillus clausii I-52 by sub-merged fermentation: Feasibility as a laundry detergent additive. *Enzym. Microb. Technol.* **2006**, *38*, 176–183. [CrossRef]
30. Singhania, R.R.; Sukumaran, R.K.; Patel, A.K.; Larroche, C.; Pandey, A. Advancement and comparative profiles in the pro-duction technologies using solid-state and submerged fermentation for microbial cellulases. *Enzym. Microb. Technol.* **2010**, *46*, 541–549. [CrossRef]
31. Mohamed, H.; El-Shanawany, A.; Shah, A.M.; Nazir, Y.; Naz, T.; Ullah, S.; Mustafa, K.; Song, Y. Comparative analysis of different isolated oleaginous Mucoromycota fungi for their γ-linolenic acid and carotenoid production. *BioMed Res. Int.* **2020**, *2020*, 3621543. [CrossRef] [PubMed]

32. Meddeb-Mouelhi, F.; Moisan, J.K.; Beauregard, M. A comparison of plate assay methods for detecting extracellular cellulase and xylanase activity. *Enzym. Microb. Technol.* **2014**, *66*, 16–19. [CrossRef] [PubMed]
33. Gomori, G. Preparation of buffers for use in enzyme studies. In *Methods in Enzymology*; Colowick, S.P., Caplan, N.O., Eds.; Academic Press: New York, NY, USA, 1955; pp. 138–146.
34. Miller, L.G. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426–428. [CrossRef]
35. Fatokun, E.N.; Nwodo, U.U.; Okoh, A.I. Classical optimization of cellulase and xylanase production by a marine Streptomyces species. *Appl. Sci.* **2016**, *6*, 286. [CrossRef]
36. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275. [CrossRef]
37. Roe, S. *Protein Purification Applications*, 2nd ed.; Oxford University Press: Oxford, UK, 2001; p. 184.
38. Lakshmi, G.; Prasad, N.N. Purification and characterization of alkaline protease from a mutant Bacillus licheniformis Bl8. *Adv. Biol. Res.* **2015**, *9*, 15–23.
39. Lakshmi, B.K.M.; Kumar, D.M.; Hemalatha, K.P.J. Purification and characterization of alkaline protease with novel properties from Bacillus cereus strain S8. *J. Genet. Eng. Biotechnol.* **2018**, *16*, 295–304. [CrossRef]
40. Wang, P.; Dudareva, N.; Morgan, J.A.; Chapple, C. Genetic manipulation of lignocellulosic biomass for bioenergy. *Curr. Opin. Chem. Biol.* **2015**, *29*, 32–39. [CrossRef]
41. Voxeur, A.; Hofte, H. Pectin-derived immune elicitors in response to lignin modification in plants. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 4442–4444. [CrossRef]
42. Francis, F.; Sabu, A.; Nampoothiri, K.M.; Ramachandram, S.; Ghosh, S.; Szakacs, G. Use of response surface methodology for optimizing process parameters for the production of alpha- amylase by Aspergillus oryzae. *Biochem. Eng. J.* **2003**, *15*, 107–115. [CrossRef]
43. Ahmed, T.; Rana, M.R.; Zzaman, W.; Ara, R.; Aziz, M.G. Optimization of substrate composition for pectinase production from Satkara (*Citrus macroptera*) peel using Aspergillus niger-ATCC 1640 in solid-state fermentation. *Heliyon* **2021**, *7*, e08133. [CrossRef] [PubMed]
44. Zahra, T.; Irfan, M.; Nadeem, M.; Ghazanfar, M.; Ahmad, Q.; Ali, S.; Siddique, F.; Yasmeen, Z.; Franco, M. Cellulase production by Trichoderma viride in submerged fermentation using response surface methodology. *Punjab Univ. J. Zool.* **2020**, *35*, 223–228. [CrossRef]
45. Pathania, S.; Sharma, N.; Handa, S. Optimization of culture conditions using response surface methodology for synergism production of cellulase, xylanase and pectinase by Rhizopus delemar F2 under solid state fermentation. *J. Pharmacogn. Phytochem.* **2017**, *6*, 1872–1878.
46. Kumar, S.; Sharma, H.K.; Sarkar, B.C. Effect of substrate and fermentation conditions on pectinase and cellulase production by Aspergillus niger NCIM 548 in submerged (SmF) and solid state fermentation (SSF). *Food Sci. Biotechnol.* **2011**, *20*, 1289–1298. [CrossRef]
47. Hoa, B.T.; Hung, P.V. Optimization of nutritional composition and fermentation conditions for cellulase and pectinase pro-duction by Aspergillus oryzae using response surface methodology. *Int. Food Res. J.* **2013**, *20*, 3269–3274.
48. Ismail, S.A.; Hassan, A.A. Optimizing the production of rice straw hydrolytic cellulase under solid-state fermentation using Aspergillus terreus RS2. *Egypt. Pharm. J.* **2020**, *19*, 7–17. [CrossRef]
49. Viikari, L.; Alapuranen, M.; Puranen, T.; Vehmaanpera, J.; Siika-Aho, M. Thermostable enzymes in lignocellulose hydrolysis. *Adv. Biochem. Eng. Biotechnol.* **2007**, *108*, 121–145. [CrossRef]
50. Bhalla, A.; Bischoff, K.M.; Sani, R.K. Highly thermostable xylanase production from a thermophilic Geobacillus sp. strain WsUcF1 utilizing lignocellulosic biomass. *Front. Bioeng. Biotechnol.* **2015**, *3*, 84. [CrossRef]
51. Siddiqui, M.A.; Pande, V.; Arif, M. Production, purification, and characterization of polygalacturonase from Rhizomucor pu-sillus isolated from decomposting orange peels. *Enzym. Res.* **2012**, *2012*, 138634. [CrossRef]
52. Sudeep, K.C.; Upadhyaya, J.; Joshi, D.R.; Lekhak, B.; Chaudhary, D.K.; Pant, B.R.; Bajgai, T.R.; Dhital, R.; Khanal, S.; Koirala, N.; et al. Production, characterization, and industrial application of pectinase enzyme isolated from fungal strains. *Fermentation* **2020**, *6*, 59. [CrossRef]