Synthesis of Water-Soluble Menthol Derivatives Using Response Surface Methodology

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Abstract: *In this study, menthol was glycosylated with starch in the presence of α-amylase to produce menthyl maltoside (MenM) as the primary product. Optimization of the experimental parameters, including the menthol-starch ratio, enzyme concentration, temperature, and time, was performed using response surface methodology (RSM). The RSM-generated model displayed adequate accuracy and reasonable predictability for the MenM yield under the specified experimental conditions. Under the optimized reaction conditions of the menthol-to-starch ratio of 1:3 (50 °C, 18 h) and enzyme concentration of 50 U, the highest yield of MenM (23.7%) was obtained. Fourier transform infrared and nuclear magnetic resonance spectroscopies were employed to analyze the synthesized menthol derivative MenM. Compared with standard menthol, the synthesized menthol derivatives displayed various biological activities, including antioxidant, antibacterial, antifungal, and insecticidal properties. The results of this study demonstrate the effectiveness of RSM-optimized synthesis of MenM from the glycosylation of menthol with starch. This study showed that MenM is highly soluble in water and can be easily stored owing to its non-volatile nature.*

Keywords: *menthol; starch; glycosylation; menthyl maltoside; optimization*

■ INTRODUCTION

Menthol is a monoterpene alcohol widely used in oral products because of its refreshing and minty flavor, including foodstuffs, medicines, cream tablets, and liquids. The consumption of menthol on an annual basis is estimated to be around 30,000 metric tons. Despite its extensive use in various fields, the low water solubility of menthol and its tendency to sublime after storage has restricted its broad application. Therefore, researchers are interested in the synthesis of menthol compounds to resolve these problems. In its free state, menthol exhibits a natural mint flavor and diverse pharmacological effects. These characteristics are lost when menthol is converted to other compounds or derivatives. Researchers are interested in the synthesis of menthol compounds with enhanced water solubility. A significant breakthrough occurred when I-menthyl-p-oglucopyranoside, a monoterpene glycoside, was extracted from mint plants. This compound exhibited excellent water solubility and minimal tendency to sublimate during storage. This finding has paved the way for the development of

suitable techniques for synthesizing monoterpene glycosides. In the present study, menthol glycosides were prepared as alternatives to menthol [1]. Menthol glucosides are also found in nature, particularly *Mentha arvensis*. However, they are present in extremely small amounts, making them ineffective for applications similar to menthol. Therefore, it is necessary to manufacture menthol glycosides [2], with glycosylation being the most commonly used method [3]. Glycosylation of non-sugar molecules is a crucial and advantageous technique for enhancing their physicochemical and biological characteristics, particularly their water solubility and stability [4].

In this study, glycosylation of menthol with starch was studied in the presence of α-amylase to obtain menthyl maltoside (MenM) as the major product. The enzymatic catalysis of glycosylation is a potent method for the synthesis of various biologically active compounds. This method yields considerable amounts of less poisonous and more active natural compounds, including glycosides, while also enabling the enantioselective and regioselective synthesis of physiologically active molecules. Numerous species include the enzyme α-amylase, which is involved in starch metabolism and is a carbon and energy source. It is extensively employed as an industrial enzyme, primarily in the production of starch derivatives owing to its exceptional efficiency and cost-effectiveness. The utilization of low-cost, high-molecular-weight starch (with a degree of polymerization of 100) as a glycosyl donor in α-amylase leads to a significant glycoside yield [5].

The use of α-amylase in glycosylation has received little attention despite extensive research on its properties and structure and numerous reports on transferase reactions in the literature. Because of its ability to act on its substrate through a "retention mechanism" and function as a "transferase," α-amylase is primarily categorized as a "saccharifying" or "liquefying" enzyme because of the degree to which it depolymerizes starch molecules. The α-amylase has great potential to participate in glycosylation events because starch is a widely available substrate. The α-amylase derived from *Aspergillus oryzae* efficiently promotes glycosylation by converting maltose to butanol, ethanol, and methanol [6]. Additionally, this enzyme transfers glucose molecules from oligosaccharides to polyols. This study aimed to produce and assess menthol derivatives with enhanced yields. Menthol was glycosylated with starch molecules in the presence of α-amylase, and the experimental conditions were optimized using response surface methodology (RSM). FTIR and ^{1}H-NMR were used to evaluate the formation of MenM.

■ EXPERIMENTAL SECTION

Materials

Reagents, such as analytical-grade starch, menthol (99%), α-amylase (from *Bacillus* sp. in powder form, with enzyme activity of ≥ 1,500 units/mg protein (biuret)), ethyl acetate, *n*-butanol, 2-propanol, methanol, 1,1-diphenyl-2-picrylhydrazyl (DPPH), silica, potassium ferricyanide, trichloroacetic acid, potassium dihydrogen phosphate, ferric chloride, menthol, dipotassium hydrogen phosphate, ciprofloxacin, and amphotericin B were purchased from Sigma-Aldrich. Bacterial and fungal strains, such as *Enterococcus*, *Escherichia coli*, *Proteus* spp., *Staphylococcus aureus, Aspergillus flavus*, and *Aspergillus niger*, were obtained from Biochemistry Department whereas *Trogoderma granarium* was provided by the Entomology Department of the University of Agriculture Faisalabad for evaluation of the bioactivities of the synthesized menthol derivatives.

Instrumentation

Multiple characterization methods, such as UV-vis spectrophotometry using a Cecil 7200 (Germany) instrument in the wavelength range of 200–800 nm, FTIR spectrometry with a Spectrum GX instrument (Perkin Elmer, USA), and NMR spectroscopy using a 600 MHz Bruker Avance spectrometer, were employed to validate the synthesis of MenM.

Procedure

Glycosylation of menthol using starch

The glycosylation of menthol using starch was performed in the presence of α-amylase. This enzyme has been extensively used for starch degradation. Studies have indicated that glycoside molecules can be generated

by α-amylase [7]. MenM was synthesized by adding menthol and α-amylase to the reaction media [6], as shown in Fig. 1.

Thirty sets of chemical reactions were performed to achieve the maximum yield of menthol derivatives, according to the evaluated parameters and their levels, as shown in Table 1. For example, 1 g menthol and 45 U α-amylase enzyme were added to a reaction vessel containing 2 g starch and 10 mL of pH of 5 potassium acetate buffers (50 mM) for the first run. The reaction was performed under vigorous shaking at 45 °C for 16 h. Following the completion of the reaction, ethyl acetate was employed to extract MenM from the reaction mixture through a solvent extraction process. Similarly, all other experiments designed using response surface methodology were performed (Table 1).

Design and analysis

The experiments were formulated using Design Expert software employing RSM technique with the central composite design (CCD) mode. The objective was to analyze the impact of various reaction parameters MenM yield. Four independent variables (menthol-to-starch ratio, residence time, temperature, and enzyme concentration) were used to determine their effects on the dependent variable (MenM yield). The RSM suggested 30 experiments for the synthesis of MenM (Table 1). The following independent variables were coded to ensure no experimental error: −2, −1, 0, +1, and +2 as lower, low, middle, high, and higher, respectively, with 6 central points. To reduce the likelihood of systematic errors caused by trends in the variable's experiments were performed in a randomized sequence. The specific number of experiments (P) required to produce MenM was determined by applying Eq. (1);

$$P = 2^N + 2N + N_r = 2^4 + 2(4) + 6 = 30 \quad (1)$$

where N signifies the no. of process parameters chosen for the experiments, and N_r denotes the no. of repeated experimental runs.

Statistical methods

Eq. (2) shows the relationship between MenM yield (dependent variable) and the interaction impact of a few factors (independent variables) [8];

$$Y = \sum_{j=1}^{k} \beta_{jj} x_j^2 + \sum_i \sum_{<j=2}^{k} \beta_{ij} x_i x_j + \beta_0 + e_i \quad (2)$$

where y represents the yield of MenM, β_0, β_{jj}, and β_{ij} are the model coefficients, x_i, and x_j are the coded factors (chosen parameters) [9]. The significance of the model was assessed using ANOVA, and a p-value exceeding 0.05 was deemed statistically significant [10] with a confidence level of 95%. To evaluate the suitability of the quadratic model proposed by the RSM as the optimal fit, coefficient of determination (R^2) and lack-of-fit values

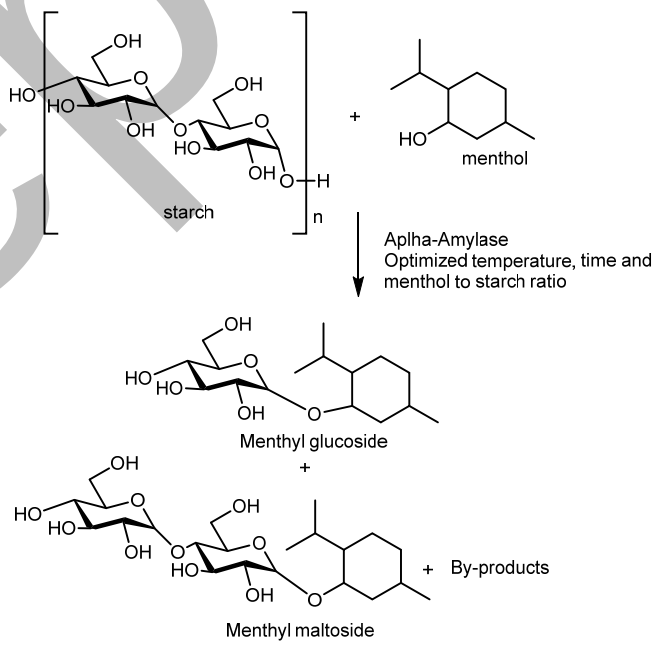


Fig 1. Starch catalyzed reaction with α-amylase to synthesize MenM

Table 1. RSM-based menthol derivative optimization with coded and uncoded factors

Processes	Parameters of reaction	Unit	Coded and uncoded factors		
			−1	0	+1
Glycosylation using starch	Menthol to starch ratio	-	1:2	1:3	1:4
	Temperature	°C	45	50	55
	Residence time	h	16	18	20
	Enzyme concentration	U	45	50	55

were examined. The utilization of the predicted model led to the generation of 3D and contour plots, which depict the correlation between two independent variables in the menthol derivative synthesis process and the resulting yield of menthol derivatives. Throughout the experiments, the third and fourth process variables were kept constant [9]. The experimental design and independent variables, both in coded and uncoded forms, proposed by CCD to improve the efficiency of menthol glycosylation using sucrose and α-glucosidase are presented in Table 2.

Purification of MenM

The synthesized product was isolated from the reaction mixture using ethyl acetate, followed by chromatography over a silica column with water:2-propanol:*n*-butanol in a 4:5:10 volume ratio [11].

Biological evaluation of synthesized derivatives

MenM has been evaluated for several biological activities, including antioxidant properties, as evidenced by DPPH and RPA assays, and insecticidal, antibacterial, and antifungal properties. MenM has antioxidant

Table 2. Experimental plan for menthol glycosylation using starch as a reactant

Run	Coded				Uncoded			
	Menthol to starch ratio	Temp. (°C)	Residue time (h)	Enzyme Conc. (U)	Menthol to starch ratio	Temp. (°C)	Residue time (h)	Enzyme Conc. (U)
1	−1	−1	−1	−1	1:2	45	16	45
2	+1	−1	−1	−1	1:4	45	16	45
3	−1	+1	−1	−1	1:2	55	16	45
4	+1	+1	−1	−1	1:4	55	16	45
5	−1	−1	+1	−1	1:2	45	20	45
6	+1	−1	+1	−1	1:4	45	20	45
7	−1	+1	+1	−1	1:2	55	20	45
8	+1	+1	+1	−1	1:4	55	20	45
9	−1	−1	−1	+1	1:2	45	16	55
10	+1	−1	−1	+1	1:4	45	16	55
11	−1	+1	−1	+1	1:2	55	16	55
12	+1	+1	−1	+1	1:4	55	16	55
13	−1	−1	+1	+1	1:2	45	20	55
14	+1	−1	+1	+1	1:4	45	20	55
15	−1	+1	+1	+1	1:2	55	20	55
16	+1	+1	+1	+1	1:4	55	20	55
17	−2	0	0	0	1:1	50	18	50
18	+2	0	0	0	1:5	50	18	50
19	0	−2	0	0	1:3	40	18	50
20	0	+2	0	0	1:3	60	18	50
21	0	0	-2	0	1:3	50	14	50
22	0	0	+2	0	1:3	50	22	50
23	0	0	0	−2	1:3	50	18	40
24	0	0	0	+2	1:3	50	18	60
25	0	0	0	0	1:3	50	18	50
26	0	0	0	0	1:3	50	18	50
27	0	0	0	0	1:3	50	18	50
28	0	0	0	0	1:3	50	18	50
29	0	0	0	0	1:3	50	18	50
30	0	0	0	0	1:3	50	18	50

properties, especially in terms of its capacity to scavenge and reduce free radicals. The ferric-reducing antioxidant power test was used to evaluate these characteristics and ascertain the RPA of MenM [12]. MenM and gallic acid (20, 40, 60, 80, and 100%) were each dissolved in 0.2 M of 2.5 mL of phosphate buffer with pH = 6.6, and 1% of 2.5 mL of potassium ferricyanide. The solution was then incubated for 20 min at 50 °C. The chemical process was stopped by introducing 2.5 mL of 1% trichloroacetic acid into the reaction environment. Subsequently, the mixture was centrifugated at 1000 rpm for 10 min. Subsequently, 0.25 mL of distilled water and 0.5 mL of a 0.1% $FeCl_3$ solution were added to the 0.25 mL supernatant. The absorbance was measured at 700 nm using a UV-vis spectrophotometer following a 30 min incubation at room temperature. The calibration curve was used to calculate the RPA, and the results were presented as g/100 mL of %gallic acid equivalent (GAE). The free radical scavenging activity was assessed using DPPH as the source of free radicals [13] according to published literature [14]. A volume of 2.5 mL of MenM was diluted in 1 mL of DPPH 0.09 mM. Subsequently, the total volume of the solution was adjusted to 4 mL by the addition of 95% methanol. A volume of 2.5 mL of MenM was diluted with 1 mL of methanol to prepare the control solutions. After keeping in the dark for 1 h, the absorbance was measured at 515 nm. Eq. (3) determined the fraction of free radicals inhibited by each sample;

$$\%\text{inhibition} = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100\% \quad (3)$$

where variables A_{blank} and A_{sample} are employed to indicate the absorbance values of the control reaction and the sample, respectively.

Six strains of microbes (comprising bacteria and fungi), including *Enterococcus*, *E. coli*, *Proteus* spp., *S. aureus*, *A. flavus*, and *A. niger*, were used to detect antimicrobial activities of MenM by adopting a previously described procedure [15]. The antibacterial effect of MenM was assessed using the disc-diffusion method. Nutrient agar (NA) was inoculated into sterile petri dishes containing test microbe cell suspensions (200 µL of cell suspension in 20 mL of NA). Filter paper discs with sterile surfaces were placed on top of the inoculated agar and impregnated with 20 µL of each sample (MenM) at various concentrations (1:1, 1:5, 1:10, and 1:20). As positive controls, 6 mm standard discs containing 25 µg per disc of amphotericin B and 25 µg of ciprofloxacin were used. Prepared sample plates were incubated for 18–24 h at 37 °C. The growth inhibitory zone diameter was expressed in millimeters. The experiments were carried out in triplicate.

The khapra beetles (*T. granarium*) were chosen to evaluate the insecticidal activities of MenM [16-17]. Before the MenM treatment, the khapra beetles were categorized into 4 distinct groups. Each group consisted of 3 replicate sets with 20 khapra beetles in each set. Different concentrations of MenM solution (125, 250, and 500 ppm) were applied to khapra beetles. Treatments were conducted by adding 20 khapra beetles to petri dishes containing filter papers that had been dipped in prepared MenM solutions of various concentrations (wheat grains were used as a food source for khapra beetles in the petri plates).

According to Abbott's formula (Eq. (4)), the mortality rate was measured at 4- and 8-day intervals;

$$\text{Corrected mortality } (\%) = \frac{M_O - M_C}{100 - M_C} \times 100\% \quad (4)$$

where M_O denotes the sample % mortality and M_C denotes the control % mortality.

■ RESULTS AND DISCUSSION

Characterization of Menthol Derivative

The synthesized MenM was characterized experimentally by evaluating its λ_{max} value. The experimental λ_{max} value for MenM was 361 nm. From the comparison of the theoretical and experimental values, it can be concluded that the targeted product may be synthesized successfully, as the synthesized product had a λ_{max} experimental value close to the theoretical value of λ_{max}.

The results of FTIR analysis of MenM were consistent with the visual observations of this study. Peak (i) in Fig. 2(a) represents menthol, whereas peak (ii) represents a derivative of MenM. The peaks that appeared between 1400 and 800 cm^{-1} were enlarged and are shown in separate graphs in Fig. 2(b). The C–H stretching

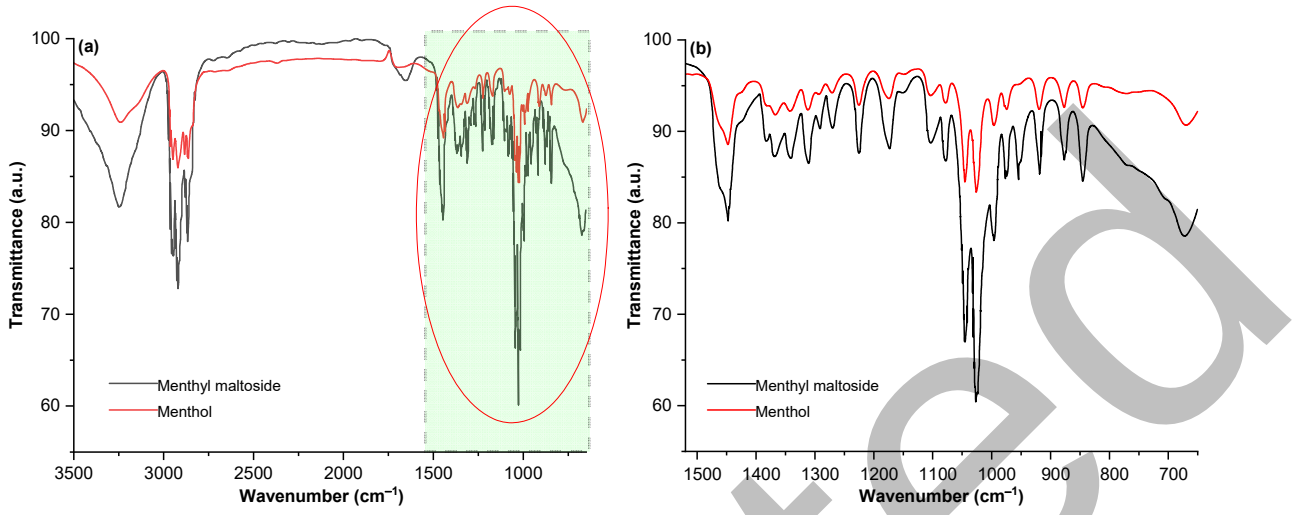


Fig 2. FTIR spectra of (a) menthol and MenM with (b) enlarged at specific regions

vibrations, both symmetrical and asymmetrical, are represented by the peaks formed in the region of 3000–2800 cm^{-1}.

Significant peaks in the menthol spectrum were observed at the following wavelengths: 3400–3200 cm^{-1} for the O–H group, 2855–2924 cm^{-1} for the CH_3 group, 1025–1045 cm^{-1} for the C–O bond, and 1368 cm^{-1} for the isopropyl group. Similar evaluations of menthol have been previously published [18]. An additional peak at 952 cm^{-1}, which was not present in the spectrum of menthol, was visible in the MenM FTIR spectrum. The glycosidic bond visible in this peak supports MenM synthesis. Prior research revealed that peaks in this range indicated the glycosidic bonds [19-20].

The ^{1}H-NMR data validated the success synthesis of MenM (Fig. 3). ^{1}H-NMR (600 MHz $CDCl_3$ ppm), 0.92 (12H, $3CH_4$), 1.36 (2H, CH_2, $C_{cyclic}H$), 1.54 (4H, $2CH_2$), 1.61 (1H, CH, $C_{cyclic}H$), 1.91 (1H, CH, $C_{cyclic}H$), 2.56 (2H, 2CHO, $O_{acyclic}$) 3.32 (5H, 5OH), 3.34 (2H, $2CH_2O$**H**, $C_{acyclic}H$), 3.36 (4H, $2CH_2$, $C_{acyclic}H$), 3.47 (5H, $5CH_2$, $C_{cyclic}H$), 4.07 (2H, 2C**H**CH_2, $C_{acyclic}H_2$), 4.24 (1H, CHO, O_{cyclic}), 5.26 (2H, 2OCHO).

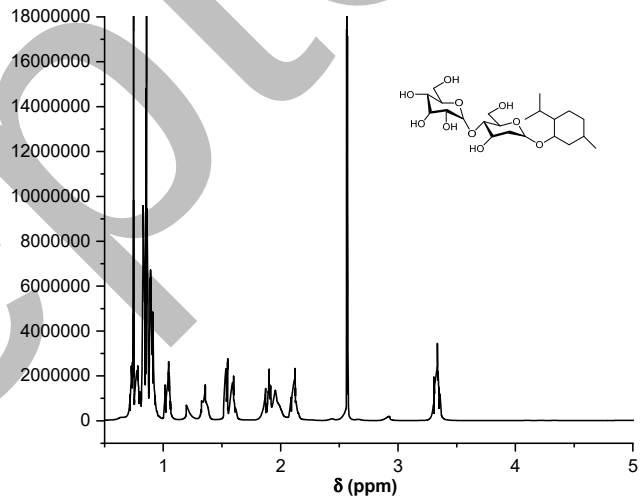


Fig 3. ^{1}H-NMR spectrum of MenM

Glycosylation of Menthol Using Starch

The α-amylase is widely utilized in the industrial sector for the processing of starch. This enzyme possesses hydrolytic and transglycosylation activities, making it highly versatile [18]. During starch degradation by α-amylase, water competes with menthol as a glycosyl acceptor [7]. In this study, MenM were obtained in 23.7% yield under optimized reaction conditions.

RSM for The Glycosylation of Menthol Using Starch

Development of regression model

The reaction variables for enzymatic glycosylation were assessed using CCD, a design approach frequently used in RSM. These variables included α_1, which is the ratio of menthol to starch (ranging from 1:2 to 1:4); α_2, which is the temperature (45–55 °C); α_3, which is the residence time (16–20 h), and α_4; the enzyme concentration (45–55 U). The 4 variables studied are listed in Table 2, along with their ranges and levels as well as the coded and real values for each process

parameter. The comprehensive CCD matrix displaying the actual process variables investigated and their corresponding results are presented in Table 3. Experiments were performed randomly to minimize the errors that could arise from systematically altering the reaction variables.

Regression analysis for MenM yield

Among the various models (cubic, linear, two-factor interaction, and quadratic polynomial) assessed for their suitability in describing the current response, the quadratic model has emerged as the most appropriate fit because of its higher-order polynomial structure and additional terms of relevance. As shown in Table 4, the RSM software suggested a quadratic model for MenM synthesis. Eq. (5) represents the model according to the encoded values α_1, α_2, α_3, and α_4 for MenM synthesis.

$$\begin{aligned} Y = {} & 21.98 + 0.9583\alpha_1 + 1.87\alpha_2 + 2.60\alpha_3 + 2.18\alpha_4 \\ & + 0.65\alpha_1\alpha_2 + 0.487\alpha_1\alpha_3 - 0.775\alpha_1\alpha_4 \\ & + 0.1625\alpha_2\alpha_3 + 0.1250\alpha_2\alpha_4 - 1.69\alpha_3\alpha_4 - 1.1\alpha_1^2 \\ & - 2.28\alpha_2^2 - 2.45\alpha_3^2 - 2.66\alpha_4^2 \end{aligned} \quad (5)$$

The MenM yield was negatively affected by the negative sign (−) in front of the terms, but the positive sign (+) demonstrated a synergistic effect [21]. The model (Eq. (5)) signified that positive signs were present

Table 3. Displays the outcomes of the experimental design matrix for the MenM yield.

Run	α_1: Menthol to starch ratio	α_2: Temperature (°C)	α_3: Time (h)	α_4: Enzyme concentration (U)	Y: MenM yield	
					Actual (%)	Predicted (%)
1	1:2	45	16	45	5.20	4.85
2	1:4	45	16	45	5.90	6.05
3	1:2	55	16	45	6.70	6.71
4	1:4	55	16	45	11.40	10.50
5	1:2	45	20	45	12.40	12.13
6	1:4	45	20	45	15.70	15.27
7	1:2	55	20	45	14.40	14.64
8	1:4	55	20	45	20.60	20.38
9	1:2	45	16	55	13.70	13.90
10	1:4	45	16	55	12.20	11.99
11	1:2	55	16	55	15.80	16.25
12	1:4	55	16	55	16.70	16.95
13	1:2	45	20	55	13.50	14.42
14	1:4	45	20	55	14.50	14.46
15	1:2	55	20	55	17.60	17.43
16	1:4	55	20	55	19.70	20.07
17	1:1	50	18	50	16.20	15.68
18	1:5	50	18	50	19.00	19.52
19	1:3	40	18	50	9.10	9.12
20	1:3	60	18	50	16.60	16.58
21	1:3	50	14	50	6.80	7.00
22	1:3	50	22	50	17.60	17.40
23	1:3	50	18	40	6.10	6.98
24	1:3	50	18	60	16.60	15.72
25	1:3	50	18	50	20.30	21.98
26	1:3	50	18	50	21.00	21.98
27	1:3	50	18	50	21.20	21.98
28	1:3	50	18	50	23.70	21.98
29	1:3	50	18	50	23.40	21.98
30	1:3	50	18	50	22.30	21.98

before the coefficient; α_1, α_2, α_3, α_4, $\alpha_1\alpha_2$, $\alpha_1\alpha_3$, $\alpha_2\alpha_3$ and $\alpha_2\alpha_4$, linearly increased MenM yield. MenM yield was negatively affected by the quadratic terms α_1^2, α_2^2, α_3^2, α_4^2, $\alpha_1\alpha_4$, and $\alpha_3\alpha_4$. The statistical ANOVA results for the MenM synthesis are shown in Table 5. The analysis was done to evaluate the suitability and significance of the quadratic model. Furthermore, Table 5 illustrates how the individual factors and their interactions influence the MenM yield.

The MenM model demonstrated statistical significance with a 95% confidence level, as indicated by its F-value of 62.96 and a p-value < 0.0001. To evaluate the significance of each regression coefficient and determine how cross-product interactions affect MenM yield, researchers utilized the p-value, which indicates the likelihood of experimental error. A lower p-value signifies a greater significance for the corresponding coefficient [22]. The main factors affecting MenM yield were identified as α_2, α_3, α_4, and interaction terms between these main factors. Additionally, the quadratic terms for MenM were identified as α_1^2, α_2^2, α_3^2, and α_4^2. The experimental results were precise, with a minimal CV value of 6.43% for the MenM yield (Table 6). The lack-of-fit test yielded a non-significant result (p-value = 0.9682), indicating that the model adequately fit the experimental data. According to the lack-of-fit test, there

Table 4. Fit Summary of the MenM yield (using menthol and starch)

Source	Sequential (p-value)	Lack of fit (p-value)	Adj. R^2	Pred. R^2	
Linear	0.0040	0.0056	0.3584	0.3002	
2FI	0.7883	0.0037	0.2742	0.1951	
Quadratic	< 0.0001	0.9682	0.9677	0.9514	Suggested
Cubic	0.8587	0.9993	0.9541	0.9836	Aliased

Table 5. ANOVA for the quadratic model of MenM yield (using menthol and starch)

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	841.2500	14	60.0900	62.9600	<0.0001	Significant
α_1: Menthol to starch ratio	22.0400	1	22.0400	23.1000	0.0002	
α_2: Temperature	83.6300	1	83.6300	87.6200	<0.0001	
α_3: Residence time	162.2400	1	162.2400	169.9900	<0.0001	
α_4: Enzyme conc.	114.4100	1	114.4100	119.8700	<0.0001	
$\alpha_1\alpha_2$	6.7600	1	6.7600	7.0800	0.0178	
$\alpha_1\alpha_3$	3.8000	1	3.8000	3.9800	0.0644	
$\alpha_1\alpha_4$	9.6100	1	9.6100	10.0700	0.0063	
$\alpha_2\alpha_3$	0.4225	1	0.4225	0.4427	0.5159	
$\alpha_2\alpha_4$	0.2500	1	0.2500	0.2619	0.6162	
$\alpha_3\alpha_4$	45.5600	1	45.5600	47.7400	<0.0001	
α_1^2	32.9400	1	32.9400	34.5100	<0.0001	
α_2^2	143.0000	1	143.0000	149.8400	<0.0001	
α_3^2	164.0800	1	164.0800	171.9200	<0.0001	
α_4^2	193.8300	1	193.8300	203.0900	<0.0001	
Residual	14.3200	15	0.9544			
Lack of fit	4.8500	10	0.4847	0.2560	0.9682	Not significant
Pure error	9.4700	5	1.8900			
Cor. total	855.5700	29				

Table 6. Fit statistics of MenM yield (using menthol and starch)

Std. dev.	Average	C.V. (%)	R^2	Adj. R^2	Pred. R^2	Adeq. precision
0.9769	15.20	6.43	0.9833	0.9677	0.9514	24.7964

is evidence of unaccounted factors in the model that may influence the relationship between the regressor and the response [23]. The predicted and adjusted R^2 values were in good agreement, indicating that the model accurately explained 96.77% of the variability in the MenM yield.

Fig. 4(a) depicts the correlation between the observed and predicted MenM yields. The close alignment between the experimental and predicted values suggests that the current study's data fit the applied model well. This correlation indicates that the response of the model within the studied range was evaluated with considerable accuracy and reliability.

To further validate the suitability of the model, a residual analysis was conducted, which provided information on the disparity between the predicted and actual reactions using the residual value. Two plots were used to evaluate the performance of the model: a normal probability residual plot (Fig. 4(b)) and a predicted versus residual response plot (Fig. 4(c)). The normal distribution of the MenM data is shown in Fig. 4(b), which shows no volatility and a close fit to a straight line. The quadratic model was deemed sufficient because the lack of structure in the predicted versus residual plot (Fig. 4(c)) did not contradict the assumption of constant or independent variance. A plot was constructed to ascertain the most appropriate model for the data, showing the relationship between the experimental runs and externally studentized residuals (Fig. 4(d)). According to the results, all data points were found to be within a reasonable range, and the empirical model was

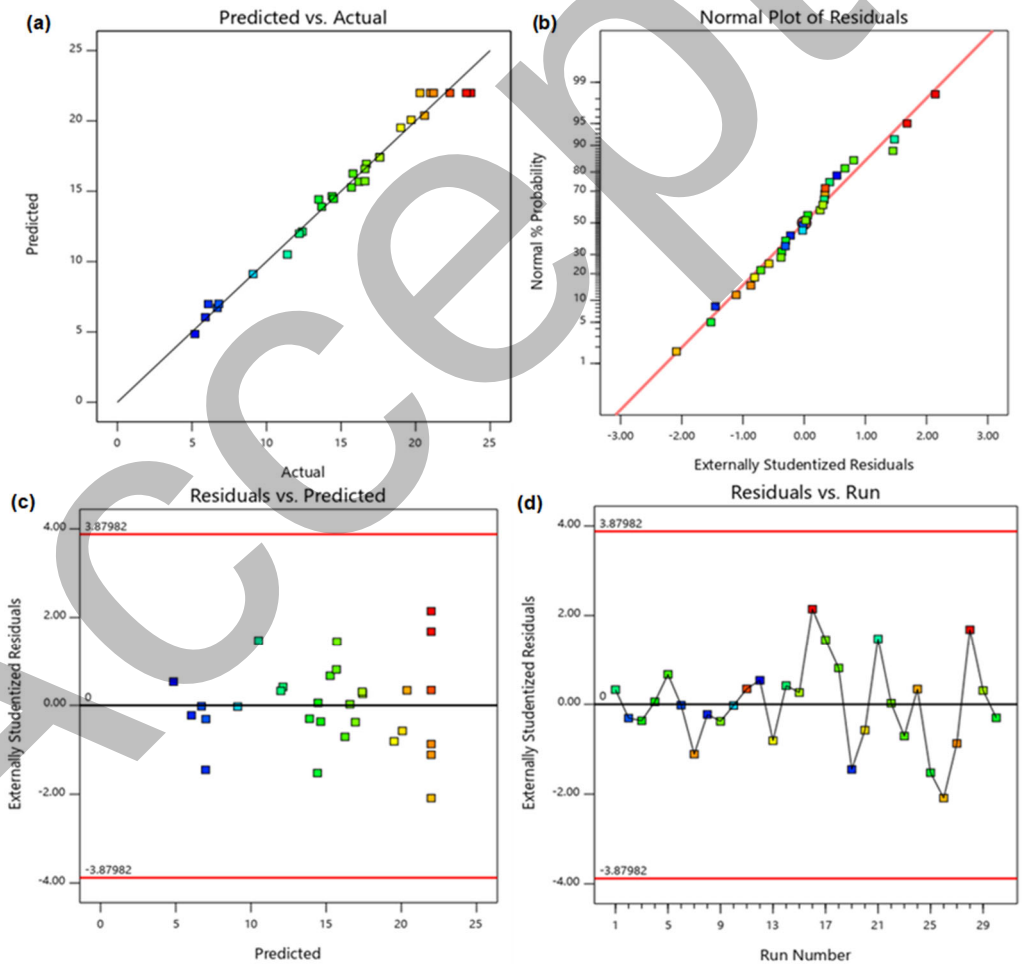


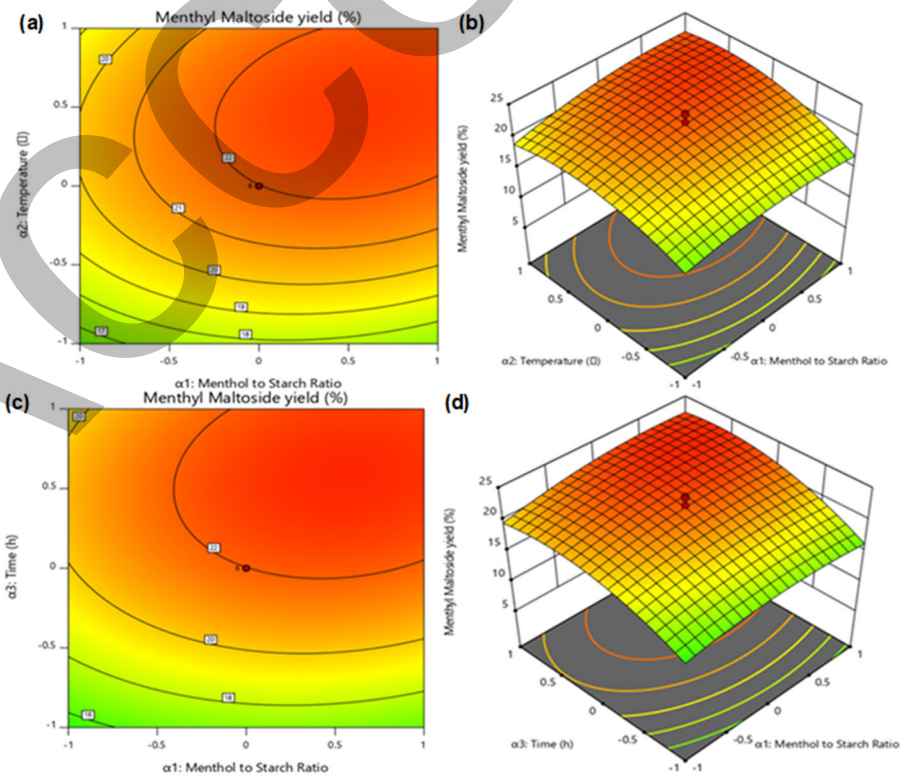
Fig 4. Diagnostic plots for the adequacy of the RSM model: (a) comparison of predicted versus actual values, (b) normal residual plots, (c) residual plots versus predicted values, and (d) residual versus run of MenM yield using menthol and starch

deemed appropriate for characterizing MenM production yield using RSM, as evidenced by the satisfactory plots in Fig. 4.

Reaction parameter's impact on MenM yield

The RSM model found that α_2, α_3, and α_4 significantly impacted MenM yield. Fig. 5(a) and 5(b) depict the contour and 3D plots, respectively, illustrating the interaction between α_1 and α_2 on the MenM yield. The α_4 and α_3 were fixed at 50 U and 18 h, respectively. Analysis of the plots shows that varying α_2 between 45–55 °C, while keeping the α_1 constant did not lead to a notable increase in MenM yield. However, the yield of MenM increased with α_1. Interestingly, an increase in both α_1 and α_2 led to a higher MenM yield, suggesting that these parameters are interdependent. The maximum MenM yield was observed at 50 °C, which is the optimal temperature for α-amylase production [18]. Similar effects on MenM yield were observed for other interaction parameters, including α_1 and α_4, at a fixed α_4 of 50 U and α_3 of 50 °C, as shown in Fig. 5(c-f). Additionally, α_1 and α_4 at a fixed α_3 of 18h and α_2 of 50 °C exhibited similar effects on the MenM yield, as illustrated in Fig. 5(c-f). Fig. 5(g-j) demonstrates the influence of α_2 and α_3 at a fixed α_1 of 1:3 and α_4 of 50 U on the MenM yield. These figures revealed that as α_2 increased, the MenM yield initially increased slightly; however, beyond a certain point, the yield decreased when α_4 remained constant. Additionally, α_4 had a similar effect on MenM yield when α_2 was kept constant.

Conversely, Fig. 5(k) and 5(l) depict the impact of α_3 and α_4 at a fixed α_1 of 1:3, and α_2 of 50 °C on the MenM yield. These graphs indicate that increasing both α_3 and α_4 led to a significant increase in MenM yield. Therefore, these interaction parameters significantly influenced each other. As mentioned in Eq. (5), the interactions between the parameters were found to reduce the MenM. Therefore, it is generally recommended that the reaction be initiated with an increase in one of these parameters when the second parameter is low. The most significant MenM yield was attained by employing α_1 of 1:3, α_3 of 18 h, α_2 of 50 °C, and α_4 of 50 U. Nevertheless, the highest MenM yield was obtained by employing α_1 of 1:3, α_2 of 50 °C, α_3 of 22 h, and α_4 of 50 U. In a previous study, the effect of α_4 on the synthesis of glycosides in the presence of α-amylase was investigated. The study revealed that the yield of the product increased above 30 °C and reached its maximum at 50 °C. However, a significant decrease in



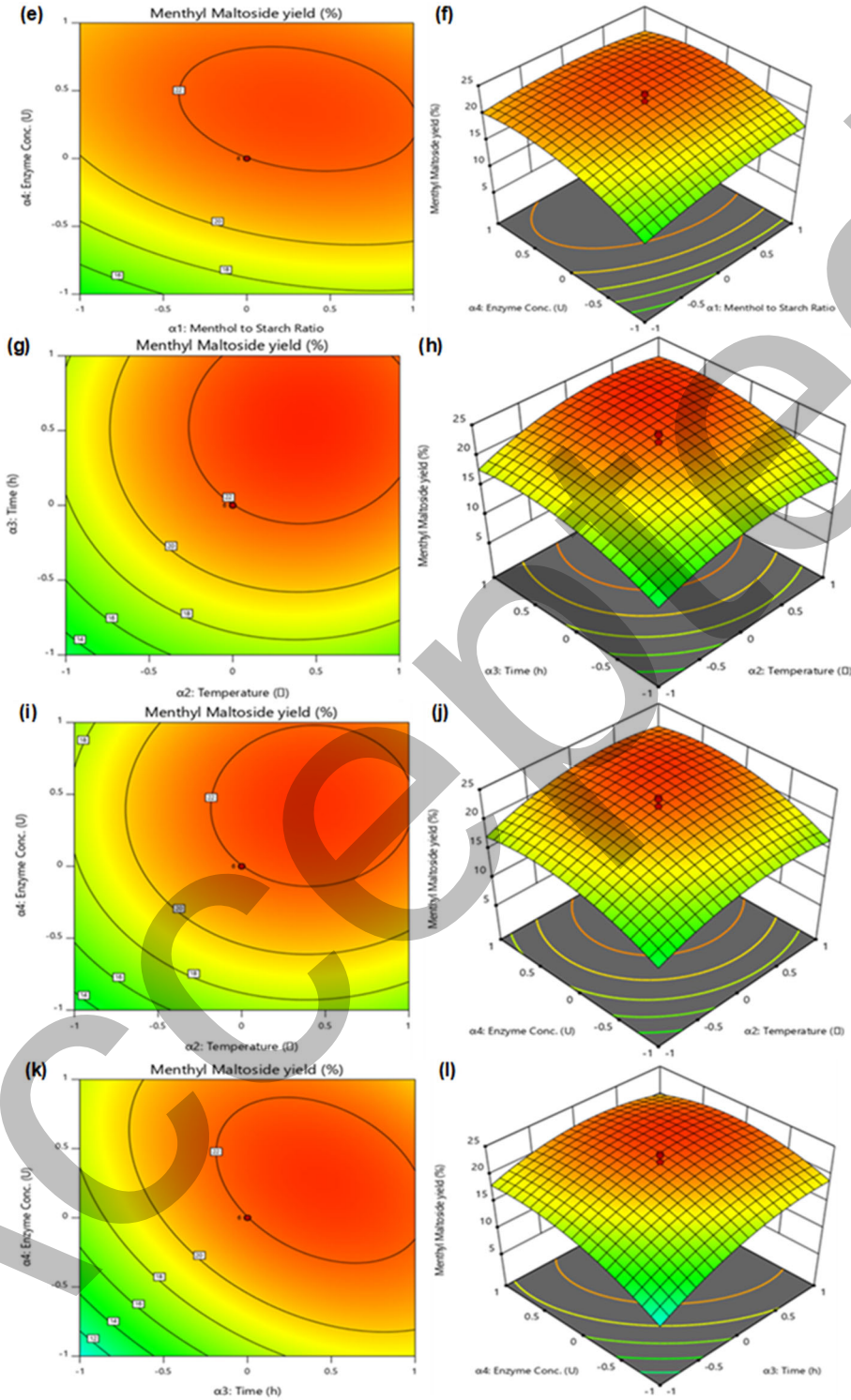


Fig 5. Contour and three-dimensional plots of reaction parameters of: (a, b) menthol-to-starch ratio × temperature; (c, d) menthol-to-starch ratio × time; (e, f) menthol-to-starch ratio × enzyme concentration; (g, h) temperature × time; (i, j) temperature × enzyme concentration; (k, l) time × enzyme concentration; and the yield of MenM, respectively

product yield was observed when α_2 was further increased to 80 °C due to the enzyme inactivation [18]. An increase in α_1 resulted in a higher MenM yield up to a certain limit. The stabilization of α-amylase can be attributed to the binding of starch molecules to their three-domain boundaries [24].

Additionally, as the starch concentration increases, the water activity decreases, which also increases the yield of the target product [18]. Previous studies have shown that an increased substrate concentration favors the transglucosylation process with α-amylase [25]. The α_3 also had a significant impact on MenM yield. As α_3 increased, the yield of the target product was also increased; however, beyond a certain point, the yield started to decrease. This decrease in the targeted product may be due to the hydrolytic activity of α-amylase because the enzyme can utilize the synthesized MenM as a substrate molecule for hydrolysis. In general, there is a competition between the hydrolysis and trans-glycosylation reactions, which are mediated by α-amylase. The relative rates of degradation and synthesis determine the final product yield. A previous study reported that the time required for glycosylation of epigallocatechin and catechin in the presence of α-amylases was 18–24 h [26].

Optimization of MenM yield

This study aimed to optimize MenM synthesis by identifying the operating conditions that yielded the highest amount of MenM. To achieve this, reaction parameters such as the α_1, α_2, α_3, and α_4 were varied within a range of high and low levels, respectively. The experimental data and model obtained from the input criteria were used to develop solutions for the MenM yield using the RSM software. The optimal chemical reaction conditions were utilized in the synthesis of MenM to validate the precision of the model prediction. With a projected MenM yield of 23.69%, the ideal reaction parameters for MenM production are as follows: α_1 of 1:3.6, α_2 of 52.63 °C, α_3 of 19.13 h, and α_4 of 50.73 U. Nonetheless, the experimental MenM yield obtained under these conditions was 24.01%. This indicates that the projected and experimental values of the RSM were similar and the MenM yield is fairly expected and accurately described by the RSM model.

Biological Activities of MenM

The antioxidant capacity of MenM was assessed based on its ability to scavenge DPPH free radicals. The results showed that MenM had a DPPH scavenging activity of 58.1 ± 0.002% and an IC_{50} value of 72.6 mg/mL. These values were comparable to previously observed values for menthol (65.2 ± 0.004%) [27]. These comparable values can be explained by several factors, such as the positioning of functional groups around the core structure, molecular configuration, substitution patterns, and the total quantity of hydroxyl groups. Additionally, the structure, number, and presence of sugar components in each compound (menthol glycosides) contribute to this similarity. According to various studies, the antioxidant properties of flavonol glycosides (found in tea) tend to decrease as the number of glycosidic moieties increases [28]. However, glucose moieties can sometimes enhance bioavailability, even though glycoside molecules are weaker antioxidant agents than aglycone compounds [28]. The IC_{50} value for MenM was determined to be 72.6 mg/mL. Previously, the DPPH activity of *M. arvensis*, with menthol as its key component, ranged from 67 to 70% after 30 min of incubation in the darkness [29]. The reducing power ability (RPA) values of MenM were 9.6 ± 0.02% of GAE. This result is similar to the findings for DPPH activity, where glycoside moieties are weaker antioxidant agents than aglycone moieties and glucose can sometimes improve bioavailability [27]. Furthermore, these results align with other reports, which indicated that mint essential oils' RPA value mostly comprised menthol, was 200 mg/mL [30].

The current investigation aimed to assess the antimicrobial properties of MenM, a synthesized menthol derivative, against a range of microbial strains, including *E. coli*, *S. aureus*, *Proteus* spp., *Enterococcus*, *A. flavus*, and *A. niger*. The disc diffusion method was employed to test the antimicrobial activity of MenM and the results were evaluated based on the zone of inhibition. These results indicated that MenM exhibited antibacterial and antifungal properties exclusively against *Proteus* species and *A. flavus*. This antimicrobial

Table 7. Antimicrobial activity of MenM

Microbes	MenM zone (mm)	Ciprofloxacin zone (mm)	Amphotericin B zone (mm)
S. aureus	0	30	-
E. coli	0	25	-
Proteus spp.	11	27	-
Enterococcus	0	30	-
A. flavus	5	-	16
A. niger	0	-	9

Table 8. Insecticidal activities of menthol and its derivatives

Samples	Concentration (ppm)	% Mortality after 4 d	% Mortality after next 4 d	Total % Mortality after 8 d
MenM	500	7	3	10
	250	5	1	6
	125	4	0	4

efficacy was similar to that observed with the standard drugs ciprofloxacin and amphotericin B (Table 7).

However, studies have revealed that menthol exhibits activity against all the antimicrobial agents used in the current study. The number of hydroxyl and methoxy groups, the existence of a sugar moiety, and the binding affinities to internal and exterior sites in bacterial strains are examples of structural characteristics that can significantly affect the membrane permeability and antimicrobial effects of substances [31-32]. Table 8 shows the mortality rates of *T. granarium* after 4 d of exposure to different substances. MenM treatment (500 ppm) resulted in the highest mortality rate (7%). Nonetheless, previous reports have suggested a 5% mortality rate associated with menthol [26]. In addition, the mortality rate increased with increasing concentrations. After 8 d, the mortality rate of MenM decreased compared to that of the first four days. The calculated value of LC_{50} for MenM menthol against *T. granarium* was 22571.8 mg/mL. The ability of these compounds to kill insects is attributed to their monoterpene units [33]. Monoterpenoids are the main components of essential oils (EOs) and plant extracts that provide insecticidal qualities [17,34]. Monoterpenoids have various effects on mammals and insect targets, including those related to the nervous system. These targets included sodium channels, nicotinic acetylcholine receptors, tyramine receptors, acetylcholine esterase, octopamine receptors, and GABA-gated chloride channels. Research has demonstrated that monoterpenoids bind to ionotropic GABA receptors in rats, humans, and insects [35]. Consistent with the findings of the current study, menthol and its constituents, menthyl chloroacetate and menthyl cinnamate, have strong insecticidal efficacy against *Anopheles tessellatus*, *Aedes aegypti*, and *Culex quinquefasciatus* [36]. Studies on the links between structure and activity have shown that even minor modifications to the chemical structure or functional groups may increase mosquitocidal effects.

■ CONCLUSION

Based on the preceding analysis, it can be inferred that enzymatic glycosylation of menthol is a promising alternative method, primarily because of its ability to control both regioselectivity and stereoselectivity. The reaction conditions utilized in the current experiment were milder than those of conventional chemical methods, suggesting its feasibility as a technique for synthesizing MenM. The optimum reaction conditions were a menthol-to-starch ratio of 1:3, temperature of 50 °C, time of 18 h, and enzyme concentration of 50 U with the maximum MenM yield of 23.7%. MenM was evaluated for its biological effects, and the results showed it had antioxidant, antibacterial, antifungal, and insecticidal properties. MenM is an outstanding alternative to menthol in various sectors including food, flavors, fragrances, pharmaceuticals, and cosmetics. This is attributed to their non-volatile nature, solubility in

water, ability to be produced through biocatalysis, and release of a non-toxic anchor molecule.

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■ CONFLICT OF INTEREST

The authors declare no conflicts of interest.

■ AUTHOR CONTRIBUTIONS

Shafaq Nisar performed investigation, visualization, methodology, formal analysis, and original draft writing. Muhammad Asif Hanif performed conceptualization, investigation, methodology, original draft writing, manuscript revision, visualization, validation, supervision, and ensured data accuracy. Umer Rashid conducted formal analysis, investigation, software, visualization, methodology, project administration, writing - review & editing. Muhammad Idrees Jilani carried out formal analysis, software, and original draft writing. Ijaz Ahmad Bhatti performed investigation, original draft writing, and provided resources. Imtiaz Ali was responsible for funding acquisition, resource management, writing review and editing, supervision, methodology, and conceptualization. Bartłomiej Zieniuk help in interpretation, analysis, and writing - review & editing of the manuscripts. All authors have reviewed and endorsed the work's published version.

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