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Isolation, **Identification, and A Statistical Optimization of Scleroglucan-Producing Athelia Rolfsii Did-2 Strain Isolated from Infected Phaseolus Vulgaris**

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Abstract

Athelia rolfsii Did-2 (OP600167) is a fungal strain that produces Scleroglucan, a non-ionic water-soluble biopolymer used in various industries. The study aimed to isolate and identify this fungal strain. Five fungal isolates from infected plants were tested, and strain Did-2 was selected as the highest producer (1.43 g/100 mL). The strain was identified using various international keys, including macroscopic, microscopic, and molecular studies. The partial sequence was deposited in GeneBank under the name *Athelia rolfsii* strain Did-2 18S ribosomal RNA gene with accession number OP600167. To maximize Scleroglucan yield, the Plackett-Burman design (PBD) and the central composite design response surface method (RSM) were employed. The optimized medium resulted in a 2-fold improvement (2.651 g/100 mL) in Scleroglucan yield.

Keywords: Athelia rolfsii, Scleroglucan, optimization Plackett-Burman, Response surface methodology.

1. Introduction:

Scleroglucan is a biopolymer produced by filamentous fungi such as *Sclerotium rolfsii* (*Athelia rolfsii*) and *Sclerotium glucanicum*. It has been established that these fungus species cause serious plant diseases. *Sclerotium rolfsii* is commonly known as the southern blight pathogen and is infamous for causing plant diseases in numerous crops including vegetables, peanuts, and ornamentals. On the other hand, it can also cause root and stem rot in plants, including maize, barley, and soybeans [1, 2].
Scleroglucan is a linear polysaccharide consisting of glucose monomers with a β-(1,3) linkage and a β-(1,6) linkage. It possesses unique physicochemical properties, such as high solubility, viscosity, and pseudoplasticity, that make it useful in various applications. For instance, Scleroglucan is utilized in cosmetic formulations due to its ability to thicken and stabilize emulsions. Similarly, it can also be used in the food industry as a thickening agent or as a fat replacer to improve texture and reduce calories in food products. Moreover, Scleroglucan is recognized as an immunomodulator and anti-inflammatory agent, making it a promising candidate for biomedical applications [3, 4]. Scleroglucan has been proven to be biodegradable, which is good for the environment. However, several challenges still limit the commercial use of Scleroglucan. For example, it has a higher production cost and a lower yield compared to xanthan gum and guar gum. In addition, Scleroglucan has poor thermal stability, which poses challenges for its application and commercialization in high-temperature processes [5].
Scleroglucan is usually produced by the submerged fermentation of fungus, with carbohydrates serving as the primary carbon source. Several factors have been proposed, including pH, temperature, agitation, aeration, concentrations, and types of carbon and nitrogen sources. In addition, other factors can affect Scleroglucan properties, including the type of fungus, the fermentation conditions, the extraction method, and the purification methods. The optimization of these factors can lead to an increase in Scleroglucan production [6].
The various statistical optimization techniques that can be employed for enhancing the properties of Scleroglucan include Response surface methodology (RSM), artificial neural networks (ANN), and genetic algorithms (GA). RSM is the most commonly used technique for optimization of the Scleroglucan process parameters. It involves selecting the most significant process parameters, assessing their impact on the product properties, and optimizing them using a mathematical model [7]. Statistical optimization techniques such as RSM not only enhance the production but also the properties of Scleroglucan. This can result in higher viscosity, broader range pH and temperature stability, and higher yield of Scleroglucan, thereby reducing the cost of production. In addition, several factors can affect Scleroglucan properties, including the type of fungus, the type of substrate, the fermentation conditions, the extraction method, and the purification methods. The optimization of these parameters

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can result in improved properties such as higher viscosity, broader range pH and temperature stability, and higher yield of Scleroglucan [8]. In recent years, advancements in the field of biotechnology have led to the discovery and development of several techniques for immobilizing enzymes. Immobilization of enzymes is a process of attaching enzymes onto a matrix or support material to enhance their functional properties, such as stability and reusability. The immobilization process comprises two essential steps: first, the enzyme is attached to the biopolymer (all polymers of biological sources) molecule, and then it is cross-linked with a crosslinking agent to provide structural support [9]. The study aimed to isolate and identify a fungal strain that produces Scleroglucan, a non-ionic water-soluble biopolymer used in various industries. To maximize the yield of Scleroglucan, the Plackett-Burman design (PBD) and the central composite design response surface method (RSM) were employed.

2. Results and Discussion

2.1. Selection and identification of the most Scleroglucan producing isolate

After eliminating potentially identical isolates based on morphological characteristics, five fungal isolates were selected (one isolate for each plant). These five isolates were coded as Did-1, Did-2, Did-3, Did-4, and Did-5. They are capable of producing biopolymers in the basal medium with capacities ranging from 1.43±0.047 to 0.9±0.036 (g/100 mL). The findings refer to the fact that the isolate Did-2 is significantly the highest biopolymer producer while the Did-4 and Did-5 are the lowest without significant difference between them. On the other hand, both isolates Did-1 and 3 have a moderate biopolymer production capacity in comparison with the other isolates **(Figure 1).** The isolate Did-2 was the only isolate obtained from a diseased Snapbean plant (*Phaseolus vulgaris*) at GPS location (24°32′44″N 27°10′24″E) in El Wadi Al Gadid governorate; hence it was symbolized as Did-2. Therefore, the Did-2 isolate was chosen for further analysis and identification. Based on the data, the five selected fungal isolates (Did-1, Did-2, Did-3, Did-4, and Did-5) show variable capacity for biopolymer production. The isolate Did-2 is the most prolific producer of biopolymers. It has a substantially higher production capacity than the other isolates. This shows that Did-2 may have a distinguishing feature or metabolic ability that permits it to produce greater quantities of biopolymers. There is no apparent agreement on whether the source of isolation or the environment impacts the formation of biopolymers by fungal isolates. Additional factors like genetic differences, climatic conditions, nutrient availability, and growth kinetics can all influence fungal metabolism and consequently biopolymer synthesis [14].

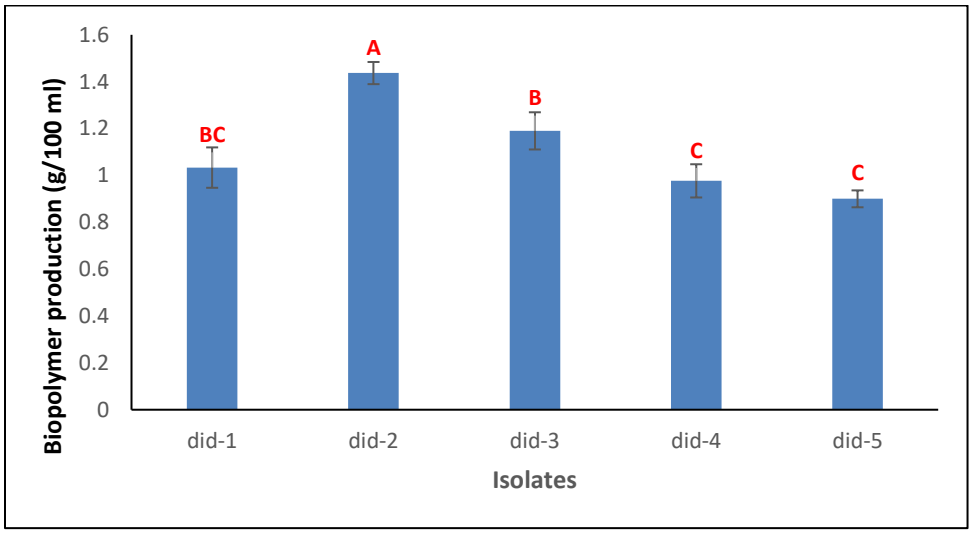

Fig. 1: Biopolymer production by different isolates.

2.2. Characterization and Identification of the potent Scleroglucan producing isolate.

The pure culture of the fungal isolate Did-2 was obtained by culturing hyphae tips on different media (PDA, CYA, SDA and creatine agar medium). Fungal growth on PDA was over-hasted to completely cover the whole plate in five days, giving arise to interwoven rhizoidal like snowy white mycelia with off-white to faint beige reverse colony. Irregularly distributed whitish sclerocia, (> 80/plate) were formed after 8 days, turning brown with age. On CYA, the culture colony reached 60 mm in diameter showing slower growth rate less than PDA with peculiar downy fluffy growth with off-white reverse color. The fungal growth diameter on SDA was nearly equal to this on CYA but much more dense mycelial growth with dull yellowish reverse color. The isolate cultured on creatine agar medium to investigate their ability to hydrolyze creatine with acid production that turned Bromocresol purple supplemented in medium from purple to yellow color, the isolate exhibited no change in purple- blue color **(Figure 2-A-D).** Both SEM and light microscopic investigation showed the intensive interwoven multi-branched septated mycelia, in addition to somatogamy sexual reproduction and the clamp connection that characterized the fungi belonging to basidiomycete **(Figure 2-E – G).** In addition, when the obtained ITS sequence was blasted in GenBank and compared with the

available sequences, it showed similarity more than 97% with *Athelia rolfsii* strains forming separate clade with them in the phylogenetic tree as shown in **(Figure 2-H).**

The fungus *Athelia rolfsii*, also known as *Sclerotium rolfsii*, is a member of the *Basidiomycota* phylum. It is a fungus known as southern blight that causes damage to a number of crops and ornamental plants. It can severely damage a plant's roots, stems, and fruits, causing wilting, rotting, and eventually plant death [15]. *Athelia rolfsii* generates a white to off-white fluffy and cottony mycelium that spreads quickly throughout the substrate. With age, the mycelium produces small, spherical, tan to dark brown sclerotia (hard, compact masses of fungal mycelium) 1-3 mm in diameter. *Athelia rolfsii* hyphae are branched, septate, which means they have cross-walls (septa) that divide the hyphae into individual cells [14]. Aside from asexual spores (conidia), *Athelia rolfsii* can create sexual structures called basidia by somatogamy (the fusing of two nucleated somatic cells). Basidia are club-shaped structures that contain basidiospores,which are the fungus' sexual spores. However, *Athelia rolfsii* sexual reproduction is less prevalent than asexual reproduction [16]. It is important to note that the macroscopic and microscopic characteristics of *Athelia rolfsii* might vary based on climatic conditions, host plant, and fungal development stage. These features serve as generic characteristics for identifying the fungus. As a result, the isolate Did-2 was named *Athelia rolfsii* Did-2 strain and was placed in GenBank with the accession number (OP600167).

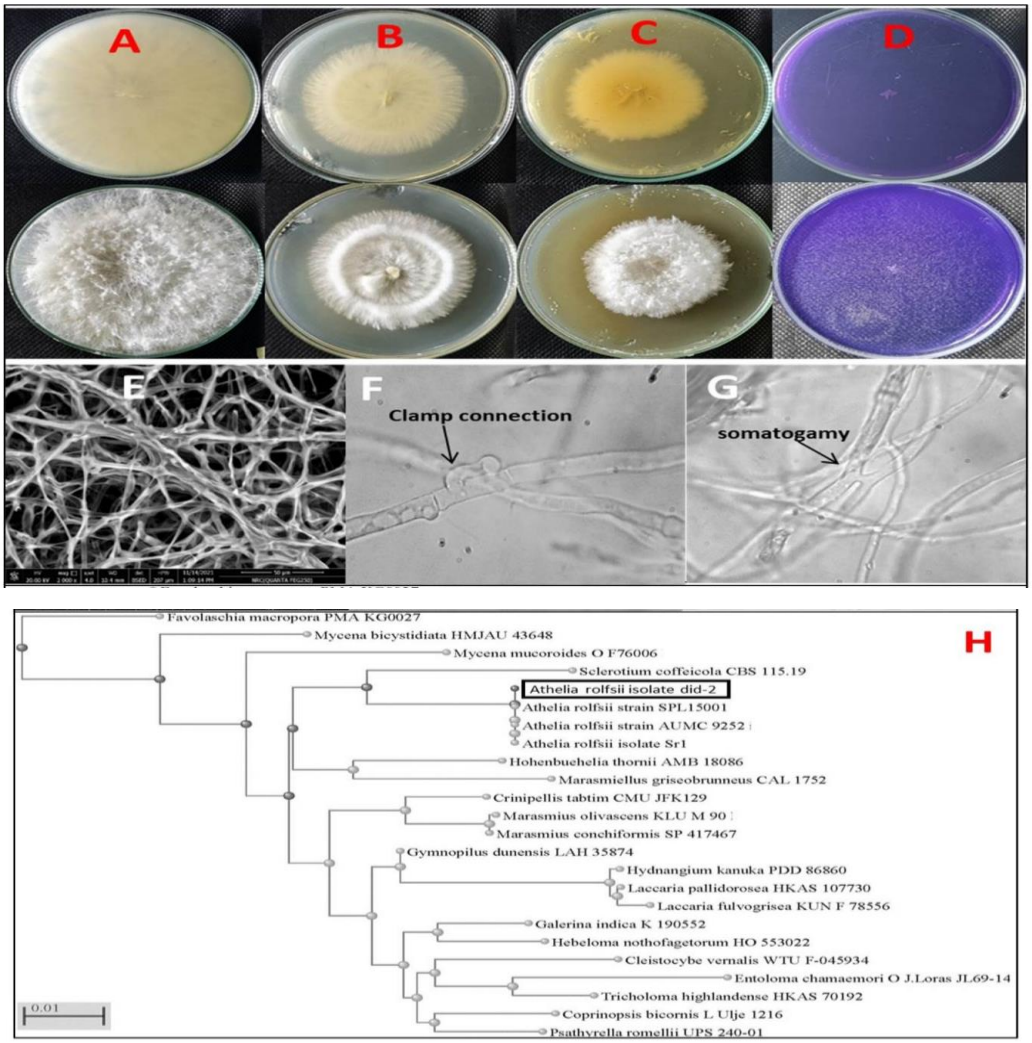

Fig. 2: Characterization of *Athelia rolfsii* Did-2, (A-D); macroscopic characteristics (front and reverse) on different media, (E); microscopic characteristics under SEM (2000X), (F-G); microscopic characteristics under light microscope (200X) and (H) phylogenetic tree of *Athelia rolfsii* Did-2 against *Athelia rolfsii* strains and other fungi.

1.1. *Plackett-Burman design optimization Scleroglucan production*

The Plackett-Burman design was used to screen the factors affecting Scleroglucan production. Twenty-eight experiments were carried out to evaluate the effect of nine factors on Scleroglucan production. The actual and predicted values for the total Scleroglucan amounts are summarized in **Table 1.** Analysis of variance (ANOVA) was applied to test the significance and adequacy of the model. The results indicated that the regression model was very significant ($P < 0.001$) **(Table 2).** According to

the design, variables that have the greatest effect on the Scleroglucan production from the *Athelia rolfsii* Did-2 strain were incubation time, sucrose concentration, temperature, inoculum size, and initial pH at a 95% confidence level as shown in the Pareto chart **(Figure 3-A).** Three of the five factors affecting the production have positive effects which are incubation time, sucrose concentrations, and inoculum size (They have a direct relationship i.e., Scleroglucan production increases as the value of the factor increases and vice versa), while incubation temperature and initial pH have negative effects on Scleroglucan production, they have an inverse relationship (i.e. it decreases as the value of the factor increases and vice versa) (**Figure 3-B).**

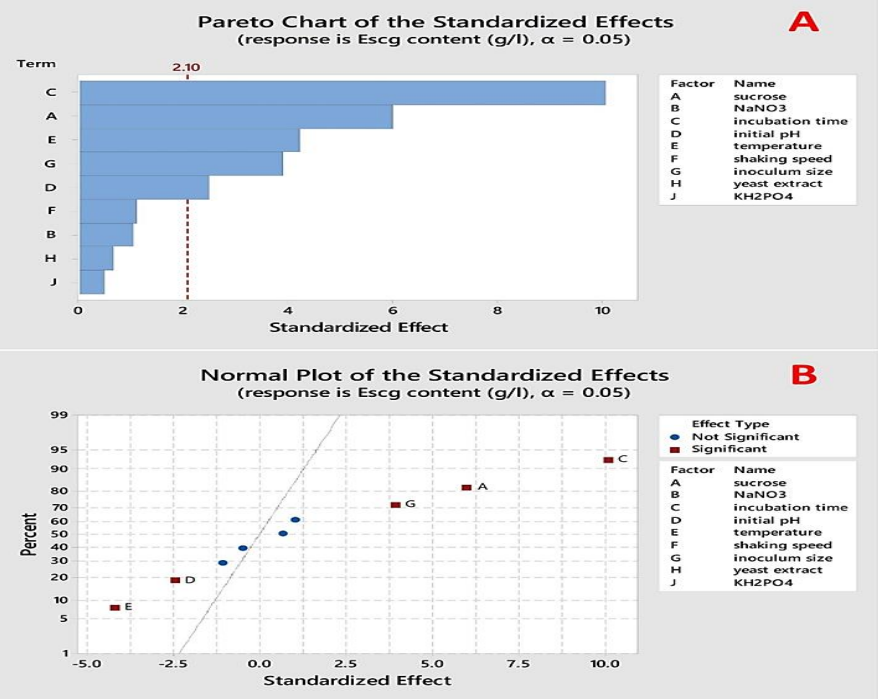

Fig. 3**: Factors affecting the production of Scleroglucan by *Atheli rolfsii* Did-2 strain, (A); Pareto chart and (B); normal plot of the standardized effect**

Scleroglucan (g/100mL) = -1.101+ 0.2222 incubation time+ 0.03070 sucrose+ 0.0664 temperature - 0.1660 inoculum size + 0.2312 initial pH- 0.00893 incubation time*incubation time - 0.000181 sucrose*sucrose- 0.001487 temperature*temperature+ 0.01872 inoculum size*inoculum size - 0.03948 initial pH*initial pH

Table 1: ANOVA Analysis of screened factors affecting the Scleroglucan production.

Source	Effect	DF	Adj SS	Adj MS	*F*-Value	*P*-Value
Model		9	921.72	102.413	20.04	0.000
Linear		9	921.72	102.413	20.04	0.000
Sucrose	5.132	1	184.39	184.388	36.08	0.000
$NaNO_3$	0.884	1	5.47	5.468	1.07	0.315
Incubation time	8.622	1	520.36	520.364	101.83	0.000
Initial pH	-2.122	1	31.53	31.531	6.17	0.023
Temperature	-3.618	1	91.61	91.611	17.93	0.000
Shaking speed	-0.943	1	6.23	6.229	1.22	0.284
Inoculum size	3.349	1	78.53	78.534	15.37	0.001
Yeast extract	0.577	1	2.33	2.328	0.46	0.508
KH_2PO_4	-0.425	1	1.27	1.266	0.25	0.625
Error		18	91.98	5.110		
Total		27	1013.70			

1.2. *Optimization of selected ingredients using RSM.*

Optimization of culture conditions is necessary to improve Scleroglucan production. The central composite experimental design was applied to find out the optimum conditions for *A. rolfthii* Did-2 Scleroglucan production through the determination of the optimum levels of the significant factor's incubation time, sucrose concentrations, inoculum, incubation temperature and initial pH. The results obtained were subjected to an ANOVA to determine the significant differences. There was a considerable variation in Scleroglucan production yield which was heavily dependent on the levels of the five independent variables involved during the production. In fact, Scleroglucan production yield varied from 0.24 g/100 mL (run 27) to 2.43 g/100 mL (run 41). The significant variables were investigated at four different levels in a set of 64 experiments, and the matrix of CCD along with the Scleroglucan yield of each run are presented in **Table (2).** By applying a least-squares method to the experimental data, the following second-order polynomial equation was found to adequately explain the Scleroglucan production yield. The effect of the interaction of various physicochemical parameters on Scleroglucan production by *A. rolfsii* Did-2 strain was investigated by plotting the response surface curves against any two independent variables while keeping the third independent variable at the "0" level. The interactive roles of incubation time, sucrose concentrations, inoculum, incubation temperature and initial pH on Scleroglucan production by *A. rolfsii* Did-2 strain are illustrated in the two-dimensional curves (contour plot) of the calculated response surface shown in **Figure 4.** Contour plots are a useful tool in response surface methodology for visualization the optimal values of used variables that will result in the best Scleroglucan production yield. The fit of the model was evaluated by the coefficient of determination R2, which was 0.9108, indicating that 91.08% of the variability in the response could be explained by the model. The ANOVA table shows that the linear component includes five factors: incubation time, sucrose, temperature, and initial pH. Of these, incubation time, sucrose, and initial pH have significant effects on Scleroglucan production, with *p*-values less than 0.005 for each while Inoculum size has a negative effect on Scleroglucan production, with a p-value of 0.079. The square component includes five factors: incubation time squared, sucrose squared, temperature squared, inoculum size squared, and initial pH squared. Of these, temperature squared dose not has significant effects on Scleroglucan production, with *p*-values of 0.089 while all other squared components have significant effects on Scleroglucan production, with p-values less than 0.005. The lack-of-fit component of the error was not significant (0.453). The performance parameters obtained for the quadratic full mode including all experimental variables, their binary interactions, and self-interactions are also listed in **Table (3).**

Table 2: optimization of factors affecting the production of Scleroglucan by *Athelia rolfsii* Did-2 strain via central composite design.

Run Order	Incubation time (day)	Sucrose (g/l)	Temperature (°C)	Inoculum size (disc)	Initial pH	Scleroglucan production(g/100mL)	
						Actual value	predicted value
1	9	60	35	3	3.0	1.75	1.87
2	6	40	30	5	4.5	1.14	1.38
3	6	40	40	5	4.5	1.22	1.0
4	9	20	35	7	3.0	1.23	1.31
5	9	60	25	3	6.0	1.56	1.73
6	6	00	30	5	4.5	0.38	0.44
7	6	40	30	5	4.5	1.12	1.38
8	9	60	25	7	3.0	2.25	2.18
9	6	40	30	5	4.5	1.63	1.38
10	6	40	30	5	4.5	1.19	1.38
11	6	40	30	5	4.5	1.55	1.38
12	6	40	30	5	4.5	1.46	1.38
13	6	40	20	5	4.5	1.29	1.46
14	3	20	25	7	3.0	1.09	0.85
15	6	80	30	5	4.5	1.72	1.73
16	6	00	30	5	4.5	0.34	0.44
17	6	40	40	5	4.5	0.92	1.0
18	9	20	25	7	6.0	0.95	1.16
19	9	20	35	3	6.0	0.99	0.85
20	9	20	25	7	6.0	1.27	1.16
21	3	20	25	7	3.0	0.89	0.85
22	6	40	30	5	7.5	0.72	0.65
23	6	40	30	5	1.5	1.27	1.39
24	6	40	30	5	4.5	1.53	1.38
25	12	40	30	5	4.5	1.77	1.75
26	3	60	35	7	3.0	1.28	1.27
27	3	20	35	7	6.0	0.24	0.25
28	6	40	30	5	4.5	1.47	1.38
29	12	40	30	5	4.5	1.86	1.75
30	6	40	30	5	4.5	1.16	1.38

31	3	60	25	3	3.0	1.35	1.41
32	6	80	30	5	4.5	2.03	1.73
33	0	40	30	5	4.5	0.34	0.37
34	3	60	25	7	6.0	1.21	1.12
35	9	60	35	3	3.0	2.09	1.87
36	6	40	30	1	4.5	1.56	1.59
37	6	40	30	5	4.5	1.20	1.38
38	9	60	35	7	6.0	1.41	1.58
39	9	20	25	3	3.0	1.41	1.45
40	6	40	30	5	4.5	1.29	1.38
41	9	60	25	7	3.0	2.43	2.18
42	3	60	35	3	6.0	0.61	0.81
43	6	40	30	1	4.5	1.72	1.59
44	6	40	30	5	4.5	1.67	1.38
45	3	20	35	7	6.0	0.25	0.25
46	6	40	30	5	7.5	0.90	0.65
47	0	40	30	5	4.5	0.37	0.37
48	6	40	30	5	1.5	1.31	1.39
49	6	40	30	9	4.5	1.87	1.76
50	3	20	35	3	3.0	0.69	0.53
51	6	40	20	5	4.5	1.59	1.46
52	9	60	25	3	6.0	1.48	1.73
53	9	20	35	7	3.0	1.22	1.31
54	9	20	25	3	3.0	1.37	1.45
55	6	40	30	9	4.5	1.67	1.76
56	3	60	35	3	6.0	0.77	0.81
57	3	20	35	3	3.0	0.48	0.53
58	3	20	25	3	6.0	0.55	0.39
59	9	20	35	3	6.0	0.86	0.85
60	3	20	25	3	6.0	0.30	0.39
61	9	60	35	7	6.0	1.65	1.58
62	3	60	35	7	3.0	1.02	1.27
63	3	60	25	3	3.0	1.52	1.41
64	3	60	25	7	6.0	0.92	1.12

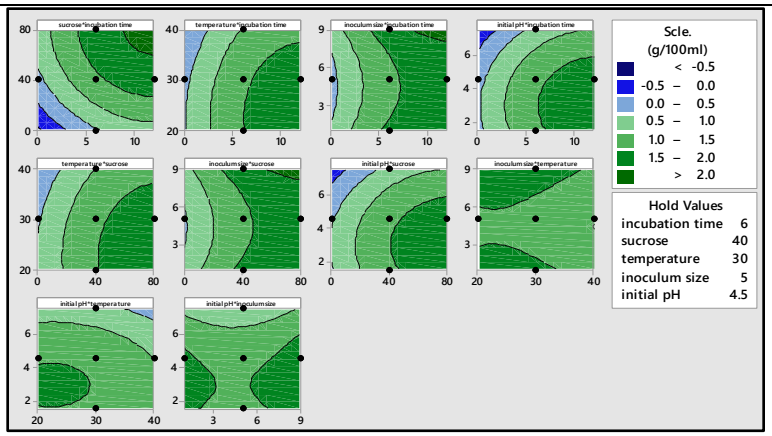

Fig. 4: Effect of different factors on the production of Scleroglucan: Contour plot.

Table 3: ANOVA Analysis of optimized factors affecting the Scleroglucan production.

Source	Coefficient	SE Coefficient	T-value	DF	Adj SS	Adj MS	F-Value	P-Value
Model				10	14.6651	1.46651	54.1	0
Linear				5	13.128	2.6257	96.86	0
Constant	1.3821	0.0464	29.76					
Incubation time	0.6904	0.0475	14.53	1	5.7192	5.7191	210.98	0
Sucrose	0.6477	0.0475	13.63	1	5.0337	5.0337	185.69	0

Temperature	-0.228	0.0475	-4.8	1	0.6237	0.6236	23.01	0
Inoculum size	0.085	0.0475	1.79	1	0.0867	0.0867	3.2	0.079
Initial pH	-0.3726	0.0475	-7.84	1	1.6657	1.6657	61.45	0
Square				5	1.5362	0.3072	11.33	0
Incubation time*	-0.3215	0.086	-3.74	1	0.3791	0.3790	13.98	0
Sucrose*	-0.2901	0.086	-3.37	1	0.3086	0.3086	11.38	0.001
Temperature*	-0.1487	0.086	-1.73	1	0.0811	0.0811	2.99	0.089
Inoculum size*	0.2996	0.086	3.48	1	0.329	0.3290	12.14	0.001
Initial pH*	-0.3554	0.086	-4.13	1	0.463	0.46303	17.08	0
Error				53	1.4367	0.02711		
Lack-of-Fit				16	0.4418	0.02761	1.03	0.453
Pure Error				37	0.995	0.02689		
Total				63	16.1018			

1.3. *Experimental Validation.*

In order to validate the statistical results, a verification experiment was performed in ten-replicates with a predicted optimal medium in shake flasks at the described conditions by response optimizer tool (Response optimizer is a statistical tool used for optimization in Minitab software. It is used to find the optimal values of input variables that will result in the best output response) as in **Figure 5.** The Scleroglucan production yield obtained experimentally was 2.651 g/100mL, which is in reasonable agreement with the maximum predicted value (2.677 g/100mL). This result proves the rightness of the model for predicting the production of Scleroglucan by *A. rolfsii* Did-2 strain. The final optimized conditions are incubation time of 12-day, sucrose concentrations of 80 g/L, inoculum size of 9 disc/100 mL, incubation temperature of 22.4 at initial pH of 2.95.

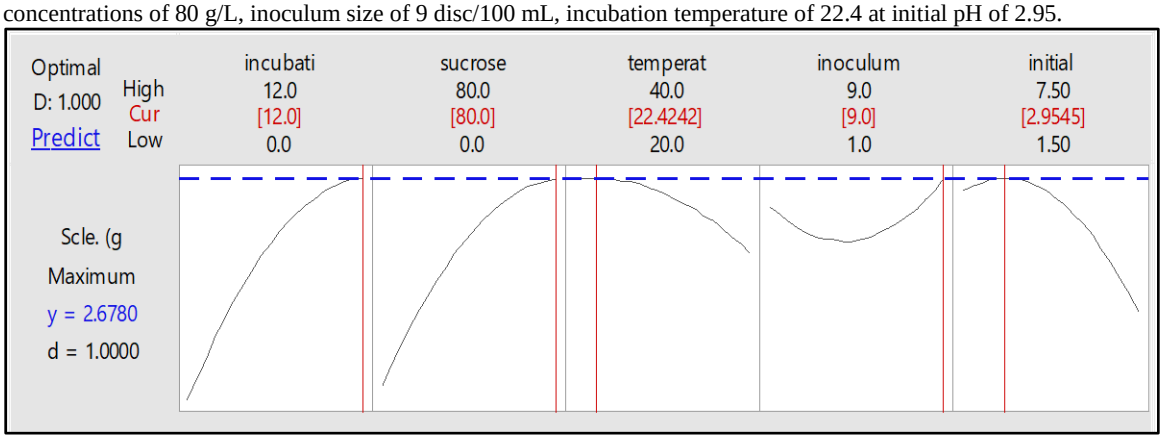

Fig. 5: Response optimizer plot illustrating the optimum levels of variables for maximum Scleroglucan production by *A. rolfsii* Did-2 strain

The predominant producer, *A. rolfsii*, may accumulate significant amounts of Scleroglucan from a variety of substrates, including glucose, sucrose, xylose, and molasses. Its production has been improved by the introduction of various strategies, the majority of which concentrated on the choice of medium components and economical optimization of managing fermentation conditions [17]. According to a prior study, Scleroglucan synthesis is influenced by variables such as phosphate levels, the starting pH, and the type and concentration of the carbon source. Additionally, altering the condition of the inoculum (sclerotia or fungal mat and its age) could enhance the development of Scleroglucan [7]. So, using the Plackett-Burman model, we first screened the fermentation-related variables to determine their impact on the formation of Scleroglucan. This model offers insightful data on how various variables affect the response variable (Scleroglucan production). Sucrose, incubation period, and inoculum size all had substantial effects, which suggest that these elements are crucial for maximizing Scleroglucan production. The detrimental effects of the initial pH and temperature may potentially be also beneficial for specifically improving Scleroglucan production. The other variables, such as shaking speed, yeast extract, $NaNO_3$ and KH_2PO_4, did not significantly affect the response variable ($P>0.05$), hence they were included in the further stages of optimization as conditions at the recommended levels by the model. Several researchers working on biopolymer production have applied factorial regression (FR) and response surface methodology as statistical tools to recognize, manipulate, and optimize influencing medium constituents and recorded the increased biopolymer production [18]. RSM with central composite design (CCD) was applied to determine the

optimal levels of the five selected variables (of incubation time, sucrose concentrations, inoculum, incubation temperature and initial pH) that affected the production of Scleroglucan by *A. rolfsii* Did-2 strain. ANOVA demonstrated that the complete model is valid based on the p-value of the Fisher F-test. In this regard, the model's p-value lower than (0.005) indicated the significance of the model's mean square compared to the residual mean square ($p < 0.05$) [19]. Also, the "lack of fit" is not statistically significant, as indicated by its p-value (0.453). Despite these findings, it can be observed that the *p*-values of some variables are not statistically significant. For additional evaluation of the models, the model correlation coefficient (R2) and adjusted R^2 (R^2 adj) were calculated and found to be 91.08 and 89.39 %, respectively. The predicted R^2 of 87.71% was in reasonable agreement with the adjusted R^2 of 89.39 %, indicating that the regression model could be used to analyze trends of responses [20].

2. Experimental

2.1. *Chemical and material used*

Potato dextrose agar (PDA) and Streptomycin antibiotic (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), Absolute ethanol, HCl, $NaNO_3$, KH_2PO_4 and yeast extract and NaOH (Merck & Co Inc, Rahway, NJ USA.), dialysis bag (molecular weight cutoff of 12 kDa; (Sigma-Ald-rich Chemie GmbH), sucrose (FLUKA CHEMICALS LIMITED, London, United Kingdom), .Czapek yeast autolysate (CYA) (HiMedia, India) and Sabouraud Dextrose Agar (SDA), agar-agar (HiMedia, India), bromocresol purple (Advent- india). Light microscope (Optika, Italy), scanning electron microscope (JEOLTechnics Ltd, Japan)

2.2. *Sample collection*

Plant samples have been collected from several Egyptian governorates (Figure 6 and Table 4) and taken immediately into the microbiological lab. Infected plant parts were chopped into 5-mm pieces and washed twice, once in distilled water and once in running water. Surface sterilization was accomplished by dipping three sample portions for five minutes in a solution of 1% sodium hypochlorite, followed by three rinses in sterile distilled water. To remove any remaining water, the plant portions were individually sandwiched between sheets of sterile filter paper.

2.3. *Isolation, maintenance and screening for biopolymer production*

Three pieces of each plant were then placed on potato dextrose agar plates (PDA). Streptomycin antibiotic was added to the media at a concentration of 0.03 g/L to inhibit bacterial growth. The plates were incubated for 7 days at 28 °C. The sclerotia were collected, surface sterilized as described above, and sub-cultured in the same medium to produce pure culture, and the observed sclerotia were picked up, subjected to surface sterilization as previously mentioned, and sub-cultured in the same medium to obtain pure culture [10]. The fungal isolates were grown on PDA medium at 28-30 °C for 5 days. After that, two agar discs of well-grown fungal culture (9 mm in diameter) were used to inoculate 50 mL of the basal medium (D-glucose 20; $NaNO_3$ 3; yeast extract 1; $MgSO_4$ 0.5; K_2HPO_4 0.9; citric acid 0.7; KCl, 0.5; and $FeSO_4$ 0.05 (g/L), pH 4.5±0.2) in 250 mL Erlenmeyer flasks and then incubated at 28 °C, 100 rpm for 7 days on a rotary shaker (New Brunswick, USA). Three replicates were performed for each isolate, and the mean± standard deviation values were calculated.

Fig.6: Some infected plants used for isolation of *Sclerotium rolfsii*.

2.4. *Recovery and purification of Scleroglucan*

The EPS Scleroglucan was recovered and extracted according to the previous work Elsehemy *et al.*, [10] with slight modification by homogenizing the whole broth in a mechanical blender for 10 min, neutralizing it to pH 6.5–7, diluting the broth culture with

distilled water (2:1 v/v) and heating it in a water bath at 80-90 °C for 1 h to precipitate the protein content of the culture medium. After heating, the broth was centrifuged at 5000 rpm for 15 min. The cell pellets were washed again and then re-centrifuged to collect the EPS-free cell pellet. The EPS was precipitated from the clear supernatant by adding an equivalent volume of absolute ethanol. The mixture was allowed to stand at 5 °C overnight in a refrigerator to complete polysaccharide precipitation. The crude EPS was further purified by absolute ethanol re-precipitation (two times). Finally, the obtained polymer was freeze-dried and milled to obtain a partially purified whitish EPS powder. The acquired EPS (5.0 g) was re-dissolved in double distilled water (1 L) and the obtained solution was dialyzed exhaustively at 4 °C to remove traces of excess salts, mono and oligosaccharides and other impurities [11].

Table 4: Host source and location of *Sclerotium rolfsii* isolates

No.	Common name	Scientific name	Governorate	GPS location
1	Tomato	*Solanum lycopersicum*	Al Sharqia	30°43′06″N 31°33′47″E
2	Snapbean	*Phaseolus vulgaris*	El Wadi Al Gedid	24°32′44″N 27°10′24″E
3	Okra	*Abelmoschus esculentus*	Beni Suef	31.097°N 29.076°E
4	Pepper	*Capsicum annuum*	Asyut	27°11′13″N 31°10′17″E
5	Onion	*Allium cepa*	Al Sharqia	30.44°N 31.48°E

2.5. *Identification of the most Scleroglucan producing fungal isolate*

The selected fungal isolate was identified morphologically according to **Kalaba et al.** [12]. A variety of media, including Potato Dextrose Agar (PDA), Czapek yeast autolysate (CYA), and Sabouraud Dextrose Agar (SDA) (HiMedia, India), were used to cultivate the fungal isolate. The culture features, such as colony color and appearance of aerial and substrate mycelia, were observed and noted after 7 days of incubation at 25 °C. In order to determine whether fungus creates an acid or a base, the fungal isolate was cultivated on creatine sucrose agar (CREA). A tip of the mycelium from the fungal isolate was cultivated on PDA for 7 days before being stained with 0.1% lactophenol blue and examined under a light microscope to look for mycelial characteristics such as branching and septation. A scanning electron microscope was utilized to look at the tiny details of the fungal isolate for further characterization. This investigation was carried out at the National Research Centre (NRC) in Giza, Egypt. The molecular characterization for the fungal isolate was determined using ITS genes according to **Sharaf et al.** [13]. The resulting nucleotide sequence was deposited into GenBank and assigned an accession number. Then, they used the BLAST tool to compare the nucleotides of distinct ITS rDNA sequences that were already published on GenBank in order to produce phylogenetic data.

2.6. *Screening of nutritional and environmental factors using Plackett-Burman design*

At the shake-flask scale, Scleroglucan production requires the selection of a suitable strain and the optimization of culture conditions. The Plackett-Burman (PB) method was used to evaluate the influence of nine independent variables on the production of Scleroglucan, including concentrations of nutrients (sucrose, $NaNO_3$, yeast extract, and KH_2PO_4), incubation time, initial pH, temperature, agitating speed, and inoculum size. Each variable was evaluated at both high and low levels. A 28-run, nine-factor experimental design was formulated. The Scleroglucan extraction was conducted according to the previous protocol. The software package "Minitab Software Version 18" was utilized to create the statistical design and analyze the results of the 28 screening experiments. As shown in **Table 5**, the data were subjected to analysis of variance (ANOVA) to determine the significance of the fitted model and the significance of each parameter's effect on Scleroglucan yield (g/L). All experimental trials were conducted in triplicate, and the mean Scleroglucan quantity was used as the response.

2.7. *Optimization of the significant parameters via central composite design*

Response surface methodology (RSM) using a central composite design was used to optimize the significant parameters identified by the Plackett-Burman design. Scleroglucan production was subjected to analysis of variance (ANOVA). Then a second-order polynomial equation was fitted to the data by multiple regression procedure. This resulted in an empirical model that related the response measured in the independent variables to the experiment. The behaviour of the system was explained by the following quadratic equation 1: $Y=\beta 0 + \sum \beta i xi + \sum \beta ij xixj + \sum \beta ii xi$ 2.

where Y is the predicted response, xi and xj are the input variables, $\beta 0$ is the intercept term, βi is the linear effects, βii is the squared effects, and βij is the interaction term. The statistical software package Minitab 18.0 (Minitab Inc., State College, PA) was used to analyze the experimental design. The response obtained was statistically evaluated and the model was built based on the variables with confidence levels of more than 95%.

2.7.1. *Validation of optimized conditions and predictive models*

An experiment (10 replicates) was carried out under the optimum conditions as determined by the response optimizer of Minitab software v.18. The model's validity and accuracy were determined by comparing experimental and predicted values.

Table 5: Screening of factors affecting the production of Scleroglucan by *Athelia rolfsii* Did-2 strain via Plackett-Burman design.

Run Order	Sucrose	$NaNO_3$ (g/l)	Incubation time (day)	Initial pH	Temperature (°C)	Agitating speed (rpm)	Inoculum size (disc/100mL)	Yeast extract (g/l)	KH_2PO_4 (g/l)	actual value (g/100 mL)	predicted value (g/100 mL)
1	20	1	9	6	25	100	7	1	0.5	1.05	1.123
2	20	5	9	6	25	200	3	3	1.5	0.84	0.797
3	60	1	9	3	35	200	3	3	1.5	0.923	1.073
4	60	1	9	6	35	100	7	1	1.5	1.12	1.232
5	20	1	9	3	25	200	3	3	0.5	0.825	0.964
6	60	1	3	3	35	100	3	3	0.5	0.307	0.347
7	60	5	3	3	35	200	7	3	0.5	0.564	0.676
8	20	1	3	3	35	200	7	3	1.5	0.099	0.032
9	20	5	9	6	35	200	3	1	0.5	0.354	0.42
10	60	5	3	6	25	200	7	1	1.5	0.427	0.726
11	60	1	3	6	25	100	3	1	1.5	0.239	0.397
12	20	5	3	3	35	100	7	1	0.5	0.284	0.200
13	60	1	3	3	25	200	7	1	0.5	0.619	0.892
14	20	5	9	6	35	100	7	3	0.5	0.448	0.907
15	60	5	9	3	25	100	7	3	0.5	2.210	1.995
16	20	1	9	3	35	100	3	1	1.5	0.491	0.596
17	20	5	3	3	25	200	3	1	1.5	0.136	0.09
18	20	5	9	3	35	200	7	1	1.5	1.064	0.925
19	60	5	3	6	35	200	3	1	0.5	0.193	0.071
20	20	1	3	3	25	100	3	1	0.5	0.265	0.138
21	20	5	3	6	25	100	3	3	0.5	0.120	0.072
22	60	5	9	3	25	100	7	1	1.5	2.162	1.894
23	60	1	9	6	25	200	7	3	0.5	1.866	1.600
24	20	1	3	6	25	200	7	3	1.5	0.237	0.182
25	60	1	9	6	35	200	3	1	0.5	1.146	0.845
26	60	5	9	3	25	100	3	3	1.5	1.490	1.617
27	60	5	3	6	35	100	3	3	1.5	0.280	0.181
28	20	1	3	6	35	100	7	3	1.5	0.148	0.000

3. Conclusion

According to this study, the local fungal strain *Athelia rolfsii* Did-2 has the highest production of Scleroglucan with a yield of 1.43±0.047 (g/100 mL) by using basal medium. In order to maximize Scleroglucan yield, Plackett-Burman design (PBD) and the central composite design (CCD) response surface method (RSM) were utilized to screen and optimize nutrient quantities, culture, and fermentation conditions in the chosen fermentation medium. Of the nine criteria tested, the PBD determined which five were the most important at a 95% confidence level, as shown in the Pareto chart. In contrast, the RSM produced a maximum Scleroglucan yield in the optimized medium with a 3-fold improvement. For the safe application of Scleroglucan delivery systems in medications, genes, and foods, further research is needed to understand the precise interaction of Scleroglucan with human organs, tissues, cells, or biomolecules, both in vitro and in vivo. Enhancing the yield of Scleroglucan also requires research on bioreactors, process optimization, strain enhancements,and regulatory network analysis for polymer biosynthesis. Lastly, the significance of looking into each of these elements as a confluence for strain-associated optimization may be emphasized.

4. Conflicts of interest

The authors declare that they have no competing interests.

5. Formatting of funding sources

Not applicable.

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7. Author Contributions Statement

A. E D: Conceptualization, Investigation, Writing – original draft, preparation., M. H. K. Conceptualization, Investigation, Writing – original draft, preparation, Supervision. I.A.E: Conceptualization, Writing – original draft, M.A.M.F: Conceptualization, Supervision., Saad A. Moghannem: Conceptualization, Supervision. All authors have read and agreed to the published version of the manuscript.

8. Ethics approval and consent to participate

Not applicable.

9. References

1. Schmid, J., Meyer, V., & Sieber, V. (2011). Scleroglucan: Biosynthesis, production and application of a versatile hydrocolloid. Applied Microbiology and Biotechnology, 91(4), 937–947. https://doi.org/10.1007/s00253-011-3438-5
2. Castillo, N. A., Valdez, A. L., & Fariña, J. I. (2015). Microbial production of Scleroglucan and downstream processing. Frontiers in Microbiology, 6(OCT), 1–19. https://doi.org/10.3389/fmicb.2015.01106
3. Mansa, R., Dzene, L., Quintela, A., Rocha, F., & Detellier, C. (2016). Preparation and characterization of novel clay/Scleroglucan nanocomposites. Applied Clay Science, 126, 235–244. https://doi.org/10.1016/j.clay.2016.03.021
4. Das, A., Ringu, T., Ghosh, S., & Pramanik, N. (2022). A comprehensive review on recent advances in preparation, physicochemical characterization, and bioengineering applications of biopolymers. In Polymer Bulletin (Vol. 80, Issue 7). Springer Berlin Heidelberg. https://doi.org/10.1007/s00289-022-04443-4
5. Lapasin, R., Abrami, M., Grassi, M., & Šebenik, U. (2017). Rheology of Laponite-Scleroglucan hydrogels. Carbohydrate Polymers, 168, 290–300. https://doi.org/10.1016/j.carbpol.2017.03.068
6. Bai, T., Wang, T., Li, Y., Gao, N. L., Zhang, L., Chen, W. H., & Yin, X. (2021). Optimization of Scleroglucan production by *Sclerotium rolfsii* by lowering pH during fermentation via oxalate metabolic pathway manipulation using CRISPR/Cas9. Fungal Biology and Biotechnology, 8(1), 1–9.
7. Schmid, J., Meyer, V., & Sieber, V. (2011). Scleroglucan: Biosynthesis, production and application of a versatile hydrocolloid. Applied Microbiology and Biotechnology, 91(4), 937–947. https://doi.org/10.1007/s00253-011-3438-5
8. Castillo, N. A., Valdez, A. L., & Fariña, J. I. (2015). Microbial production of Scleroglucan and downstream processing. Frontiers in Microbiology, 6(OCT), 1–19. https://doi.org/10.3389/fmicb.2015.01106
9. Mansa, R., Dzene, L., Quintela, A., Rocha, F., & Detellier, C. (2016). Preparation and characterization of novel clay/Scleroglucan nanocomposites. Applied Clay Science, 126, 235–244. https://doi.org/10.1016/j.clay.2016.03.021
10. Das, A., Ringu, T., Ghosh, S., & Pramanik, N. (2022). A comprehensive review on recent advances in preparation, physicochemical characterization, and bioengineering applications of biopolymers. In Polymer Bulletin (Vol. 80, Issue 7). Springer Berlin Heidelberg. https://doi.org/10.1007/s00289-022-04443-4
11. Lapasin, R., Abrami, M., Grassi, M., & Šebenik, U. (2017). Rheology of Laponite-Scleroglucan hydrogels. Carbohydrate Polymers, 168, 290–300. https://doi.org/10.1016/j.carbpol.2017.03.068
12. Bai, T., Wang, T., Li, Y., Gao, N. L., Zhang, L., Chen, W. H., & Yin, X. (2021). Optimization of Scleroglucan production by *Sclerotium rolfsii* by lowering pH during fermentation via oxalate metabolic pathway manipulation using CRISPR/Cas9. Fungal Biology and Biotechnology, 8(1), 1–9. https://doi.org/10.1186/s40694-021-00108-5
13. Zeng, W., Wang, J., Shan, X., Yu, S., & Zhou, J. (2021). Efficient Production of Scleroglucan by *Sclerotium rolfsii* and Insights into Molecular Weight Modification by High-Pressure Homogenization. Frontiers in Bioengineering and Biotechnology, 9(September), 1–10. https://doi.org/10.3389/fbioe.2021.748213
14. Valdez, A. L., Delgado, O. D., & Fariña, J. I. (2021). Cost-effective optimized Scleroglucan production by *Sclerotium rolfsii* ATCC 201126 at bioreactor scale. A quantity-quality assessment. Carbohydrate Polymers, 260(December 2020).https://doi.org/10.1016/j.carbpol.2020.117505
15. Lyu, X., Gonzalez, R., Horton, A., & Li, T. (2021). Immobilization of enzymes by polymeric materials. Catalysts, 11(10). https://doi.org/10.3390/catal11101211
16. Elsehemy, I. A., Noor El Deen, A. M., Awad, H. M., Kalaba, M. H., Moghannem, S. A., Tolba, I. H., & Farid, M. A. M. (2020). Structural, physical characteristics and biological activities assessment of Scleroglucan from a local strain *Athelia rolfsii* TEMG. International Journal of Biological Macromolecules, *163*, 1196–1207. https://doi.org/10.1016/j.ijbiomac.2020.06.272
17. Fariña, J. I., Viñarta, S. C., Cattaneo, M., & Figueroa, L. I. C. (2009). Structural stability of *Sclerotium rolfsii* ATCC 201126 β-glucan with fermentation time: A chemical, infrared spectroscopic and enzymatic approach. Journal of Applied Microbiology, *106*(1), 221–232. https://doi.org/10.1111/j.1365-2672.2008.03995.x
18. Kalaba, M. H., Sultan, M. H., Elbahnasawy, M. A., El-didamony, S. E., El, N. M., & Sharaf, M. H. (2022). First report on isolation of Mucor bainieri from honeybees , Apis mellifera : Characterization and biological activities. Biotechnology Reports, *36*(September), e00770. https://doi.org/10.1016/j.btre.2022.e00770
19. Sharaf, M. H., Abdelaziz, A. M., Kalaba, M. H., Radwan, A. A., & Hashem, A. H. (2022). Antimicrobial, Antioxidant, Cytotoxic Activities and Phytochemical Analysis of Fungal Endophytes Isolated from *Ocimum Basilicum*. Applied Biochemistry and Biotechnology, *2022*, 1–19. https://doi.org/doi.org/10.1007/s12010-021-03702-w
20. Mahapatra, S., & Banerjee, D. (2013). Fungal Exopolysaccharide: Production, Composition and Applications. Microbiology Insights, *6*, MBI.S10957. https://doi.org/10.4137/mbi.s10957

21. Jebaraj, M. D., Aiyanathan, K. E. A., & Nakkeeran, S. (2017). Virulence and genetic diversity of *Sclerotium rolfsii* SACC., infecting groundnut using nuclear (RAPD & ISSR) markers. *Journal of Environmental Biology*, *38*(1), 147–159. https://doi.org/10.22438/jeb/38/1/ms-274
22. Aalami, A., Masigol, H., Fisheries, I., Grossart, H., & Fisheries, I. (2021). Population genetics and gene sequence analyses of *Athelia rolfsii* collected Population genetics and gene sequence analysis of *Athelia rolfsii* collected from Northern Iran. Mycologia Iranica, *7*(2). https://doi.org/10.22043/MI.2021.124012
23. Survase, S. A., Saudagar, P. S., & Singhal, R. S. (2007). Use of complex media for the production of Scleroglucan by *Sclerotium rolfsii* MTCC 2156. Bioresource Technology, 98(7), 1509–1512. https://doi.org/10.1016/j.biortech.2006.05.022
24. Arul Jose, P., & Jebakumar, S. R. D. (2014). Successive Nonstatistical and Statistical Approaches for the Improved Antibiotic Activity of Rare Actinomycete *Nonomuraea* sp. JAJ18. BioMed Research International, 2014. https://doi.org/10.1155/2014/906097
25. Evangeline, S., & Sridharan, T. B. (2019). Biosynthesis and statistical optimization of polyhydroxyalkanoate (PHA) produced by *Bacillus cereus* VIT-SSR1 and fabrication of biopolymer films for sustained drug release. International Journal of Biological Macromolecules, 135, 945–958. https://doi.org/10.1016/j.ijbiomac.2019.05.163
26. Ghribi, D., Abdelkefi-Mesrati, L., Mnif, I., Kammoun, R., Ayadi, I., Saadaoui, I., Maktouf, S.,&Chaabouni-Ellouze, S. (2012). Investigation of antimicrobial activity and statistical optimization of *Bacillus subtilis* SPB1 biosurfactant production in solid-state fermentation. Journal of Biomedicine and Biotechnology, 2012. https://doi.org/10.1155/2012/373682