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Research article

Response Surface Optimization of *Rhizopus* sp. Immobilization onto Loofah Sponge Using Potato Dextrose

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Abstract

Keywords

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loofah sponge;

response surface methodology;

Rhizopus sp.;

immobilization;

scanning electron microscope

The immobilization process has garnered significant attention in recent years, as it can help avoid the complex procedures of isolation and purification during the fermentation process. Loofah sponge (*Luffa cylindrica*) has gradually been used as an immobilizing matrix for various microorganisms due to its renewability and pore structure. In this study, the weight of immobilized *Rhizopus* sp. onto loofah sponge was investigated using a face-centered central composite design (FCCCD). Studied parameters included dextrose concentration (0.02-1.5 M), agitation speed (0-150 rpm), and incubation time (24-72 h). After two sets of experiments consisting of 17 and 11 runs, respectively, and based on the parameter proposed by FCCCD, a maximum biomass weight of 0.252 g was achieved onto 0.3 g of loofah at dextrose concentration of 0.45 M, agitation speed of 100 rpm, and incubation time of 24 h. The SEM results showed well-formed mycelia within and on the loofah surface with no sign of structural degradation.

1. Introduction

Immobilizing microbial producers is one of the practical approaches that have been applied as systems of long-term fermentation [1]. Many fermentation reactions have shown enhanced productivity when processed using immobilized cell systems [2]. The system offers many benefits over usual suspended-cell fermentations including higher cell densities, and enhancement of the tolerance and efficiency of microorganisms, increases cell productivities, lowers the production cost,

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simplifies the process, and provides preferable cell separation from the reaction system, making continuous and semi-continuous processes applicable in various types of reactors [3-5].

Fungal cells can be immobilized by adsorption on a support matrix, entrapment, or by embedding with natural polymers like alginate gels and synthetic polymers like polyurethane foam or sponges [6]. The main requirements for the immobilization matrix to be employed are that the cell-carrying material is non-reactive, non-toxic, highly porous, mechanically strong enough to withstand long-term usage, and easy to use during the immobilization procedure [4, 7].

Cell immobilization on natural support could be an effective way for fungal cells to grow under submerged cultural conditions. Loofa is a vegetable sponge made of an open network of fibrous support from *Luffa cylindrica*'s dry fruit. The low density and high porosity (> 90%) of the loofah sponge make it a unique structure that can minimize tensity and the difficulty of movement of air and nutrients around the cells and the aqueous system [3, 8]. Therefore, the physicochemical features of loofa make it a perfect eco-friendly bio support. Loofah has become preferrable to other matrices used for cell immobilization. It has been successfully used to immobilize diverse cells, including bacteria, yeast, fungi, microalgae, and plants. A cheap loofah sponge can be used as the immobilization method, and this technology can be easily adapted in developing tropical countries that have an abundance of loofa sponge [8, 9].

Loofah sponge has been used previously as an immobilization matrix for *Rhizopus* sp. during various fermentation processes such as biodiesel [10, 11], lactic acid [8, 12], and fumaric acid productions [13]. However, to our knowledge, no studies were focused on maximizing the immobilized fungal cell density onto the loofah sponge. The aim of this study was to determine the optimum conditions for immobilizing *Rhizopus* sp. onto a loofah sponge by examining the effects of dextrose concentration, agitation, and incubation period using Response Surface Methodology (RSM) using face centered central composite design (FCCCD) as the tool for the design of experiment.

2. Materials and Methods

2.1. Microorganism and inoculum preparation

Rhizopus sp. was previously isolated from ragi tapai and grown on potato dextrose agar (PDA) under an incubation temperature of 37°C for 7 days [14]. After growth and sporulation, 15 ml distilled water was added to each agar plate, which was then scratched to release spores. This spore suspension was filtered to remove fungal hyphae [15]. Then, the number of spores was determined by a hemocytometer [4]. Finally, a 1 ml spore suspension containing 10^6 spores was used to deliver a constant spore quantity in each experiment

2.2. *Rhizopus* sp. immobilization on loofah sponge

A 0.3 g of loofah sponge (in rectangular form 3×4 cm) was cut and placed in boiling distilled water for 1 h, with several changes of water. After that, the pieces were dried in an oven at 70°C for 12 h and stored in a desiccator [8]. The immobilization process was prepared as previously described by Sattari *et al*. [4] with slight modifications. As shown in Figure 1, each loofah piece was placed in a 250-ml Erlenmeyer flask containing 50 ml of culture medium (0.02, 0.11, and 0.2 M dextrose (the dextrose concentration had been calculated using the dextrose molar mass of 180.2 g/mol and the molarity equation: M = mol/l)). The initial pH of the culture medium was adjusted to 5.6, and these flasks were sterilized in an autoclave at 121°C for 20 min. Then, 1 ml of the spore suspension

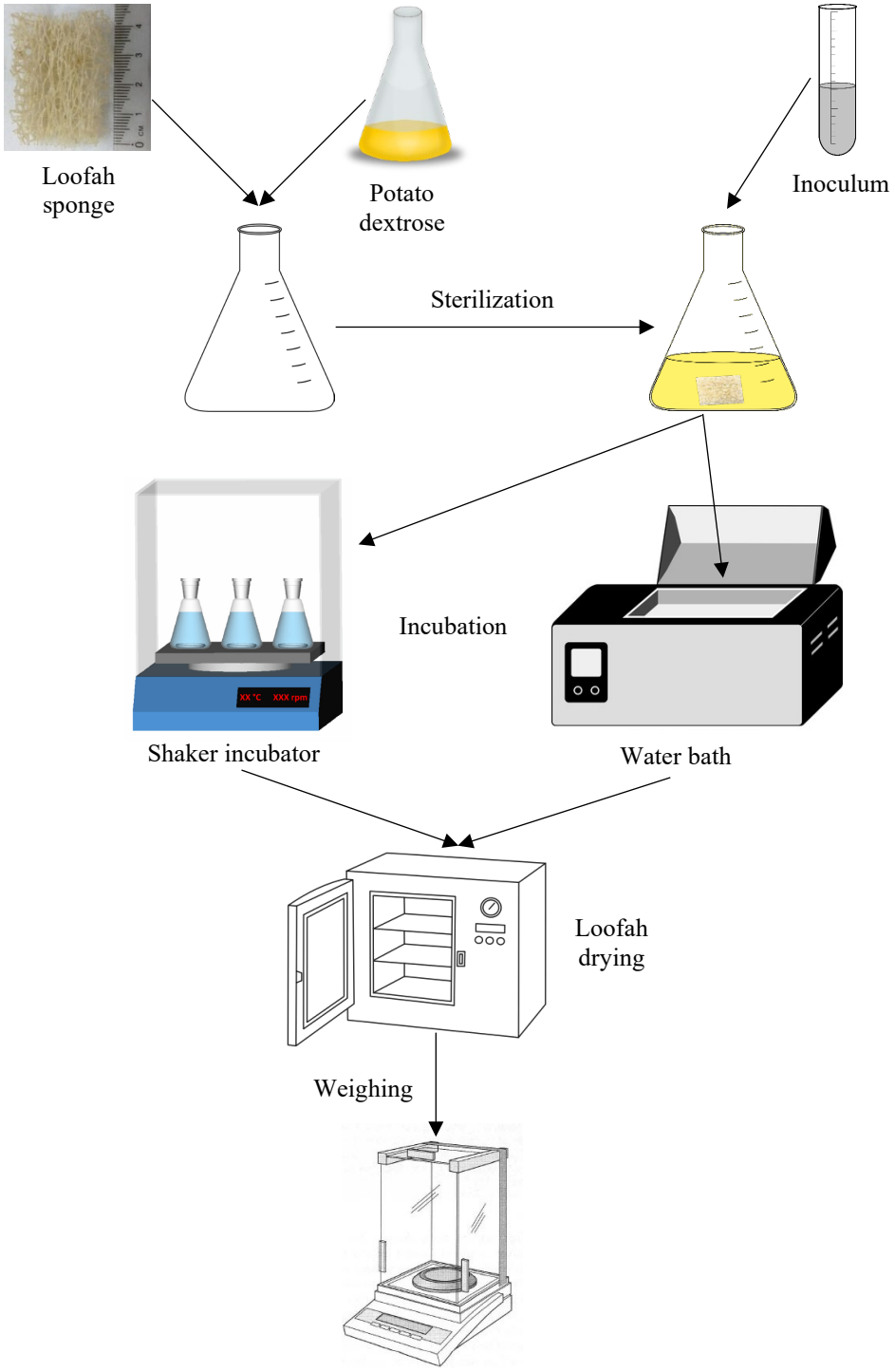

Figure 1. A scheme diagram of *Rhizopus* sp. immobilization onto loofah sponge process

prepared in Section 2.1 was added to the flasks containing loofah pieces. The inoculated flasks were incubated in an incubator shaker (agitation speed ≠ 0) and in a water bath (agitation speed = 0) with controlled process parameters of dextrose concentration (0.02 to 0.2 M), agitation speed (0 to 150), and incubation time (24 to 72 h) which were adjusted accordingly based on experimental design (Table 1). The immobilized cells were washed twice with distilled water to remove dextrose residues then dried using an oven at 50°C for 24 h. The biomass weight on each piece of loofah was determined by measuring the weight differences between the loofah immobilized cells and the neat loofa pieces [4]. The optimization results were not satisfactory because the attached fungus weight kept rising along with increasing dextrose concentration. Therefore, similarly to the above-described method, a second experiment under the following conditions: 0.1, 0.8, and 1.5 M dextrose; 0, 75, and 150 rpm of agitation speed for 24 h was conducted to find the optimum dextrose concentration.

Table 1. Actual and coded values of variables used in FCCCD for optimizing *Rhizopus* sp. immobilization on the loofah

Run	Dextrose concentration (M)	Agitation (rpm)	Time (h)	Fungal biomass weight (g)	
				Actual value	Predicted value
1st Experiment					
1	0.02 (-1)	150 (+1)	72 (+1)	-	-
2	0.2 (+1)	0 (-1)	24 (-1)	-	-
3	0.02 (-1)	0 (-1)	72 (+1)	-	-
4	0.11 (0)	75 (0)	48 (0)	-	-
5	0.2 (+1)	150 (+1)	72 (+1)	-	-
6	0.2 (+1)	0 (-1)	72 (+1)	-	-
7	0.02 (-1)	75 (0)	48 (0)	-	-
8	0.2 (+1)	150 (+1)	24 (-1)	-	-
9	0.2 (+1)	75 (0)	48 (0)	-	-
10	0.11 (0)	75 (0)	72 (+1)	-	-
11	0.11 (0)	0 (-1)	48 (0)	-	-
12	0.11 (0)	75 (0)	24 (-1)	-	-
13	0.11 (0)	150 (+1)	48 (0)	-	-
14	0.02 (-1)	0 (-1)	24 (-1)	-	-
15	0.11 (0)	75 (0)	48 (0)	-	-
16	0.11 (0)	75 (0)	48 (0)	-	-
17	0.02 (-1)	150 (+1)	24 (-1)	-	-

Table 1. Actual and coded values of variables used in FCCCD for optimizing *Rhizopus* sp. immobilization on the loofah (continued)

Run	Dextrose concentration (M)	Agitation (rpm)	Time (h)	Fungal biomass weight (g)	
				Actual value	Predicted value
		2nd Experiment			
1	1.5 (+1)	150 (+1)	24	-	-
2	1.5 (+1)	75 (0)	24	-	-
3	0.1 (-1)	75 (0)	24	-	-
4	0.8 (0)	75 (0)	24	-	-
5	0.8 (0)	75 (0)	24	-	-
6	0.1 (-1)	0 (-1)	24	-	-
7	0.8 (0)	75 (0)	24	-	-
8	0.8 (0)	0 (-1)	24	-	-
9	0.8 (0)	150 (+1)	24	-	-
10	0.1 (-1)	150 (+1)	24	-	-
11	1.5 (+1)	0 (-1)	24	-	-

2.3. Response surface methodology (RSM) for immobilization

RSM was used to optimize both immobilization experiments by software Design-Expert v11.1.2.0 using face-centered central composite design ($\alpha = 1$), as shown in Table 1. The central design point was adjusted at 3 center points to give 17 and 11 runs for the first and second experiments, respectively. The model's validity was confirmed by conducting three runs of each experiment with its suggested values and comparing them with the predicted values.

2.4 Scanning electron microscope (SEM)

SEM was applied on a low vacuum scanning electron microscope (JSM-IT100, JEOL, Ltd., Japan) after sputtering the samples with a gold/palladium layer. SEM was used to evaluate the immobilized fungal cells on the loofah sponge and to check the loofah structure before and after immobilization.

3. Results and Discussion

3.1. FCCCD for optimization of *Rhizopus* sp. immobilization on the loofah

A three-level response of face centered central composite design (FCCCD) was evaluated for a maximum weight of immobilized biomass. The results of both optimization experiments are shown in Table 2. Different combinations of dextrose concentration, agitation speed, and incubation time were examined.

From the first experiment among 17 runs, the maximum attached biomass weight was achieved in run 8, where the dextrose concentration, agitation speed, and incubation time were 0.2

M, 150 rpm, and 24 h, respectively. Under these conditions, the attached biomass weight was 0.2155 g. On the other hand, the maximum attached biomass weight from the second experiment was achieved in run 4, where the dextrose concentration, agitation speed, and incubation time were 0.8 M, 75 rpm, and 24 h, respectively, and the attached biomass weight was 0.2241 g.

A quadratic (second-order polynomial) model that fitted the experimental design for the first set of the experiment was achieved using the following regression equation (Eq. 1).

$$y^{0.52} = 0.0216 + 3.8006A + 0.0020B - 0.0020C - 0.0022AB + 0.0005AC - 0.000004BC - 8.8359A^2 - 0.000008B^2 + 0.00002C^2 \quad (1)$$

Similarly, a quadratic (second-order polynomial) model that fitted the experimental design for the second set of the experiments was achieved using the following regression equation (Eq. 2).

$$y^{0.42} = 0.273191 + 0.528639A + 0.003457B - 0.000517AB - 0.470003A^2 + 0.000018B^2 \quad (2)$$

Where Y represents fungal biomass weight (g) and A, B and C represent dextrose concentration (M), agitation speed (rpm), and time (h), respectively. The predicted fungal biomass weights attached on the loofah at each run by Eq. 1 and Eq. 2 are tabulated in Table 2, along with actual values gathered during the test.

Analysis of variance (ANOVA) was used to interpret the experiment response values. As shown in the summary of ANOVA in Table 3, the generated regression model's significant standard was tested. The first and second experiments' R^2 values were 0.9767 and 0.9961, indicating a high correlation between the predicted and tested values. Furthermore, both models were significant because the adjusted and predicted R^2 were within the sensible accord (the difference was less than 0.2) and the insignificant lack of fit.

The significant demand level of $P = 0.05$ suggests that a model is significant. According to experiments results, the observed P-value was < 0.0001, indicating that it was significant, meaning that the created regression model used to predict the significance pattern for optimizing *Rhizopus* sp. immobilization on the loofah was acceptable and accurate.

The linear (A) and the quadratic (A^2) terms of dextrose in both experiments, along with the quadratic terms of agitation speed (B^2) in the second experiment, were highly significant ($p < 0.01$). Meanwhile, the linear term of agitation (B) in both experiments and the interactive term between dextrose concentration and agitation speed (AB) in the second experiment were significant ($p < 0.05$). On the other hand, in the first experiment, the linear term of incubation time (C), interactive term between dextrose concentration and agitation speed (AB), interactive term between dextrose concentration and incubation time (AC), interactive term between agitation speed and incubation time (BC), and the quadratic terms of agitation speed (B^2) and incubation time (C^2) were not significant. Linear and quadratic effects of dextrose concentration were significant, indicating that a small change of dextrose concentration would alter the attached biomass weight onto loofah.

Generally, the residual from the least-squares is a valuable tool to ensure that this model provides a precise approximation of the highest value of desired response (biomass weight). Normal probability was screened by plotting the normal probability plot of residuals for both experiments. The normality presumption is sufficient as normal residuals shaped a straight line, as shown in Figures 2a, 2c. Figures 2b, 2d show the plot of predicted against the residual response. The residual plots of both models are randomly spread without any directions. This result represents useful predictions of maximum biomass weight and constant variance and sufficiency of the quadratic models [16].

Table 2. Central composite design runs for optimization of *Rhizopus* sp. immobilization on the loofah

Run	Dextrose concentration (M)	Agitation (rpm)	Time (h)	Fungal biomass weight (g)	
				Actual value	Predicted value
1st Experiment					
1	0.02	150	72	0.0162	0.0205
2	0.2	0	24	0.183	0.1673
3	0.02	0	72	0.0031	0.0036
4	0.11	75	48	0.1496	0.1405
5	0.2	150	72	0.1904	0.1842
6	0.2	0	72	0.1817	0.1673
7	0.02	75	48	0.0335	0.021
8	0.2	150	24	0.2155	0.2089
9	0.2	75	48	0.166	0.2095
10	0.11	75	72	0.1387	0.1428
11	0.11	0	48	0.0733	0.0898
12	0.11	75	24	0.149	0.1555
13	0.11	150	48	0.1434	0.1323
14	0.02	0	24	0.0037	0.0042
15	0.11	75	48	0.1569	0.1405
16	0.11	75	48	0.1363	0.1405
17	0.02	150	24	0.0265	0.0314
2nd Experiment					
1	1.5	150	24	0	0
2	1.5	75	24	0.007	0.0051
3	0.1	75	24	0.149	0.1706
4	0.8	75	24	0.2241	0.2133
5	0.8	75	24	0.2166	0.2133
6	0.1	0	24	0.0733	0.067
7	0.8	75	24	0.2097	0.2133
8	0.8	0	24	0.1073	0.1097
9	0.8	150	24	0.1424	0.1478
10	0.1	150	24	0.1434	0.1329
11	1.5	0	24	0	0

Table 3. Analysis of variance (ANOVA) for the optimization of *Rhizopus* sp. immobilization on the loofah

Source	Sum of Squares	Degree of Freedom	Mean Square	*F*-value	*p*-value
1st Experiment					
Model	0.2892	9	0.0321	32.61	< 0.0001
A-dextrose	0.2394	1	0.2394	242.98	< 0.0001
B-agitation	0.0102	1	0.0102	10.31	0.0148
C-time	0.0007	1	0.0007	0.685	0.4352
AB	0.0017	1	0.0017	1.76	0.2263
AC	0	1	0	0.0111	0.919
BC	0.0004	1	0.0004	0.3971	0.5486
A^2	0.0137	1	0.0137	13.93	0.0073
B^2	0.005	1	0.005	5.03	0.0599
C^2	0.0003	1	0.0003	0.3403	0.578
Residual	0.0069	7	0.001		
Lack of Fit	0.0065	5	0.0013	6.97	0.1302
Pure Error	0.0004	2	0.0002		
Cor Total	0.2961	16			
R^2 = 0.9767, Adj-R^2 = 0.9468, Pred-R^2 = 0.8265, Adeq-precision = 16.2103					
2nd Experiment					
Model	0.4154	5	0.0831	257.14	< 0.0001
A-Dextrose	0.2021	1	0.2021	625.36	< 0.0001
B-Agitation	0.0042	1	0.0042	12.89	0.0157
AB	0.003	1	0.003	9.13	0.0294
A^2	0.1344	1	0.1344	415.83	< 0.0001
B^2	0.0258	1	0.0258	79.9	0.0003
Residual	0.0016	5	0.0003		
Lack of Fit	0.0015	3	0.0005	9.33	0.0984
Pure Error	0.0001	2	0.0001		
Cor Total	0.4171	10			
R^2 = 0.9961, Adj-R^2 = 0.9923, Pred-R^2 = 0.9628, Adeq-precision = 38.8360					

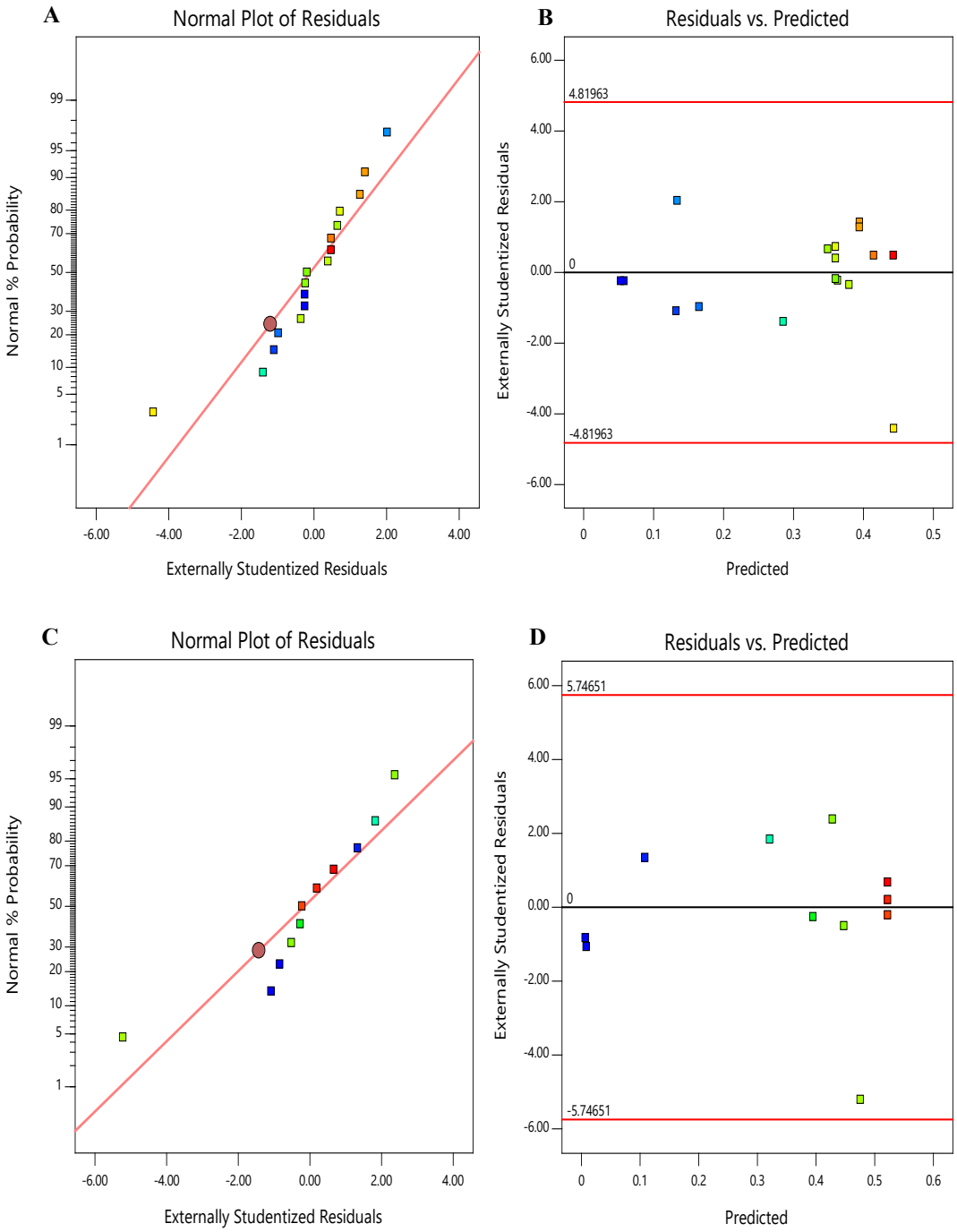

Figure 2. A, C Normal probability of externally studentized residuals B, D and plot of predicted response vs externally studentized residuals for first and second *Rhizopus* sp. immobilization on the loofah experiments, respectively

From Eq. 1 and Eq. 2, the maximum immobilized biomass could be determined by carrying out several series of tests listed from the design, where all the variables were diverse in a slit framework. The design generated a 3D surface plot (Figure 3) based on Eq. 1 and Eq. 2. The graphical plots show the relation of two variables while keeping others fixed.

The first experiment shows the effect of dextrose concentration, agitation speed, and incubation time on immobilized *Rhizopus* sp. weight (Figures 3a, 3b, and 3c). It can be observed from Figure 3a that the immobilization of *Rhizopus* sp. at a higher dextrose concentration (0.2 M) is preferable to the lower concentration (0.02 M) since a higher dextrose concentration provided an adequate amount of substrate for *Rhizopus* to grow and attach onto the loofah. On the other hand, from the analysis, maximum and minimum immobilized biomass weight of 0.227 g and 0.159 g could be reached when the agitation speed was adjusted at 100 rpm and 0 rpm, respectively, at a dextrose concentration of 0.2 M. It was also observed that at all dextrose concentration levels, the low level (0 rpm) and the high level (150 rpm) of agitation speed resulted in lower immobilized biomass weight. This occurred due to the unequal attachment of hyphae on both sides of loofah at the absence of agitation (0 rpm) (Figures 4c and 4g) and the harsh mixing that led to damage the fungal hyphae at high-speed agitation (150 rpm), as shown in Figures 4d and 4h.

Meanwhile, Figure 3b represents the incubation time and dextrose concentration influence with the agitation speed at the center point. Apparently, according to the graph, dextrose had been utilized since the first day of the immobilization process. Longer incubation time did not increase the weight of the fungi on the loofah (Figures 4b and 4f). The weight increased only when the concentration of dextrose increased. The maximum biomass weight of 0.227 g was predicted at 0.2 M of dextrose, agitation speed of 100 rpm, and 24 h of incubation. Finally, the role of agitation speed and incubation time on the immobilized biomass were shown in Figure 3c by fixing the dextrose concentration at the high level (0.2 M). Clearly, the effects of agitation and incubation were not as significant as dextrose concentration, which can be seen in Table 3. However, since the weight of the attached fungal biomass kept increasing with the increasing of dextrose concentration, the optimum immobilization conditions could not be determined from the first set of experiments. Therefore, a second set of experiments was conducted by increasing the dextrose concentration to 1.5 M, and a concave 3D graph was successfully obtained as shown in Figure 4d. This graph shows that the maximum weight of the immobilized fungi was achieved at 0.4 M of dextrose, and it started to decrease until the fungi stopped growing at 1.5 M of dextrose. The maximum attached biomass weight of 0.252 g was achieved at dextrose concentration and agitation speed at 0.45 M and 100 rpm, respectively, in 24 h of incubation.

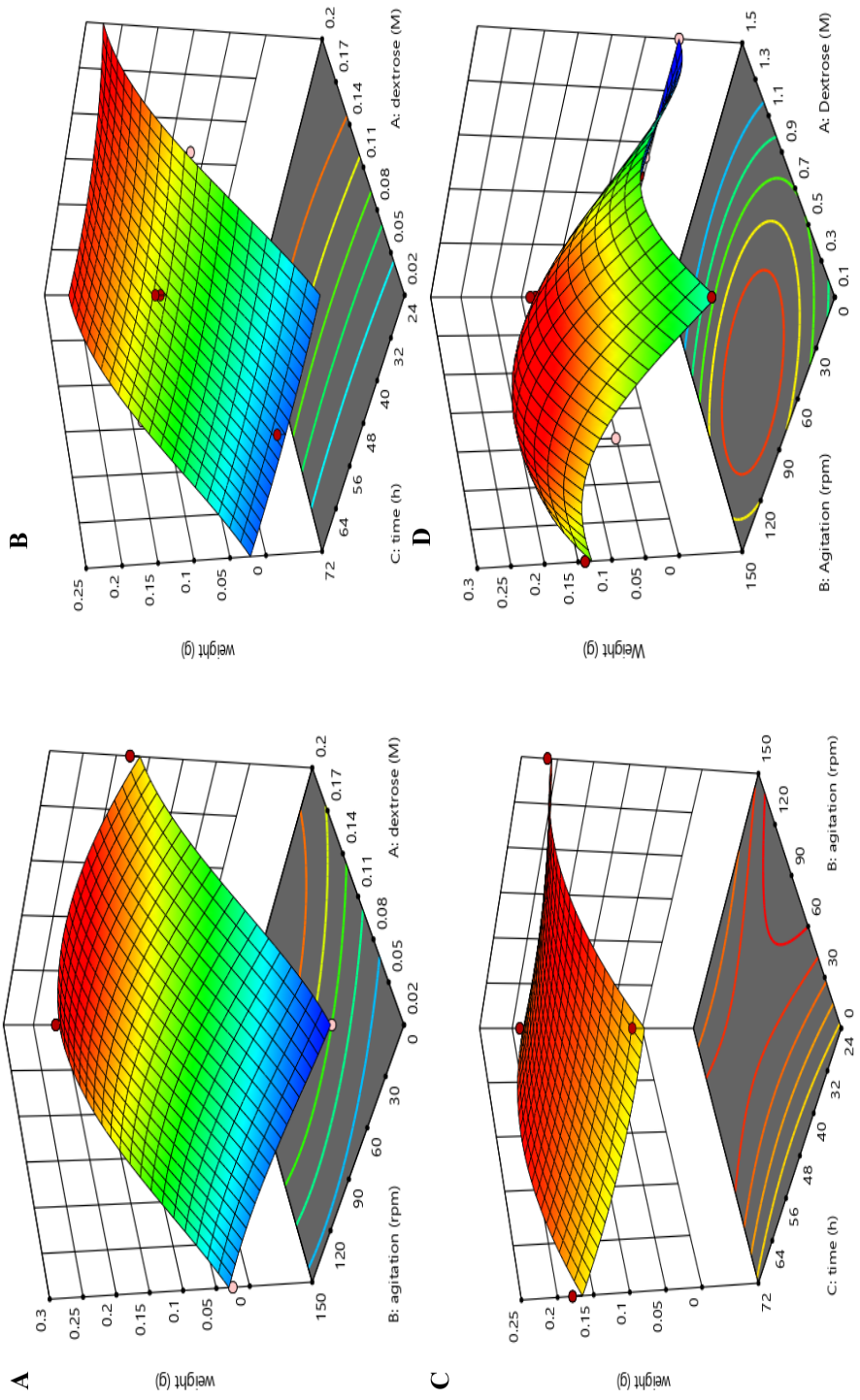

Figure 3. Response surface plot of optimization of A, B, and C first and D second *Rhizopus* sp. immobilization on the loofah from model equation

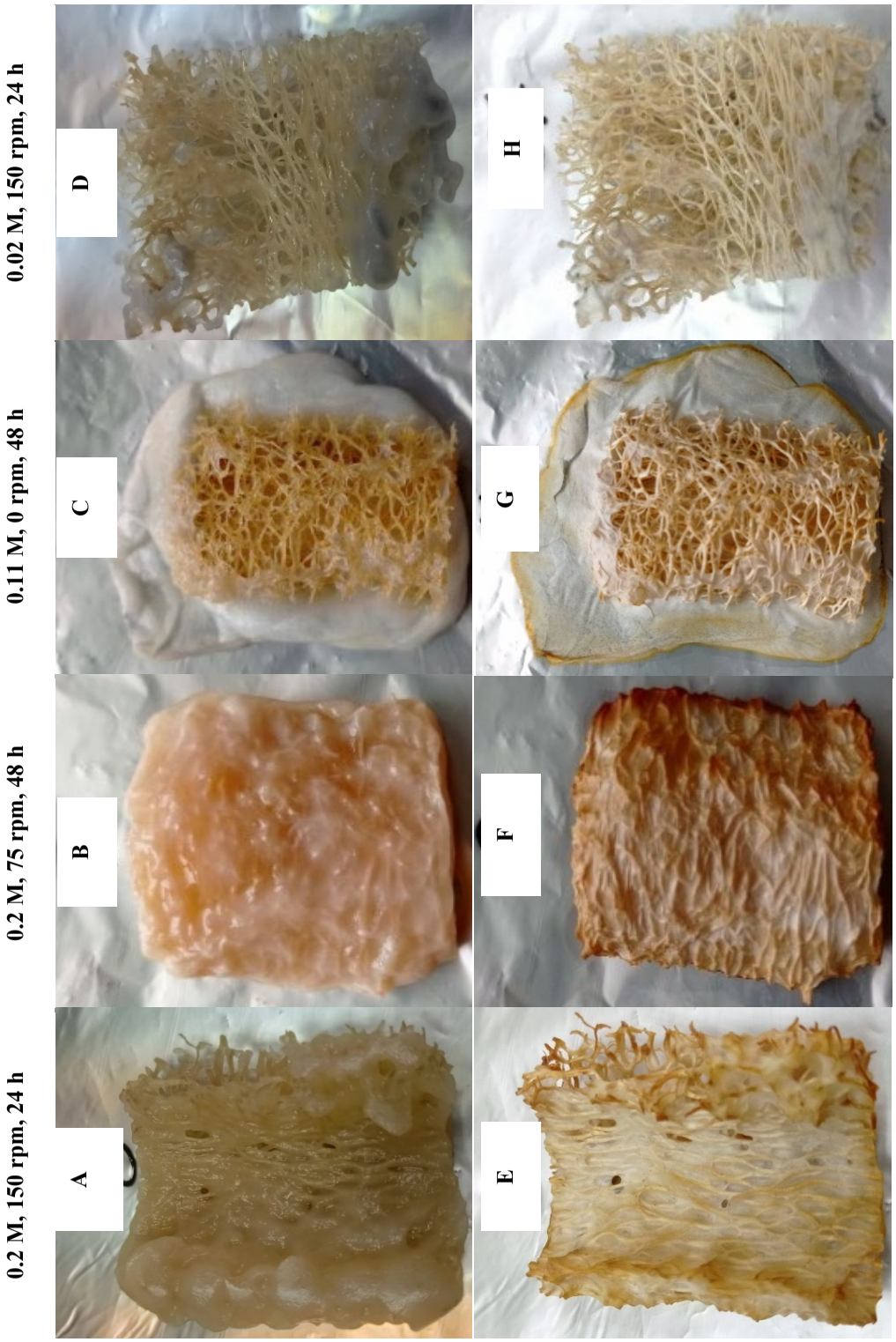

Figure 4. Immobilized cells on loofah sponges under different conditions; A, B, C, D Before and E, F, G, H After drying at 60°C for 24 h

3.2. Validation experiments

The mathematical regression models (Eq. 1 and Eq. 2) generated by RSM optimization were validated. Table 4 shows data from three selected runs of the model. The validation suggested by the software for the first experiment predicted that 0.213, 0.143, and 0.022 g of immobilized biomass would be achieved using 0.2, 0.11, and 0.02 M of potato dextrose, respectively, at an agitation speed of 75 rpm for 72 h. Under these conditions, the actual immobilized biomasses were 0.209, 0.138, and 0.021 g, respectively (Table 4). And for the second experiment, the software predicted that 0.106, 0.175, and 0.202 g of immobilized biomass would be achieved using 0.3, 0.3, and 0.2 M of potato dextrose, at an agitation speed of 0, 150, and 75 rpm, respectively, for 72 h. Under these conditions, the actual immobilized biomasses were 0.1013, 0.1829, and 0.1982 g, respectively. Since the predicted and the actual values are close (less than 4.5% difference), both models could accurately predict optimum immobilization of *Rhizopus* sp. on the loofah.

Table 4. Model validation of *Rhizopus* sp. immobilization on loofah sponge

Dextrose concentration (M)	Agitation (rpm)	Time (h)	Biomass weight (g)		Difference (%)
			Actual value	Predicted value	
1st Experiment					
0.2	75	72	0.209	0.213	1.9
0.11	75	72	0.138	0.143	3.5
0.02	75	72	0.021	0.022	4.5
2nd Experiment					
0.3	0	24	0.1013	0.106	4.4
0.3	150	24	0.1829	0.175	4.3
0.2	75	24	0.1982	0.202	1.9

3.3. Scanning electron microscope (SEM) results

The surface morphology of the loofah before and after immobilization was analyzed using SEM (Figure 5). Three samples were analyzed under 100 X and 200 X magnifications; neat loofah (Figures 5a and 5b), first experiment's 8th run (Figures 5c and 5d), and second experiment's 4th run of *Rhizopus* sp. immobilized loofah (Figures 5e and 5f). When the neat loofah before (Figures 5a and 5b) and after (Figures 5c and 5d) immobilization images were compared, it could be clearly seen that the open structure of the neat loofah sponge provided a suitable and comfortable structure for mycelial attachment. The fungal mycelia grew both within and on the surface of the loofah. Moreover, the network of *Rhizopus* sp. mycelia distributed on the loofah fibers showed that *Rhizopus* sp. had successfully inhabited and was populating the loofah structure (Figure 5e). Furthermore, a comparison of the loofah surfaces under 200 X magnification before (Figure 5b) and after (Figure 5f) immobilization, the loofah sponge surface was still intact without sign of degradation by *Rhizopus* sp., as shown in Figure 5f.

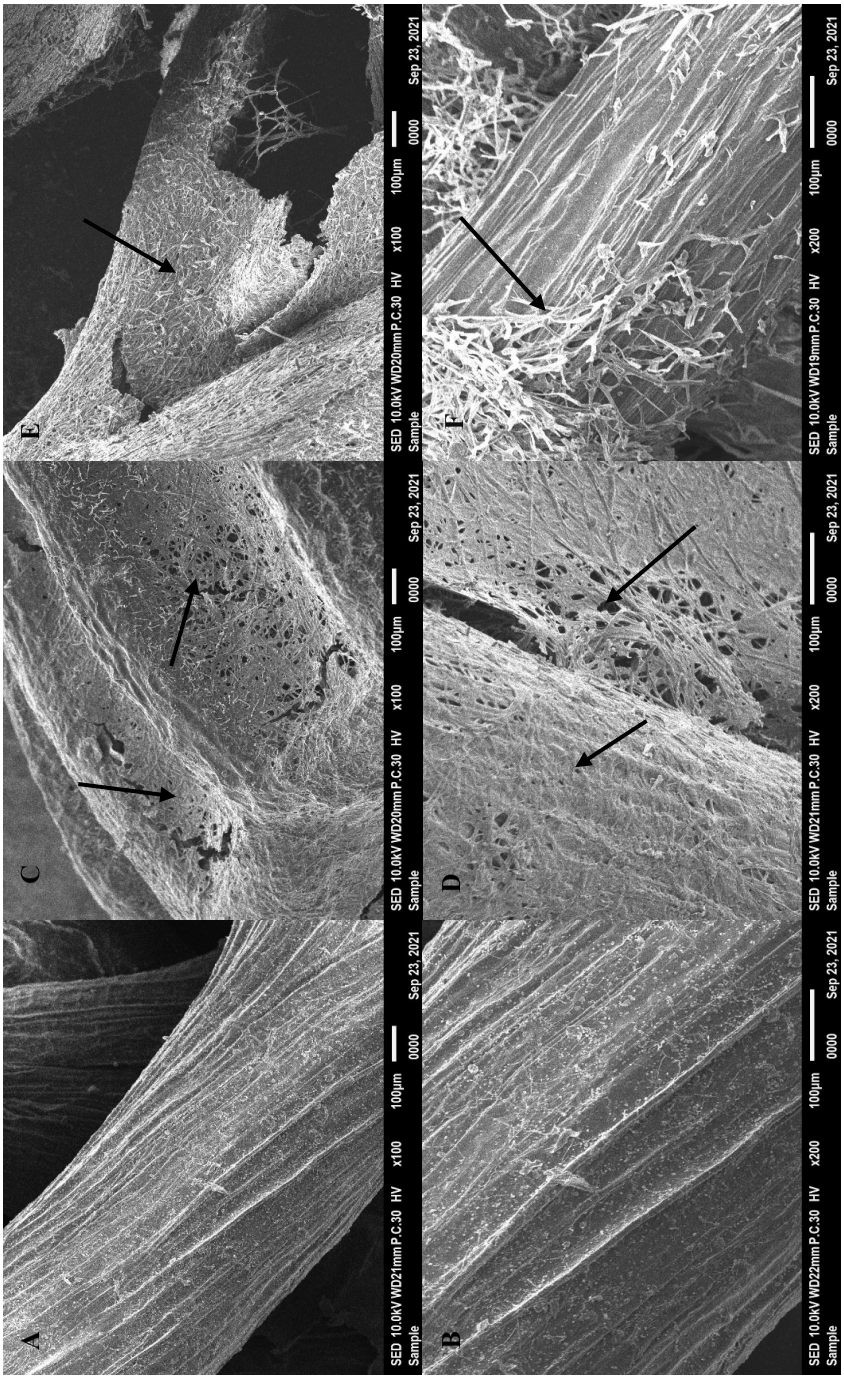

Figure 5. SEM micrographs of A and B neat loofah, C and D first experiment's 8th run of *Rhizopus* sp. immobilized loofah, and E and F second experiment's 4th run of *Rhizopus* sp. immobilized loofah. The networks of mycelium are marked with arrows.

4. Conclusions

This study investigated the immobilization of *Rhizopus* sp. onto loofah sponge using potato dextrose using FCCCD as the tool for the design of the experiment. After conducting two set of experiments on immobilizing *Rhizopus* sp. onto loofah sponge with 17 and 11 runs under three different variables, the results and ANOVA analysis showed that both models were significant and a maximum biomass weight of 0.252 g attached to 0.3 g of loofah at the optimum conditions of potato dextrose, agitation speed and incubation time at 0.45 M, 100 rpm, and 24 h, respectively. The model validation showed good agreement between actual and predicted values, with less than 5% difference. The SEM images confirmed that loofah surface was a good matrix for *Rhizopus* attachment, and that it could be used as whole-cell biocatalyst in submerged fermentation.

5. Acknowledgements

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