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Simultaneous production of an exopolysaccharide and chitosan by *Aspergillus quadrilineatus* using response surface methodology

Magdy Mohamed Khalil Bagy, Nivien Allam Nafady, Elhagag Ahmed Hassan, and Marwa Mahmoud Hashem*

Botany and Microbiology Department, Faculty of Science, Assiut University, 71516 Assiut, Egypt.

*Corresponding Author: marwa011721@science.au.edu.eg, marwa2hashem@gmail.com, and ORCID: 0000-0001-8798-3238

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ABSTRACT

This study was aimed to reduce the production cost of fungal exopolysaccharides by increasing the yield and extracting chitosan from the fungal wastes produced simultaneously. *Aspergillus quadrilineatus* was isolated as well as identified morphologically and by molecular analyses (GenBank Accession No. MT742630.1). One-variable-at-a-time method was combined with response surface methodology to explore the effects of medium components and fermentation conditions on both exopolysaccharide and biomass yields. Sucrose and the inorganic nitrogen $(NH_4)_2SO_4$ had the greatest stimulating effect on biomass and EPS production. The highest biomass and EPS production were found after 14 and 4 days of fermentation, respectively. Statistical analyses of Plackett–Burman experimental design indicated that the models were significant; in particular, $(NH_4)_2SO_4$, K_2HPO_4, tween 80, $CaCl_2.2H_2O$, sucrose, and the incubation interval were the most significant for EPS production and biomass. Responses of the central composite design for biomass and exopolysaccharide production cleared the strong similarity between the experimental and predicted values. The optimized medium boosted the exopolysaccharide by 329.73% and the biomass by 31.44% of the primary yield. The waste fungal biomass, obtained after production of the enhanced EPS, was used to extract chitosan, yielding 3.52 mg/g with about 88.84% deacetylation degree (DD).

1. INTRODUCTION

Microorganisms are abundant in nature and considered exceptional sources for various metabolites that fortify them to combat the stressful conditions and survive in various habitats **[1]**. Although biomolecules produced by plants reach billion tons

annually, microorganisms reveal less production time for safe, nontoxic, and biodegradable molecules **[2]**. Fungi are a broad group, among these microorganisms, with a fast growth rate and cell walls composed of various polysaccharides in parallel with those produced as metabolites **[3]**.

Submerged fermentation is a rapid and effective method to produce bioactive metabolites and fungal mycelia, simultaneously. Among these metabolites, polysaccharides are considerable substances mainly found in the surrounding medium, such as exopolysaccharides (also known as extracellular polymeric substances (EPSs)), or as a constituent of fungal biomass including chitosan **[4]**. Fungal polysaccharides have been mentioned as biomacromolecules with high value; they have many applications in medicine, pharmaceuticals, foods, and other industrial sectors **[5,6]**. Fungal EPSs are different from those of other sources, such as archaea and bacteria, in properities and activities **[7]**. In addition, production of natural chitosan from the cell walls of filamentous fungi has shown more advantages than the traditional extraction by deacetylation of shrimp residues. These advantages include that the biotechnological strategies need moderate temperatures and simple chemical methods, without the demineralization step, due to the lower level of inorganic matters compared with crustacean wastes **[8]**. Besides, fungal chitosan has lower viscosity than chitosan obtained by deacetylation of chitin from shrimp shells **[9]**. Accordingly, satisfactory simultaneous production of EPSs and chitosan by filamentous fungi in shorter time can decrease the production cost.

Enhancement of biomass and exopolysaccharide production has been achieved by optimizing medium components, mainly carbon and nitrogen sources, as well as culture conditions including temperature, initial pH, and agitation **[10–12]**. One-variable-at-a-time (OVAT) is a time-consuming method and unable to determine the interaction between independent variables involved in the fermentation process; however, OVAT method helps in selection of different nutrients and conditions that boost the yield **[13]**. In contrast, response surface methodology (RSM) is an important statistical technique to optimize several variables, simultaneously, and achieve the desired responses through limited number of designed experiments and the desirability function approach **[14]**. Previously, several studies had been performed to optimize medium components, utilized

by microorganisms during the fermentation process for desired natural products, using different statistical experimental designs **[5]**.

Hence, the OVAT optimization process can be reduced by statistical experimental designs blended with RSM, such as Plackett–Burman design (PBD) followed by a central composite design (CCD). In the present study, the main efficient medium components for EPSs and biomass were firstly established by OVAT method, then experiments of PBD and CCD followed by RSM statistical analyses were carried out to find the optimized critical variables for achieving the maximal ESP yield by *Aspergillus quadrilineatus*. After that, the obtained fungal biomass waste was analyzed for chitosan content.

2. MATERIALS AND METHODS

2.1. Isolation and identification of the fungal strain

The fungus was isolated, in the laboratory, from soil collected from Assiut Governorate, Egypt. For isolation, 10 g of the soil sample were homogenized in 90 mL of sterilized distilled water and serially diluted up to 10^{-4} dilution. After that, the pour plate method was performed using 3% sucrose – Czapek's agar (CzA) medium, then the Petri-dishes were incubated at 28 ℃ for 7 days. The isolate was purified and stored on potato dextrose agar (PDA) slants at 28 ℃ and 4 ℃, respectively.

For identification, the EPS-producing isolate was reactivated by culturing on PDA medium in a Petri-dish at 28 ℃ for 7 – 15 days, then the identification was performed based on the macroscopic and microscopic features using a light microscope. In addition, the identification was confirmed by rDNA-based molecular techniques. The internal transcribed spacer (ITS) region (including 5.8S rDNA) was amplified from the genomic DNA by polymerase chain reactions (PCR) using ITS1 and ITS4 primers. Forward and reverse DNA sequencing reactions were carried out by SolGent company (South Korea). The obtained sequence was compared to the GenBank database using the basic local alignment search tool (BLAST) at the national center for biotechnology information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/). The resulting nucleotide sequence of the strain was deposited in the GenBank, and an accession number was assigned. The phylogenetic tree was constructed from the aligned sequences by MEGA software (version 10.1.8) using the neighbor-joining algorithm with 1000 bootstrap replicates.

2.2. Preparation of the fungal inoculum and the basal medium

The fungal strain was cultured on PDA medium at 28 ℃ for 7 days. After that, the spores were gently scraped with a sterilized loop and dispersed in sterilized distilled water. The number of spores was counted using a hemocytometer and adjusted to approximately 0.18×10^6 spore/mL. Then, 1 mL of the spore suspension was used to inoculate 25 mL of the basal medium described by Li *et al.* **[15]** with some modifications (40 g/L sucrose, 2 g/L yeast extract, 1.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4.7H_2O$; pH 8.0 ± 0.1) in 100 mL-Erlenmeyer flasks, which were incubated for 7 days at 28 ℃ on a rotary shaker (100 rpm). All experiments were performed in triplicate.

2.3. Optimization of medium components for elevated biomass and EPS production

2.3.1. One-variable-at-a-time (OVAT) method

To estimate the optimal medium components and culture conditions on time, primary screening was done, using one-variable-at-a-time (OVAT) method, to explore the impact of carbon sources, nitrogen sources, initial pH, incubation temperatures, incubation intervals, and agitation on biomass and EPS production by the fungal strain under-study.

The basal medium composition was modified by using separately 1.68 g % (w/v) carbon from five different sources: glucose, galactose, fructose, sucrose, and maltose. Furthermore, yeast extract, peptone, ammonium sulfate, ammonium nitrate, potassium nitrate, and sodium nitrate were used separately with weights equivalent to 0.018 g % (w/v) of nitrogen. After identifying the preferred carbon and nitrogen source, different concentrations of these two selected nutrients were applied to the basal medium to determine the effective concentration for biomass and EPS production.

In addition, the fungus was cultured in separate Erlenmeyer flasks containing the basal medium with different initial pH (2.5, 3, 4, 6, 8, 10, and 11.5) at various incubation temperatures (25, 30, 35, and 40 ℃) for diverse incubation intervals (2, 4, 6, 8, 10, 12, and 14 days) either under the static culture condition or on a rotary shaker at 100 rpm.

2.3.2. Plackett – Burman experimental design

Plackett – Burman design (PBD) is an efficient method to detect variables that have significant contributions, by performing a small number of experiments, assuming there are no interactions between them **[16]**. In this investigation, PBD was applied to study the key variables, prior to the optimization, using Design-Expert 11.1.2 software (Stat-Ease Inc., Minneapolis, USA).

The independent variables and their levels were selected based on the primary screening (OVAT method) and the literature data **[13,17,18]**; tween 80, $FeSO_4$, $CaCl_2.2H_2O$, KCl, and NaCl were added to the medium composition as supplementary components and mineral elements to investigate their effects on biomass and EPS production by the fungal strain under-study. Altogether, 11 assigned variables were defined at two levels: low level (−1) and high level (+1), as shown in **Table 1**, and screened in 12 experiments. All experiments were performed in triplicate under the static culture condition and medium pH 6. The dry weights averages for each of EPS and biomass were taken as responses, and the models were demonstrated by the following first-order polynomial equation

$$Y = \beta_0 + \sum \beta_i X_i \qquad (1)$$

where Y is the predicted response, β_0 is the regression coefficient of the model, β_i is the linear coefficient, and X_i is the level of the independent variable.

Table 1: Levels of variables used in Plackett – Burman design.

Variable	Unit	Type	Low level (-1)	High level (+1)
A: Sucrose	(g/L)	Numeric	40.00	60.00
B: $(NH_4)_2SO_4$	(g/L)	Numeric	0.85	4.00
C: $MgSO_4.7H_2O$	(g/L)	Numeric	0.50	1.00
D: K_2HPO_4	(g/L)	Numeric	1.50	3.00
E: Tween 80	(g/L)	Numeric	4.00	7.00
F: $FeSO_4$	(g/L)	Numeric	0.10	0.20
G: NaCl	(g/L)	Numeric	0.50	1.00
H: KCl	(g/L)	Numeric	0.50	1.00
J: $CaCl_2.2H_2O$	(g/L)	Numeric	0.50	1.00
K: Temperature	(℃)	Numeric	25.00	28.00
L: Incubation interval	(Days)	Numeric	4	7

2.3.3. Response surface methodology

Response surface methodology was used to detect effects of the significant variables, identified by PBD, and their interactions on the responses. The significant medium components studied as independent variables were sucrose, $(NH_4)_2SO_4$, K_2HPO_4, $CaCl_2.2H_2O$, and tween 80. The combination of their concentrations, at five different levels shown in **Table 2**, was determined by a central composite design (CCD). The CCD was composed of 32 experiments, of which 6 replicates at the center point, using Design-Expert 11.1.2 software (Stat-Ease Inc., Minneapolis, USA). All experiments were performed in triplicate, under the static culture condition, medium pH 6, for 7 days, and at 28 ℃. The dry weights averages for each of EPS and biomass were taken as the responses. The data were analyzed by multiple regression to correlate the response to the independent variables as the following second-order polynomial equation

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \qquad (2)$$

where Y is the predicted response, X_i and X_j are the levels of the independent variable, β_0 is the regression coefficient of the model, as well as β_i, β_{ij}, and β_{ii} are the linear, interaction, and quadratic coefficients, respectively.

After that, the desirability function approach was performed using an optimization method developed by Derringer and Suich as well as applied by Design-Expert 11.1.2 software (Stat-Ease Inc., Minneapolis, USA) **[19]**. After specifying the optimal levels of the variables, a confirmatory experiment was performed in triplicate.

Table 2: Levels of variables used in the central composite design.

Variable	Actual levels of coded variables (g/L)				
	-2	-1	0	+1	+2
A: Sucrose	50.0	55.0	60.0	65.0	70.0
B: $(NH_4)_2SO_4$	2.0	3.0	4.0	5.0	6.0
C: K_2HPO_4	2.0	2.5	3.0	3.5	4.0
D: $CaCl_2.2H_2O$	0.0	0.5	1.0	1.5	2.0
E: Tween 80	0.0	3.5	7.0	10.5	14.0

2.4. Determination of biomass and exopolysaccharides

The fungal mycelium was separated from the fermented broth through filtration by a pre-weighed layer of gauze followed by Whatman No. 1 filter paper. After that, the biomass was washed with distilled water and dried at 60 ℃ till a constant weight, then the dry weight of fungal biomass was calculated.

The resulting filtrate was thoroughly mixed with chilled absolute ethanol (1:3) and kept overnight in a refrigerator. After centrifugation, the supernatant was collected for prospective studies, while the precipitate was dried at 60 ℃ and weighed. The total sugars content of the precipitate was determined according to the phenol-sulfuric acid method **[20]**, using glucose as the standard. The absorbance of the characteristic yellow-orange color was measured at 490 nm by a spectrophotometer (Ray Wild Ltd., Germany).

2.5. Chitosan extraction

Fungal biomass, obtained under the optimized conditions, was utilized to extract chitosan with sodium hydroxide (NaOH) and acetic acid (CH_3COOH) solutions used by Synowiecki and Al-Khateeb (1997) **[21]** with some modifications.

The dried fungal biomass was ground finely, then deproteinized with NaOH (0.5 M), in a ratio of 1/40 (g/mL), for 8 h at 40 ℃ using an orbital shaking water bath (200 rpm). After that, the alkali-insoluble materials (AIMs) were collected and washed repeatedly, with distilled water followed by centrifugation, until reaching to a neutral pH value (7 ± 0.2). AIMs were dried at 60 ℃ till a constant weight and ground finely.

Chitosan reflux was achieved by mixing the obtained AIMs with CH_3COOH solution (15%) in a ratio of 1/100 (g/mL) for 8 h at 40 ℃ and 200 rpm. After that, the insoluble fraction in the acetic acid solution was separated by centrifugation, while the supernatant was re-centrifuged, its pH was adjusted to 13 by NaOH (4 M), and kept in a refrigerator overnight. The solution was centrifuged and the precipitated chitosan was washed with distilled water to a neutral pH value, then dried at 60 ℃ till a constant weight. The functional groups and the deacetylation degree (DD) of the extracted chitosan was determined by using a Fourier-transform infrared spectrometer (Nicolet

IS10 FTIR spectrometer, Thermo Fisher Scientific Inc., USA) and the following equation **[22,23]**

$$DD\% = 97.67 - (26.486 \frac{A_{1655}}{A_{3450}}) \quad (3)$$

where A_{1655} and A_{3450} are the absorbance values of amide-I band and OH^- band at wave numbers 1655 cm^{-1} and 3450 cm^{-1}, respectively.

The FTIR analysis was achieved in collaboration with the Instrumental Organic Chemistry Laboratory – Faculty of Science, Assiut University, Egypt. After that, the FTIR spectrum was graphed using OriginPro 2018 software (OriginLab Corporation, USA).

3. RESULTS AND DISCUSSION

3.1. Identification of the tested fungus and phylogenetic analyses

The morphological characteristics of the isolated fungus were investigated based on the macroscopic and microscopic features. Colonies of the fungus were plane or slightly wrinkled with a tendency toward floccosity, and expanded on CzA medium. In addition, the olive-green conidial areas revealed a brownish orange reverse **(Figure 1 a,b)**. The microscopic morphology elucidated that conidial heads were biseriate and metulae covered only the upper third to half of the pyriform vesicles. Conidiophores were dull brownish in color, sinuous, and smooth-walled. Conidia were globose, slightly roughened, and pale yellow-green in color **(Figure 1 c,d)**. In addition, **Figure 1 (e-h)** shows globose to sub-globose ascomata, which appear pale brown due to surrounding hulle cells. Hulle cells were globose. Asci were 8-spored and globose to ellipsoidal. Ascospores were red, lenticular, with smooth wall, and four equatorial crests (two quite indistinct). All these morphotypes of the isolate are identical to those of *Aspergillus quadrilineatus* **[24,25]**.

Molecular techniques confirmed the identification. The basic local alignment search tool (BLAST) revealed that the sequence was 100% identical to recorded sequences of *Aspergillus quadrilineatus*. Accordingly, the fungal isolate was named *Aspergillus quadrilineatus* (*A. quadrilineatus*), and the assigned accession number is MT742630.1. In addition, the phylogenetic tree has shown that the strains are clustered into one species as shown in **Figure 1 (i)**.

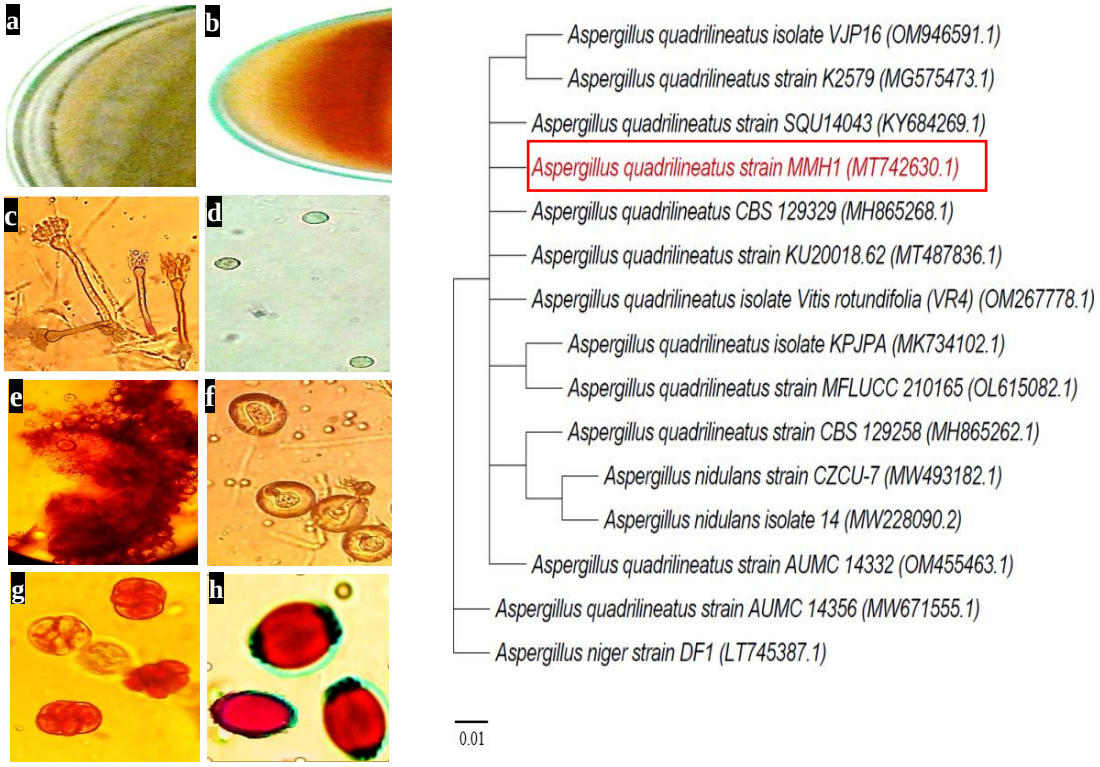

Figure 1: The morphotypes and phylogenetic tree of *Aspergillus quadrilineatus*. **a:** Olive-green conidial area; **b:** Brownish orange reverse; **c:** Conidiophores; **d:** Conidia; **e:** Ascomata surrounded by hulle cells; **f:** Hulle cells; **g:** Asci; **h:** Ascospores; (**c-g**: 40x; **h:** 100x); **i:** Phylogenetic tree constructed based on the neighbor-joining method.

Aspergillus quadrilineatus (MT742630.1) showed the highest exopolysaccharide production among 60 tested fungal isolates **(Supplementary file 1)**. So, *Aspergillus quadrilineatus* was selected for further experiments.

3.2. Effects of OVAT method on biomass and EPS production

3.2.1. Effects of carbon sources

Various monosaccharides (glucose, galactose, and fructose) and disaccharides (sucrose and maltose) were screened separately as carbon sources in the medium constituents to optimize the biomass and EPS production by *Aspergillus quadrilineatus* (MT742630.1). Exceptionally, some carbon sources (such as starch) were excluded to avoid the interference with EPS yield. Although the fungal strain had the ability to grow

using these carbon sources, sucrose had the highest stimulating effect on production of EPS and biomass (1.25 ± 0.45 g/L EPS and 7.34 ± 0.50 g/L biomass), while maltose repressed remarkably the EPS production (0.25 ± 0.05 g/L) as shown in **Figure 2A**.

Different concentrations of sucrose were tested to evaluate its effect on biomass and EPS production. It was observed that biomass and EPS production increased, simultaneously, with increasing sucrose concentrations up to 60 g/L, but above which EPS production decreased. **Figure 2B** shows that at 60 g/L of sucrose, the maximum EPS yield was 1.80 ± 0.50 g/L and the biomass was 7.81 ± 0.60 g/L.

These findings were in concordance with *Penicillium commune* **[26]**, *Syncephalastrum* sp. **[27]**, and *Sclerotium rolfsii* **[28]** that preferred sucrose as a carbon source for EPS production. In addition, *Paecilomyces hepiali* revealed the highest concurrent production of EPS and biomass (4.57 g/L EPS and 12.16 g/L biomass) after culturing with 50 g/L sucrose **[10]**, while *Hirsutella* sp. utilized 25 g/L sucrose to produce 2.17 g/L EPS and 10.06 g/L biomass **[15]**.

3.2.2. Effects of nitrogen sources

Nitrogen is a considerable nutrient for both biomass and EPS production. Among the tested inorganic and organic nitrogen sources, the maximum EPS production (3.50 ± 0.10 g/L) was estimated when using $(NH_4)_2SO_4$ and secondly (2.50 ± 0.90 g/L) when using NH_4NO_3, while the maximum biomass production (14.04 ± 0.17 g/L) was found after NH_4NO_3 utilization **(Figure 2C)**.

Different concentrations of $(NH_4)_2SO_4$ were also tested. The maximum EPS production (4.25 ± 0.25 g/L) was observed at 4 g/L $(NH_4)_2SO_4$, while the maximum biomass (9.16 ± 2.25 g/L) was at 2 g/L $(NH_4)_2SO_4$ **(Figure 2D)**.

Previously, the maximum EPS (1.34 g/L) was produced by *Aspergillus terreus* using 9.57 g/L of NH_4NO_3 as a nitrogen source **[29]**. In contrast, organic nitrogen sources (yeast extract and peptone) were preferred, frequently, to produce the maximum biomass and EPS by *Alternaria tenuissima* **[30]**, *Antrodia cinnamomea* **[31]**, *Aspergillus clavatus* **[32]**, *Fusarium solani* **[17]**, *Hirsutella* sp. **[15]**, *Penicillium commune* **[26]**, and *Phellinus nigricans* **[11]**. Probably, that occurred because these compounds caused metabolic shifting.

3.2.3. Effects of culture conditions

Biomass and EPS production by *A. quadrilineatus* were assessed during 14 days **(Figure 2E)**. It was noticed that there was a slight increase in EPS production from 2 to 4 days and a decrease from 4 to 14 days, while the biomass increased till the 14th day. The highest biomass (10.96 ± 0.58 g/L) and EPS production (4.40 ± 2.20 g/L) were found after 14 and 4 days of fermentation, respectively. Fermentation for 4 days was recorded as the optimum to reach the highest EPS and biomass yield (2.17 and 10.06 g/L) by *Hirsutella* sp. **[15]**. In addition, EPS produced by *Alternaria tenuissima* increased until the 5th day, then the yields of EPS decreased gradually **[30]**. In contrast, incubation for 9 days was the optimum when using *Penicillium commune* **[26]** or *Aspergillus clavatus* **[32]**.

Aspergillus quadrilineatus was able to grow at incubation temperatures ranged from 25 to 40 ℃, whereas the maximum production of EPS occurred at 25 ℃ (3.65 ± 1.15 g/L) and the maximum biomass was at 30 ℃ (12.66 ± 0.57 g/L) as shown in **Figure 2F**. It was comparable to many other fungi, such as *Alternaria tenuissima* **[30]**, *Aspergillus clavatus* **[32]**, *Aspergillus terreus* **[29]**, *Fusarium solani* **[17]**, *Ganoderma lucidum* **[12]**, and *Penicillium commune* **[26]** that had similar optimum temperatures between 25 ℃ and 30 ℃ for biomass and EPS production in submerged culture. It might be due to a main role of the temperature in enzymatic induction or inhibition of fungal growth and EPS production.

Initial pH of the fermentation medium usually affects the production of microbial metabolites; at definite pH ranges, some metabolic channels become available based on the influence of pH on enzymes activity. Initial medium pH, ranging from 2.5 to 11.5, was taken into consideration to investigate its effect on biomass and EPS production by *A. quadrilineatus*. The results indicated that initial medium pH 6 was the preferred value to produce the maximum EPS (4.70 ± 1.80 g/L) and biomass (12.31 ± 0.81 g/L), simultaneously **(Figure 2G)**. The slightly acidic pH values, ranging from 5 to 6.5, were indicated as the optimum for biomass and EPS production by *Aspergillus clavatus* **[32]**, *Fusarium solani* **[17]**, *Ganoderma lucidum* **[12]**, *Hirsutella* sp. **[15]**, and *Penicillium commune* **[26]**.

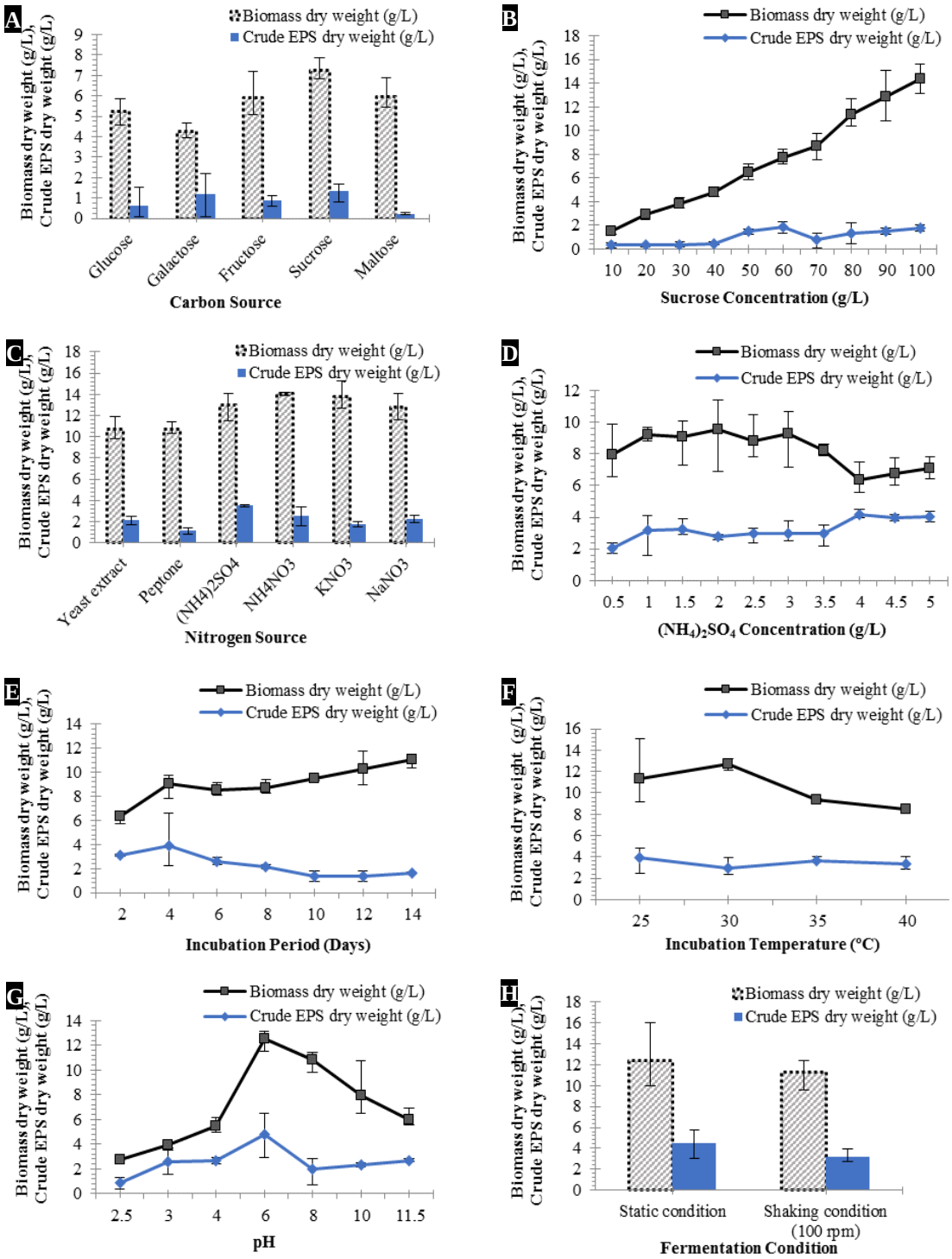

Figure 2: OVAT optimization for biomass and EPS production by *Aspergillus quadrilineatus*. **A:** The effects of different carbon sources; **B:** The effects of sucrose concentrations; **C:** The effects of different nitrogen sources; **D:** The effects of $(NH_4)_2SO_4$ concentrations; **E:** The effects of incubation interval; **F:** The effects of incubation temperature; **G:** The effects of medium pH; **H:** The effects of static and shaking conditions.

Also, **Figure 2H** shows effects of the static and shaking culture conditions on biomass and EPS production by *A. quadrilineatus*. The maximal production of both EPS (4.40 ±1.40 g/L) and biomass (12.97 ± 2.99 g/L) were found under the static culture condition. Previously, the static culture condition was preferred by *Penicillium commune* to produce EPS and biomass, simultaneously **[26]**. In contrast, *Aspergillus clavatus* produced the maximum EPS and biomass using shaking culture condition at 150 rpm **[32]**.

OVAT optimization indicated that the static culture condition and medium pH 6 were the optimum to produce simultaneously EPS and biomass by *Aspergillus quadrilineatus*, while the other remaining variables need further optimization to achieve the synchronous production.

3.3. Significant independent variables affecting biomass and EPS production

Eleven independent variables were screened at two levels: low level (−1) and high level (+1) through 12 experimental runs designed by Plackett – Burman method, and the dependent responses (yield of EPS and biomass) are shown in **Table 3**.

Table 3: Plackett – Burman experimental design and the corresponding responses of EPS and biomass.

Std. order	Variables											Responses	
	A (g/L)	B (g/L)	C (g/L)	D (g/L)	E (g/L)	F (g/L)	G (g/L)	H (g/L)	J (g/L)	K (℃)	L (Days)	Crude EPS dry weight (g/L)	Biomass dry weight (g/L)
1	60.0	4.00	0.5	3.0	7.0	0.2	0.5	0.5	0.5	28.0	4	5.9	6.7
2	40.0	4.00	1.0	1.5	7.0	0.2	1.0	0.5	0.5	25.0	7	5.1	6.7
3	60.0	0.85	1.0	3.0	4.0	0.2	1.0	1.0	0.5	25.0	4	2.8	4.9
4	40.0	4.00	0.5	3.0	7.0	0.1	1.0	1.0	1.0	25.0	4	6.5	3.3
5	40.0	0.85	1.0	1.5	7.0	0.2	0.5	1.0	1.0	28.0	4	4.7	6.2
6	40.0	0.85	0.5	3.0	4.0	0.2	1.0	0.5	1.0	28.0	7	4.4	7.7
7	60.0	0.85	0.5	1.5	7.0	0.1	1.0	1.0	0.5	28.0	7	3.6	8.9
8	60.0	4.00	0.5	1.5	4.0	0.2	0.5	1.0	1.0	25.0	7	4.6	8.4
9	60.0	4.00	1.0	1.5	4.0	0.1	1.0	0.5	1.0	28.0	4	5.8	8.0
10	40.0	4.00	1.0	3.0	4.0	0.1	0.5	1.0	0.5	28.0	7	6.1	6.0
11	60.0	0.85	1.0	3.0	7.0	0.1	0.5	0.5	1.0	25.0	7	5.4	11.4
12	40.0	0.85	0.5	1.5	4.0	0.1	0.5	0.5	0.5	25.0	4	2.7	3.8

A: Sucrose; **B:** $(NH_4)_2SO_4$; **C:** $MgSO_4.7H_2O$; **D:** K_2HPO_4; **E:** Tween 80; **F:** $FeSO_4$; **G:** NaCl; **H:** KCl; **J:** $CaCl_2.2H_2O$; **K:** Temperature; **L:** Incubation interval. **Space type:** Factorial.

Pareto chart was used to show the effect of each independent variable on the production of EPS and biomass by *A. quadrilineatus*, as shown in **Figure 3**. $(NH_4)_2SO_4$, $CaCl_2.2H_2O$, tween 80, and K_2HPO_4 had the significant positive effect on EPS production **(Figure 3A)**, while the incubation interval and sucrose had significant positive effects on the biomass **(Figure 3B)**.

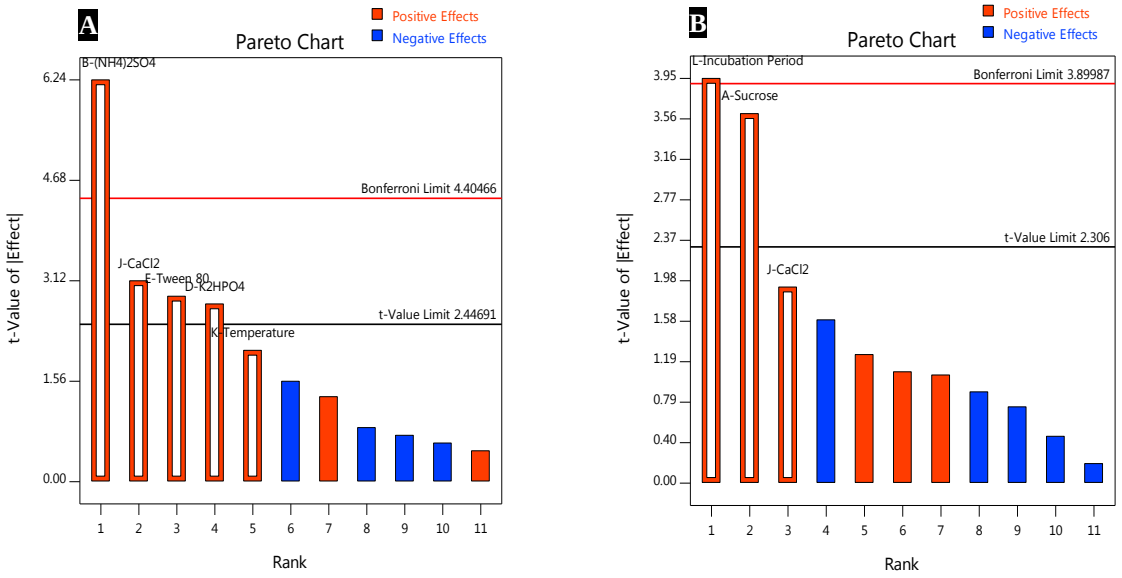

Figure 3: Pareto chart showing the rank of significant variables affecting production of EPS **(A)** and biomass **(B)** by *Aspergillus quadrilineatus*.

In addition, two separate first-order polynomial equations elucidated the models of EPS and biomass as follows

$$\text{Crude EPS dry weight} = 4.80 + 0.8667B + 0.3833D + 0.4000E + 0.4333J + 0.2833K \quad \textbf{(4)}$$

$$\text{Biomass dry weight} = 6.82 + 1.23A + 0.6523J + 1.35L \quad \textbf{(5)}$$

where **B:**$(NH_4)_2SO_4$, **D:**K_2HPO_4, **E:**Tween 80, **J:**$CaCl_2.2H_2O$, **K:**Temperature, **A:**Sucrose, and **L:**Incubation interval. The coefficient of each variable in the equations represents the extent of effect of this variable on the response.

The results of statistical analyses of variance (ANOVA) for both models are shown in **Table 4**. The models F-values (13.77 and 10.76) indicated that the models were significant and there were only 0.31% and 0.35% chance that an F-value could occur due to noise, respectively. The coefficient of determination (R^2) was close to 1, and the

Table 4: ANOVA for EPS and biomass models elucidated by first-order polynomial equations.

Source	SS	DF	MS	F-value	p-value
Response 1: Crude EPS dry weight					
Model 1	15.91	5	3.18	13.77	0.0031
B: $(NH_4)_2SO_4$	9.01	1	9.01	39.00	0.0008
D: K_2HPO_4	1.76	1	1.76	7.63	0.0328
E: Tween 80	1.92	1	1.92	8.31	0.0280
J: $CaCl_2.2H_2O$	2.25	1	2.25	9.75	0.0205
K: Temperature	0.96	1	0.96	4.17	0.0872
Residual	1.39	6	0.23		
Corrected total	17.30	11			
R^2: 0.9198; Adj. R^2: 0.8531; Pred. R^2: 0.6794					
Response 2: Biomass dry weight					
Model 2	44.98	3	14.99	10.76	0.0035
A: Sucrose	18.13	1	18.13	13.00	0.0069
J: $CaCl_2.2H_2O$	5.11	1	5.11	3.66	0.0920
L: Incubation interval	21.75	1	21.75	15.60	0.0042
Residual	11.15	8	1.39		
Corrected total	56.13	11			
R^2: 0.8013; Adj.R^2: 0.7269; Pred. R^2: 0.553					

SS: sum of squares; **DF:** degree of freedom; **MS:** mean square.

predicted R^2 was in reasonable agreement with the adjusted R^2 (the difference is less than 0.2). These results indicated that the models were fit to the data.

P-values less than 0.05 indicate that model terms are significant. Accordingly, $(NH_4)_2SO_4$, K_2HPO_4, tween 80, $CaCl_2.2H_2O$, sucrose, and the incubation interval were the most significant for biomass and EPS production by *A. quadrilineatus*.

Previously, mineral elements and surfactants were effective supplements to stimulate microbial growth and EPS production. It has been demonstrated that metal ions (Ca^{2+}) could act as cofactors of key enzymes in EPS biosynthesis and increase cell membrane permeability to accelerate EPS excretion which also achieved by tween 80. In addition, phosphate might perform an important role in phosphorylation of important enzymes required for EPS biosynthesis **[13,33,34]**.

3.4. Central composite design (CCD) using the significant medium components

According to the PBD screening results, medium components showing the significant effect on biomass and EPS production (sucrose, $(NH_4)_2SO_4$, K_2HPO_4, $CaCl_2.2H_2O$, and tween 80) were selected for further optimization to investigate their individual and interactive effects on the simultaneous production of EPS and biomass by *A. quadrilineatus* (MT742630.1) using CCD in the response surface methodology. The central composite design and the corresponding responses are shown in **Table 5**.

After multiple regression analysis, the following two polynomial equations were obtained to elucidate EPS and biomass dry weights as a function of sucrose **(A)**, $(NH_4)_2SO_4$ **(B)**, K_2HPO_4 **(C)**, $CaCl_2.2H_2O$ **(D)**, and tween 80 **(E)**

$$\text{Crude EPS dry weight} = 7.61 - 0.1917A - 0.3500B + 0.0000C + 0.5750D + 0.1667E - 0.6375AE - 0.2000BC - 0.1500CD - 0.1750CE - 0.3134D^2 - 0.3009E^2 \quad (6)$$

$$\text{Biomass dry weight} = 6.37 - 0.0297A - 0.5680B - 0.1260C - 0.6497D + 0.0680E - 0.2970AC + 0.2600BC + 0.4335BD - 0.3720CE \quad (7)$$

The models F-values were 25.41 and 14.35, thus the models were significant and there was only 0.01% chance (p-value < 0.0001) that an F-value could occur due to noise. For each model, the lack of fit was non-significant (p-value > 0.05), the coefficient of determination (R^2) was close to 1, and the predicted R^2 was in reasonable agreement with the adjusted R^2 (the difference was less than 0.2). Therefore, the models were fit to the data.

The adequate precision measures the signal to noise ratio, and ratio greater than 4 is desirable. Accordingly, ratio of 19.31 and 13.97 indicated to adequate signals and these models could be used to navigate the design space. In addition, the coefficient of variation (C.V.%) was 4.24 and 7.06 that revealed satisfactory adjustment of each model to the predicted data.

Table 5: The central composite design and the corresponding responses of EPS and biomass.

Std. order	Space Type	Variables					Responses	
		A (g/L)	B (g/L)	C (g/L)	D (g/L)	E (g/L)	Crude EPS dry weight (g/L)	Biomass dry weight (g/L)
1	Factorial	55.0	3.0	2.5	0.5	10.5	7.8	8.5
2	Factorial	65.0	3.0	2.5	0.5	3.5	6.5	8.1
3	Factorial	55.0	5.0	2.5	0.5	3.5	5.1	4.6
4	Factorial	65.0	5.0	2.5	0.5	10.5	5.5	6.6
5	Factorial	55.0	3.0	3.5	0.5	3.5	6.5	8.0
6	Factorial	65.0	3.0	3.5	0.5	10.5	6.6	6.9
7	Factorial	55.0	5.0	3.5	0.5	10.5	6.5	6.1
8	Factorial	65.0	5.0	3.5	0.5	3.5	6.4	6.4
9	Factorial	55.0	3.0	2.5	1.5	3.5	7.1	5.7
10	Factorial	65.0	3.0	2.5	1.5	10.5	7.0	6.8
11	Factorial	55.0	5.0	2.5	1.5	10.5	8.7	5.9
12	Factorial	65.0	5.0	2.5	1.5	3.5	7.4	5.6
13	Factorial	55.0	3.0	3.5	1.5	10.5	8.6	5.5
14	Factorial	65.0	3.0	3.5	1.5	3.5	8.2	5.1
15	Factorial	55.0	5.0	3.5	1.5	3.5	6.3	5.9
16	Factorial	65.0	5.0	3.5	1.5	10.5	5.8	4.8
17	Axial	50.0	4.0	3.0	1.0	7.0	8.0	6.2
18	Axial	70.0	4.0	3.0	1.0	7.0	7.3	5.9
19	Axial	60.0	2.0	3.0	1.0	7.0	8.2	8.2
20	Axial	60.0	6.0	3.0	1.0	7.0	7.3	5.7
21	Axial	60.0	4.0	2.0	1.0	7.0	7.7	6.6
22	Axial	60.0	4.0	4.0	1.0	7.0	7.8	6.7
23	Axial	60.0	4.0	3.0	0.0	7.0	5.2	8.0
24	Axial	60.0	4.0	3.0	2.0	7.0	8.0	5.2
25	Axial	60.0	4.0	3.0	1.0	0.0	6.4	6.5
26	Axial	60.0	4.0	3.0	1.0	14.0	6.9	6.4
27-32*	Center	60.0	4.0	3.0	1.0	7.0	7.7	6.3

*Average of 6 center point values.

A: Sucrose; **B:** $(NH_4)_2SO_4$; **C:** K_2HPO_4; **D:** $CaCl_2.2H_2O$; **E:** Tween80.

The relationship between the actual and predicted values of EPS and biomass dry weights are shown in **Figure 4-A**. Most of the data points are nearby the straight line and that reveals the strong similarity between the experimental and predicted values.

Box-Cox plot (**Figure 4-B**) determines the most appropriate power transformation. For each model, the ratio of maximum to minimum values was less than 3 and Lambda (λ) = 1. Consequently, the transformation had a little effect and no transformation was recommended.

These results showed that the two proposed models were acceptable to estimate the responses of EPS and biomass yield within the range of the employed variables.

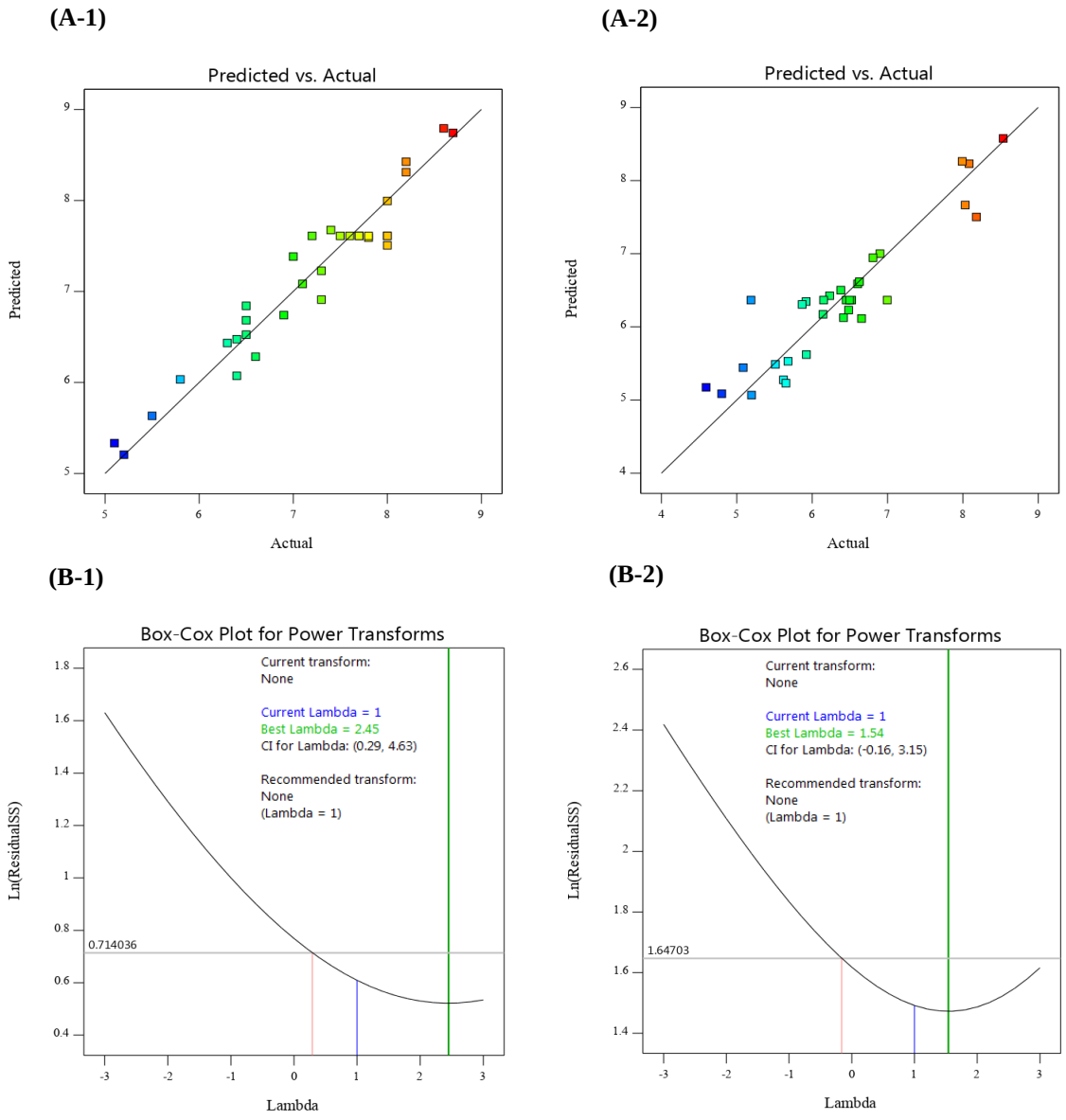

Figure 4: Diagnostic plots for the central composite model adequacy of EPS yield **(1)** and biomass yield **(2)**.

3.5. The synchronous effect of independent variables on the synchronous production of exopolysaccharides and biomass by *Aspergillus quadrilineatus*

Based on the configured models, **Figure 5** shows the three-dimensional (3D) response surface curves and the contour plots that were used to elucidate the dependency of EPS production **(Figure 5-a)** and biomass **(Figure 5-b)** on the independent variables.

EPS production was approaching to the maximum whenever sucrose concentration was lower than 60 g/L, on condition that the concentration of tween 80 was higher than 7 g/L **(Figure 5 a-1)**.

The same interpretation was suitable for the interaction between $(NH_4)_2SO_4$ and K_2HPO_4; EPS production was increasing whenever $(NH_4)_2SO_4$ concentration was lower than 4 g/L, on condition that the concentration of K_2HPO_4 was higher than 3 g/L **(Figure 5 a-2)**.

In contrast, EPS production was increasing whenever K_2HPO_4 concentration was lower than 3 g/L, on condition that the concentration of $CaCl_2.2H_2O$ was higher than 1 g/L **(Figure 5 a-3)** or the concentration of tween 80 ranged between 7 g/L and 12 g/L **(Figure 5 a-4)**.

Biomass yield was increasing whenever sucrose concentration was higher than 60 g/L, on condition that the concentration of K_2HPO_4 was lower than 3 g/L **(Figure 5 b-1)**.

In addition, using the minimal level of concentrations, $(NH_4)_2SO_4$ maximized the biomass yield when combining either with K_2HPO_4 **(Figure 5 b-2)** or $CaCl_2.2H_2O$ **(Figure 5 b-3)**.

The interaction between K_2HPO_4 and tween 80 boosted the biomass yield when tween 80 concentration was close to the maximum, while K_2HPO_4 concentration was at the minimal level **(Figure 5 b-4)**.

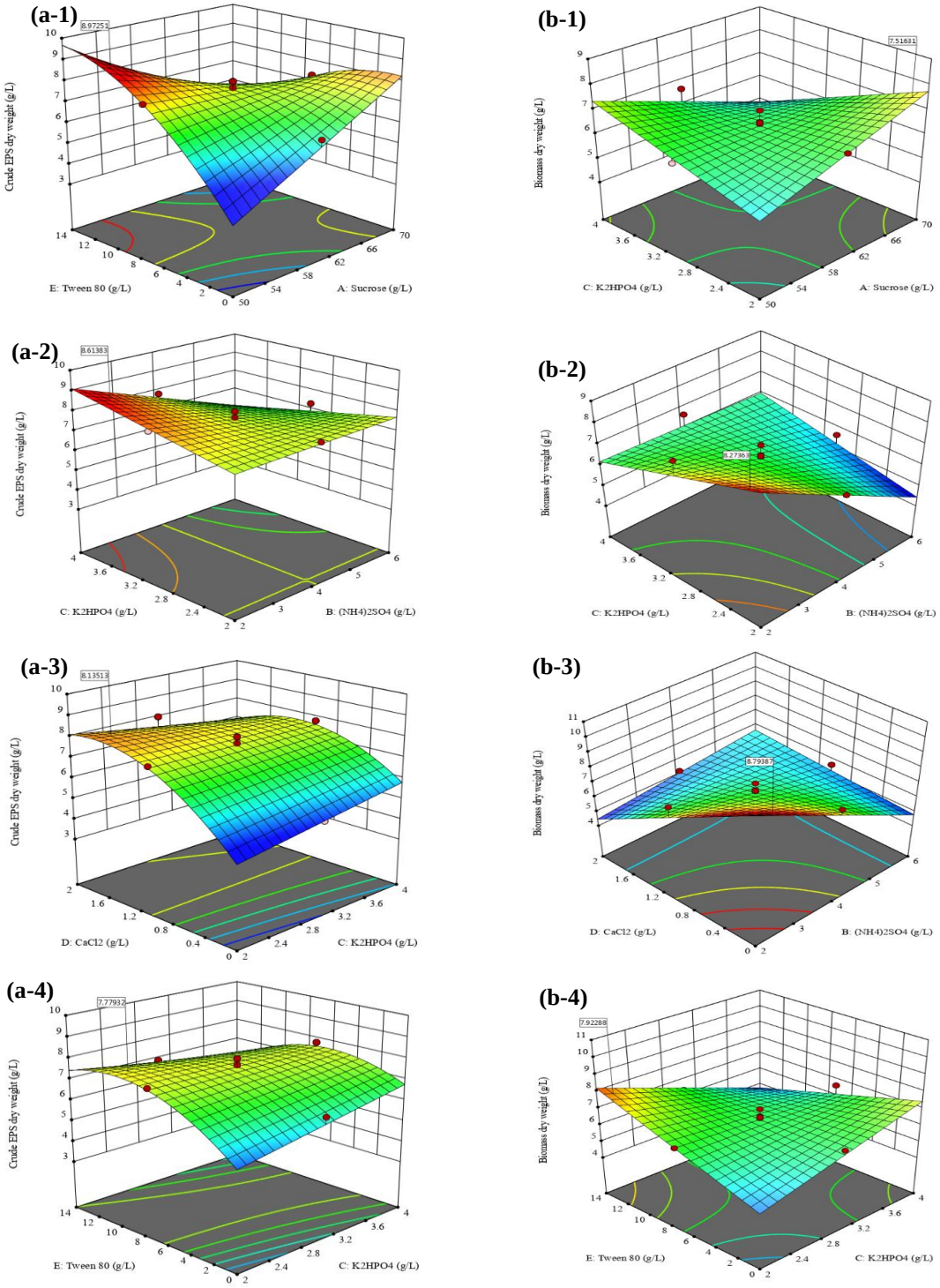

Figure 5: Three-dimensional (3D) response surface plots showing the effect of interactions between medium components on exopolysaccharide yield **(a)** and biomass yield **(b)**.

The desirability function approach searches to satisfy the requirements for each response in the design and to find a set of the optimal conditions for all responses together, by specifying the goals and boundaries required for each response **[19]**. In this study, the goal for EPS and biomass yield was assigned as "maximize" and the obtained results are shown in **Figure 6**. The maximum yield of EPS (8.24 g/L) and biomass (7.98 g/L) was predicted when using 55.02 g/L sucrose, 3 g/L $(NH_4)_2SO_4$, 2.5 g/L K_2HPO_4, 0.78 g/L $CaCl_2.2H_2O$, and 10.5 g/L tween 80.

The confirmatory experiment was performed in triplicate under the optimized concentrations of these significant variables at 28 ℃ and initial pH 6 for 7 days under static culture conditions. The obtained EPS and biomass yields were 7.95 ± 0.05 g/L and 8.88 ± 0.01 g/L, respectively. Accordingly, the results revealed good agreement with the predicted values, which indicated to the adequacy of the models and the configured optimum conditions to enhance EPS production by *Aspergillus quadrilineatus* with high biomass yield.

In comparison with the yield that was obtained using the basal medium (1.85 ± 0.15 g/L EPS and 6.76 ± 0.44 g/L biomass), the optimization process enhanced the EPS production by 329.73%, while biomass increased by only 31.44%.

In addition, the phenol-sulfuric acid method revealed that the total sugars content of the enhanced EPS was 94.8% (standard equation: $y = 778.94x - 0.0123$, $R^2 = 0.9842$).

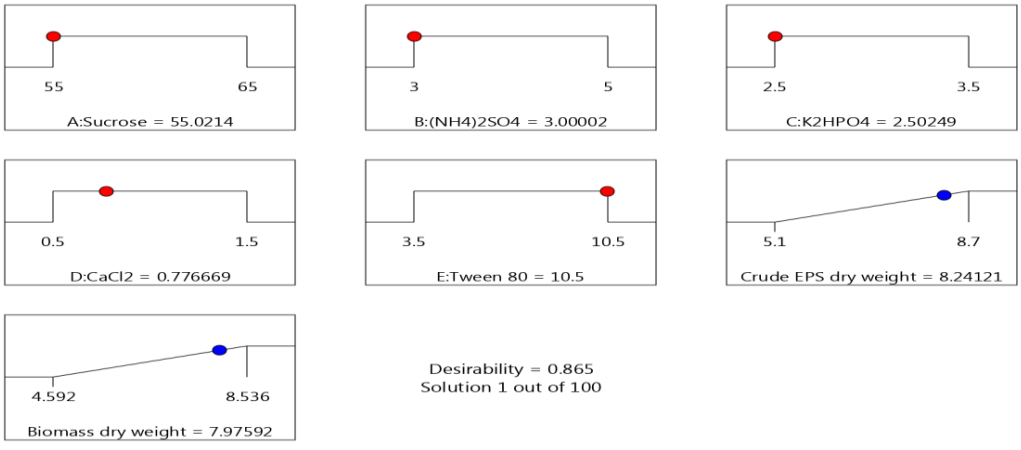

Figure 6: Desirability ramps plot for EPS and biomass enhancement.

Although the optimization of biomass and EPS production by *Aspergillus quadrilineatus* has been rarely investigated, response surface methodology (RSM) and desirability function approach were used to reach the maximum EPS and biomass yield by several EPS-producing fungi. *Neopestalotiopsis* sp. production of EPS was enhanced to 2.02 g/L after selection of the most influencing variables using PBD then applying RSM using CCD **[14]**. In the same way, *Ganoderma lucidum* produced its highest biomass (3.12 g/L) and EPS (1.96 g/L) **[12]** and *Aspergillus terreus* (1.34 g/L EPS), as well **[29]**. In another study, orthogonal matrix method and Box-Behnken experimental design were used to optimize the medium components and culture conditions for EPS production by *Alternaria tenuissima*, and the maximum yield was 3.42 g/L **[30]**.

3.6. Chitosan extraction

The fungal biomass, obtained as wastes after production of the maximum EPS, was used to extract chitosan. The FTIR spectrum of the extracted chitosan demonstrated the characteristic bands **(Figure 7A)**. The broad band at 3400 cm^{-1} indicates to O-H overlapping N-H vibrations, while the C-H bond is at around 2920 cm^{-1}. The band at 1650 cm^{-1} is the characteristic of amide group (amide I band) from N-H vibration and corresponding to C=O stretching. The band at 1430 cm^{-1} attributes to CH_2 bending vibration, while band at 1040 cm^{-1} attributes to C-O-C stretching vibration **[35–37]**.

In addition, the yield of the extracted chitosan was 20 mg/g AIMs (approximately 3.52 mg chitosan /g biomass) with about 88.84% DD (according to absorbance values shown in **Figure 7B**).

Cell walls of fungi are composed of various polysaccharides (including chitin and chitosan), glycoproteins, as well as minor amounts of polyuronides, lipids, and melanin **[38]**. Fungal chitosan had deacetylation degree between 70 – 90% depending on fungal species and extraction conditions, of which chitosan extracted from *Rhizopus oryzae* biomass that had 86 – 90% DD **[39]**.

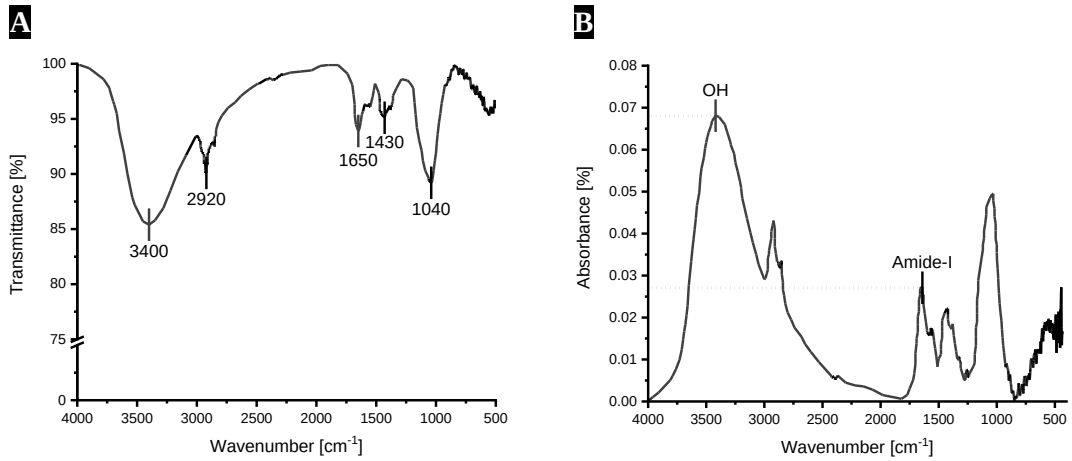

Figure 7: FTIR spectra of the extracted chitosan; **A:** Transmittance and **B:** Absorbance.

4. CONCLUSION

In this study, the strain *Aspergillus quadrilineatus* (GenBank Accession No. MT742630.1) was used for the simultaneous production of an exopolysaccharide and chitosan. One-variable-at-a-time method was combined with response surface methodology to enhance EPS and biomass yield, in submerged fermentation, by applying the central composite design. The optimum concentrations, obtained by the desirability function approach, were 55.02 g/L sucrose, 3 g/L $(NH_4)_2SO_4$, 2.5 g/L K_2HPO_4, 0.78 g/L $CaCl_2.2H_2O$, and 10.5 g/L tween 80. The use of this medium enhanced the EPS and biomass yield by 329.73% and 31.44% of the primary yield, respectively. The total sugars content of the enhanced EPS was 94.8%, and the simultaneously enhanced biomass was proven to contain 20 mg chitosan /g AIMs (approximately 3.52 mg chitosan /g biomass) with about 88.84% DD. This work may be helpful to decrease the production cost of several polysaccharides by producing them simultaneously.

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