Egyptian Journal of Aquatic Biology & Fisheries Zoology Department, Faculty of Science, Ain Shams University, Cairo, Egypt. ISSN 1110 – 6131 Vol. 25(5): 799 – 819 (2021) www.ejabf.journals.ekb.eg



Statistical optimization of chitosan production using marine-derived *Penicillium* chrysogenum MZ723110 in Egypt

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ARTICLE INFO

Keywords:

Penicillium

chrysogenum,

characterization.

Chitosan,

Chitosan

Article History: Received: Aug. 22, 2021 Accepted: Oct. 19, 2021 Online: Nov. 11, 2021

Statistical optimization,

ABSTRACT

Fungal chitosan is highly demanded because of its better physico-chemical characteristics compared to crustaceans' chitosan. In the current study, a marine-derived fungus was isolated from the Mediterranean Sea in Egypt and was identified according to microscopic and molecular characteristics of Penicillium chrysogenum MZ723110. The fungal strain was used for chitosan extraction. The resultant chitosan was characterized by Fourier transform infrared spectroscopy (FT-IR) analysis and the degree of deacetylation (DD%). Statistical optimization of fungal growth conditions for maximum chitosan yield was done using Plackett Burman design (PBD) and central composite design (CCD). The statistical optimization revealed that the type of fungal culture, peptone concentration, and incubation period were the most significant factors. Upon their optimization, there was a 3.1-fold increase in chitosan yield by P. chrysogenum MZ723110. The yield of chitosan was increased from 4.4% to 7.5 % with DD% equal to 94%. The results of this study proved the importance of optimizing fungal growth conditions for the maximum yield of chitosan.

INTRODUCTION

Chitin is the second highly abundant polymer present naturally on earth after cellulose, and it is present in nature as the main constituent of crustacean's skeleton and in the cuticles of insects (**Kaur & Dhillon, 2014**). Chitin is also found as a major component of the cell walls of microorganisms such as yeast, mushrooms, and some other fungi for maintaining the integrity, strength, and shape of cell structure (**Hon, 1996**; **Sitanggang** *et al.*, **2012**). Chitosan is derived from chitin by the deacetylation process (**Cai** *et al.*, **2006**). It is composed of two monosaccharides; d-glucosamine and *N*-acetyl-d-glucosamine, linked together by b- $(1\rightarrow 4)$ glycosidic linkages (**Raafat & Sahl, 2009**). The distinct difference between chitin and chitosan is the acetyl content of the polymer so chitosan is considered very reactive for its free amino group (**Rinaudo, 2006**). The

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physical and chemical characteristics of chitosan rely mainly on its deacetylation degree (DDA) and molecular weight (**Paul, 2000**). The great properties of chitosan, including its biodegradability, biocompatibility, and non-toxicity have offered a vast array of prospects in the pharmaceutical and biological industries (**Puvvada** *et al.*, **2012**).

Industrially, chitosan was mainly extracted from the crustaceans' exoskeleton via strong alkali for a long time at high temperature. Despite of the high yield produced, there have been some restrictions encountered by using crustacean waste as a source of chitosan as the limited and seasonal crustaceans waste supply, high production cost and the variable physicochemical properties of the produced chitosan (Dhillon et al., 2013; **Kumari & Rath, 2014**). For continuous industrial chitosan production, it is essential to search for an alternative source for chitosan. Chitin and chitosan production from fungal mycelium has received increased attention due to several advantages, such as the availability of fungal mycelium all over the year avoiding seasonal fluctuations adding to the fact that it can be obtained by simple fermentation process (Santos, 2013). The extraction of chitosan from fungal mycelia does not require demineralization treatment due to the lower level of inorganic materials present in fungal mycelia compared to crustacean wastes and the consistency of physico-chemical properties of produced chitosan (Khor, 2001; Nwe & Stevens, 2002a). The presence of chitin deacetylase genes in nearly all fungi shows that chitosan is a ubiquitous component of the fungal cell wall (Ruiz-Herrera, 2012). Many fungi have been reported to contain chitin and chitosan in their cell walls, including Rhizopus oryzae, Mucor rouxii, Gongronella butleri, Aspergillus niger, Trichoderma reesei, Penicillum verruculosum, and Penicillium chrysogenum (Mario et al., 2008; Wu et al., 2009; Kaur & Dhillon, 2014; Osarumwense et al., 2017).

The quantity of chitosan in the cell wall relies on many factors, such as the fungal grouping, strain, fermentation techniques and growth conditions (**Akila, 2014**). Bioprocess parameter optimization is critical in the development of any fermentation process, since it has a substantial impact on the process's economy and efficiency. Statistical experimental designs provide a set plan for optimizing variables through experimentation and can overcome the constraints of the one-factor-at-a-time approach. Statistical data analysis entails a small number of experimental trials in which the interactions between multiple experimental variables can be seen at the same time (**Bas & Boyaci, 2007**).

The objective of this study was focused on the production of chitosan from the mycelium of a marine-derived *Penicillium chrysogenum* MZ723110 in Egypt. The fungal growth conditions were statistically optimized using Placket Burman design (PBD) followed by response surface methodology (RSM) based on the central composite design (CCD) for the highest yield of chitosan in submerged culture. Furthermore, the physical properties of the generated fungal chitosan were characterized.

MATERIALS AND METHODS

Fungus isolation

From the Mediterranean Sea (Alexandria governorate) in Egypt, 0.2 ml of marine water samples were spread without dilution into agar plates using three different isolation media; namely, Czapek- Dox agar medium (Thom and Church, 1926), Sabouraud dextrose agar medium (Feng *et al.*, 1990), and yeast extract medium (Wickerham, 1951). The plates were incubated at 28°C for three weeks. The inoculated agar plates were examined periodically for the growing colonies. The different colonies were selected, purified, and maintained in fresh slants for further study (Amer & Ibrahim, 2019).

Identification of marine-derived fungal isolate Morphological identification

Macromorphological and micromorphological characteristics of the isolated marinederived fungus were determined using the universal manual (**De Hoog** *et al.*, 2000). The macroscopic characteristics were based on the texture and color of the colony (obverse and reverse). Microscopic characteristics were attained by applying the slide culture technique (**Riddell**, 1950).

Molecular identification

The ITS1–5.8S–ITS2 genomic region was amplified from genomic DNA by polymerase chain reaction (PCR) carried out in Sigma company using forward primer ITS1: (5' -TCC GTA GGT GAA CCT GCG G- 3') and reverse primer ITS 4: (5' - TCC TCC GCT TAT TGA TAT GC- 3'). The PCR reaction was performed using 25μ L of MyTaq Red Mix, 8μ L DNA Template, 1μ l forward primers (ITS1), 1μ l reverse primers (ITS4) and 15 μ L nuclease free water as follows: initial denaturation was done at 94°C for 6 minutes, denaturation at 95°C for 45 seconds, annealing at 56°C for 45 seconds, extension at 72°C for 1 minute and final extension at 72°C for 5 minutes. The number of cycles was 35. Finally, the PCR product was ready for sequencing in ABI Prism 3730XL DNA sequencer and analyzed on GATC Company (Elshahawy *et al.*, 2018).

The evolutionary history was inferred by using the maximum likelihood method and Kimura 2-parameter model (**Kimura, 1980; Nasser** *et al.*, **2021**). Phylogenetic and molecular evolutionary analyses were conducted in Molecular Evolutionary Genetics Analysis (MEGA) X (**Kumar** *et al.*, **2018**).

Total chitosan extraction

Submerged fermentation of fungal isolate

Erlenmeyer flasks (250 mL), containing 100 mL of yeast extract peptone glucose (YPG) media (1% yeast extract; 2% peptone; 2% glucose), were inoculated with one milliliter of spore suspension. It was prepared by washing the surface of a fresh mature

culture of fungal isolate with 10 mL distilled water amended with Tween 20 (0.1%, v: v). It was adjusted to 10^6 spores mL⁻¹ using a Neubauer counting chamber. The flasks were incubated for 10 days at $28\pm2^{\circ}$ C without shaking (**Osarumwense** *et al.*, **2017; Darwesh** *et al.*, **2018**). The fungal mycelia were then harvested by filtration, washed many times with water, and dried in an oven overnight at 70°C. Fungal dry weight was ground and then weighed (**Khayria** *et al.*, **2016**).

Extraction of Chitosan

Extraction of total chitosan was carried out by deproteination of dried fungal biomass using1M sodium hydroxide (NaOH) solution (1:40 w/v) at 121°C for 20 min (ElMekawy *et al.*, 2013). The the alkaline-insoluble material (AIM) was filtered and washed several times with water till a neutral pH was attained, and it was dried in an oven at 40°C. The alkaline insoluble material (AIM) was then deacetylated with 50% NaOH for 2 h at 110°C and washed several times with water till a neutral pH was determined. Finally, AIM was treated with 10% v/v acetic acid (1:40 w/v) at 60°C for 6 h. The acid-insoluble residues were separated by filtration through Whatman No.1 filter paper. The fungal chitosan was further obtained by adjusting the pH of the supernatant to pH 9-11 with 4 M NaOH, then centrifugation at 7000 rpm for 15 minutes. The precipitated chitosan was washed with distilled water, 95% ethanol, and acetone, respectively, and then it was dried at 60°C to a constant weight (Tayel *et al.*, 2015; Khayria *et al.*, 2016).

The total chitosan yield was calculated according to the following equation:

Chitosan yield (%) = [dry weight of the obtained chitosan / dry weight of sample] \times 100 (**Khayria** *et al.*, **2016**).

Statistical optimization of chitosan production Plackett–Burman design

In a total of 24 experimental runs, a nine-variable design was used to screen the important nutritional and physical parameters affecting fungal growth for the highest yield of chitosan. The parameters tested and their effects were evaluated at two levels (low and high level) and are represented in Table (1). All experiments were conducted in duplicates; the chitosan extraction was performed as the previously given protocol. The statistical software package "Minitab software Version 18" was used for analyzing the experimental data. The data were subjected to analysis of variance (ANOVA) to determine the significance of the fitted model and to test the importance of each parameter's effect on chitosan yield (%).

Response surface methodology using central composite design (CCD)

Response surface methodology using central composite design was used to optimize the significant parameters identified by the Plackett-Burman design. The significant variables were investigated at five different levels $(-\alpha, -1, 0, +1, +\alpha)$ with $\alpha = 1.41421$ in a set of eleven experiments, coded values of variables, and matrix of CCD along with chitosan yield of each run are presented in Table (4). The goodness of fit of the model was investigated by the coefficient of determination (R^2) and the analysis of variance (ANOVA) using the same software package previously mentioned.

Table 1. Experimental range and levels of independent nutritional and physical variablesstudied for maximum chitosan yield (%) from *Penicillum chrysogenum* MZ723110 usingPlackett–Burman design

Code of	Factor (variable)	Levels	Unit	
factor		Low level (-1)	High level (+1)	
A	Glucose concentration	20	30	g/L
В	Peptone concentration	20	30	g/L
С	Yeast extract concentration	10	20	g/L
D	Temperature	28	32	°C
E	pH	5.5	6.5	-
F	Incubation period	4	7	days
G	Volume of medium in 250mL flasks	50	100	mL
H	Inoculum size	104	106	mL
J	Type of fungal culture	Static	Shaked (agitated)	-

Validation of optimized conditions and predictive models

An experiment 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.

Chitosan characterization

Fourier-transform infrared (FTIR) spectroscopy

Thermo Electron Nicolet iS10 (Waltham, Massachusetts, USA) infrared spectrometer with OMNIC FTIR software was used to record FTIR spectra for chitosan using KBr disc. FTIR data were recorded in the absorbance mode with a resolution of 4 cm⁻¹ in the wavenumber range of 4000–400 cm⁻¹ (**Kumirska** *et al.*, **2010**).

Degree of deacetylation (DD)

According to the IR spectra obtained on an FTIR spectrometer, the degree of acetylation (DA%) of produced fungal chitosan was examined using the absorbance ratio (A1655/A3450); the following equation was used to determine DA:

Degree of acetylation (DA%) = $(A1655/A3450) \times 100 / 1.33$

The deacetylation was calculated using the following equation:

Deacetylation degree (DD%) =100-% acetylation.

The amide I band at 1655 cm⁻¹ and the hydroxyl group absorption band at 3450 cm⁻¹ were used as an internal reference. The value 1.33 represents the ratio of this absorbance for a fully acetylated compound (**Ebrahimzadeh** *et al.*, **2013**).

RESULTS

1. Identification of marine-derived fungal isolate

From the morphological identification of the selected marine-derived fungal isolate, it was observed that it was related to the *Penicillium* genus. Fig. (1) illustrates the macroscopic characteristics (a) and the microscopic examination (b) under a binocular light microscope. The colonies were fast-growing, velvety in texture, color was initially white and turn bluish green with conidial production, the reverse was mostly pale brown. Conidia were subglobose, smooth and arranged in basipetal chains. Conidiophores were smooth, branched following the terverticillate pattern. Phialides were flask shaped.

The morphological identification was confirmed molecularly using PCR sequencing. Sequenced data were aligned against the 18S rRNA sequences of the National Center for Biotechnology Information (<u>http://blast.ncbi.nlm.nih.gov/Blast.cgi</u>) and displayed that this fungal isolate had a similarity of 100% for its 18S rRNA sequence to *Penicillum chrysogenum* and was deposited in GenBank with accession number MZ723110. Furthermore, the phylogenetic tree was constructed and is presented in Fig. (2).



Fig. 1. (a) macroscopic characteristics on PDA plates, (b) micrograph of microscopic features of the marine-derived fungal isolate *Penicillium chrysogenum* MZ723110





2. Fungal total chitosan extraction

The results revealed that the yield of total chitosan from *Penicillium chrysogenum* MZ723110 upon growing on basal media using 50% NaOH (11M NaOH) at 110°C for two hours was 0.044 g/g of the dry cell weight (4.4%).

3. Statistical optimization of chitosan production

3.1. Placket- Burman Design (PBD)

In this study, the Plackett-Burman design was used to determine the most important growth culture conditions influencing P. chrysogenum MZ723110 chitosan yield. The chitosan yield (response) of the different trials (24 experimental runs) in coded values together with the predicted response is shown in Table (2). Based on Plackett-Burman's design results, the Pareto chart illustrates the order of significance of the variables affecting the chitosan yield (Fig. 3). Among the nine tested parameters, the factors showing a significant positive effect on chitosan yield (P < 0.05) were: peptone concentration, incubation period, and type of fungal culture. The data showed wide variation in the yield of chitosan ranging from 0 to 5.7%. Result presented in Table (3) shows that, the type of fungal culture was the most significant variable followed by peptone concentration and incubation period (P=0.001, 0.012, and 0.020, respectively). Non-significant factors were not investigated in the next optimization experiment and were used at their (-1) and (+1) values. The coefficient of determination (R^2) for chitosan yield was calculated to be 0.7580, which represented 75.80% variability of the response. The obtained results were represented in a polynomial equation which described the correlation between the nine studied parameters and the response as follows:

Chitosan = 21.3 + 0.0425 glucose conc. + 0.1358 peptone conc. + 0.0592 yeast extract conc. yield - 0.144 temperature - 0.325 pH + 0.414 incubation period + 0.01750 rate of aeration

-0.214 inoculum size +1.029 type of fungal culture



Fig. 3. Paretograph of chitosan yield identifying type of fungal culture (J), peptone concentration (B), and incubation period (F) as the major significant factors.

3.2. Response surface methodology using central composite design (CCD)

The central composite design matrix for the studied factors given in coded values together with the predicted and experimental chitosan yield (response) of the different trials (experimental runs) are presented in Table (4). Model terms with P values less than 0.05 were considered significant, whereas those with P values more than 0.05 were regarded as insignificant. ANOVA analysis showed that the incubation period was highly statistically significant influencing chitosan yield (p = 0.000) (Table 5).

The model fitting's validity was examined by the coefficient of determination (R^2) which was calculated to be 0.9790, and the model adjusted R^2 (R^2 adj) was 0.9580. The second-order polynomial equation in coded values by the model regression analysis emerged as follows:

chitosan yield = $-23.23 + 1.075 \text{ A} + 3.557 \text{ B} - 0.00460 \text{ A}^{*}\text{A} + 0.1267 \text{ B}^{*}\text{B} - 0.1547 \text{ A}^{*}\text{B}$

The three-dimensional response surface plot and the corresponding 2 D contour plot were plotted to determine the optimum level of each variable and the effect of their interactions on chitosan yield (Fig. 4). The data obtained from the response optimizer indicated that maximum chitosan yield was 7.6 % at peptone concentration equal to 17.9 g/L and incubation period 8 days with the desirability of 100%. Chitosan yield by *P*. *chrysogenum* MZ723110 using optimized conditions was found to be 7.5%.

(%)



Fig. 4. Three-dimensional response surface plot (a) and 2D contour plot of the interacted effect between incubation period and peptone concentration and their optimum levels (b).

4. Chitosan characterization

In this study, the results presented in Fig. (5) indicated the presence of different absorption bands within a range from 4000-400 cm⁻¹. The bands in the FTIR spectra represent the obtained chitosan. Broadband at 3442.99 cm⁻¹ was assigned to hydrogenbonded O-H stretching vibrations and overlapped with the N-H stretching band. The main characteristic absorption peaks, at wavelengths of 1640.83 cm⁻¹ were corresponding to the bending band of N-H (amide I band). The significant peak observed at 1559.68 cm⁻¹ and the band at 1339.27 cm⁻¹ were due to the stretching vibration of Amide II and Amide III, respectively due to the formation of the CO–NH group. The bands at 1414.28 cm⁻¹, 1081.70 cm⁻¹ and 1021.13 cm⁻¹ may be attributed to CH2, C–O–C, and C–O stretching vibrations, respectively. Based on FTIR spectroscopic measurements of obtained chitosan, the degree of deacetylation was equal to 94%



Fig.5. FTIR spectra of optimized chitosan form P. chrysogenum MZ723110

Table 2. Plackett–Burman experimental design matrix for screening significant variablesaffectinggrowthcultureconditionsofPenicilliumchrysogenumMZ723110formaximumchitosanyielddialacticdialacticchrysogenumMZ723110for

Coded values of factors												
Run	Run A	ВС	D	Е	F	G	Н	J	dry weight	chitosan yield (%)		
										(g/L)	Experimental	Predicted
1	+	-	+	-	-	-	+	+	+	13.2	1.7	2.46667
2	+	+	-	+	-	-	-	+	+	13.4	2.8	1.78333
3	-	+	+	-	+	-	-	-	+	0.8	2	2.84167
4	+	-	+	+	-	+	-	-	-	0.08	0	0.84167
5	+	+	-	+	+	-	+	-	-	0.02	0	0.91667
6	+	+	+	-	+	+	-	+	-	12.8	2.7	1.80833
7	-	+	+	+	-	+	+	-	+	7.4	5.7	4.70833
8	-	-	+	+	+	-	+	+	-	0.006	0	-0.91667
9	-	-	-	+	+	+	-	+	+	0.04	0	0.91667
10	+	-	-	-	+	+	+	-	+	5.4	4.4	3.43333
11	-	+	-	-	-	+	+	+	-	6.8	1.2	1.99167
12	-	-	-	-	-	-	-	-	-	1	0	-0.84167
13	+	-	+	-	-	-	+	+	+	14	1.4	2.46667
14	+	+	-	+	-	-	-	+	+	13.6	2.6	1.78333
15	-	+	+	-	+	-	-	-	+	0.8	2	2.84167
16	+	-	+	+	-	+	-	-	-	0.06	0	0.84167

17	+	+	-	+	+	-	+	-	-	0.012	0	0.91667
18	+	+	+	-	+	+	-	+	-	14.2	2.6	1.80833
19	-	+	+	+	-	+	+	-	+	7.4	5.4	4.70833
20	-	-	+	+	+	-	+	+	-	0	0	-0.91667
21	-	-	-	+	+	+	-	+	+	0.02	0	0.91667
22	+	-	-	-	+	+	+	-	+	5.5	4.3	3.43333
23	-	+	-	-	-	+	+	+	-	7.4	1.1	1.99167
24	-	-	-	-	-	-	-	-	-	0.9	0	-0.84167

(A) Glucose concentration (g/L), (B) peptone concentration (g/L), (C) yeast extract concentration (g/L), (D) Temperature ($^{\circ}$ C), (E) pH, (F) Incubation period (days), (G) volume of medium (ml), (H) inoculum size (ml) and (J) type of fungal culture. The following values are – and + levels of each variable respectively.

Table 3. Statistical analysis of the main effect of each tested variable on chitosan yield (%) from *P. chrysogenum* MZ723110 using PBD

Variable	Effect Coefficient	T-Value	F-Value	P-Value
A: Glucose conc.	0.212	0.90	0.81	0.383
B: Peptone conc.	0.679	2.88	8.28	0.012
C: Yeast extract conc.	0.296	1.25	1.57	0.230
D: Temperature	-0.288	-1.22	1.48	0.243
E: pH	-0.162	-0.69	0.47	0.502
F: Incubation period	0.621	2.63	6.92	0.020
G: Rate of aeration	0.437	1.85	3.44	0.085
H: Inoculum size	-0.321	-1.36	1.85	0.195
J: Type of fungal culture	1.029	4.36	19.02	0.001
Model P -value	0.004			
Model R ²	0.7580			
Model adjusted R^2	0.6025			

At 95% confidence intervals, the analysis of variance (ANOVA) was used; variables and models would be statistically significant at levels of significance, P-value < 0.050. A negative T value suggests a negative effect, while a positive T value indicates a positive one.

	Coded value	s of factors	dur woight	ahitasan	viold (94)
Run	А	В	(g/L)	Cintosan	yiciu (70)
				Experimental	Predicted
1	-1.00000	-1.00000	11	0.36	0.31183
2	1.00000	-1.00000	9.33	2.5	2.57718
3	-1.00000	1.00000	11.66	6	5.88282
4	1.00000	1.00000	17.33	3.5	3.50817
5	-1.41421	0.00000	16.66	2.6	2.70864
6	1.41421	0.00000	14.66	2.7	2.63136
7	0.00000	-1.41421	5.66	1.2	1.17120
8	0.00000	1.41421	14	5.7	5.76880
9	0.00000	0.00000	14.33	3.2	2.90000
10	0.00000	0.00000	11.33	2.3	2.90000
11	0.00000	0.00000	16.66	3.2	2.90000

Table 4. Central Composite design matrix with two significant factors in coded values together with experimental and predicted chitosan yield by *P. chrysogenum* MZ723110

(A) Peptone concentration (g/L), (B) Incubation period (days). The following values are - (low level), + (high level), 0 (center points), -1.412 (low star point) and +1.412 (high star point) of each variable respectively.

Table 5. Statistical analysis of the main, interacted, and squared effect of two factors on chitosan yield (%) from *P. chrysogenum* MZ723110 using CCD

Source	Effect Coefficient	T-Value	F-Value	P-Value
A: peptone concentration	-0.027	-0.23	0.05	0.830
B: incubation period	1.625	13.45	180.93	0.000
Square				
A*A	-0.115	-0.80	0.64	0.460
B*B	0.285	1.98	3.93	0.104
2- Way interaction				
A*B	-1.160	-6.79	46.07	0.001
Lack-of-Fit				0.979
Model P value	0.000			
Model R^2	0.9790			
Model adjusted R ²	0.9580			
Predicted R ²	0.9450			

DISCUSSION

Marine fungi have been shown to be a significant source of novel biologically active everyday products (Ghanem et al., 2010). The great majority of these microbes thrive in an intriguing and unusual habitat, and as a result, they can produce outstanding and unusual secondary metabolites (Fang et al., 2005). From various environments including marine substrates, such as sponges, sediment, and algae *Penicillium* species have been commonly isolated (Paz et al., 2010). In addition, *Penicillium* species from the Eastern coast of Alexandria, Egypt have been isolated by Amer et al. (2019). *Penicillium* has been found to have a wide range of bioactive natural compounds (Gomes et al., 2014). The present results assessed that, the marine-derived fungal isolate was identified as *Penicillum chrysogenum* MZ723110. Notably, the ITS region is the most sequenced DNA region in fungus, and it is important for molecular systematics both at the species level and within species (Fajarningsih, 2016).

Upon chitosan extraction from *Penicillum chrysogenum* MZ723110; the yield of chitosan was 4.4% that is slightly lower than the yield obtained from *P. chrysogenum* in the study of **Wang et al. (2007)** which was 5.7%. In fungal mycelia, the chitosan is accumulated in the cell wall where it exists as both freely (free chitosan) and as a complex (chitin/chitosan), which is covalently bound to glucan. Thus, an effective extraction method would have been to involve breaking not only the cell wall but chitosan glucan complexes as well (**Zininga, 2018**).

The first step in fungal chitosan extraction as mentioned previously in this study was the deproteination step using 1M NaOH for 15 minutes at 121°C. This step breaks down the cell wall, releasing the internal components and extracting free chitosan as well (Nwe *et al.*, 2008; Dos Santos *et al.*, 2009). Due to the high temperature used in this step, proteins were denatured beside the degradation of carbohydrates and sugars (AIM) and result in the formation of soft brown slurry. Through successive washes with water, the degraded sugars were separated from the slurry leaving behind chitin/chitosan microfibrils untouched (Zuluaga *et al.*, 2015). For the extraction of chitosan from the individual chains of aggregated chitin/chitosan microfibrils, the chitin microfibrils must be deacetylated to make the chitosan-glucan complex soluble in acetic acid (Robson, 1999). Thereafter, the chitosan–glucan complex must be split by breaking the covalent bond between the chitosan and glucan.

It was concluded from the results of **Nwe** *et al.* (2008) that the breakdown of the matrix could be obtained by 11M NaOH treatments thus, successfully extracting the chitosan-glucan complex. In this condition, chitin present in the mycelial cell wall is converted into chitosan and by decreasing the pH by using acetic acid to a range of 3.4-3.8, solubilizing the free amino groups of the chitosan, allowing it to be soluble and easily separated (Zuluaga *et al.*, 2015). Several methods have been developed for the

extraction of chitosan from fungal mycelia (Nwe & Stevens, 2002a; New & Stevens, 2002b). The choice of the proper chitosan extraction procedure is important for the high yield production of fungal chitosan. It is essential to free the chitin/chitosan from its anchorage in the membrane and the glucan. A high concentration of NaOH is required in the first step of this solubilization. Nwe and Stevens (2002b) obtained a high yield of chitosan by applying 11M NaOH, followed by enzymatic treatment with amylase.

Remarkably, the chemical composition of the growth media and the experimental conditions influence the development of fungi and the accumulation of chitosan in the fungal cell wall (Kannan *et al.* 2010). It is worthy to mention that, Plackett–Burman design is a promising approach for screening important medium components that have been effectively used in optimization studies by numerous researchers (Singh & Tripathi, 2008; Fu *et al.*, 2009). Zininga (2018) reported that the highest chitosan yield was recorded using media containing glucose, peptone, and yeast extract. Usually, the media recommended by many authors to produce chitosan from fungi contain yeast extract, D-glucose and peptone (YPG) (Kannan *et al.*, 2010; Tai *et al.*, 2010). However, Chatterjee *et al.* (2004) examined the yield and physicochemical characteristics of chitosan and chitin produced using different media and found no significant differences.

Fungi require an organic or inorganic source of nitrogen in their nutrition to synthesize nitrogen in their walls because chitin/chitosan are nitrogen-containing biopolymers (ElMekawy et al., 2013; Abasian et al., 2019) which explains the significance of peptone concentration detected in this study. It has been reported that the highest extractable chitosan was produced during the late exponential growth phase (ElMekawy et al., 2013). The late exponential growth phase of P. chrysogenum in the study of Violeta et al. (2014) was anticipated to be between days 6 and 9, which explains the significant positive effect of the incubation period on chitosan yield optimization. Agitation provides adequate mixing, mass, heat transfer, and improving dissolved oxygen in the culture medium. At lower agitation speed, insufficient oxygen in the culture medium usually affects microbial growth. Agitation, on the other hand, produces shear forces, which cause morphological changes in the fungi by destroying the surface and interior cell structures, as well as variance in fungal growth (Darah et al., 2011; Zhu et al., 2012). Moreover, Streit et al. (2009)stated that, the curve for chitosan production has the same profile as the microorganism's growth curve. Since chitosan is a component of filamentous fungal cell wall, the amount of chitosan produced increases as the growth increases upon agitation.

The significant factors identified from PB design as major variables were presented in a central composite design (CCD) to determine their optimum level and the effect of their interactions for maximum yield of chitosan by *P. chrysogenum* MZ723110. The lack of fit data provided by ANOVA indicates the fitness of the experimental data with the selected model. In this study, the value of lack of fit was non-significant (P > 0.05). It is

desired to be insignificant to represent the model (**Khayria** *et al.*, **2016**). The model fitting's validity was examined by the coefficient of determination (R^2) and the model adjusted R^2 (R^2 adj) which was calculated to be 0.9790 and 0.9580, respectively. This indicated a strong correlation between the experimental and theoretical values predicted by the model at a 95% confidence level, and that the model was accurate and can explain 97.90% variability of the data. Only a poor fraction of data remained unexplained by the model.

Data obtained with respect to response optimizer revealed that, the chitosan yield was increased 3.1-fold compared to that in the basal level medium (4.4%). The actual response (7.5%) was found to be quite close to the predicted response (7.6%). The response model's validity is supported by the good correlation between the predicted and the experimental values. Many factors influence the synthesis of fungal chitosan, including the fungal strain, culturing method, culture medium, growth temperature and extraction methodology (**Nwe** *et al.*, **2010**).

IR spectroscopy is a frequently used analytical method for chitin and chitosan characterization and to study the possible functional groups present in the samples (**Kasaai, 2010**). The FTIR spectra of the obtained chitosan in this study indicate the presence of the main characteristic absorption peaks, at wavelengths of 1640.83 cm⁻¹. It has been reported by **Pavia** *et al.* (2009) that the band identified as an amine group (N-H bending bands) absorbs infrared between 1640 cm⁻¹ to 1550 cm⁻¹. The absorbance peak at 1640.83 cm⁻¹ indicates that most secondary amides were transformed into primary amine because of alkaline deacetylation and suggested to be an effective deacetylation band (**Tayel** *et al.*, 2015). Paul *et al.* (2014) revealed that the major absorption band was observed between 1220-1020 cm⁻¹, which represents the free amino group (–NH2) at the C2 position of glucosamine; a major group present in chitosan. The literature for spectral analyses supports the infrared transmission spectral profile of the extracted chitosan. Thus, the product was confirmed to be chitosan.

One of the main parameters characterizing chitosan and influencing its chemical reactivity, solubility, and biodegradability is the degree of deacetylation (DD) (Abdel-Salam, 2013). Optimized chitosan from *P. chrysogenum* MZ723110 was 94%. Chitosan DD% from *Rhizopus oryzae* was found to be 75.21% in the study of Gachhi and Hungund (2018). These degrees were good and were found in the highest group in DD classifications based on properties (Mohamed *et al.*, 2015). High DD% in chitosan was accompanied by high positive charge density, which makes chitosan a material with many industrial applications such as a coagulating agent in wastewater treatment and as an antimicrobial agent (He *et al.*, 2016).

CONCLUSION

Chitosan extraction from fungi has gained great attention over its extraction from other sources. In this study, it has been observed that medium composition and environmental conditions play a critical role in the yield of chitosan from *P. chrysogenum* MZ723110. Optimization of the growth culture conditions, by statistical experimental designs as Placket Burman and central composite design, enables studying the effect of several factors, and at the same time has led to a 3.1-fold increase in chitosan yield from *P. chrysogenum* MZ723110. This study also revealed that, based on FTIR spectra and deacetylation degree, the extracted product was chitosan with a DD% equal to 94%. Chitin and Chitosan are found in varying levels in fungi, depending on the fungal species and, most likely, the extraction method used, as well as the culture conditions.

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