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Enhancement of β-1,3-1,4-Glucanase Production by Marine *Halomonas meridiana* ES021 via Statistical Optimization and Cell Immobilization

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ABSTRACT

β-1,3-1,4-glucanase (β-glucanase) is an enzybiotic enzyme that cleaves linear β-glucans with β-1,3 and β-1,4 linkage, including cereal β-glucans. It has several applications in industry, agriculture, and medicine in which it has been observed with a broad antimicrobial spectrum. A local marine strain with high activity of β-glucanase was screened and isolated from Gabal El-Zeit offshore in the Red Sea of Egypt. It was successfully identified through a partially 16S rRNA gene sequencing technique as *Halomonas meridiana* ES021. The medium was optimized using a multifactorial matrix of Plackett-Burman design, and the most influencing components were barley flour, urea and KH_2PO_4. The enzyme activity was positively affected by barley flour, urea and negatively affected by KH_2PO_4. The optimal medium obtained through statistical design increased enzyme activity by 1.4-fold from 455.83±20.45 to 597.50±24.50U/ mL. In addition, a Box-Behnken experimental design was studied to investigate interactions between barley flour, urea and KH_2PO_4 concentrations. In the optimized medium, the enzyme activity was increased by about 1.8-fold from 597.50±24.50 U/mL to 1095.50±31.00 U/mL. Moreover, cell immobilization by adsorption and entrapment was investigated and immobilized cultures of *Halomonas meridiana* ES021, which were entrapped in a 3% agarose gel produced the maximal β-glucanase activity, which was about 1111.67 ± 29U/ mL, followed by cultures adsorbed on clay particles, which was about 916.97 ±29.47U/ mL.

INTRODUCTION

Cereal β-glucans and lichenan are types of linear β-glucans through β-1,3 and β-1,4 bonds that are hydrolyzed by β-1,3-1,4-glucan lysis enzyme (β-glucanase) or lichenase (EC 3.2.1.73). The enzyme has a stringent selectivity for cleaving β-1,4 glycosidic bonds on 3-O-substituted glycosyl residues **(Planas, 2000)**. β-glucanase is widely used to lower the backbone polymer units to small molecular weight of β-glucan, while increasing its water solubility and bioactivity **(Roubroeks *et al*., 2001)**. The most common dervatives of breaking barley β-glucan are the oligomers of trisaccharides (3-O-cellobiosyl-D-glucopyranose) and tetrasaccharides (3-O-cellotriosyl-D-glucopyranose) **(Henrissat & Bairoch, 1996)**. Cellotriosyl and cellotetraosyl byproducts are characterized by a single β-

1, 3 glycosidic linkage and representing about 90 percent of the total water-soluble barley β-glucan **(Stone & Clarke, 1992)**.

The enzyme β-1,3-1,4-glucanase may serve as a source of low-toxic antitumor drugs, and it has been reported with potent antimicrobial efficacy. β-1,3-1,4-glucanase produced from bacterial strains revealed a significant lethal effect against different cell wall types of bacteria in terms of highly polysaccharide content (gram negative bacteria) and lower polysaccharide content (gram positive bacteria) such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella typhi*, *Salmonella paratyphi* A, *Enterococcus faecalis* and *Shigella dysenteriae*, as well as restricted the growth and the germination of *Candida albicans* and *Cryptococcus neoformans* **(Jin *et al*., 2011)**. Moreover, they destroyed the mycelial structure of fungi-causing plant diseases as *Cryphonectria parasitica*, *Cylindrocladium quinqueseptatum* and *Helicobasidium purpureum* **(Xu *et al*., 2016)** and inhibited the growth of the phytopathogenic fungus *Alternaria alternata* as well **(Zalila-Kolsi *et al*., 2018)**. Mixed linkage β-oligosaccharides produced from β-glucan cleavage by β-1,3-1,4-glucanase have antioxidant and antibacterial activities **(Chaari *et al*., 2016)**. Moreover, β-1,3-1,4-glucan hydrolase has different agricultural applications as animal feed industry **(Hua *et al*., 2010; Ribeiro *et al*., 2012)**. Additionally, it has industrial applications as brewing **(Celestino *et al*., 2006)**, detergent industry **(Chaari *et al*., 2012; Maktouf *et al*., 2013)**, wine clarification and filtration **(Chandra *et al*., 2013)**, and oligosaccharides production **(Chaari *et al*., 2015; Cho *et al*., 2018)**.

Diversity of enzymes with commercial and pharmacological applications can be detected in marine ecosystem **(Hamdache *et al*., 2011)**. The unique ecological features of the Red Sea habitats with respect to the high temperature, high salinity and strong UV radiation are remarkable. Accordingly, the Red Sea is viewed as a "laboratory" for investigating life in the event of "global warming" **(Renn *et al*., 2021)**. In marine ecosystems, the organic carbon and nitrogen cycles are significantly influenced by microbial communities via different metabolic pathways using extracellular enzymes to facilitate breaking high molecular weight molecules into low molecular weight molecules, which afterwards could be easily uptaken through the cytoplasmic membrane for nutritional cycles **(Alderkamp *et al*., 2007)**. Polysaccharides are significant components of algae-produced HMW organic matter **(Biersmith & Benner, 1998)**. In order to break down polysaccharides, glycoside hydrolases (EC 3.2.1.-) hydrolyze the glycosidic backbone connection with two or even more carbohydrate groups **(Alderkamp *et al*., 2007)**.

The inducer and nutrient sources had an effect on the productivity of the bacterial β-glucanase enzyme **(Tang *et al*., 2004)**. The increased β-glucanase synthesis required an optimization of cultural conditions. The fitting matrix of Plackett-Burman is a fractional factorial design with upper and lower levels for each factor, which is particularly helpful for screening and investigations since it allows the estimation of the main effects of k factors using a linear model in only k + 1 experiments. However, the interaction of factors is not taken into account in this approach **(Plackett and Burman, 1946)**. With 3D illustration on x, y and z axis, the response surface methodology (RSM) is applied as a statistical and mathematical optimization method that can be used to optimize the most three significant factors selected by Plackett-Burman design **(Myers *et al*., 2004)**. Medium optimization through statistical experimental designs has received a lot of attention in

recent years, and there have been a lot of published studies about how to utilize them in enzymes production **(Felse & Panda, 1999; Roohi & Kuddus, 2015)**.

The use of immobilized enzymes or entire cells to modify biotechnology processes has many advantages since these types of biocatalysts have superior operational stability and catalysis efficiency and can be reused. Cell immobilization offers numerous benefits versus free cell culture. It can accelerate the reaction offering a continouos fermentation with high dilution rate without cell wash. Besides, the immobilized cells are more resistant towards blocking chemicals and deficient nutrition than free cells. Furthermore, when compared to batch fermentation, the immobilized cells can be used as industrial reusable catalysts, which can be beneficial. Various immobilization strategies have been widely applied to produce ethanol, organic acids, enzymes, amino acids and in bioremediation of hazardous wastes as well **(Kar *et al*., 2009; Abd El-Zaher *et al*., 2017)**.

The proposed target of the present work was to identify a marine-origin strain capable of producing β-1,3-1,4-glucanase, as well as optimizing cultural conditions for maximum production using statistical approaches and various cell immobilization methods.

MATERIALS AND METHODS

2.1 Cultivation and molecular identification

Bacterial strains capable of yielding β-1,3-1,4-glucan lysis enzyme were obtained from different locations in Egypt; namely, the Red Sea and Wadi El-Natrun. Sediment samples were collected in sterile glass containers, 1.0mL of diluted sediment samples was transferred to Zobell marine broth containing the following ingredients (g/L): yeast extract, 1; peptone, 5; 800mL; distilled water; filtered sea water, 200mL; $FeSO_4.7H_2O$, traces; final pH level at 7.5 **(Zobell, 1942)** and incubated overnight in shaken condition at 120rpm and 37°C. After enrichment, 1.0mL of enrichment cultures was then transferred to fermentation medium composed of (g/L): barley flour, 63.5; corn flour, 44.8; KH_2PO_4, 1.0; $CaCl_2$, 0.1; $MgSO_4.7H_2O$, 0.1 and pH adjusted to 7.0 **(Guo-qing *et al*., 2003)**. Cultures were then transferred to Zobell agar medium and incubated overnight at 37°C. The bacterial colonies differing in morphological characteristics were selected and checked for purity using streaking method.

For the production of β-1,3-1,4-glucanase, one millilitre of an overnight bacterial batch growth in Zobell medium was introduced into 100mL for cultivation, which were then shaked for 24 hours at 37°C and 180rpm. The most potent strain was isolated from Gabal El-Zeit-off shore, the Red Sea, Egypt, showing the highest β-1,3-1,4-glucanase yield. The strain was used for further investigations and identified by NCBI tools including the pair-wise alignment, the tree method (fast minimum evolution) and the blast search for the given sequence.

2.2 β-Glucan extraction

Whole barley flour was used to extract β-glucan using a modified version of the technique of **Wood *et al*. (1977)**. 30g barley flour was stirred into 300mL distilled water, and then the pH was set to 7, followed by adding sodium carbonate (20% w/v), and then the mixture was rapidly shaken for 30 minutes at 55°C. To dispose the solid particles, the mixture was centrifuged for 20 minutes at 2000×g at 4°C. The adjusted pH of supernatant at 4.5 level was done by dropping carefully 2M HCl before centrifugation (25 min at 2000

×g, 4°C). The proteins were precipitated, separated and then discarded. Adding equal volume of ethyl alcohol (99.9%) as an organic solvent to the supernatant, gradually with stirring, provided the precipitate of β-glucan. After allowing the precipitate to settle over 12 hours at 4°C, the β-glucan was obtained by centrifugation (10min at 2000 ×g). After being suspended again in ethanol, the rubbery particle was homogenized for 1– 2 minutes at room temperature (21°C) to achieve a uniform dispersion. With 100mL of 99.9% ethanol, the β-glucan sample was filtered, rinsed, allowed to air dry and let to equilibrate for 24 hours in a desiccator before being weighed and ground to pass 0.25mm screen. β-Glucan was stored at 4°C ready for use.

2.3 Protein content determination

Lowry technique was used to determine the enzyme preparation's protein concentration. **(Lowry *et al*., 1951)** as follows: To 1mL crude preparation, 5mL reagent C {50 ml Lowry A (2% Na_2CO_3 in 0.1N NaOH): 1mL Lowry B (1% cupric Sulphate; 2% Na,K tartarate)} were added and allowed to stand at room temperature for 10 minutes; 0.5mL Folin-Ciocalteu reagent (diluted 1:1) was then added and shaked immediately. After 20min, the produced blue color was measured via spectrophotometer at a wavelength of 750nm. A standard curve was prepared with standard bovin serum albumin.

2.4 Assay for β-1,3-1,4-glucanase activity

The technique of **Miller (1959)** for reducing sugars measurement was applied using the dinitrosalicylic acid technique, targeting the liberated reducing sugars from β-1,3-1,4-glucan hydrolysis. Culture supernatant (0.1 mL) was left for incubation mixed with 0.9mL of 0.2% barley β-glucan dissolved in 0.1M phosphate buffer pH 6.0 for 10min at 50°C. After incubation, the mixture was added to 1mL dinitrosalycilic acid reagent (1% NaOH; 0.2% Phenol; 1% DNS). This mixture was boiled for 15min for color development. After boiling, 0.5mL of rochell salt solution (40% Na, K tartrate) was immediately added to the mixture to stop the reaction and was left to cool. The absorbance was measured at 575nm. The concentration of the liberated reducing sugars was evaluated using glucose calibration curve (100 to 1000µg/ mL). The amount of enzyme required to produce 1µ mol of reducing sugar (glucose equivalent) per minute is known as one activity unit of β-glucanase activity (U).

2.5 Plackett-Burman design for seven factors

Applying a comprehensive factorial matrix would need more experiments, if n factors are required to be examined. This study used the Plackett-Burman experiment design in a sense of having a multifactorial design to reflect the relevance of several environmental conditions on β-1,3-1,4-glucanase production. In the present work, nine different combinations of seven independent factors were tested using the Plackett-Burman design matrix. Table (1) shows the levels of seven culture factors that were investigated. Upper (+) and lower (-) levels were examined for every variable **(Plackett & Burman, 1946)**.

The main effect of different variables was calculated using the following equation:

$E_{xi} = (\Sigma M_{i+}/- \Sigma Mi-) / N$

Where, E_{xi} represents the variable main effect; M_{i+} and M_{i-} represent the efficiency of each trial in precent; xi represents the independent variable in high and low levels, and N

represents the total number of trials divided by 2. The main effect column chart with upper positive side implies that the variable with highest attitude is close to the optimum; whereas, the lower negative side suggests that the variable with the lowest level is close to the optimum. In order to assess the variable's significance, the function of t-test in microsoft excel was applied to assist the statistical t-values under condition of equal unpaired samples.

Table 1. Upper and lower levels of independent variables in Plackett-Burman matrix

Variable (g/L)	Symbol	Level		
		Lower (+)	**Basal (0)**	**Higher (-)**
Barley flour	Ba	90	63.5	30
Wheat flour	Wh	60	44.8	20
Urea	Ur	8	5.4	2.7
Fructose	Fr	7.5	5	2.5
KH_2PO_4	Kh	1.5	1.0	0.5
$MgSO_4$	Mg	0.15	0.1	0.05
$CaCl_2$	Ca	0.15	0.1	0.05

2.6 Box-Behnken experimental design for three factors

Box-Behnken experimental matrix was hired for this study in order to determine the relationship between barley flour, urea and KH_2PO_4. The Box-Behnken design, coming under RSM, was described I the study of **Box and Behnken (1960)**. The optimum culture settings for maximizing *Halomonas meridiana* ES021 β-1,3-1,4-glucanase production were evaluated by three factors. Table (2) displays the tested levels of three culture-related factors. Statistically, 14 analytical software was used for drawing 3D histogram charts to determine the relationship between fermentation factors (barley flour, urea and KH_2PO_4 concentrations).

Table 2. Examined culture variables and their levels in Box-Behnken experiment

Symbol code	Factor	Levels of coded factors		
		Low (-1)	**Basal (0)**	**High (+1)**
X_1 (g/L)	**Barley flour**	45	90	135
X_2 (g/L)	**Urea**	4	8	12
X_3 (g/L)	**KH_2PO_4**	0.25	0.5	0.75

2.7 Production of extracellular β-glucanase by immobilized *Halomonas meridiana* ES021 cells

2.7.1 Adsorption on different solid porous materials

Three mL of pre-activated bacterial cells were introduced into 100mL of sterilized production medium with solid supports (natural and synthetic luffa, sponge, clay and pumice) in Erlenmeyer flasks. Flasks were incubated statically at 37°C for 16h.

2.7.2 Entrapment in agar and agarose

The gel was made by mixing 0.5g of agar or agarose with 25mL of distilled water as described in the work of **Chapatwala *et al*. (1993)**. After sterilization, 3mL of pre-activated culture was added and mixed; the obtained mixture was then poured aseptically into sterilized Petri-dish. After the gel was solidified, it was divided into similar cubes of about 0.5cm in length using a sterile cutter; the cubes were then relocated to 100mL of sterile mineral broth medium and incubated statically at 37°C for 16 h.

2.7.3 Entrapment in calcium alginate and potassium k-carrageenan

For entrapment in calcium-alginate and potassium k-carrageenan, bacterial cells were entangled in 2% calcium alginate or potassium k-carrageenan of small diameter of circular gel beads as discussed in the study of **Eikmeier *et al*. (1984)**. Approximately, 3mL of activated culture was added to the sterile solutions. A sterile syringe was used to draw the gel-mixing bacteria, dropped into a cross linking solution (100mL of 2% $CaCl_2$ solution for calcium-alginate and 2% KCl solution for potassium k-carrageenan) to get circular beads (3 - 4 mm diameter). The resulting beads were left in their different solutions for 1.0 h for adequate hardening. Later, several washing in sterile distilled water was processed. The produced beads were added to 100mL of production medium and cultivated statically at 37°C for 16h.

2.7.4 Different agarose gel concentrations

Different concentrations of agarose solution (1, 2, 3, 4 and 5%) were prepared; 3mL of activated culture was entrapped in sterile agarose solutions. Gel-bacterial cell mixtures were prepared as previously described then transferred to 100mL sterile mineral media and cultivated statically at 37°C for 16h. Enzyme activity as well as protein content were determined.

2.8 Scanning electron microscopy

Free, entrapped, and adsorbed *Halomonas meridiana* ES021 cells were scanned using a Jeol electron microscope (Electron Microscope Unit in Faculty of Science, Alexandria University). Free and immobilized cells on sponge cubes or in agaros gel was harvested, rinsed by phosphate buffer and stabilized with 2% glutaraldehyde before treated with 1% osmium tetraoxide. After being rinsed in a buffer solution, the samples were dehydrated in ethanol. Before being gold-coated, the critical point dryer was used to completely dry the samples. A scanning electron microscope with a 45° beam angle, and a voltage of 20 kV was used to examine the specimens.

2.9 Statistical analysis

Statistical analysis methods were applied for Plackett-Burman and Box-Behnken output using regression, t-test and analysis of variance. Results were presented as mean ± SD.

RESULTS

3.1 Strain identification

Real Bio Gene Company in Egypt partially sequenced the isolate's 16S rRNA. The sequence similarity to the *Halomonas* sp. H-141 16S ribosomal RNA gene was 99.61% similar, followed by 99.52% similarity to the *Halomonas aquamarina* strain CON4 16S ribosomal RNA gene, *Halomonas meridiana* strain SCSIO 43005 chromosome, and *Halomonas* sp. strain 13-8 16S ribosomal RNA gene (Fig. 1).

Consequently, the given name of the starin was proposed to be *Halomonas meridiana* ES021 at the genus level. The accession number for the sequence was MT322833 (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

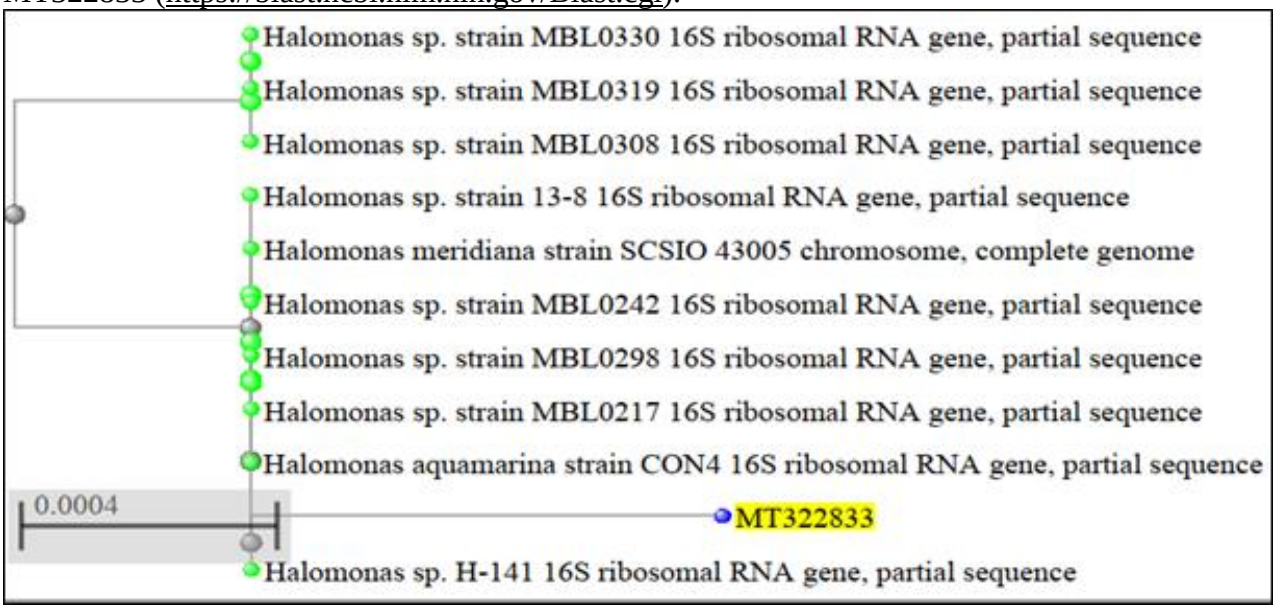

Fig.1. Phylogenetic relationships between *Halomonas meridiana* ES021 strain and the most closed relatives upon multiple alignments of 16S rDNA sequences

3.2 Plackett-Burman experimental design

The matrix of combining factors following the Plackett-Burman protocol was used to interpret the impact of various fermentation factors in nine combinations of fermentation conditions. β-1,3-1,4-glucan hydrolase activity and protein content of each trial were measured, and the output calculations were presented in Table (3). Trial 8 yielded the highest measurement of glucan lysis activity occurring by β-1,3-1,4-glucanase (664.17±29.77 U/mL) followed by trial 4, which yielded the highest protein content (502.50±25.23 U/mL). Table (4) shows the main statistical analysis of this trial. With an 85 % tested degree of significance (α= 0.15), barley flour was the most significant factor giving a proportional relation between barlely concentration and glucanase activity. This indicates that the more barley concentarion, the more glucanase activity and vice versa.

The main effect of each factor on the β-1,3-1,4-glucan hydrolase action was calculated and graphically represented (Fig. 2). The high enzyme activity observed in the culture media of bacterial cells was caused by high levels of barley flour and urea in the medium with basic levels of wheat flour, fructose, $MgSO_4.7H_2O$, $CaCl_2$ and low levels of KH_2PO_4. Based on the Plackett-Burman experiment's findings, the most ideal medium for

extracellular glucan hydrolase enzyme production from *Halomonas meridiana* ES021 was (g/L): barley flour, 90; wheat flour, 44.8; urea, 8; fructose, 5; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1 and $CaCl_2$, 0.1; initial pH = 8.

A verification experiment was used to quantify the β-1,3-1,4-glucanase using predicted optimum medium, non-optimized medium, and anti-optimized medium and giving a clear evaluation window for the Plackett-Burman design accuracy. Table (5) reveals that optimum culture medium resulted in more enzyme units of β-1,3-1,4-glucanase when compared to non- and anti-optimized medium settings, yielding 597.50±24.50U/ mL enzyme activity, while non-optimized and anti-optimized cultures yielded 455.83±20.45 and 310.00±16.50U/ mL enzyme activity, respectively. The optimized medium's enzyme production was about 1.3-fold higher than the basic medium.

Table 3. The outcome data of Plackett-Burman matrix for yielding extracellular β-glucanase by *Halomonas meridiana* ES021 cultures

Trial	Ba	Wh	Ur	Fr	Kh	Mg	Ca	β-glucanase activity (U/mL)	Protein content (mg/mL)
1	-	-	-	+	+	+	-	331.67±19.85	0.44±0.04
2	+	-	-	-	+	+	+	312.50±18.12	0.86±0.09
3	-	+	-	-	-	+	+	178.33±6.05	0.34±0.03
4	+	+	-	-	-	-	-	502.50±25.23	1.94±0.19
5	-	-	+	+	+	-	+	390.00±25.10	0.70±0.07
6	+	-	+	+	-	-	-	439.17±24.79	1.13±0.11
7	-	+	+	+	+	-	-	336.67±20.30	0.15±0.02
8	+	+	+	-	-	+	+	664.17±29.77	0.38±0.04
9	0	0	0	0	0	0	0	456.67±21.20	0.64±0.03

Table 4. Statistical analysis of the Plackett-Burman design

Factor	β-glucanase activity		Protein content	
	Main Effect	**t-test**	**Main Effect**	**t-test**
Barley flour	170.417±6.652	1.978	0.670±0.07	1.935
Wheat flour	52.082±0.940	0.479	-0.080±0.01	-0.181
Urea	126.252±4.433	1.288	-0.305±0.03	-0.720
Fructose	-39.997±1.569	-0.365	-0.275±0.03	-0.644
KH_2PO_4	-103.333±0.356	-1.009	-0.410±0.04	-1.004
$MgSO_4$	-45.417±3.122	-0.416	-0.475±0.05	-1.198
$CaCl_2$	-16.252±1.606	-0.147	-0.345±0.03	-0.825

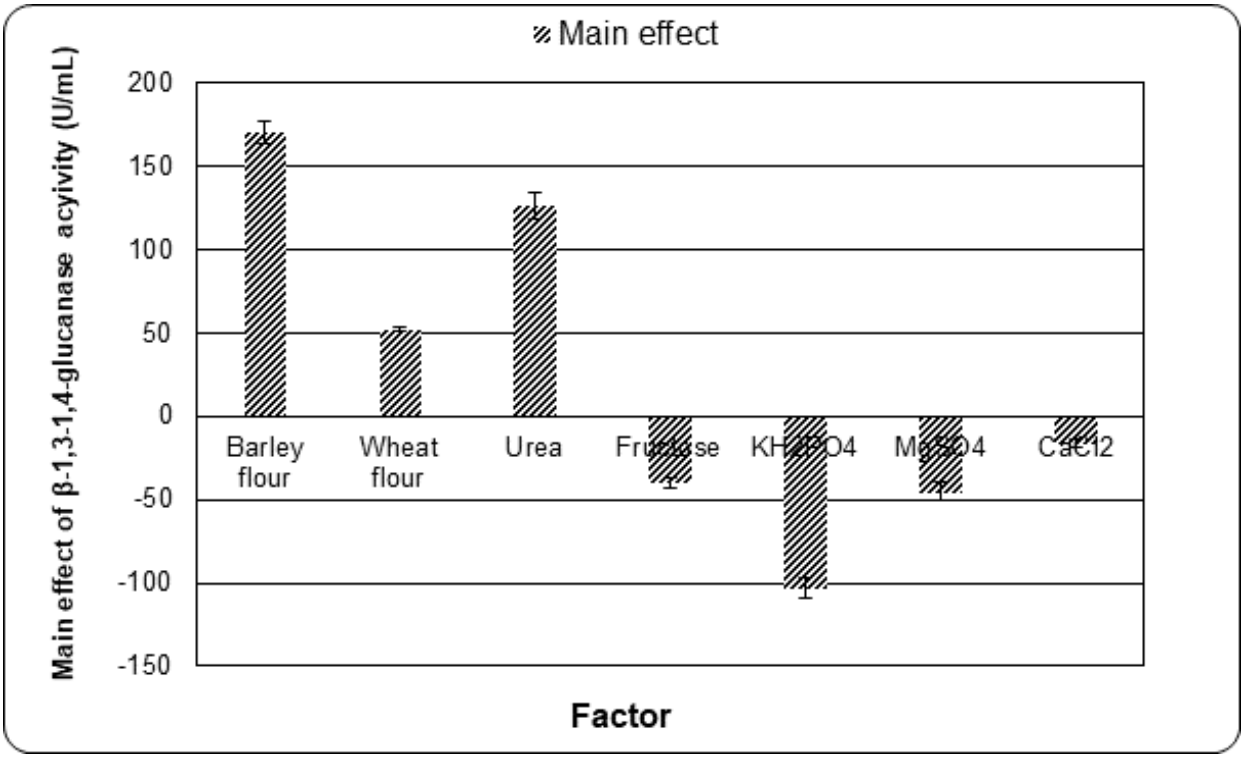

Fig. 2. The main effect of each variable on β-glucanase production by *Halomonas meridiana* ES021

Table 5. A verification experiment for extracellular β-glucanase production by *Halomonas meridiana* ES021 prepared on basal, optimized and anti-optimized medium

Medium	β-glucanase activity (U/mL)	Protein content (mg/L)
Non-optimized medium	455.83±20.45	0.64±0.04
Optimized medium	597.50±24.50	1.03±0.06
Anti-optimized medium	310.00±16.50	0.56±0.01

3.3 Box-Behnken experimental design

With resoect to the results of the two levels applied in Plackett-Burman design, the most influencing factors (barley flour, X_1; urea, X_2; and KH_2PO_4, X_3) were further optimized at three levels. The Box–Behnken experimental design was applied to evaluate the relationship between the independent variables involved in yielding β-1,3-1,4-glucan hydrolase production at three levels (+1, 0, −1). The design was used with 15 different fermentation settings, and the measurements of β-1,3-1,4-glucan cleavage activity and the protein content of each experiment were calculated as shown in Table (6).

The results of the design's statistical analysis are shown in Tables (7, 8). As a result, the model may be used to identify the optimal conditions for variables listed as barley flour, urea, and KH_2PO_4 concentrations affecting the activity of β-1,3-1,4-glucanase.

The following equation was discovered to describe β-1,3-1,4-glucanase production using quadratic regression analysis on the experimental data:

$$Y=530.42+227.29X_1-58.33X_2+103.75X_3-92.81X_1X_2+111.36X_1X_3-2.39X_2X_3+178.69X_1^2 4.22X_2^2 -217.34X_3^2$$

Where, Y is the dependent variable (β-1,3-1,4-glucanase yield) in U/mL, and X_1, X_2 and X_3 are the concentrations of the independent variables.

It was found that maximum production yield for the enzyme was at high level (+1) of barley flour, low level (−1) of urea and near to standard level (0) of KH_2PO_4. By applying a solver program found in Microsoft Excel as a prediction tool, the optimum levels of the three components were calculated and found to be in g/L (Barley flour, 135; Urea, 8; KH_2PO_4, 0.625). By providing the three-dimensional graphics via STATISTICA 14.0, the impacts of the three tested significant independent factors on each response were depicted in Figs. (3, 4, 5). The results in Fig. (3) depict the effect of barley flour and KH_2PO_4 on the yield of β-glucan-degrading enzyme. Maximum β-glucan-hydrolase activity was designated by the red color region, and this occurred at high level (+1) of barley flour and low level (−1) of urea. Moreover, Fig. (4) showed that, the maximum β-1,3-1,4-glucanase production occurred at high level of barley flour (+1) and near to zero level (0) of KH_2PO_4. In Fig. (5), the top peak of β-1,3-1,4-glucan hydrolase production occurred at low level (−1) of urea and near to zero level (0) of KH_2PO_4.

The most perferable medium for β-glucan-hydrolase activity from *Halomonas meridiana* ES021 growth, as determined by the findings of the applied Box-Behnken experiment, was (g/L): barley flour, 135; wheat flour, 44.8; urea, 4; fructose, 5; KH_2PO_4, 0.625; $MgSO_4.7H_2O$, 0.1; $CaCl_2$, 0.1 and pH adjusted to 8 with a predicted β-glucanase action equals to 1134.37±67.76U/ mL.

A verification experiment was conducted to assess the accuracy of the applied Box-Behnken experiment. The non-optimized setting was compared to the expected optimum levels of independent variables (optimum conditions of Plackett-Burman design). β-glucan cleavage activity was about 1095.50±31.00U/ mL, which was nearly 1.8-fold higher than that of the medium optimized by Plackett-Burman design (597.50±24.50U/ mL). Furthermore, the protein content of the new medium (1.78±0.11 mg/mL) was around 1.7-fold higher than that of the Plackett-Burman-optimized medium (1.03±0.06mg/ mL). The model's accuracy and validity were demonstrated by comparing observed (1095.50±31.00U/ mL) and predicted (1134.37±67.76U/ mL) activity of β-glucan-hydrolysing enzyme at the most ideal conditions; this demonstrated a strong correlation between the experimental and predicted results (Table 9).

3.4 Production of extracellular β-1,3-1,4-glucanase by immobilized *Halomonas meridiana* ES021 cultures

For cell adsorption, different supporting materials were used for cell immobilization. Table (10) figures out that, the top reading of β-glucan cleavage activity (916.97±29.47 U/mL) was given by supporting the bacterial cells on clay particles; however, the activity was still less than that observed from cultures of free cells (1064.25±30.75U/ mL). The lowest activity (305.30±19.47U/ mL) was obtained from cells adsorbed on pumice particles.

For cell entrapment, different gel materials were used. The highest reading of β-1,3-1,4-glucanase activity (1111.67±29.00U/ mL) was given by entrapping the bacterial cells in

agarose gel, which was slightly higher than that obtained from free cells cultures (1081.67±23.33U/ mL), approximately reached about 1.03-fold. The lowest activity (671.67±16.80U/ mL) was obtained from cells entrapped in kappa-carrageenan beads.

Table 6. Experimental results of the Box-Behnken design for yielding extracellular β-glucanase by *Halomonas meridiana* ES021

Trial	X_1	X_2	X_3	X_1X_2	X_1X_3	X_2X_3	X_1X_1	X_2X_2	X_3X_3	β-1,3-1,4-glucanase activity (U/mL)	Protein content (mg/mL)
1	0	+1	-1	0	0	-1	0	+1	+1	185.83 ± 11.15	1.11 ± 0.05
2	+1	+1	0	+1	0	0	+1	+1	0	785.83 ± 47.15	1.13 ± 0.06
3	+1	-1	0	-1	0	0	+1	+1	0	1100.83 ± 66.05	1.98 ± 1.00
4	-1	-1	0	+1	0	0	+1	+1	0	438.33 ± 26.30	1.68 ± 0.08
5	+1	0	+1	0	+1	0	+1	0	+1	953.33 ± 57.20	1.13 ± 0.04
6	0	-1	+1	0	0	-1	0	+1	+1	436.67 ± 26.20	1.65 ± 0.08
7	+1	0	-1	0	-1	0	+1	0	+1	462.5 ± 27.82	1.21 ± 0.06
8	-1	0	+1	0	-1	0	+1	0	+1	298.33 ± 17.99	1.91 ± 0.09
9	-1	0	-1	0	+1	0	+1	0	+1	252.92 ± 15.17	0.84 ± 0.04
10	-1	+1	0	-1	0	0	+1	+1	0	494.58 ± 29.67	0.99 ± 0.08
11	0	-1	-1	0	0	+1	0	+1	+1	285.00 ± 17.10	0.83 ± 0.04
12	0	+1	+1	0	0	+1	0	+1	+1	327.92 ± 19.67	1.25 ± 0.06
13	0	0	0	0	0	0	0	0	0	528.33 ± 35.37	1.04 ± 0.05
14	0	0	0	0	0	0	0	0	0	530.42 ± 33.28	0.99 ± 0.05
15	0	0	0	0	0	0	0	0	0	532.5 ± 31.20	1.04 ± 0.07

Table 7. Analysis of variance for the fitted quadratic polynomial model

	Coefficients	Standard error	t-Stat	*P*-value
Intercept	530.42±33.28	24.049	22.056	0.000
X_1	227.29±13.64	14.727	15.434	0.000
X_2	-58.33±3.50	14.727	-3.961	0.010
X_3	103.75±6.23	14.727	7.045	0.001
X_1X_2	-92.81±5.57	20.827	-4.456	0.007
X_1X_3	111.35±6.64	20.827	5.347	0.003
X_2X_3	-2.39±0.14	20.827	-0.115	0.913
X_1X_1	178.69±10.01	21.677	8.243	0.000
X_2X_2	-4.22±1.01	21.677	-0.195	0.853
X_3X_3	-217.34±13.75	21.677	-10.026	0.000

Table 8. The analysis of variance (ANOVA) for the response quadratic model

	Df	SS	MS	F	Significance F
Regression	9	926964.7	102996.1	59.36281	0.000151
Residual	5	8675.135	1735.027		
Total	14	935639.8			

DF: Degree of freedom; SS: Sum of square; MS: Mean square; F:F-test

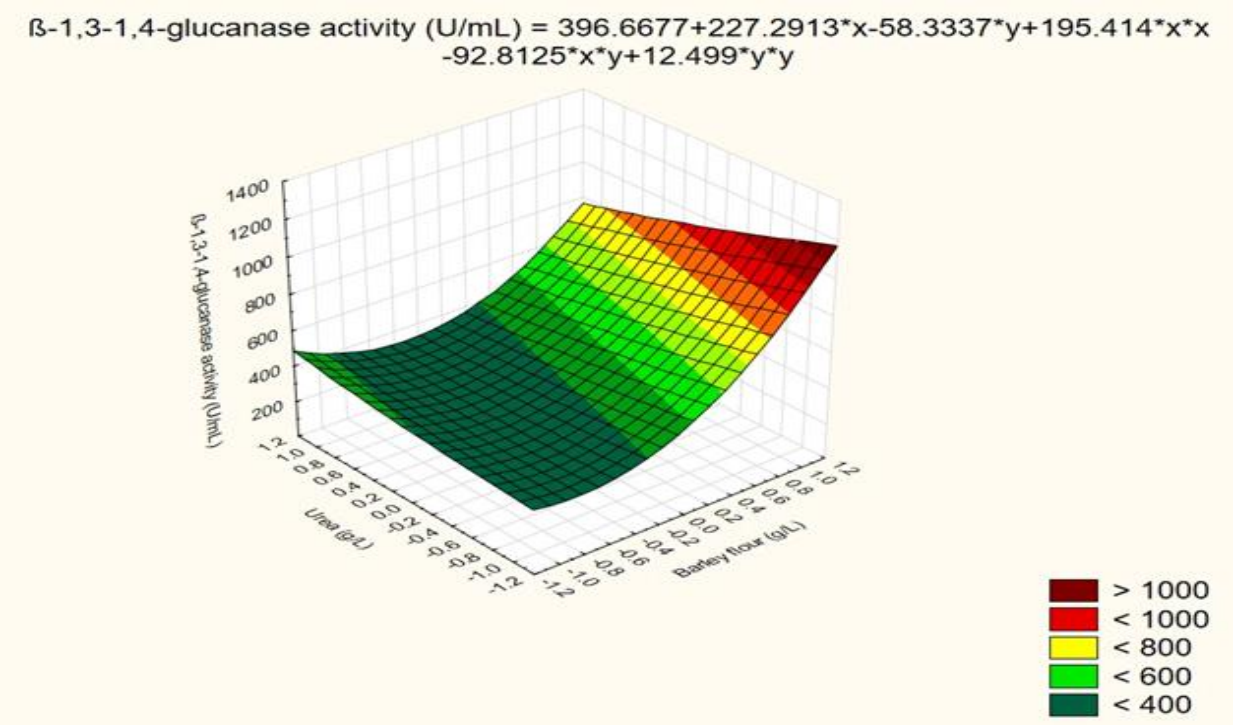

Fig. 3. 3D Surface Plot depicting the interaction effect of ß-glucanase activity (U/mL) against barley flour concentration (g/L) and urea concentration (g/L) on yielding extracellular β-glucanase by *Halomonas meridiana* ES021

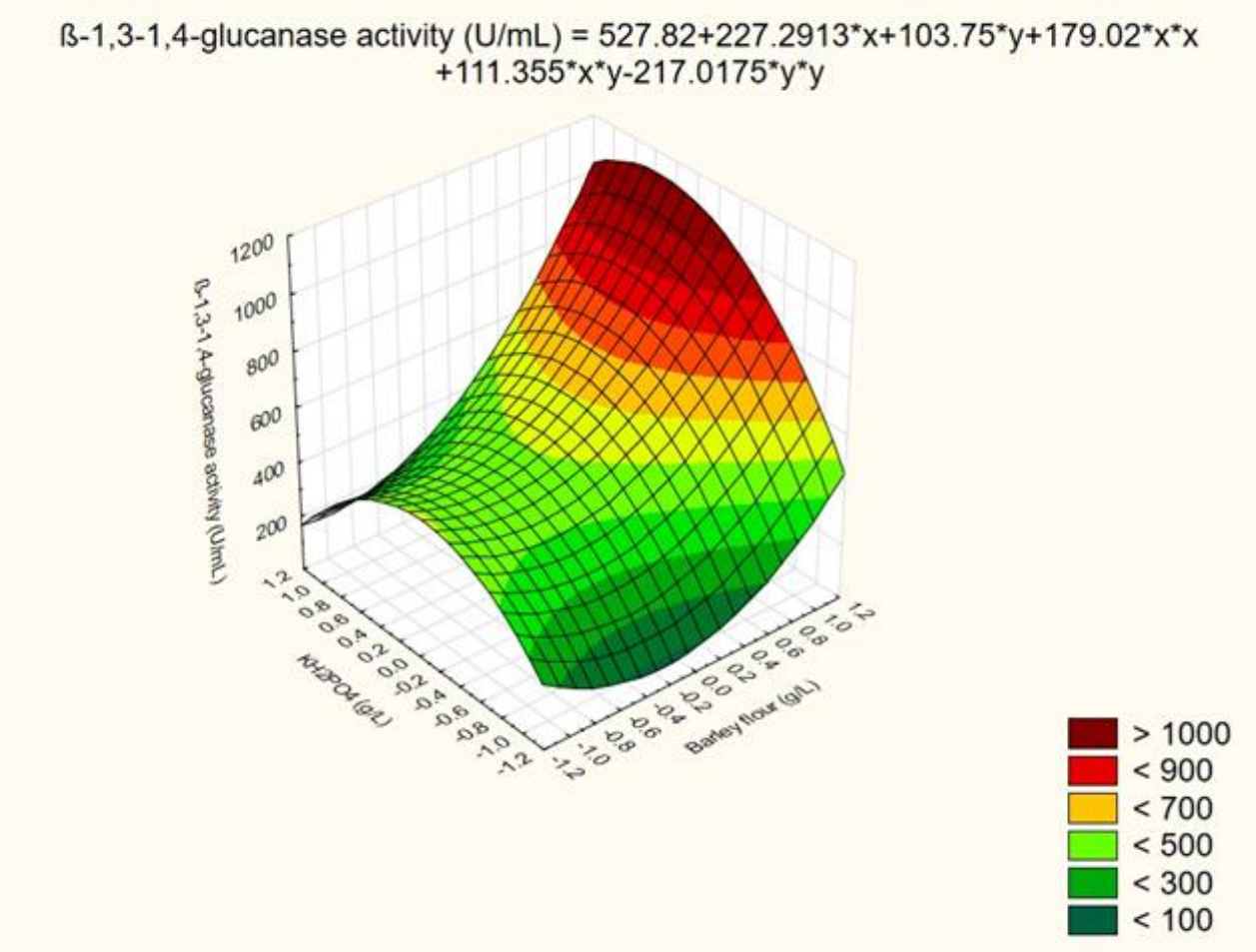

Fig.4. 3D Surface Plot demonestrating the interaction effect of ß-glucanase activity (U/mL) against barley flour concentration (g/L) and KH_2PO_4 concentration (g/L) on producing extracellular β-1,3-1,4-glucanase by *Halomonas meridiana* ES021

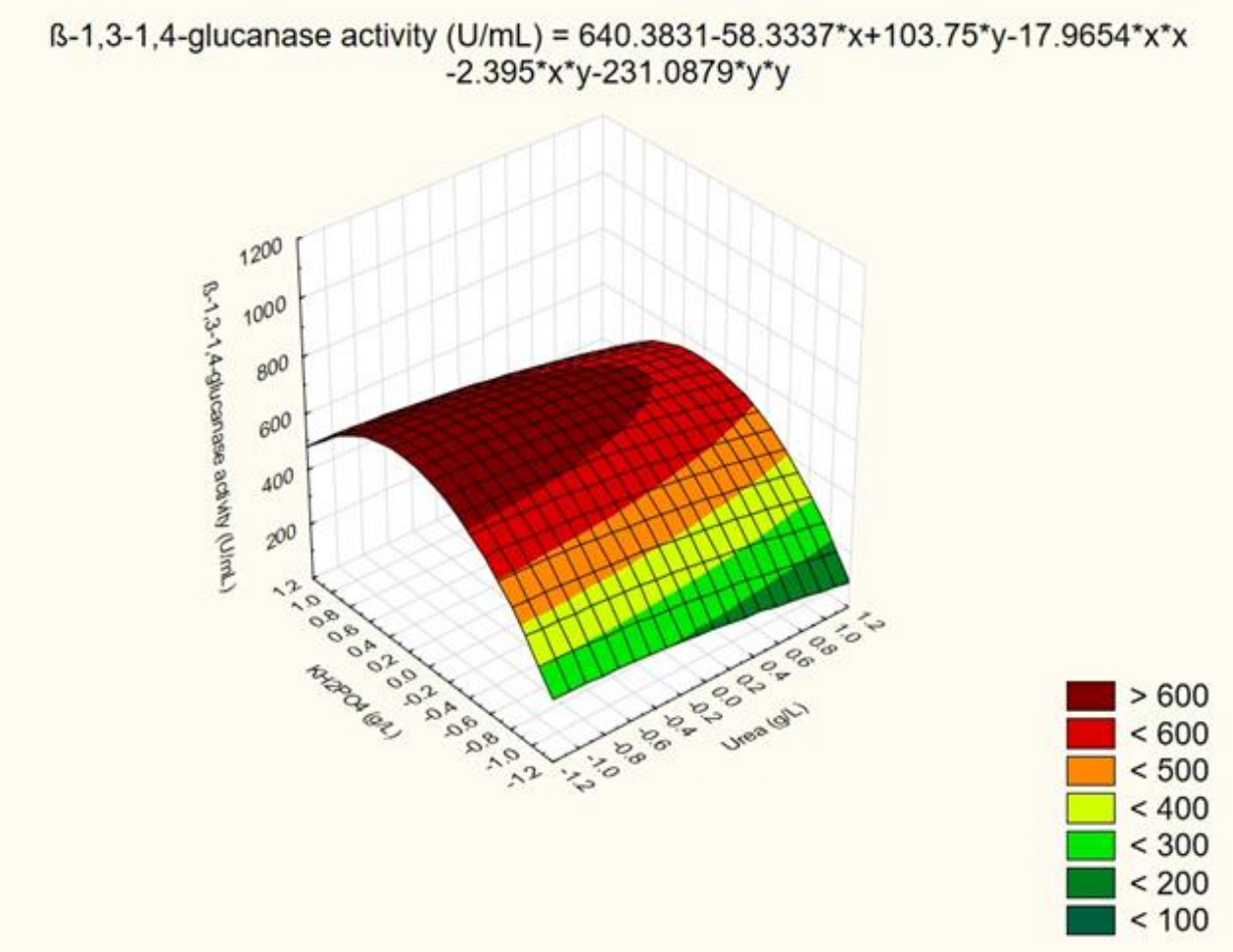

Fig. 5. 3D Surface Plot illustrating the interaction effect of ß-glucanase activity (U/mL) against urea concentration (g/L) and KH_2PO_4 concentration (g/L) on production of extracellular β-1,3-1,4-glucanase by *Halomonas meridiana* ES021

Table 9. A verification experiment for extracellular β-glucanase expressed by *Halomonas meridiana* ES021 prepared on basal and optimized media

Medium	β-1,3-1,4-glucanase activity (U/mL)	Protein content (mg/mL)
Non-optimized medium	597.50±24.50	1.03±0.06
Optimized medium	1095.50±31.00	1.78±0.11

Table 10. Wild type cell expression of β-glucanase by *Halomonas meridiana* ES021 immobilized on or in different materials

Immobilization material	β-glucanase activity(U/mL)
Free cells	1064.25±30.75
Adsorption	
Natural luffa	720.25±22.33
Synthetic luffa	0.000
Sponge	758.95±23.53
Clay	916.97±29.47
Pumice	305.30±19.47
Entrapment	
Agar	790.00±19.75
Agarose	1111.67±29.00
Ca-Alginate	783.33±19.58
Kappa-Carrageenan	671.67±16.80

3.4.1 Entrapment in different agarose gel concentrations

Different concentrations of agarose gel (from 1 to 5%) were used for the entrapment of *Halomonas meridiana* ES021 cells. Data represented graphically in Fig. (6) show that the best concentration for producing much improved activity of β-1,3-1,4-glucan hydrolase enzyme (1225.00±29.00U/ mL) was obtained at level 3%, which approximately reached about 1.08-fold of that obtained by 2% agarose (1128.75±27.75U/ mL).

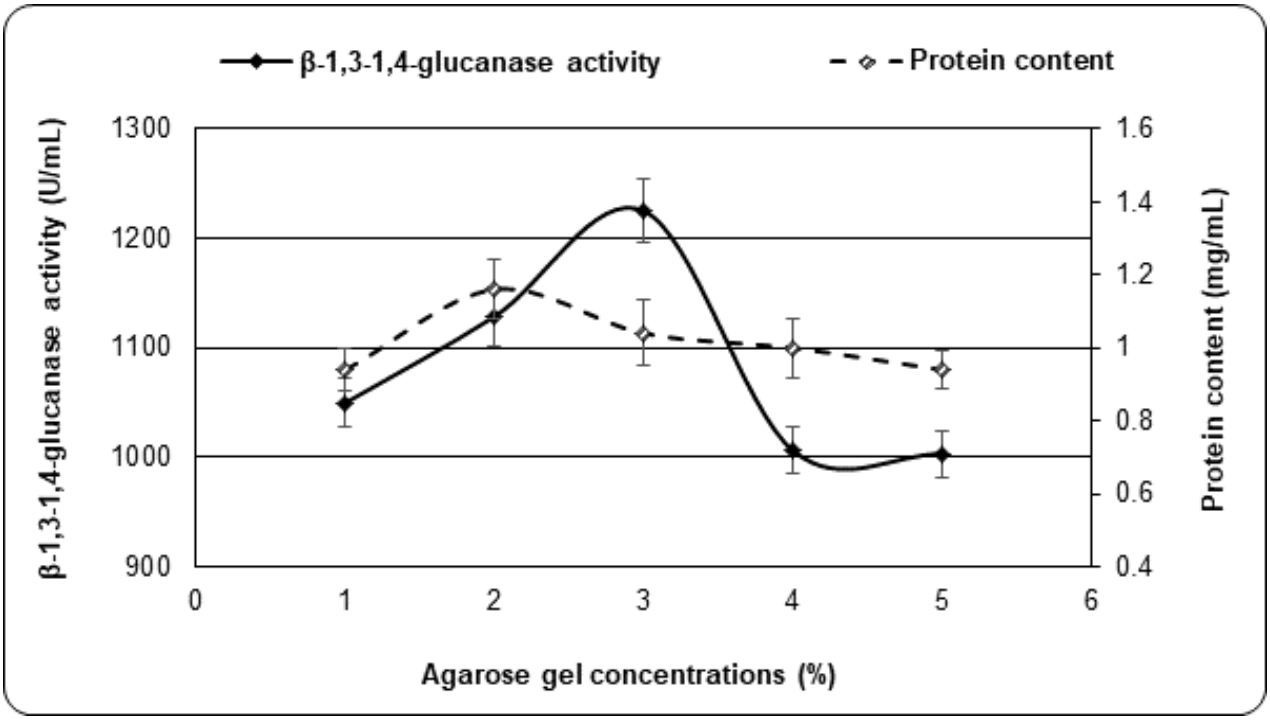

Fig.6. Effect of different agarose gel concentrations on the production of β-glucanase enzyme by entrapped *Halomonas meridiana* ES021 cells

3.5 Scanning electron microscopy observations of immobilized *Halomonas meridiana* ES021 cells

Scanning electron micrographs represented in Fig. (7a, b, c) shows some details of morphological characteristics of free cell of *Halomonas meridiana* ES021 and immobilized cells by adsorption on sponge and entrapment in 3% agarose gel. Micrographs showed a good growth with much high number of bacterial cells on sponge cubes and also penetrating the open pores. Moreover, a high number of cells entrapped in agarose gel indicates a successful immobilization of cells on or in the studied matrices. Furthermore, the morphological characteristic of *Halomonas meridiana* ES021 was illustrated as it was found to be rod shaped bacteria.

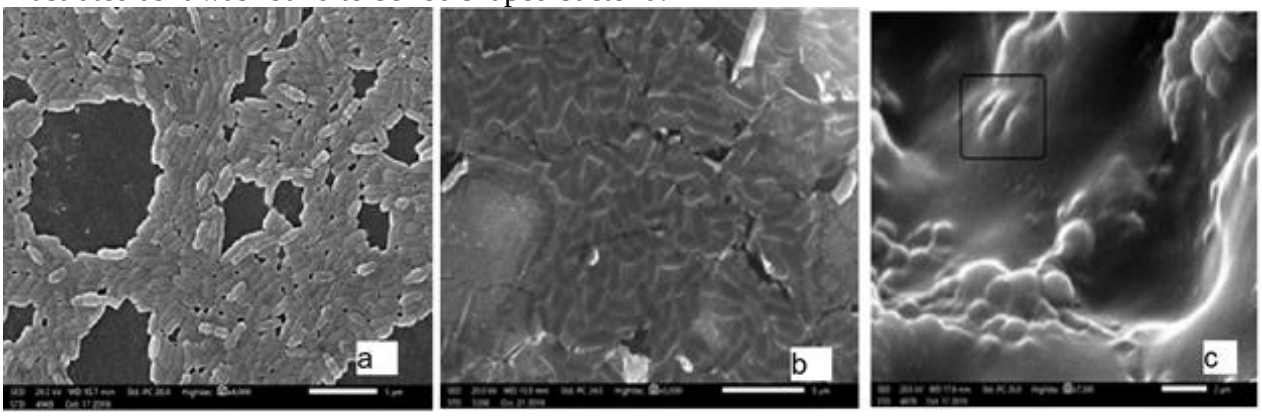

Fig.7 (a) Scanning electron micrograph of free cells of *Halomonas meridiana* ES021; (b) adsorbed on sponge; (c):entrapped in agarose gel

DISCUSSION

Many studies have reported that the enzyme systems break down substrates in marine habitat. Current research is based on the hypothesis that bacteria residing in sediments of seaweeds, which contain the storage polysaccharides laminarin, may produce an exo **β**-1,3-glucanase alongside with endo-acting enzymes to entirely breakdown the 1,3-glucan **(Nakatani *et al*., 2010)**. β-1,3-1,4-glucan hydrolase enzyme was previously discovered from marine bacteria as *Pseudoalteromonas* sp. BB1, *Saccharophagus degradans* and *Paenibacillus barengoltzii* **(Nakatani *et al*., 2012; Lafond *et al*., 2016; Zhang *et al*., 2017)** and also cloned from the marine bacterium *Zobellia galactanivorans* **(Labourel *et al*., 2014)**. However, the present work is the first (so far) to detect β-1,3-1,4-glucanase enzyme from *Halomonas* species.

Plackett-Burman design studies showed the impact of the three medium ingredients; barley flour, urea and, KH_2PO_4 on increasing the enzyme production. In a study by **Yinghua *et al*. (2007)**, Plackett-Burman statistical design showed that nitrogen-providing sources such as yeast extract was the only significant variable for β-1,3-1,4-glucanase production from the heterologous expression of *Escherichia coli*. Multifactorial statistical design as Plackett-Burman was also hired to maximize productivity for several enzymes as dextransucrase **(Abedin *et al*., 2013)**, cellulase **(Korany *et al*., 2017; Darabzadeh *et al*., 2019)**, chitinase **(Jha and Modi, 2018)**, uricase **(Pustake *et al*., 2019)** and α-amylase **(Saeed *et al*., 2021)**. In another study, a fractional factorial design (2^{6-2}) was used by **Tang *et al*. (2004)** to determine which medium components had a substantial impact on production of *Bacillus subtilis* ZJF-1A5 beta-glucanase. Consequently, concentrations of soybean flour, corn flour, and barley flour, were found to be significant parameters in the medium.

The Box–Behnken experimental design was also applied to analyse the relationship between the independent variables and the results showed that the 'Predicted R^2', which was 0.9907, and the 'Adjusted R^2', which was 0.974, were reasonably in agreement with the regression model for production of β-1,3-1,4-glucanase. This finding was comparable to that of **Guo-qing *et al*. (2003)**, who obtained a satisfactory R^2 = 0.917 value for the determination coefficient. According to the present study, barley flour was more important than urea and KH_2PO_4. Barley flour is a source of β-glucan, which was needed in adequate amounts to stimulate the synthesis of β-1,3-1,4-glucanase suggesting that the loading level of the inducer had a major impact on enzyme synthesis **(Khare and Upadhyay, 2011).** Urea is a nitrogen source that is vital in moderate amounts to promote the growth of bacteria. However, limited levels of nitrogen will cause nitrogen deficiency as a nutrient source for bacterial growth, which in turn would produce β-glucanase to breakdown β-glucan as an alternate source of nutrition **(Tang *et al*., 2004; Dewi *et al*., 2016)**. In an investigation by **Dewi *et al.* (2016)**, the model indicated that yeast extract concentration, oat β-glucan concentration, and inoculum size were significant factors affecting the production of β-glucanase.

Immobilized bacterial cells are recently used in several biotechnological applications, as bioremediation, biocontrol and producing various compounds include enzymes, antibiotics, steroids or amino acids **(Żur *et al*., 2016)**. Adsorption was found to be desirable method of immobilization because nutrients come into direct contact with immobilized cells and enhancing the efficiency of substrate conversion **(Martins *et al*.,**

2013). It is rapid, easy, low cost, effective, and no need for more adding chemicals; nevertheless, due to the weak binding force between cells and support, the adsorbed cell leaks from the carrier at a high rate while processing **(Bayat *et al*., 2015)**. In present study, different support materials namely; natural luffa, synthetic luffa, sponge, clay and pumice were used, while clay particles as a support material for adsorption of *Halomonas meridiana* ES021 cultures gave the highest β-glucanase activity among different supporting materials, however, lower than that obtained from free cultures. **Beshay *et al*. (2011)** have hired different inorganic supports like pumice, ceramic and porous sintered glass for cell immobilization in order to improve the production of β-glucanase from the recombinant *E. coli* strain. The variation in porosity and subsequent oxygen transfers, among the different matrices, which improved the enzyme productivity from immobilized cells, could be the most probable cause of the difference in enzyme activity (**Beshay *et al*., 2011).**

Halomonas meridiana ES021 cultures were also immobilized by cell entrapment using different gel materials namely; agar, agarose, Ca-alginate and kappa-carrageenan. Generally, critical factors are influencing the efficiency of various microbial metabolic pathways and the effectiveness of the entrapping processes including the diffusion of necessary nutrients, the gel chemical and physical characteristics, and the process of immobilization **(El-Borai *et al*., 2013)**. The highest *Halomonas meridiana* ES021 β-glucanase production was reported by agarose with a slight increase than free cultures. Production of cellulase by the archaeal strain *Haloarcula* 2TK2 immobilized in various polymeric supports was investigated by **Ogan *et al.* (2012),** and the results revealed that immobilization in Na-alginate beads improved the enzymatic activity compared to free cells and other supports.

CONCLUSION

In the current study, β-1,3-1,4-glucanase was produced from a local halotolerant bacterial strain isolated from marine habitat and was identified as *Halomonas meridiana* ES021. The medium used for enzyme production was optimized via Plackett-Burman and Box-Behnken statistical designs giving a significant 1.8- fold increase in the enzyme activity. The study also revealed the possibility of producing β-1,3-1,4-glucanase using immobilized *Halomonas meridiana* ES021 cells with an enhancement in enzyme production by cultures entrapped in agarose beads, and this afford for industrial application and recycling.

Compliance with Ethical Standards

Conflict of interest No conflict of interests was facing

Ethical Approval No human studies in the current work

Consent to participate: I agree to participate information and data release

Consent for publication: It is my pleasure to publish in your journal

Availability of data and material: data openly available repository that issues datasets with DOI

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