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Response Surface Methodology-Based optimization of Inulinase Production from New *Bacillus* Isolates

Sercan ÖZBEK YAZICI*[1], Selmihan ŞAHİN[2], Bahadır TÖRÜN[3], Hacı Halil BIYIK[3], İsmail ÖZMEN[2]

Abstract

This study aimed to investigate new bacterial sources with the ability to produce inulinase and to optimize fermentation conditions for inulinase production. The inulinase production was carried out using newly isolated *Bacillus licheniformis* and *Bacillus velezensis* among identified sixteen bacterial strains. The four essential variables incubation time (1-3 days), temperature (25-40°C), pH (5-7), and Wheat Bran **(**WB**)** level (1-5%) determined by the Plackett–Burman design (PB) were selected for optimization studies with Box-Benhken Design (BBD). These parameters were found to be very effective on inulinase production**.** The maximum inulinase activities for *B. licheniformis* and *B. velezensis* were 401.18 EU/mL and 344.61 EU/mL. Both inulinases sustained 50% of their initial activity at 30°C for 9 days. The results point out that bacteria are an important source as inulinase producer. The new isolates can be used in production of inulinase for industrial processes.

Keywords: Bacteria inulinase, response-surface methodology, optimization, submerged fermentation

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1. INTRODUCTION

Inulin is present in many plants and is a fructose polymer consisted of fructose residues and a glucose moiety at the terminal end. It is an essential source in the production of fructose and fructooligosaccharides, which are used in industrial areas such as food, pharmaceutical, and medical applications [1]. Moreover, fructose is 1.5 times sweeter than sucrose and it is generally considered the preferred safe sweetener in several foods and beverages. In addition, fructose has advantages such as less production costs and increased flavor, color, and product stability [2]. Fructose can be obtained from inulin by acid hydrolysis. However, that process leads to fructose degradation and low yield production. Therefore, the use of inulinases is considered to be a desirable approach to obtain high fructose syrups from inulin because of 95% pure fructose yield in a single-stage process [3, 4].

Inulinases are industrially significant enzymes, but the production cost of the enzyme continues to be a major problem in its large-scale application. Using microorganisms as potential sources for inulinase production can result in high enzyme yield and stability, which reduces the cost [5]. Although fungi such as *Aspergillis*, *Penicillium*, *Kluyveromyces sp.* are the most common microorganisms to produce inulinase, bacteria and yeast also can produce inulinase. There are previous reports on the production of inulinase from bacteria such as *Bacillus* sp., *Streptomyces* sp., *Xanthomonas* sp., *Clostridium* sp. that can grow in environmental conditions such as high temperature, alkalinity, acidity, and salinity [1]. However, there is still a need to explore microorganisms for inulinase production with high yield and superior properties. Moreover, the synthesis of the enzyme by microorganisms is dependent on growth conditions, mainly the carbon source. The type of carbon source used in the medium affects growth, sporulation, and cell wall structure in microorganisms, which in turn can affect the secretion of the enzyme [1,6]. Inulinases have different catalytic properties depending on the microbial origin such as molecular mass, optimum pH, optimum temperature, thermal and pH stabilities [7]. Therefore, the appropriate carbon source and optimal fermentation conditions should be determined to increase enzyme production. Previously, the conventional "one-factor-at-a-time" approach or statistical approaches have been used to determine optimal conditions for enzyme production. Statistical optimization facilitates determining parameters effective in enzyme production and the interactions between these parameters with lesser experiments while conventional methods are both laborious and time-consuming. Factor design and response surface analysis, which are among the current statistical methods, are considered significant tools for optimization analysis. Especially, the statistical approach using PB design and response surface methodology (RSM) have a larger and wider application for optimization due to their advantages [8, 9]

Based on all these findings, this study aims to investigate new bacterial sources with the ability to produce inulinase with high activity. For this, the fermentation conditions of these microorganisms are optimized for the production of inulinase with a low-cost substrate using statistical methods and increase the yield of enzyme production. After that, the obtained inulinases from *B.licheniformis* and *B. velezensis* were characterized by determining their optimum pH, temperature, and thermal stability.

2. MATERİALS AND METHODS

2.1. Materials

Inulin from Alfa Aesar, Sucrose from Sigma, glucose from Sigma, fructose from Merck, and 3.5-dinitrosalicylic acid from Sigma were purchased. Other chemical reagents used in experiments were analytical grade.

2.2. Identification of bacterial strains

Materials and Methods

Soil samples were collected aseptically from Buharkent, Aydın geothermal sources and brought back to laboratory. Soil samples for isolation of microbes were obtained from the 15 to 20 cm layers below soil surface.

Nutrient agar was used for all experiments concerning screening, isolation and culture of microbes. Ten grams of each soil sample was added to 90 ml of autoclaved physiologic saline water (0.85%) and homogenized with shaker (150 rpm). Ten-fold dilutions were prepared from 10^{-1} to 10^{-7} and 100 µl of these dilutions were inoculated on growth media and incubated at 55°C for 24-48 hours. Each different colony were selected from the mixed culture and inoculated and incubated at 55°C for 24-48 hours until pure cultures were obtained [10]. Each isolate were stored in 20 % Skim milk solution at -20°C.

DNA isolation of the samples were made with GeneMark Bacterial Genomic DNA purification kit according to the manufacturers instructions. After isolations DNA concentration and purity was measured with nanodrop spectrometer (Thermo Scientific). 16S universal rDNA primers were used (27F: 5'-AGA GTT TGA TCM TGG CTC AG-3', 1492R: 5'-CGG TTA CCT TGT TAC GAC TT-3') [11]. PCR reactions were carried out at initial denaturation 95ºC 5 min, denaturation 94ºC 30 sec, annealing 58ºC 30 sec, extension 72ºC 45 sec with 35 cycles and final extension at 72ºC 10 mins. Reagents concentrations were 10X Taq Buffer, 0.5M dNTP mix, 10 pM from each primer, 7.5 mM MgCl2 and 1U Taq polymerase with the final volume of 25 µl. PCR products were sent to sequencing (Atlas Biotechnology, Turkey).

Evolutionary analysis and tree construction were made with MEGA X software. The evolutionary history was inferred using the Maximum Likelihood method [12, 13, 14].

2.3. Determination of bacterial strain producing inulinase

A rapid plate screening assay was used to screen for inulinase-producing bacterial strains [14, 15]. For this, nutrient agar (NA) plates containing 1% w / v inulin were used to grow the bacterial strains, incubating at 40°C for 2 days. Lugol's iodine solution (5 mL) was added to plates and incubated for 10 min at room temperature. Afterward, the plates were washed with distilled water. The hydrolytic zone around the colony obtained is evidence of extracellular inulinase.

2.4. Submerged fermentation medium and inoculum preparation

Before starting the study, the strains were grown on NA slants at 40°C for two days.

Table 1
The activity of enzyme obtained with basal mediums selected according to the previous literature

Medium	**Enzyme activity (EU/mL)**		**Reference**
	B.licheniformis	***B. elezensis***	
1%inulin, 0.3% K_2HPO_4, 0.1% KH_2PO_4, 0.3% yeast extract, 0.3% peptone	35.21 ± 0.01	45.77 ± 0.01	[17]
0.5 % inulin, 0.3% $NaNO_3$, 0.5% $CaCl_2$,0.3% yeast extract	47.32 ± 0.09	66.32 ± 0.2	[18]
2%inulin, , 0.5% $(NH_4)_2SO_4$, 0.5% yeast extract	11.49 ± 0.03	15.78 ± 0.06	[19]
0.02%inulin, 0.04% K_2HPO_4, 0.008%$MgSO_4$ 0.2%peptone, 0.2%yeast extract	24.11 ± 0.9	45.21 ± 0.11	[20]

1% inulin, 0.1% K_2HPO_4, 0.05% $MgSO_4$ $7H_2O$, 0.015%$CaCl_2.2H_2O$, 0.05% NaCl, 0.2% yeast extract, 0.3% peptone	64.012 ± 0.7	69.23 ± 0.14	[21]
1% inulin, 0.2%KH_2PO_4, 0.05% $MgSO_4$, 0.5% $NaNO_3$	101.45 ± 0.04	89.13 ± 0.07	[22]

In 250 ml Erlenmeyer flask, a loopful of bacteria was used to inoculate nutrient broth (NB, 100 ml).After that, the flasks were incubated at 37°C and 150 rpm for one day. The optical density of NB was measured at 600 nm until it reached at 1.0. 1 mL of NB was used for inoculation of the inulinase production medium [21, 23]

In SmF where the production of enzyme takes place, the media components were determined according to the literature research (Table 1). After 100 mL of medium was prepared in 250 mL Erlenmeyer flask, the pH was adjusted to 6.5. The media was incubated at 30 ºC, and 150 rpm for 2 days for enzyme production after the medium was autoclaved. Among those tested the medium which provided the highest activity was used as medium components in SmF for enzyme production.

2.5. Optimization of inulinase production from isolates

2.5.1. Determining carbon source and fermentation parameters by PB experimental design

PB design was used to test the effect of eleven variables (carbon sources in media and fermentation parameters). For this, the effect of fructose, glucose, maltose, sucrose, lactose, onion peel, wheat bran, oat bran concentrations as carbon sources on enzyme production were evaluated, ranging from low to high. Concentrations of 0.1% and 1% levels Fermentation parameters including incubation period (1-4 days), incubation temperature (25-45 °C), and initial pH (5-8) were also tested at 2 levels as high and low. For all tests, inulinase activity was taken as a response. The variables and their levels are presented in Supplementary Table S1.

2.5.2. Optimization of fermentation parameters by using BBD

The four variables obtained from PB Design were further analyzed for optimization of inulinase production using BBD. The variables determined were incubation time (24-72 hours), incubation temperature (25-40°C), initial pH (5-7), and WB concentration (1-5%) as carbon source. For BBD analyses, three levels as high (+1), medium (0) and, low (-1) were described (Table 2).

Table 2
Levels of the factors tested in Box-Behnken Design independent variables

Symbol	Variable	Units	Coded levels		
			-1	0	1
A	Initial pH		5	6.25	7.5
B	Incubation temperature	^{0}C	25	32.5	40
C	Wheat Bran (WB)	%	1	3	5
D	Incubation time	h	24	48	72

The experimental design method was carried out by 29 experiments in duplicates. The response function coefficients were identified by regression using the experimental data and Design-Expert V7 trial version. The model equation for 4 variable systems is as follows:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD \quad (1)$$

Where Y is the predictable response (inulinase activities of *B. licheniformis* and *B. velezensis*), β_0 is the model constant; independent variables are A, B, C, and D term; β_1, β_2, β_3, and β_4 are linear

coefficients, and β_{11}, β_{22}, $\beta 3_3$, and β_{44} are the quadratic coefficient.

2.6. Enzyme assay

Activity assays were performed based on the previous study [24]. The crude enzyme was used to analyze inulinase activity. One unit of inulinase activity was the amount of enzyme that catalyzes the release of 1 µmol of fructose per minute per mL.

2.7. Effect of pH and temperature on enzyme activity

To determine the effect of pH on inulinase activity, three buffer solutions including Na-acetate (0.1 M, pH 4.0-5.0), K-phosphate (0.1 M, pH 6.0-7.0), and Tris-HCl (0.1M, pH 8.0-9.0) were used in activity assay.

The effect of temperature on the inulinase activity was determined at different temperatures between 30-60 ºC. Also, the thermal stability of enzymes was evaluated at 60ºC by the standard assay procedure after 1h incubation at different temperatures between 30-60 ºC. Enzyme activities were determined as relative activities by taking 100% of the activity at the start of the experiment.

3. RESULTS AND DISCUSSION

3.1. Identification of bacterial strains

Sixteen bacteria were isolated and identified by molecular characterization (Supplementary Table S2). From 16 samples, 1 Genus and 4 different species were found.

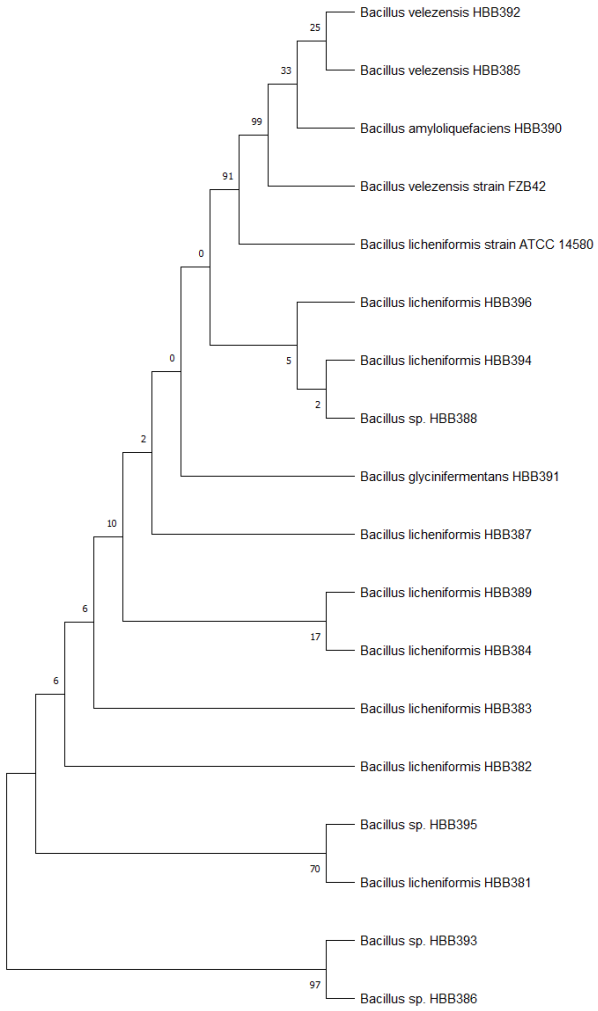

Figure 1 Phylogenetic relationships of *Bacillus* species

The evolutionary history was inferred by using the Maximum Likelihood method and Hasegawa-Kishino-Yano model (Fig. 1). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Evolutionary analyses were conducted with MEGA X software.

All species isolate were *Bacillus* species. Different strains of *Bacillus licheniformis* were abundant in soil samples. This result is similar to the results of Llarch et al [25], Balsam et al [26].

3.2. Bacteria strains selection for inulinase production and identification

The microorganism and medium selection are significant factors in determining the outcome of

inulinase production. Extracellular hydrolytic enzymes are detected by plate screening assay [16]. In this work, bacteria were screened out based on the assay of plate screening. Among the sixteen molecularly identified microorganisms, *Bacillus licheniformis* strain SCRSW1 HBB387 and *Bacillus velezensis* strain KKLV HBB392 shown a clear zone indicating the presence of extracellular inulinase on their agar plates on agar plates (Supplementary Fig. S1). Hence, the bacteria strains were used to produce inulinase through the study.

3.3. Optimization of inulinase production

3.3.1. Determination of basal medium

Micronutrients are critical for enzyme production for making significant contribution to maintain the osmotic pressure of the environment. Moreover, suitable concentrations of trace elements are reported to be essential for the substrate usage of microorganisms [27]. In previous studies, a medium consisting of different components was used for inulinase production, and enzyme production was successfully performed. In this study, the media used in the previous studies given in Table 1 were tested to obtain maximum enzyme production. Maximum inulinase activity was observed as 101.45 ± 0.04 for *B.licheniformis* and 89.13 ± 0.07 U/mL for *B. velezensis* in medium containing 0.5% $NaNO_3$, 0.2% $MgSO_4$, 0.2% KH_2PO_4, 0.4% yeast extract, 1%inulin on a rotary shaker at 30°C, 150 rpm, pH 7.0 for two days [22]. Hence, this medium was selected as a basal medium for further experiment.

The complex nitrogen sources have been reported to be better than inorganic nitrogen sources. Jain et al. [28] found that yeast extract was the best nitrogen source to conjunct with dahlia extract for inulinase production, followed by the beef extract. The lowest inulinase activities from *B. licheniformis* and *B. velezensis* when $(NH_4)_2SO_4$ was used as a nitrogen source in the fermentation medium. It can be associated with acidic conditions caused by ammonium salts because highly acidic conditions may inhibit the synthesis of inulinase [29].

3.3.2. Determination of significant variables by PB design

Various bacterial sources as inulinase producers have been reported to exploit sucrose, starch inulin, and inulin-rich plant extracts to produce inulinase. Characteristically, inulinases syntheses require an inducer. Inulin takes a function as a carbon source as well as an inducer for inulinase production [1, 30]. However, catabolic suppression may occur due to a high inulin concentration, which reduces enzyme yield [31]. In this study, 1% raw inulin was used in the production medium.

Plackett Burman design was carried out to identify factors that can affect inulinase production from bacterial sources. All cultivations were performed at 30°C under agitation (150 rpm) in a rotary shaker. Eleven variables were screened for their influence on enzyme production, and the inulinase activities are illustrated in Table 3. The maximum activities for *B. licheniformis* and *B. velezensis* were observed at run number 12 for two bacteria, with 14.41 and 34.3 EU/mL, respectively.

In the PB design, the p values of the model by ANOVA analysis show that the model is significant ($p < 0.05$). Also, the productions of inulinase from both bacteria were found to be affected by WB, incubation temperature, initial pH, and incubation time ($p<0.05$) (Supplementary Table S3). Hence, other insignificant variables were neglected, and the optimum levels of these four variables were determined by the BBD design. The effects of variables are presented in Pareto charts (Supplementary Figures S2 and S3). One of the four significant variables that screened, WB concentration, exerted a positive effect. However, incubation temperature, initial pH, and incubation time exerted negative effects on enzyme production. In another study where 11 parameters were scanned affecting inulinase production from *Arthrobacter mysorens* strain no.1 by PB, pH and temperature were not significant [32]. It has been reported that the cost of enzyme production is reduced by the use of low-cost complex substrates as a carbon source [33]. This study indicates that WB was more effective than other carbon sources. In previous studies in which inulinase production was

optimized, the effects of different carbon sources were evaluated.

Table 3
Real values of the variables in PB experimental design with values of inulinase activity

Run order	Fruktoz	Sucrose	Glucose	Maltose	Lactose	Onion peel	Oat bran	Wheat bran	Temperature	pH	Time	Experimental value for	Experimental value for *B.*
	%	%	%	%	%	%	%	%	ºC		Day	EU/mL	EU/mL
1	1.00	1.00	1.00	0.10	0.10	1.00	3.00	1.00	45.00	8.00	1.00	0.39	0.61
2	1.00	0.10	0.10	0.10	1.00	1.00	3.00	3.00	25.00	8.00	4.00	2.1	15.1
3	0.10	0.10	0.10	1.00	0.10	3.00	3.00	1.00	45.00	8.00	4.00	0.18	2.66
4	1.00	1.00	0.10	1.00	1.00	3.00	1.00	1.00	25.00	8.00	1.00	3.52	15.1
5	1.00	1.00	0.10	0.10	0.10	3.00	1.00	3.00	45.00	5.00	4.00	5.64	13.99
6	0.10	0.10	1.00	0.10	1.00	3.00	1.00	3.00	45.00	8.00	1.00	5.65	10.8
7	0.10	1.00	1.00	0.10	1.00	3.00	3.00	1.00	25.00	5.00	4.00	5.05	15.9
8	0.10	1.00	0.10	1.00	1.00	1.00	3.00	3.00	45.00	5.00	1.00	8.32	18.09
9	0.10	1.00	1.00	1.00	0.10	1.00	1.00	3.00	25.00	8.00	4.00	5.75	15.4
10	0.10	0.10	0.10	0.10	0.10	1.00	1.00	1.00	25.00	5.00	1.00	8.34	25.64
11	1.00	0.10	1.00	1.00	1.00	1.00	1.00	1.00	45.00	5.00	4.00	0.81	7.3
12	1.00	0.10	1.00	1.00	0.10	3.00	3.00	3.00	25.00	5.00	1.00	14.41	34.3

Rawat et al. [33] obtained maximum inulinase activity in medium with the dahlia extract. In another study, *Tithonia rotundifolia* was reported to be effective in inulinase production [34]**.** Trivedi et al. [35] obtained maximum inulinase production using chicory root powder (21.9 EU/mL). However, glucose, fructose, and sugarcane bagasse were reported to be poor inducers for inulinase production, 5.8 EU/mL, 5.1 EU/mL, and 4.7 EU/mL, respectively. The highest inulinase activity from *X. campestris* pv. *Phaseoli* KM 24 mutant was observed in sucrose (5.19 EU/mL) [36]. The maximal activities of *B. polymyxa* 722, *B. subtilis* 68, and *B. polymyxa* 29 were observed in the presence of sucrose, starch/molasses, and starch, respectively [37]. There are previous studies in parallel with our study, which support that WB is an important source of carbon to increases production of inulinase [31, 38]. WB contains 1–4% inulin and has a high nutritional value for microorganisms [35]. Apart from this, WB is a cheap and available substrate. All these features make them an essential resource for large scale fermentation.

3.3.3. BBD for optimization of fermentation medium

The relation between the significant variables (initial pH, incubation temperature, incubation time, and WB concentration) obtained by PB experiment was developed using BBD to produce maximum enzyme [34]. The enzyme productions from both bacteria were carried out by experimental designs and the results are shown in Table 4.

ANOVA analysis showed the significant of the model ($p < 0.0001$) (Supplementary Table S4).

The regression equations for inulinase activity responses (Y) were obtained, and these equations are given below:

$Y_{\text{Inulase activity for } B.licheniformis}$=+374.18 -22.54 *A-25.14 *B -88.56*C+26.15 *D-4.46 *A*B+ 9.08 *A *C -5.36*A*D+32.87 *B*C-16.23 *B*D-17.23*C*D-145.54 *A^2-112.43*B^2-111.8 *C^2-123.31*D^2 (2)

$Y_{\text{Inulase activity for } B.\ velezensis}$=+328.65+82.42 *A +34.98 *B+34.10 *C+15.27 *D+17.18*A*B+ 11.70*A*C+48*A*D-5.80*B*C+ 15.63 *B*D +32.58*C*D-108.8*A^2-102.57*B^2-94.23 *C^2-82.11 *D^2 (3)

The coefficients of determination (R^2) for inulinase activities of *B. licheniformis* and *B. velezensis* were calculated as 0.99.

Table 4
Experimental design-based BBD for the optimization of inulinase production

Run order	Initial pH	Incubation Temperature (^{0}C)	Incubation time (h)	WB (%)	*B.licheniformis* Inulinase activity(EU/mL)	*B. velezensis* Inulinase activity(EU/mL)
1	7.5	25.00	48.00	3.00	105.54	147.4
2	6.25	32.50	72.00	1.00	40.09	132.22
3	6.25	32.50	48.00	3.00	375.18	325.25
4	5	32.50	72.00	3.00	39.1	74.66
5	6.25	40.00	48.00	5.00	121.3	221.74
6	6.25	32.50	48.00	3.00	385.11	315.25
7	6.25	40.00	48.00	1.00	108.36	159.74
8	6.25	32.50	48.00	3.00	371.27	335.25
9	6.25	25.00	24.00	3.00	315.57	33.51
10	7.5	32.50	48.00	1.00	73.24	205.85
11	6.25	25.00	72.00	3.00	59.24	168.14
12	5	32.50	24.00	3.00	236.3	14.85
13	6.25	25.00	48.00	1.00	123.03	102.25
14	7.5	32.50	24.00	3.00	176.34	158
15	5	40.00	48.00	3.00	123.88	55.33
16	7.5	32.50	72.00	3.00	15.45	264.6
17	6.25	32.50	72.00	5.00	64.42	233.29
18	6.25	25.00	48.00	5.00	200.9	101.74
19	6.25	40.00	72.00	3.00	62.12	151.25
20	5	25.00	48.00	3.00	155.79	23.25
21	5	32.50	48.00	5.00	160.18	61.33
22	6.25	40.00	24.00	3.00	186.96	159.81
23	6.25	32.50	24.00	1.00	167.34	139.04
24	7.5	32.50	48.00	5.00	115.21	231.77
25	6.25	32.50	48.00	3.00	371.11	335.25
26	5	32.50	48.00	1.00	96.76	37.33
27	6.25	32.50	48.00	3.00	368.24	332.25
28	6.25	32.50	24.00	5.00	260.57	109.79
29	7.5	40.00	48.00	3.00	55.78	248.22

* Experimental results

The “Predicted R-Square” of 0.97 and 0.98 was in reasonable agreement with the “Adjusted R-Square” of 0.99 and 0.97 for *B. licheniformis* and *B. velezensis*, respectively. This model can be used in navigating the design space.

These variables have been shown by statistical analysis to significantly affect inulinase activity. In this case, the model F-value of 206.27 and 185.51, for *B. licheniformis* and *B. velezensis*, respectively, imply that the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values

of "Prob > F" less than 0.05 indicate model terms are significant. In this case, for *B. licheniformis*; the linear, square, and interaction coefficients for all parameters were statistically significant for all parameters, except for the interaction of pH and incubation temperature, time, and WB concentration (AB, AC, and AD). When analyzed for *B. velezensis*, significant model terms are statistically significant for all parameters, except for the interaction of pH and WB concentration (AD). The "Lack of Fit" of 0.1029 and 0.3210 for *B. licheniformis* and *B. velezensis*, respectively, implie the Lack of Fit is not significant relative to the pure error. Non-significant lack of fit is good for the model fitness (Table 5).

All parameters had the same effective impacts on enzyme production (based on the p-value). The interactions between parameters are analyzed with contour graphics. The two-dimensional contours (2D) were drawn by using BBD.

Table 5
ANOVA analyses for models obtained from the BBD

Factor	**df**	**Inulinase activity of *B.licheniformis***		**Inulinase activity of *B. velezensis***	
		Coefficient Estimate	**Prob > F**	**Coefficient Estimate**	**Prob > F**
β_0	14	374.18	< 0.0001*****	328.65	< 0.0001*****
B_1	1	-22.54	< 0.0001*****	82.42	< 0.0001*****
B_2	1	-25.14	< 0.0001*****	34.98	< 0.0001*****
B_3	1	-88.56	< 0.0001*****	34.10	< 0.0001*****
B_4	1	26.15	< 0.0001*****	15.27	0.0002*****
B_{12}	1	-4.46	0.4498	17.18	0.0054*****
B_{13}	1	9.08	0.1361	11.70	0.0420*****
B_{14}	1	-5.36	0.3660	0.48	0.9281
B_{23}	1	32.87	< 0.0001*****	-35.80	< 0.0001*****
B_{24}	1	-16.23	0.0134	15.63	0.0097*****
B_{34}	1	-17.23	0.0095	32.58	< 0.0001*****
B_{11}	1	-145.54	< 0.0001*****	-108.80	< 0.0001*****
B_{22}	1	-112.43	< 0.0001*****	-102.57	< 0.0001*****
B_{33}	1	-123.31	< 0.0001*****	-94.23	< 0.0001*****
B_{44}	1	-111.80	< 0.0001*****	-82.11	< 0.0001*****
Lack of Fit	14		0.1029		0.3210

*****Significantly different at $\mathbf{p} < 0.05$. β_0: intercept; β_1, β_2, β_3, and β_4: linear regression coefficients for pH, temperature, time, and WB; β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, and β_{34}: regression coefficients for interaction between pH and temperature, pH and time, pH and WB concentration, temperature and time, temperature and WB concentration; Time and WB concentration; β_{11}, β_{22}, β_{33} and β_{44}: quadratic regression coefficients for pH x pH, temperature x temperature, time x time, WB concentration x WB concentration.

2D contour plots enable visualizing the relationship between responses and each variable as well as the type of interactions between two test variables. The circular contour plots show the insignificant interactions between the correspondent variables; whereas an elliptical

contour plots show important interactions [32]. Therefore, the relationships between parameters for optimization of fermentation conditions are analyzed based on the results obtained through experiments and their interpretation is made based on diagrams. It was determined that enzyme productions were remarkably affected by all parameters in the model ($p < 0.05$).

The inulinase activity from *B. licheniformis* increased evidently at first as pH increase as well as incubation temperature and time also increased. However, these values decreased when the two variables ascended continually (Fig. 2).

The linear effect of the initial pH (**A**) was significant ($p< 0.0001$), and its quadratic ($\mathbf{A}^2$) had a significant **(**$p<0.05$), which is the negative effect on the enzyme activity of *B. licheniformis*. Moreover, the negative and insignificant effects of pH and incubation time, pH and incubation temperature and pH and WB concentration on inulinase activity ($p>0.05$) could be seen from Fig. 2a, c, e for the presence of circular contour, which was consistent with results of coefficients of the regression equation in Table 5. The maximum activity was obtained at pH 6.25 (385.11 EU/mL). However, Fig. 2b, c, and d demonstrate that inulinase activity was maximum at 32.5°C. Inulinase activity remarkably decreased up 36°C. It is seen that high temperature and long incubation time negatively affect enzyme production. Differently, the singular effect of WB concentration on enzyme activity was positive for its plus coefficient. The activity of inulinase was maximum between 3% and 4% WB concentrations (Fig. 2d, e, and f). Fig 2d and f graphed the effects of incubation temperature and incubation time with WB concentration on enzyme activity and their interactions. However, interactions of WB concentration with incubation temperature and incubation time had a negative effect on enzyme activity (Table 5).

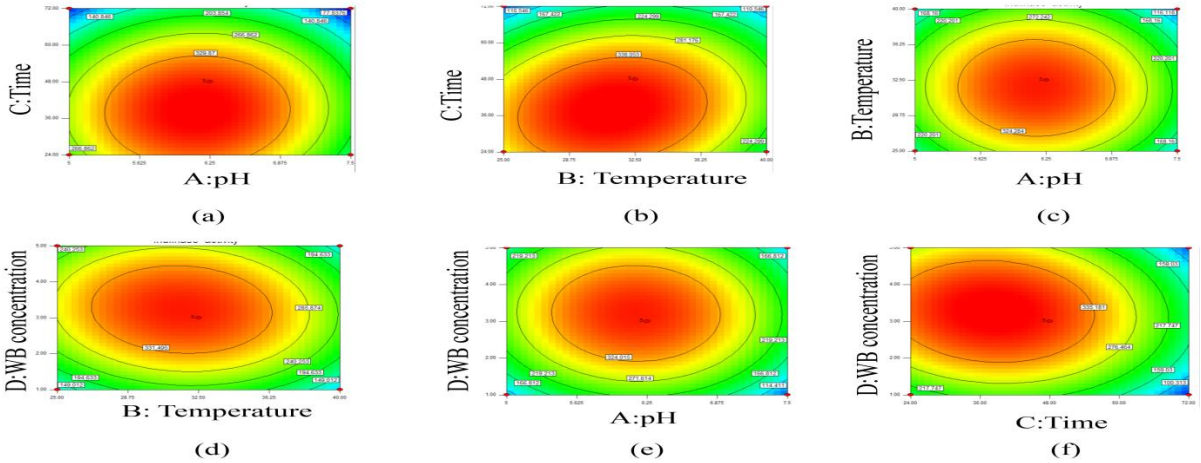

Figure 2 2-D plots of the combined effect of two variables on the inulinase activity from *B.licheniformis*

The enzyme activity was the maximum at 3% WB concentration at 32.5°C and 36h, and then exhibited a decreasing trend.

In the study, all parameters affected the enzyme production from *B. velezensis* ($p < 0.05$). However, the interaction between WB concentration and pH (AD) was insignificant ($p > 0.05$) (Table 5). Inulinase activity from *B. velezensis* was constant, almost in the range of pH 6.25-6.8. The highest activity was found at pH 6.25, like inulinase activity from *B. licheniformis* (see in Fig. 3a, c, and e). Also, Fig. 3b, c, and d illustrate that the highest inulinase activity from *B. velezensis* is obtained at ranged of 32.5°C (335.25 EU/mL). The relationship between temperature and time was negative; it could be seen that it retained its activity for a longer time at low temperatures as seen in Fig. 3b. However, the inulinase activity from *B. velezensis* decreased significantly after 60h of incubation. Furthermore, inulinase productions of both bacterial decreased significantly at higher temperatures than 36°C.

The result shows that the increase in temperature may have caused the denaturation of enzymes.

The pH and temperature of the fermentation medium in inulinase production are the most significant variables. Generally, bacterial inulinases are produced in fermentation media with pH between 4.8 and 7.0, and a temperature of 35-37°C is optimal for bacterial growth and enzyme production [30]. Zherebtsov et al. [37] suggested that *Bacillus* sp. viz. *B. polymyxa* 29, *B. polymyxa* 722, and *B. subtilis* 68 were obtained inulinase in the optimal parameters for the growth to be pH 7.0 and 33-35°C. In addition, optimal culture conditions for inulinase production from *Marinimicrobium* sp. LS-A18, were pH 7.5, 37°C, and 96 h with inulinase activity of 14.6 EU/mL [39]. According to experimental results, the maximum inulinase activities were obtained in the midpoint of the response plot as 385.11 EU/mL for *B. licheniformis*, and 335.25 EU/mL for *B. velezensis*. The findings were acquired at 48h of incubation time, 32.5°C of incubation temperature, initial pH of 6.25, and 3% (v/v) WB (Table 4).

By employing the software of Design-Expert, the optimum values of the independent variables tested for *B. licheniformis* were predicted as 6.1 of initial pH, 30.5°C of incubation temperature, 35.05h of incubation time, and 3.1% of WB concentration with 396.504 EU/mL of inulinase activity. For *B. velezensis*, the optimum values were predicted as 6.7 of pH, 33.9°C, 55.7h, and 3.59% WB concentration with 350.171 EU/mL of inulinase activity. These predictions were validated by experiments in which 401.18 EU/mL of inulinase activity for *B .licheniformis* and 344.61 EU/mL of inulinase activity for *B. velezensis.* Experimental activity results were close to the activity predictions for both bacteria. The statistical model made a strong prediction of the experimental results.

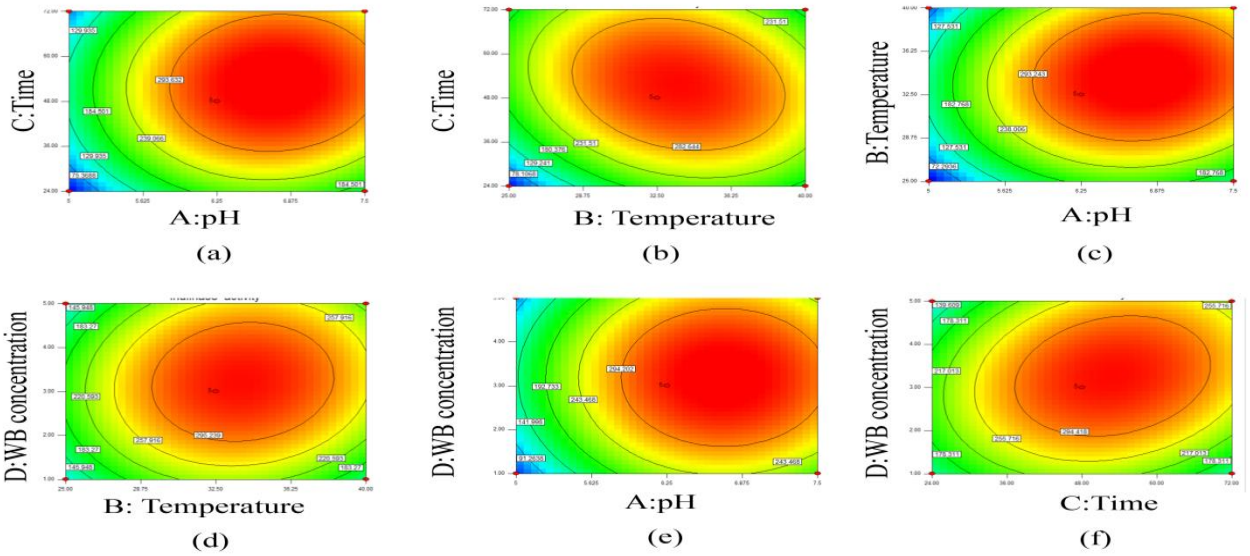

Figure 3 2-D plots of the combined effect of two variables on the inulinase-specific activity *B. velezensis*

In another study, the highest level of inulinase production (9.24 EU/mL) was observed at 35-40, pH 7.0, and medium with 3% sucrose [36]. Kamble et al. [32] tested *Tithonia rotundifolia* as a substrate for inulinase production from *Arthrobacter mysorens* and obtained the maximum inulinase activity with 1669.45 EU/mL 30^0C in the rotary shaker at 120 rpm after 48 h.

3.4. Effect of pH and temperature on enzyme activity

To date, all microbial inulinases have generally been shown to have optimum pH ranging from

3.5 to 7.0 and temperature stability in the range 10-80°C [1, 39]. As seen in Fig. 4a, the inulinase from *B. licheniformis* showed optimum activity at pH 5.0, while optimum activity at pH 6.0 was observed for inulinase from *B. velezensis*. Bacteria inulinase generally exhibit optimal activity in the pH range of 6.0-7.0, while the optimal pH ranges of 3.0-6.0 for fungal inulinases [39]. The Inulinase enzyme from *Bacillus* sp. demonstrated optimum activity at 55 °C and pH 6.5. [22] while inulinase from *Acetobacter diazotropicus* SRT4 exhibit optimal activity at pH 5.5 [40].

In order to determine the optimum temperatures of the enzymes obtained in this study, enzyme activities were tested at 30ºC-60ºC. The activities of inulinase from both bacteria were preserved at 30ºC for 120 minutes (Figure 4b).

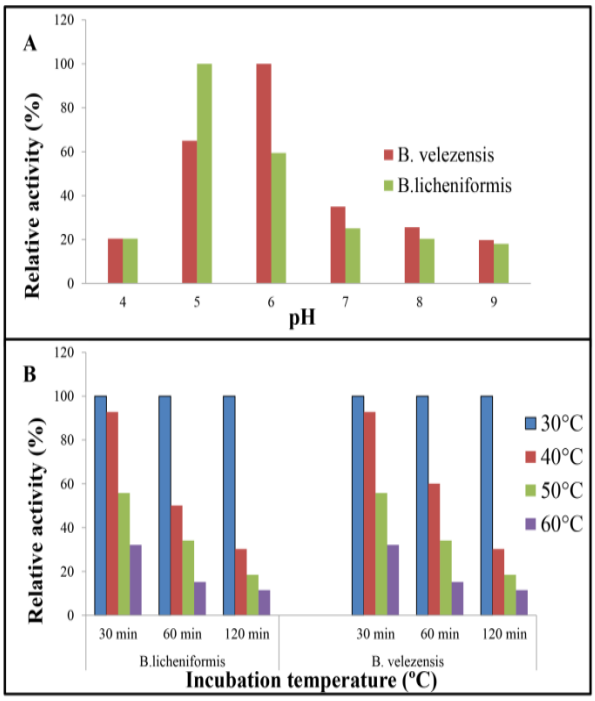

Figure 4 Effect of pH and temperature on inulinases activity from *B.licheniformis* and *B. velezensis*

Similarly, Kwon et al. [41] found the optimum temperature for the inulinase obtained from *B. polymyxa* MGL21 as 35ºC. In the study, enzyme thermostability was investigated by incubating both inulinases at 30ºC for 11 days and the remaining activity was determined. The enzymes retained 50% of their activities until 9 days, indicating that they were quite stable at 30ºC (Fig. 5). After 11 days, the inulinase from *B.licheniformis* and *B. velezensis* sustained 28% and 33% of its initial activity, respectively. This study found that both inulinases have remarkable thermostability.

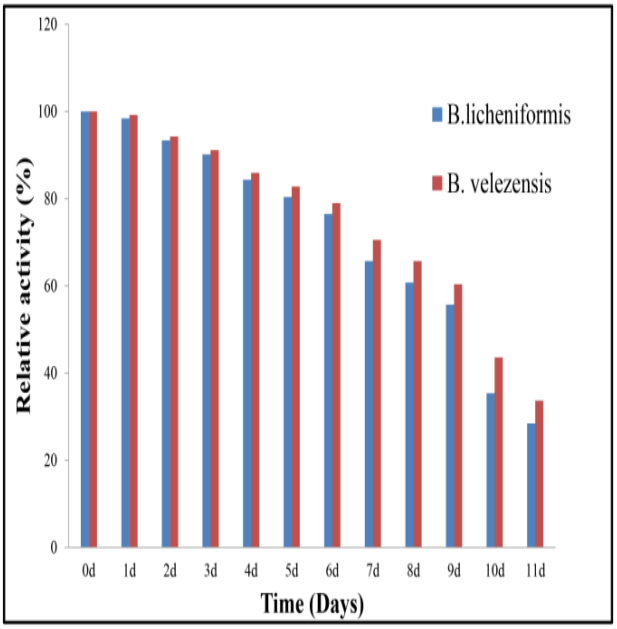

Figure 5 Thermalstability of inulinases from *B.licheniformis* and *B. velezensis* at 30°C

4. CONCLUSION

In this study, sixteen bacteria were isolated from soil samples, and molecular identifications were made. All species isolated were *Bacillus*. Among the tested microorganisms for inulinase activity, *B. licheniformis* strain SCRSW1 and *B. velezensis* strain KKLV showed a clear zone on agar plate containing 1% w/v inulin. The bacteria strains were selected to produce inulinase. Fermentation parameters and different substrates were evaluated to find the fermentation conditions supporting maximum enzyme productions by PB. WB and the fermentation parameters (pH, temperature, and time) were found to be very effective on inulinase production**.** Also, fermentation conditions were optimized by using BBD for the maximum value of inulinase for both bacteria. The maximum inulinase activities for *B. licheniformis* and *B. velezensis* were 401.18 EU/mL and 344.61

EU/mL under predicted optimum conditions by the software of Design-Expert. The obtained inulinases were found to be quite stable at 30ºC.

Moreover, optimizations were achieved successfully with high enzyme activities. The results point out that the bacteria are significant source as inulinase producers. The new isolates *B. licheniformis* and *B. velezensis* can be used to produce inulinase for industrial processes. Moreover, the highest inulinase production was obtained with WB, which is an inexpensive carbon source. It means that industrial use of WB is possible.

The Declaration of Conflict of Interest/ Common Interest

No conflict of interest or common interest has been declared by the authors.

The Declaration of Ethics Committee Approval

Ethics Committee Approval is not required.

The Declaration of Research and Publicatıon Ethics

The authors of the paper declare that they comply with the scientific, ethical and quotation rules of SAUJS in all processes of the paper and that they do not make any falsification on the data collected. In addition, they declare that Sakarya University Journal of Science and its editorial board have no responsibility for any ethical violations that may be encountered, and that this study has not been evaluated in any academic publication environment other than Sakarya University Journal of Science.

REFERENCES

[1] R. S. Singh, K. Chauhan, and J. F. A Kennedy, "Panorama of bacterial inulinases: production, purification, characterization and industrial applications," International Journal of Biological Macromolecules, vol. 96, pp. 312-322, 2017.

[2] F. Gong, T. Zhang, Z. Chi, J. Sheng, J. Li, and X. Wang, "Purification and characterization of extracellular inulinase from marine yeast *Pichia guilliermondii* and inulin hydrolysis by the purified inulinase," Biotechnology and Bioprocess Engineering, vol. 34, pp. 179-185, 2008.

[3] P. K. Gill, R. K. Manhas, and P. Singh, "Purification and properties of a heat-stable exoinulinase isoform from *Aspergillus fumigatus*," Bioresource Technology, vol. 97, no. 7, pp. 894-902, 2006.

[4] M. Germec and I. Turhan, "Partial purification and characterization of *Aspergillus niger* inulinase produced from sugar-beet molasses in the shaking incubator and stirred-tank bioreactors," International Journal of Biological Macromolecules, vol. 64, pp. 3789-3799, 2020.

[5] D. Das, M. R. Bhat, and R. Selvaraj, "Review of inulinase production using solid-state fermentation," Annals of Microbiology, vol. 69, pp. 201–209, 2019.

[6] R. A. Pessoni, M. R. Braga, and R. D. C. L. Figueiredo-Ribeiro, "Purification and properties of exo-inulinases from *Penicillium janczewskii* growing on distinct carbon sources," Mycologia, vol. 994, pp. 493-503, 2007.

[7] M. Beran, J. Pinkrová, M. Urban, and J. Drahoras, "Immobilisation of endoinulinase on polyhydroxybutyrate microfibers," Czech Journal of Food Sciences, vol. 34, pp. 541-546, 2016.

[8] S. M. Basheer, S. Chellappan, P. S. Beena, R. K. Sukumaran, K. K. Elyas, and M. Chandrasekaran, "Lipase from marine *Aspergillus awamori* BTMFW032: Production, partial purification and application in oil effluent treatment," New Biotechnology, vol. 286, pp. 627-638, 2011.

[9] Y. Zhang, X. Zhang, W. Qi, J. Xu, Z. Yuan, and Z. Wang, "Ann and Rsm Based Optimization of Cellulase Production by *Hypocrea sp* Z28 by Submerged Fermentation," Cellulose Chemistry and

Technology**,** vol. 523, no. 4, pp. 259-264, 2018.

[10] Y. Zhang, W.P. Wu, D.M. Hu, Y.Y. Su, L. Ca, "A new thermophilic species of *Myceliophthora* from China, Mycological Progress, vol. 13(1), pp.165–170, 2014.

[11] H. Jiang, H. Dong, G. Zhang, B. Yu, L.R. Chapman, M.W. Fields, "Microbial Diversity in Water and Sediment of Lake Chaka, an Athalassohaline Lake in Northwestern China," Applied and Environmental Microbiology, vol. 72(6), pp.3832-3845, 2006.

[12] M. Hasegawa, H. Kishino, and T. Yano, "Dating the human-ape split by a molecular clock of mitochondrial DNA," Journal of Molecular Evolution, vol. 22, pp. 160-174, 1985.

[13] J. Felsenstein, "Confidence limits on phylogenies, an approach using the bootstrap," Evolution, vol. 39, pp. 783-791, 1985.

[14] S. Kumar, G. Stecher, M. Li, C. Knyaz, and K. Tamura, "MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms," Molecular Biology and Evolution, vol. 35, pp. 1547-1549, 2018.

[15] J. J Allais, S. Kammoun, P. Blanc, C. Girard, and J. C. Baratti, "Isolation and characterization of bacterial strains with inulinase activity," Applied and Environmental Microbiology, vol. 52, pp. 1086-1090, 1986.

[16] X. Li, L. Z. Guo, Q. Fu, and W. D. Lu, "A simple and rapid plate assay for screening of inulin degrading microorganisms using Lugol's iodine solution," African Journal of. Biotechnology, vol. 1046, pp. 9518-9521, 2011.

[17] K. Keto, T. Araki, T. Kitamura, N. Morita, M. Moori, and Y. Suzuki, "Purification and Properties of a Thermostable Inulinase β-D-Fructan Fructohydrolase from *Bacillus stearothermophilus* KP1289," Starch-Stärke, vol. 517, pp. 253-258, 1999.

[18] E. Y. Tohamy, "Purification and characterization of exoinulinase enzyme from *Sterptomyces grisenus*," Pakistan Journal of Biological Sciences, vol. 95, pp. 911-916, 2006.

[19] W. Gao, Y. Bao, Y. Liu, X. Zhang, J. Wang, and L. An, "Characterization of thermo-stable endoinulinase from a new strain *Bacillus smithii* T7," Applied Biochemistry and Biotechnology, vol. 1573, pp. 498-506, 2009.

[20] S. Gavrailov and V. Ivanova, "Effects of nitrogen and carbon sources on the production of inulinase from strain *Bacillus sp.* SG113," Acta Scientifica Naturalis, vol. 31, pp. 69-74, 2016.

[21] S. Jeza, S. B. Maseko and J. Lin, "Purification and characterization of exo-inulinase from *Paenibacillus sp*. d9 strain," Protein Journal, vol. 37, no. 1, pp. 70-81, 2018.

[22] R. Ramapriya, A. Thirumurugan, T. Sathishkumar, and D. R. Manimaran, "Partial purification and characterization of exoinulinase produced from *Bacillus sp*.," Journal of Genetic Engineering and Biotechnology, vol. 162, pp. 363-367, 2018.

[23] Y. Suberu, I. Akande, T. Samuel, A. Lawal, and A. Olaniran, "Optimization of protease production in indigenous *Bacillus* species isolated from soil samples in Lagos, Nigeria using response surface methodology," Biocatalysis and Agricultural Biotechnology, vol. 18, 101011, 2019, https://doi.org/10.1016/j.bcab.2019.01.049.

[24] M. Ilgın, M. Germec, and I. Turhan, "Inulinase production and mathematical modeling from carob extract by using

Aspergillus niger," Biotechnology Progress, vol. 36, p. e2919, 2019.

[25] À. Llarch, N. A. Logan, J. Castellví, M. J. Prieto, and J. Guinea, "Isolation and Characterization of Thermophilic *Bacillus* spp. from Geothermal Environments on Deception Island, South Shetland Archipelago," Microbial Ecology, vol. 34(1), pp. 58-65, 1997.

[26] B. T. Mohammad, H. I. Al Daghistani, A. Jaouani, S. Abdel-Latif, and C. Kennes, "Isolation and Characterization of Thermophilic Bacteria from Jordanian Hot Springs: *Bacillus licheniformis* and *Thermomonas hydrothermalis* Isolates as Potential Producers of Thermostable Enzymes," International Journal of Microbiology, vol. 2017, pp.1-12, 2017.

[27] H. Sattar, Z. Bibi, A. Kamran, A. Aman, and S. A. U. Qader, "Degradation of complex casein polymer: Production and optimization of a novel serine metalloprotease from *Aspergillus niger* KIBGE-IB36," Biocatalysis and Agricultural Biotechnology, vol. 21, p. 101256, 2019, https://doi.org/10.1016/j.bcab.2019.101256

[28] S. Jain, P. Jain, and N. Kango, "Production of inulinase from *Kluyvermyces marxianus* using dahlia tubers extract," Brazilian Journal of Microbiology, vol. 43, no. 1, pp. 62-69, 2012.

[29] S. N. Muslim, A. M. Ali, I. A. Salman, I. M. A. L. Kadmy, and S. N. Muslim, "Detection of the optimal conditions for inulinase productivity and activity by *Acinetobacter baumannii* isolated from agricultural *rhizosphere* soil," Biological Sciences, vol. 21, pp. 1-7, 2015.

[30] R. Singh and R. Singh, "Inulinases. Current Developments in Biotechnology and Bioengineering Current Developments in Biotechnology and Bioengineering: Production, Isolation and Purification of Industrial Products," Elsevier, pp. 423-446, 2017, http://dx.doi.org/10.1016/B978-0-444-63662-1.00018-X.

[31] C. Xiong, W. Jinhua, and L. Dongsheng, "Optimization of solid-state medium for the production of inulinase by *Kluyveromyces* S120 using response surface methodology," Biochemical Engineering Journal, vol. 342, pp. 179-184, 2007.

[32] P. P. Kamble, M. V. Kore, S. A. Patil, J. P. Jadhav, and Y. C. Attar, "Statistical optimization of process parameters for inulinase production from *Tithonia* weed by *Arthrobacter mysorens* strain no.1," Journal of Microbiological Methods, vol. 149, pp. 55-66, 2018.

[33] H. K. Rawat, S. Chand Jain, and N. Kango, Production and properties of inulinase from *Penicillium sp.* NFCC 2768 grown on inulin-rich vegetal infusions," Biocatalysis and Biotransformation, vol. 331, pp. 61-68, 2015.

[34] P. P. Kamble, S. S. Suryawanshi, J. P. Jadhav, and Y.C. Attar, "Enhanced inulinase production by *Fusarium solani* JALPK from invasive weed using response surface methodology," Journal of Microbiological Methods, vol. 159, pp. 99-111, 2019.

[35] S. Trivedi, J. Divecha, and A. Shah, "Optimization of inulinase production by newly isolated *Aspergillus tubingensis* CR16 using low cost substrates," Carbohydrate Polymers, vol. 901, pp. 483-490, 2012.

[36] K. Naidoo, M. Ayyachamy, K. Permaul, and S. Singh, "Enhanced fructooligosaccharides and inulinase production by a *Xanthomonas campestris* pv.phaseoli KM 24 mutant," Bioprocess and Biosystems Engineering, vol. 325, pp. 689-695, 2009.

[37] N. A. Zherebtsov, S. A. Shelamova, and I. N. Abramova, "Biosynthesis of inulinases

by *Bacillus* bacteria," Applied Biochemistry and Microbiology, vol. 386, pp. 544-548, 2002.

[38] A. A. Onilude, I. F. Fadaunsi, and E. O. Garuba, "Inulinase production by *Saccharomyces sp.* in solid state fermentation using wheat bran as substrate," Annals Microbiology, vol. 622, pp. 843-848, 2012.

[39] X. Li, L. Z. Guo, and W. D. Lu, "Alkaline inulinase production by a newly isolated bacterium *Marinimicrobium sp.* LS–A18 and inulin hydrolysis by the enzyme," World Journal of Microbiology and Biotechnology, vol. 281, pp. 81-89, 2012.

[40] Y. Tambara, J. V. Hormaza, C. Perez, A. Leon, J. Arrieta, and L. Hernandez, "Structural analysis and optimized production of fructo-olgiosaccharides by levansucrase from *Acetobacter diazotrophicus* SRT4," Biotechnology Letter, vol. 21, pp. 117–121, 1999.

[41] H. J. Kwon, S. J. Jeon, D. J. You, K. H. Kim, Y. K. Jeong, Y. H. Kim, Y. M. Kim, and B. W. Kim, "Cloning and characterization of an exoinulinase from *Bacillus polymyxa,"* Biotechnology Letter, vol. 25, pp. 155-159, 2003.