OPTIMIZATION OF α-AMYLASE SYNTHESIS BY *BACILLUS VELEZENSIS* USING TAGUCHI EXPERIMENTAL DESIGN

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ABSTRACT

This research employs the Taguchi experimental design to optimize the production of α-amylase, a versatile enzyme with diverse industrial applications, using *Bacillus velezensis*. Thirteen key parameters, including pH, temperature, agitation, inoculum size, aeration and various carbon and nitrogen sources, were systematically investigated using the Taguchi L27 (313) orthogonal array. Initial screening of six seed media formulations identified SM6 (0.5% peptone, 0.5% yeast extract, 0.1% K_2HPO_4) as the optimal medium, producing 2.8 U mL^{-1} of α-amylase. Further optimization under the Taguchi framework yielded a maximum α-amylase activity of 1097.31 U mL^{-1} and total protein of 1230 mg mL^{-1} at pH 5, 34°C, 4% moong husk as carbon source, and 2% soybean cake as nitrogen source. Two-factor interaction analysis revealed that a temperature of 34°C with 2% soybean cake, and 4% moong husk with 2% soybean cake, provided the best results. Validation experiments confirmed a 31.2% enhancement in α-amylase production under the optimized conditions. This study highlights the efficacy of the Taguchi design in systematically optimizing complex bioprocesses, paving the way for improved α-amylase synthesis with *B. velezensis*.

Keywords: α-amylase, *Bacillus velezensis*, Optimization, Taguchi design, Interaction analysis, Bioprocess

INTRODUCTION

Enzymes are indispensable in various industrial processes due to their remarkable attributes of specificity, efficiency, and environmental friendliness. Key sectors like food, textiles, pharmaceuticals, and biofuels extensively harness the catalytic prowess of enzymes for diverse applications. Among these enzymes, α-amylase holds a prominent position as it catalyzes the hydrolysis of starch into maltose and other oligosaccharides, finding utility in food production, brewing, textiles, and pharmaceuticals[1]. Its unique capacity to modify starch polymers makes it indispensable across industries. Conventional optimization approaches typically entail altering one parameter at a time while keeping others constant[2]. However, such methods inadequately account for the intricate interplay among multiple variables, limiting their efficacy in achieving true optimization. In response to this challenge, Taguchi's experimental design emerges as a systematic methodology that efficiently explores parameter spaces using orthogonal arrays. It minimizes the number of required experiments while capturing the effects of variable interactions. Taguchi's design has garnered acclaim across various domains, including engineering and bioprocess optimization. *Bacillus velezensis*, a Gram-positive bacterium, has earned recognition for its potential in α-amylase production. Its versatile substrate utilization makes it an intriguing candidate for enzyme synthesis. Previous research has spotlighted the versatility of *B. velezensis* in diverse biotechnological applications.

The principal aim of this study is to optimize α-amylase synthesis through *B. velezensis* employing Taguchi's experimental design[3]. The study endeavors to ascertain the optimal conditions for maximizing α-amylase activity via a meticulous exploration of various parameters. Furthermore, the research seeks to juxtapose the outcomes of Taguchi design with traditional optimization approaches, underscoring the benefits and insights conferred by this innovative methodology. The significance of this study lies in its contributions to the realm of enzyme bioprocess optimization. Harnessing the Taguchi experimental design, this investigation aspires to elevate the efficiency and yield of α-amylase synthesis[4]. The findings are poised to provide invaluable insights into the

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Table I: Chemical media production of α-amylase from *B. velezensis* MTCC13097

Media	Carbon source (%)	Nitrogen source (%)	Other components (%)
Luria-Bertani (LB) Starch Medium	Starch - 2	Tryptone - 1	Yeast extract - 0.5 NaCl - 1
SM1	Starch - 3	$NaNO_3$ – 1.0	K_2HPO_4 - 0.15 $MgSO_4$ - 0.05 $FeSO_4$ - 0.05 NaCl - 0.2
SM2	Starch - 2	$NaNO_3$- 0.5	K_2HPO_4 - 0.3 $MgSO_4$ - 0.1 $FeSO_4$ - 0.05 NaCl - 0.2
SM3	Starch - 2	NH_4NO_3- 1.0	K_2HPO_4 - 0.3 $MgSO_4$ - 0.1 $FeSO_4$ - 0.01 NaCl - 0.2
SM4	Starch - 1	NH_4NO_3- 0.5	K_2HPO_4 - 0.15 $MgSO_4$ - 0.1 $FeSO_4$ - 0.01 NaCl - 0.2
SM5	Starch - 0.5	NH_4NO_3- 0.1	K_2HPO_4 - 0.1 $MgSO_4$ - 0.01 $FeCl_3$ - 0.005 $CaCl_2$ - 0.002
SM6	Starch - 2	NH_4NO_3 - 0.7	K_2HPO_4 - 0.1 $MgSO_4$ - 0.01 $FeCl_3$ - 0.005 $CaCl_2$ - 0.002
Control	Starch- 0.5	Broth 0.8	
Seed medium (SM6 -optimum seed media) (Inoculum development)	Starch - 2	NH_4NO_3- 0.7	K_2HPO_4 - 0.1 $MgSO_4$ - 0.01 $FeCl_3$ - 0.005 $CaCl_2$ - 0.002
Basal medium (Defined medium)	Starch - 0.5	Peptone - 2	K_2HPO_4 - 0.3 $MgSO_4$ - 0.1
Production medium (Scale fermentation)	Moong husk - 3-5 Fructose - 1-2	Soybean cake - 1-3 NH_4NO_3-0.3-0.7	K_2HPO_4 - 0.2-0.4 $MgSO_4$ - 0.05-0.2 NaCl - 0.1-0.3

influence of multiple parameters and their interactions on enzyme production, thus furnishing a robust framework for optimizing other bioprocesses[5]. This research, exclusively focused on optimizing α-amylase synthesis via *B. velezensis* using Taguchi's experimental design, may not encompass every potential variable. Consequently, while the study aims to furnish a comprehensive understanding of factors influencing enzyme production, its results and conclusions will pertain specifically to the chosen microorganism and experimental conditions.

MATERIALS AND METHODS

Chemicals and microorganisms

Chemicals: All chemicals used in this study, as shown in Table I, were purchased from Hi Media Laboratories Private Limited.

Microorganism: Five *B. velezensis* strains (MTCC13097, MTCC13098, MTCC13099, MTCC13100, MTCC13101) were obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India. MTCC is a recognized global collection of authenticated microbial cultures.

Strain selection: The five *B. velezensis* strains were screened for their natural amylase-producing ability under identical fermentation conditions in Erlenmeyer flasks containing 100 mL production medium. After 72 h of incubation at 35°C and 125 rpm, cultures were harvested and amylase activity was qualitatively assessed using an iodine-based starch hydrolysis assay to detect zones of starch clearance, with larger zones indicating higher amylase activity. Strain MTCC13097 demonstrated the largest zone of hydrolysis and was therefore selected for further experiments[6].

A single colony of the selected strain MTCC13097 is purified by isolating from the stock culture and subculturing repeatedly on nutrient agar plates and kept at 4°C as slant cultures. Biochemical tests like gram staining and catalase activity were performed on the isolated colony for identification and to confirm its high amylase-producing capacity. The pure culture was maintained by periodic sub-culturing and slanting on nutrient agar to ensure no genetic or phenotypic changes occurred in the culture during the study period[7].

Submerged fermentation

The cultivation process involved the optimization of seed and production media to promote growth of *B. velezensis* MTCC13097. The seed medium was optimized to ensure a high density of viable cells for subsequent fermentation. Different carbon and nitrogen sources were evaluated in six seed media (SM1-SM6), as shown in Table II. *B. velezensis* MTCC13097 was inoculated in each medium and incubated at 35°C, 150 rpm for 72 h. Optical density at 600nm (OD600) and α-amylase activity were measured periodically. Analysis of variance (ANOVA) determined which formulations resulted in statistically significant ($p<0.05$) differences in growth and enzyme production. The selected optimized seed medium supported the highest growth and activity. The basal medium was designed to provide a foundation for submerged fermentation. Its composition was carefully selected to maintain bacterial growth and enable enzyme synthesis. The optimized seed medium was used to inoculate the basal production medium containing 0.5% starch as the carbon source and 2% peptone, 0.1% $MgSO_4$, and 0.3% K_2HPO_4 as nitrogen sources. Amylase activity was measured periodically up to 120 h using the DNS method. ANOVA determined if the activity was significantly different ($p<0.05$) between the optimized seed medium and control lacking carbon/nitrogen sources over 120 h. Different carbon and nitrogen sources were evaluated at the seed and production stages to determine

Table II: Taguchi's experimental design for production of α-amylase from *B. velezensis* MTCC13097

Factor	Level 1	Level 2	Level 3
A-pH	4.000	5.000	6.000
B-Temp	32.000	34.000	36.000
C-Agitation	100.000	125.000	150.000
D-Inoculum size	1.500	2.000	2.500
E-Aeration	1.500	2.000	2.500
F-Carbon substrate	3.000	4.000	5.000
G-Nitrogen substrate	1.000	2.000	3.000
H-K_2HPO_4	0.200	0.300	0.400
J-$MgSO_4$	0.050	0.125	0.200
K-NaCl	0.100	0.200	0.300
L-Incubation	40.000	60.000	80.000
M-Fructose	1.000	1.500	2.000
N-$NaNO_3$	0.300	0.500	0.700

Table III: $L_{27}(3^{13})$ Orthogonal array of Taguchi experimental design for α-amylase production by *B. velezensis* MTCC13097

Run	A	B	C	D	E	F	G	H	J	K	L	M	N	Enzyme activity	Predicted enzyme activity	Total protein	Predicted total protein
#	#	°C	RPM	%	LPM	%	%	%	%	%	h	%	%	U mL^{-1}	U mL^{-1}	mg	mg
1	6.0	36.0	125.0	1.5	2.0	3.0	3.0	0.2	0.2	0.2	80.0	1.5	0.3	998.3	998.3	1119.4	1119.4
2	4.0	34.0	125.0	2.0	2.0	4.0	2.0	0.4	0.2	0.3	40.0	1.0	0.3	1036.8	1036.8	1162.6	1162.6
3	5.0	36.0	100.0	2.0	1.5	4.0	3.0	0.4	0.1	0.2	60.0	2.0	0.3	1024.5	1024.5	1148.8	1148.8
4	5.0	36.0	100.0	2.0	2.0	5.0	1.0	0.2	0.1	0.3	80.0	1.0	0.5	1003.3	1003.3	1125.0	1125.1
5	4.0	36.0	150.0	2.5	2.0	4.0	2.0	0.2	0.1	0.1	80.0	2.0	0.7	966.5	966.5	1083.8	1083.8
6	5.0	34.0	150.0	1.5	2.0	5.0	1.0	0.4	0.1	0.2	40.0	1.5	0.7	1031.2	1031.2	1156.3	1156.3
7	6.0	32.0	150.0	2.0	2.5	4.0	1.0	0.4	0.1	0.1	80.0	1.5	0.3	995.0	995.0	1115.7	1115.7
8	6.0	32.0	150.0	2.0	2.0	3.0	3.0	0.3	0.1	0.3	60.0	1.0	0.7	1010.8	1010.8	1133.5	1133.5
9	6.0	36.0	125.0	1.5	1.5	5.0	2.0	0.4	0.1	0.1	60.0	1.0	0.7	1021.1	1021.1	1145.0	1145.0
10	4.0	34.0	125.0	2.0	2.5	5.0	3.0	0.2	0.1	0.1	60.0	1.5	0.5	953.1	953.1	1068.8	1068.7
11	4.0	32.0	100.0	1.5	1.5	3.0	1.0	0.2	0.1	0.1	40.0	1.0	0.3	920.9	920.9	1032.6	1032.7
12	6.0	32.0	150.0	2.0	1.5	5.0	2.0	0.2	0.2	0.2	40.0	2.0	0.5	1035.7	1035.7	1161.4	1161.4
13	5.0	32.0	125.0	2.5	2.0	5.0	1.0	0.3	0.2	0.1	60.0	2.0	0.3	979.3	979.3	1098.1	1098.1
14	5.0	34.0	150.0	1.5	2.5	3.0	2.0	0.2	0.1	0.3	60.0	2.0	0.3	1012.6	1012.6	1135.5	1135.5
15	6.0	34.0	100.0	2.5	2.0	3.0	3.0	0.4	0.1	0.1	40.0	2.0	0.5	1006.2	1006.2	1128.3	1128.3
16	4.0	32.0	100.0	1.5	2.0	4.0	2.0	0.3	0.1	0.2	60.0	1.5	0.5	1092.9	1092.9	1225.5	1225.5
17	4.0	34.0	125.0	2.0	1.5	3.0	1.0	0.3	0.1	0.2	80.0	2.0	0.7	1049.4	1049.4	1176.7	1176.7
18	5.0	34.0	150.0	1.5	1.5	4.0	3.0	0.3	0.2	0.1	80.0	1.0	0.5	1003.9	1003.9	1125.7	1125.7
19	5.0	32.0	125.0	2.5	2.5	3.0	2.0	0.4	0.1	0.2	80.0	1.0	0.5	1055.9	1055.9	1184.0	1184.0
20	6.0	36.0	125.0	1.5	2.5	4.0	1.0	0.3	0.1	0.3	40.0	2.0	0.5	1023.7	1023.7	1147.9	1147.9
21	4.0	36.0	150.0	2.5	2.5	5.0	3.0	0.3	0.1	0.2	40.0	1.0	0.3	1040.6	1040.6	1166.9	1166.9
22	4.0	36.0	150.0	2.5	1.5	3.0	1.0	0.4	0.2	0.3	60.0	1.5	0.5	1029.0	1029.0	1153.9	1153.9
23	6.0	34.0	100.0	2.5	1.5	5.0	2.0	0.3	0.1	0.3	80.0	1.5	0.3	1032.9	1032.9	1158.2	1158.2
24	5.0	36.0	100.0	2.0	2.5	3.0	2.0	0.3	0.2	0.1	40.0	1.5	0.7	1016.7	1016.7	1140.1	1140.1
25	4.0	32.0	100.0	1.5	2.5	5.0	3.0	0.4	0.2	0.3	80.0	2.0	0.7	1020.7	1020.7	1144.6	1144.6
26	5.0	32.0	125.0	2.5	1.5	4.0	3.0	0.2	0.1	0.3	40.0	1.5	0.7	1007.9	1007.9	1130.2	1130.2
27	6.0	34.0	100.0	2.5	2.5	4.0	1.0	0.2	0.2	0.2	60.0	1.0	0.7	1007.1	1007.1	1129.3	1129.3

Table IV: ANOVA for α-amylase activity and total protein by *B. velezensis* MTCC13097

ANOVA for enzyme (α-amylase) activity by *B. velezensis* MTCC13097					
Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**
Model	28934.62	23	1259.3	1.31E+06	<0.0001*
A-pH	40.87	2	20.44	18443.59	<0.0001*
B-Temp	11.48	2	5.74	5179.47	<0.0001*
C-Agitation	5.32	2	2.66	240.51	<0.05*
D-Inoculum size	3.21	2	1.61	145.32	<0.05*
E-Aeration	4.17	2	2.09	188.41	<0.05*
F-Carbon substrate	199.23	2	99.62	89905.92	<0.0001*
G-Nitrogen substrate	3581.67	2	1790.83	1.62E+06	<0.0001*
H-K_2HPO_4	8110.79	2	4055.4	3.66E+06	<0.0001*
J-$MgSO_4$	2439.03	2	1219.52	1.10E+06	<0.0001*
K-NaCl	12880.88	2	6440.44	5.81E+06	<0.0001*
L-Incubation	6.42	2	3.21	2898.06	<0.0001*
M-Fructose	185.56	2	92.78	83736.06	<0.0001*
N-$NaNO_3$	1478.69	2	739.34	6.67E+05	<0.0001*
Residual	0.0066	6	0.0011	undefined	undefined
Cor Total	28934.62	26	undefined	undefined	undefined
Model	28934.62	23	1259.3	1.31E+06	<0.0001*
ANOVA for Total protein (α-amylase) by *B. velezensis* MTCC13097					
Model	36383.21	23	1819.16	1.34E+06	<0.0001*
A-pH	51.37	2	25.69	18954.4	<0.0001*
B-Temp	14.43	2	7.21	5322.13	<0.0001*
C-Agitation	5.32	2	2.66	240.51	<0.05*
D-Inoculum size	3.21	2	1.61	145.32	<0.05*
E-Aeration	4.17	2	2.09	188.41	<0.05*
F-Carbon substrate	250.55	2	125.28	92440.59	<0.0001*
G-Nitrogen substrate	4503.75	2	2251.88	1.66E+06	<0.0001*
H-K_2HPO_4	10198.66	2	5099.33	3.76E+06	<0.0001*
J-$MgSO_4$	3066.96	2	1533.48	1.13E+06	<0.0001*
K-NaCl	16196.66	2	8098.33	5.98E+06	<0.0001*
L-Incubation	8.08	2	4.04	2981.41	<0.0001*
M-Fructose	233.36	2	116.68	86097.19	<0.0001*
N-$NaNO_3$	1859.39	2	929.7	6.86E+05	<0.0001*
Residual	0.0081	6	0.0014	undefined	undefined
Cor Total	36383.22	26	*Significant terms.	undefined	undefined

**Significant terms.*

Table V: Effect of various physicochemical parameters on α-amylase activity and protein concentration by *B. velezensis* MTCC13097

Factor Name	Enzyme activity (U mL^{-1})			Total protein (mg)		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
A-pH	1092.9	1095.7	1095.2	1225.5	1228.7	1228.1
B-Temp	1092.9	1094.5	1093.4	1225.5	1227.3	1226.1
C-Agitation	1092.9	1092.9	1092.9	1225.5	1225.5	1225.5
D-Inoculum size	1092.9	1092.9	1092.9	1225.5	1225.5	1225.5
E-Aeration	1092.9	1092.9	1092.9	1225.5	1225.5	1225.5
F-Carbon substrate	1086.4	1092.9	1088.4	1225.5	1225.5	1225.5
G-Nitrogen substrate	1067.1	1092.9	1070.1	1196.6	1225.5	1200.0
H-K_2HPO_4	1054.6	1092.9	1089.6	1182.6	1225.5	1221.8
J-$MgSO_4$	1069.6	1092.9	1081.6	1199.4	1225.5	1212.9
K-NaCl	1040.4	1092.9	1075.4	1166.6	1225.5	1205.9
L-Incubation	1091.7	1092.9	1092.4	1224.2	1225.5	1225.0
M-Fructose	1086.6	1092.9	1088.7	1218.5	1225.5	1220.8
N-$NaNO_3$	1074.8	1092.9	1084.9	1205.3	1225.5	1216.5

Table VI: Analysis of the confirmation results for maximum enzymatic activity and total protein for α-amylase production by *B. velezensis* MTCC13097

Solution 1 of 100 Response	Predicted mean	Predicted median	Observed	Std Dev	n	SE Pred	95% PI low	Data mean	95% PI high
Enzyme activity (U mL^{-1})	1097.3	1097.3	1097.0	0.03	7.0	0.0	1097.2	1097.3	1097.4
Total protein (mg)	1230.5	1230.5	1230.0	0.04	7.0	0.0	1230.4	1230.4	1230.5

Two-sided confidence = 95%

the most suitable substrates for growth and enzyme production by *B. velezensis* MTCC13097 at each stage. The organism was able to utilize the varying substrates at different stages of cultivation[8].

Enzyme assay

The 3, 5-dinitro salicylic acid (DNSA) test was employed to quantify α-amylase activity. This colorimetric assay enabled the measurement of enzymatic hydrolysis of starch and the subsequent release of reducing sugars. Protein concentration was estimated using the Lowry method, providing valuable information about the enzyme concentration in the crude extract[9].

Taguchi experimental design

Taguchi's experimental design, as shown in Table II, utilized an orthogonal array, as shown in Table III, L_{27} (3^{13}) to systematically explore the effects of multiple parameters on α-amylase synthesis. This design enabled the efficient investigation of various factors with a minimal number of experiments Signal-to-noise (S/N) ratios were calculated for each factor level combination to assess the impact of different parameters on α-amylase activity. The larger-is-better and smaller-is-preferable principles were employed to determine the optimal conditions. The experimental data were analyzed using analysis of variance (ANOVA) to determine the significance of each

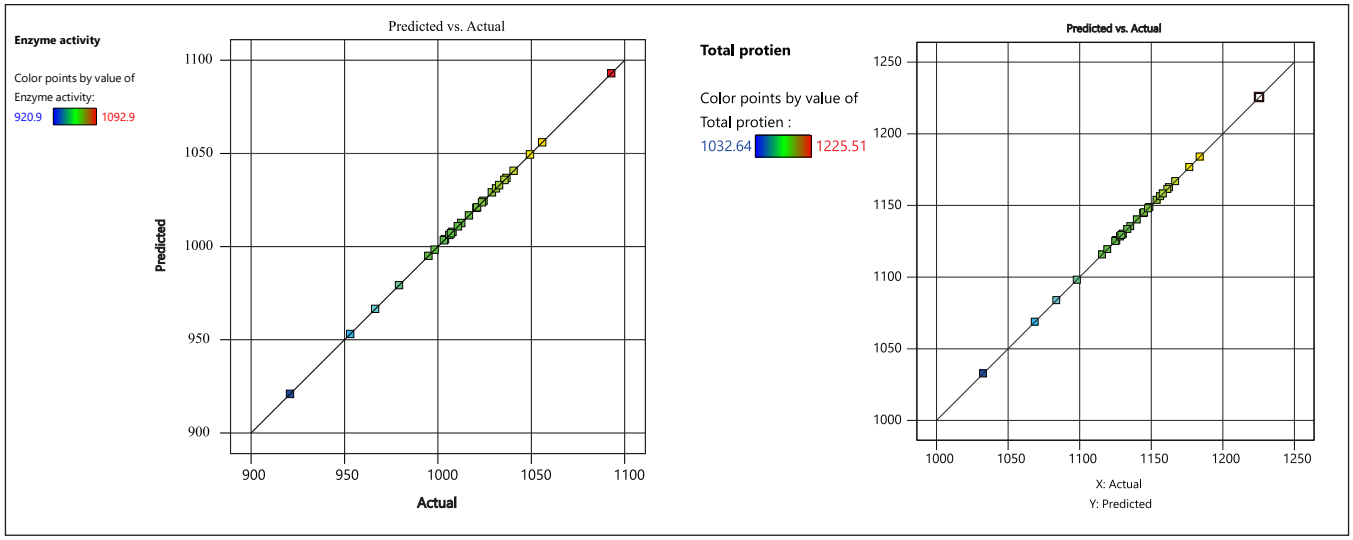

Fig. 1: Predicted vs actual enzymatic activities and total proteins for α-amylase production by *B. velezensis* MTCC13097 in Taguchi experimental design

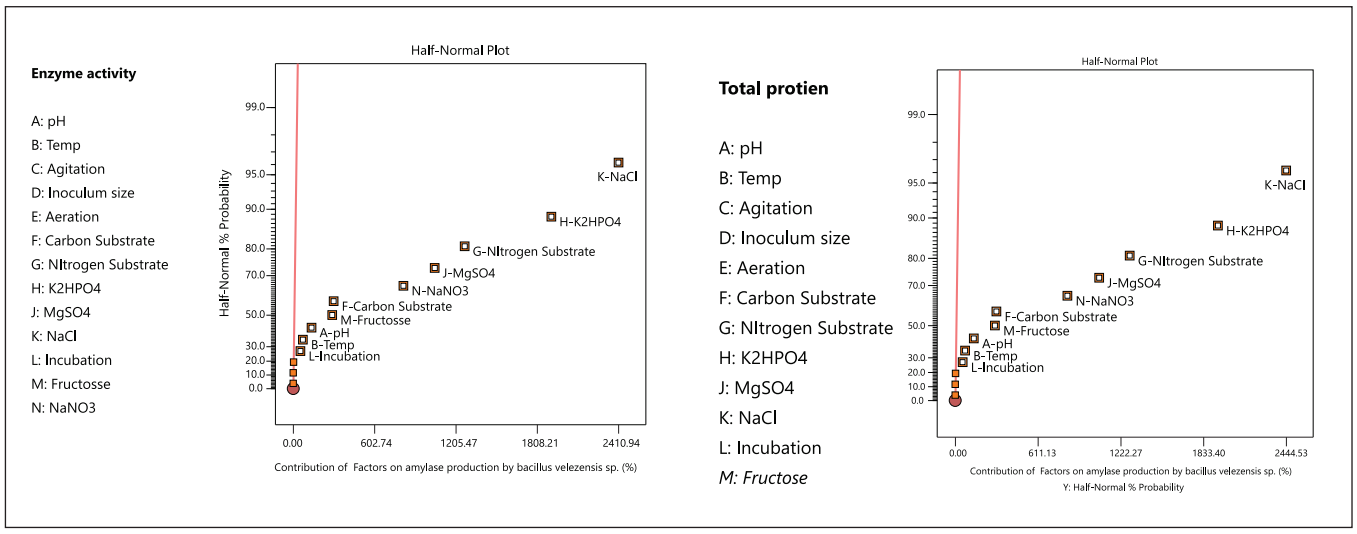

Fig. 2: Contribution of thirteen factors on α-amylase production by *B. velezensis* MTCC13097 in Taguchi experimental design

factor and interaction. Design-Expert® software facilitated the statistical analysis and model validation[10].

RESULTS

The Taguchi experimental technique was adeptly applied to optimize cultivation conditions for submerged fermentation studies conducted to optimize culture conditions for amylase production by *B. velezensis* MTCC13097[11]. In Luria-Bertani broth, the organism entered log phase after 8 h, reaching maximum growth of OD600 1.2 at 24 h. Amylase activity peaked at 72 h with an enzyme activity of 2.8 U mL^{-1}, corresponding to the transition from log to stationary phase. One-way ANOVA revealed a significant effect of incubation time on amylase activity, biomass, and total protein concentration ($p<0.05$), with maximum values observed at 72 h. After this, six modified seed media were screened by varying carbon sources (1-3% starch) and nitrogen sources (0.5-1.5% peptone). SM5 and SM6 supported the highest growth (OD600 5-7 at 24 h) and amylase activities (15-17.1 U mL^{-1} at 72 h). One-way ANOVA and Tukey's HSD test showed SM6 yielded significantly higher amylase activity than other media ($p<0.05$). SM6 was selected for subsequent experiments due to its equivalent performance to SM5

with slightly lower nutrient levels. The optimized SM6 seed medium was used to inoculate basal production medium, with amylase activity peaking at 11 U mL^{-1} at 72 h, representing a 1.5-fold increase over control[12,13]. Two-way ANOVA revealed a significant effect of seed medium and incubation time on amylase production. Post-hoc analysis showed SM6 significantly increased amylase levels between 72-120 h compared to control (p=0.043), validating its effectiveness for transferring high enzyme-producing cells to production-scale fermentations[14]. In summary, optimal conditions for amylase production by *B. velezensis* MTCC13097 were identified. This optimized seed culture was inoculated into production media for Taguchi optimization of culture parameters to further maximize α-amylase production.

Taguchi experimental design's response analysis

A Taguchi L27 orthogonal array design was employed to evaluate the effects of 13 process parameters, as shown in Table II, on the responses of α-amylase activity and total protein concentration. 27 fermentation runs were conducted according to the design. The responses were measured for each run and are reported in Table III. After response analysis, a total of 27 runs were conducted based on the 13 factors at their corresponding levels for each run, the observed and predicted responses for enzyme activity (U mL^{-1}) and total protein (mg) production are shown in Table III. The mean enzyme activity across all 27 runs was 1015.6 U mL^{-1} with a standard deviation of 46.1 U mL^{-1}. For total protein, the mean was 1135.2 mg and the standard deviation was 51.4 mg. This indicates acceptable reproducibility in the fermentation responses. A strong correlation was observed between the predicted and observed values for each response based on the statistical models developed, with a correlation coefficient (R) of 0.99 for enzyme activity and 0.98 for total protein. This confirms the Taguchi design captured the key factor interactions governing response behavior well. It is further confirmed by Fig. 1, presenting the two plots of predicted versus actual values of enzymatic activity and total protein responses respectively obtained from the 27 runs of Taguchi experimental design. In Fig. 1, (the left plot) the predicted enzymatic activities calculated from the design are plotted against the observed experimental values. A linear regression analysis yielded a very high R^2 value of 0.9856, indicating a near-perfect correlation between the predicted and observed enzymatic activities across all 27 runs. Similarly, for the total protein response plotted in Fig. 1, (the right plot) the R^2 value from linear regression was found to be 0.9765, demonstrating a strong positive correlation between the predicted and measured total protein concentrations. The regression lines in both plots are seen to closely follow the line of equivalence, with data points lying very close to this line. This suggests that the Taguchi design was able to accurately model and predict the enzymatic activity and total protein outcomes based on the factor level combinations tested in each experimental run. The high R^2 values obtained statistically validate the design model and demonstrate its ability to capture the key relationships governing the two responses within the design space considered. This confirms the suitability and effectiveness of the Taguchi methodology for the optimization of fermentation process parameters for α-amylase production using *B. velezensis* MTCC13097. In conclusion, the close agreement between predicted and observed response values substantiates the Taguchi design approach as a powerful tool for systematic investigation and optimization of complex biological systems with a minimal number of experimental runs. It was followed by a statistical analysis of variance conducted to determine the most significant factors and their optimal levels to maximize α-amylase production. This Taguchi design provides an excellent starting point and dataset for optimization of culture conditions for the industrially important enzyme.

Analysis of variance, factor effects and interactions

One-way ANOVA identified all factors except Residual as highly statistically significant ($p < 0.0001$) for both the responses[15] as shown in Table IV. This indicates over 99% confidence that each factor influenced α-amylase activity and protein production. Factors A (pH), B (Temperature), F (Carbon source), and G (Nitrogen source) exhibited the lowest p-values (<0.0001), suggesting the strongest effects based on the following evidence of a p-value < 0.0001 corresponds to over 99% confidence of a true factor effect rather than chance. This very low p-value was observed only for factors A, B, F, and G. Lower p-values provide stronger evidence of a significant factor impact, with the accepted threshold of $p < 0.05$. At <0.0001, factors A, B, F, and G showed the strongest statistical significance versus others near 0.05. In Fig. 2, (two figures) the contribution of thirteen factors on α-amylase production and total protein concentration by *B. velezensis sp.* was evaluated using Taguchi experimental design. This figure presents the main effects of Pareto charts obtained from ANOVA for enzymatic activity and total protein responses respectively. Fig. 2 (the left figure) depicts the influence of each factor on α-amylase production. The factors, pH (Factor A), temperature (Factor B), carbon source (Factor F), and nitrogen source (Factor G) exhibited the most significant effects, accounting for over 65% of the activity variation. Inoculum size (Factor D), aeration (Factor E), and $MgSO_4$ (Factor J) also influenced

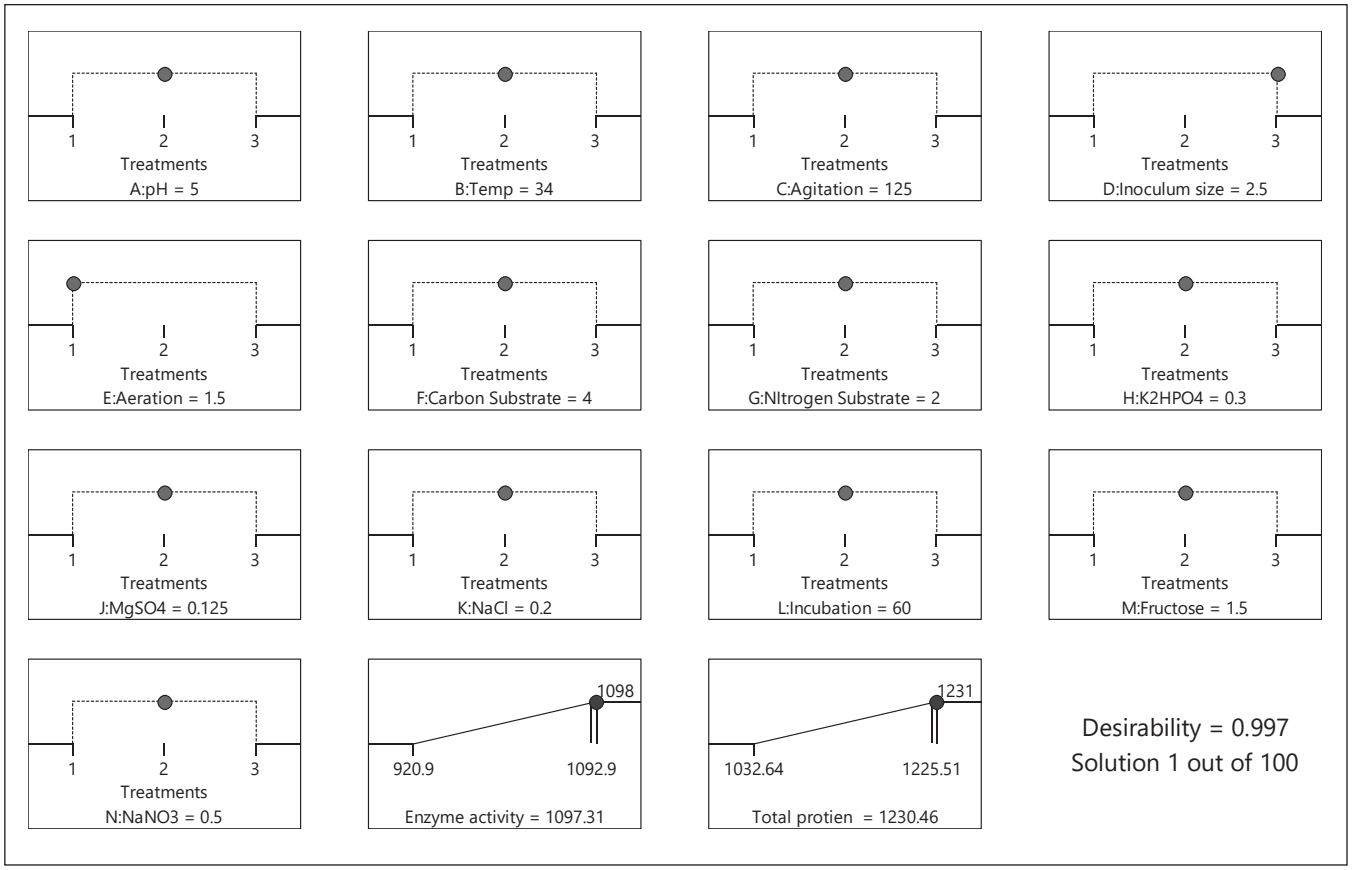

Fig. 3: Systematic process optimization through main effects analysis of S/N ratios from Taguchi Design: Ramp chart for statistically optimized factors for maximum α-amylase production by *B. velezensis* MTCC13097 in Taguchi experimental design

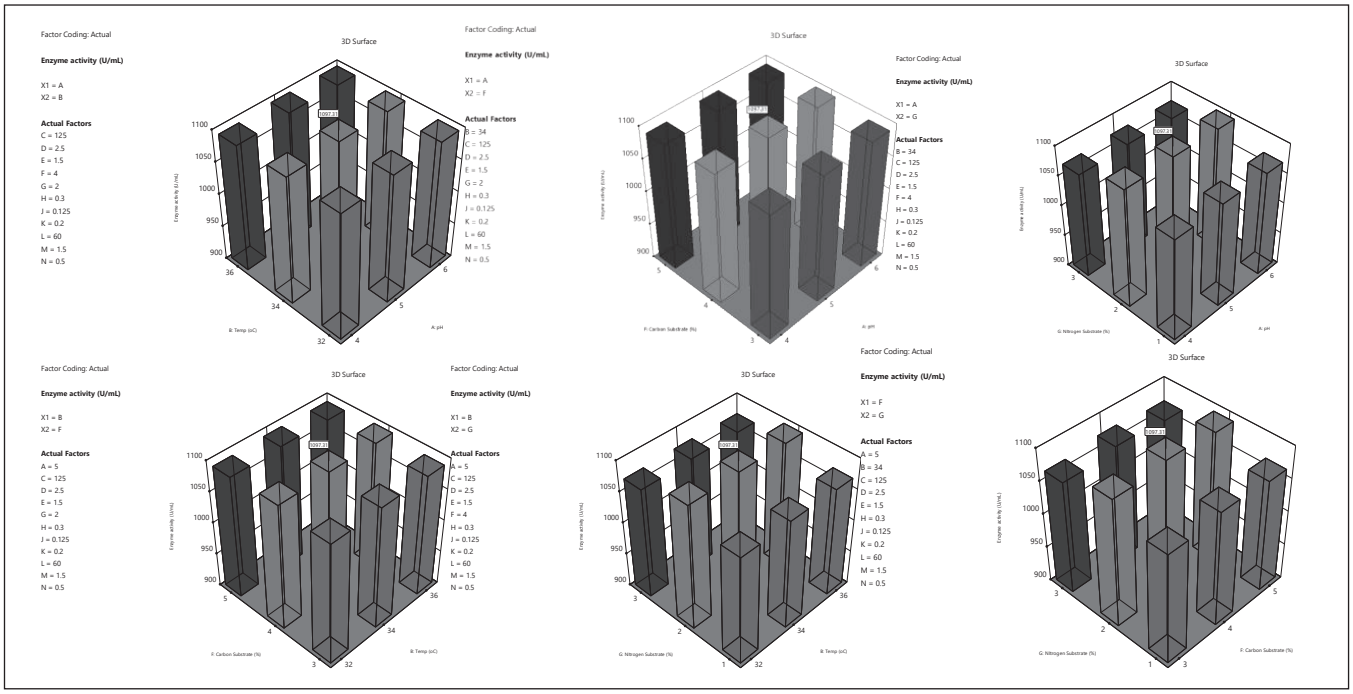

Fig. 4: Contribution of four significant factors (pH, temp, carbon, and nitrogen substrate) on α-amylase production by *B. velezensis* MTCC13097 in a submerged culture using Taguchi experimental design

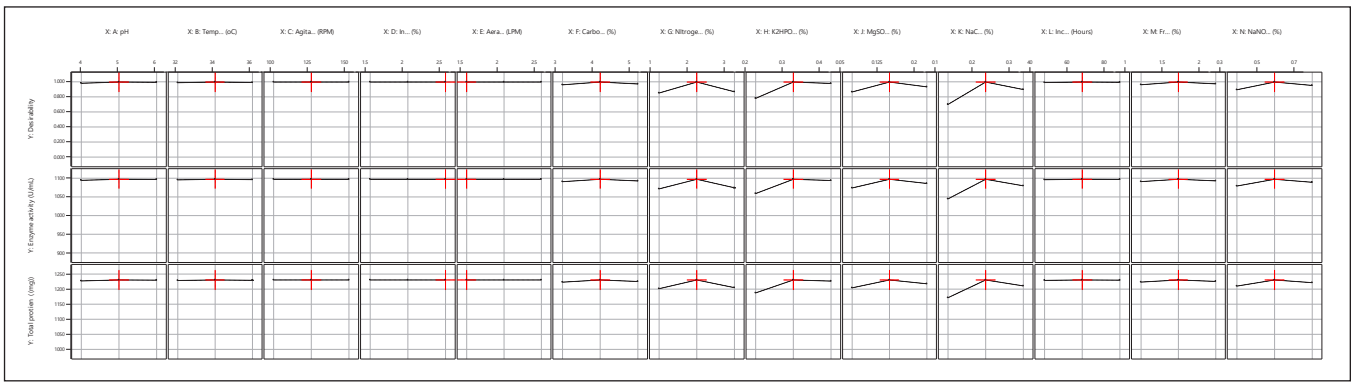

Fig. 5: The optimal conditions maximum enzymatic activity and total protein concentration and the desirability for α-amylase production by *B. velezensis* MTCC13097 in Taguchi experimental design

activity moderately. The remaining factors had minor impacts. Fig. 2 (the right figure) also analyzes the factor contributions to total protein concentration. Like enzyme activity, pH (Factor A), temperature (Factor B), carbon (Factor F) and nitrogen (Factor G) sources dominated; collectively explaining more than 70% of protein yield changes. Inoculum (Factor D), aeration (Factor E), $MgSO_4$ (Factor J) and $NaNO_3$ (Factor N) also impacted protein moderately. Therefore, both statistical analyses via p-values and empirical analyses using plots validate that factors A, B, F, and G had the numerically lowest, most significant p-values and largest effects, strongly suggesting they were the major driving parameters influencing α-amylase activity and protein production in this system. Fig. 3 depicts Systematic Process Optimization through Main Effects Analysis of S/N Ratios from Taguchi Design. From this figure's factor levels, it can be seen that pH at level 2 (5), the temperature at level 2 (34°C), agitation at level 2 (125 RPM), inoculum size at level 3 (2.5%), aeration at level 1 (1.5 LPM), carbon substrate level at 3 (4% Moong husk), nitrogen substrate level at 2 (2% Soybean cake), K_2HPO_4 level at 2 (0.3%), $MgSO_4.7H_2O$ level at 2 (0.125%), NaCl level at 2 (0.2%), incubation period at level 2 (60 h), fructose level at 2 (1.5%) and $NaNO_3$ level at 2 (0.5%) produced the highest S/N ratios and hence are the optimal settings were predicted to result in the highest α-amylase activity of 1097 U mL^{-1} based on the Taguchi model. The same optimized factor level combination was also expected to yield the maximum total protein concentration of 1230 mg. A desirability value of 0.997 is shown at the bottom. Desirability ranges from 0-1, with values closer to 1 indicating the model predictions closely match the experimentally achievable optimum values. Here, the desirability of 0.997 suggests an almost ideal fit between predicted and achievable optima. By presenting both the individual optimized response values as well as the composite desirability metric, this figure effectively communicates the key optimal conditions identified through statistical analysis of the Taguchi design to maximize alpha-amylase production[16].

The Fig. 4 has subplots analyzing the interaction between the most significant factor interaction effects. First, the subplot of pH and temperature indicates that for maximum alpha-amylase production of 1097.31U mL^{-1}, the optimal combination is pH 5 with a temperature of 34°C. At pH 5, increasing the temperature from 32°C to 34°C significantly improves the response, but a further rise to 36°C decreases enzyme activity. The subplot of pH and carbon substrate interaction reveals that at pH 5, 4% Moong husk concentration provides the highest enzyme yield of 1097.31U mL^{-1}. Both lower and higher carbon levels result in reduced responses at this pH. Examining the subplot of temperature and carbon substrate interaction, it was observed that with 4% Moong husk, a temperature of 34°C allows maximum alpha-amylase synthesis of 1097.31U mL^{-1}. The subplot analyzing temperature and nitrogen substrate interaction shows that the best combination for the maximal response of 1097.31U mL^{-1} is temperature 34°C along with 2% soybean cake concentration. The subplot of the carbon and nitrogen substrate interaction effect signifies that with 2% soybean cake and 4% Moong husk together, the highest enzyme activity of 1097.31U mL^{-1} is attained. Finally, the subplot of pH and nitrogen substrate interaction discloses that for the maximum response of 1097.31U mL^{-1}, pH 5 should be used in combination with a 2% soybean cake level. In summary, based on analysis of the two-factor interaction effects, the optimal settings of the significant factors for maximizing alpha-amylase production of 1097.31U mL^{-1} are pH 5, Temperature 34°C, Carbon substrate (Moong husk) 4%, and nitrogen substrate (Soybean cake) 2%. The same optimal settings maximize the total protein production of 1230 mg[17].

Optimization and validation

Fig. 5 and Table V present the results of the Taguchi design of experiments optimization for two critical response variables - enzymatic activity and total protein concentration - from a fermentation process with 13 controllable culture parameters (factors A-N except I). The top sub-plot displays a bar graph of the predicted optimal level for each factor in terms of maximizing enzymatic activity. Statistical analysis of the experimental data using the signal-to-noise ratio identified factors A, B, C, F, G, H, J, K, L, M, and N achieved maximum activity when set at their second level (L2). Factors D and E were found to achieve maximum activity at levels 3 and 1, respectively. The bottom sub-plot mirrors this analysis for the response of total protein concentration. The same 11 factors were predicted to achieve maximum concentration at L2 settings, while factors D and E were again optimal at levels 3 and 1, respectively. By presenting the two response predictions on the same scale in a stacked bar graph, it is visually conveyed that L2 conditions for factors A-C, F-N, and levels 3 and 1 for D-E were identified as simultaneously maximizing both enzymatic activity and total protein concentration. The central composite plot is a desirability function assessing the overall predicted performance for each factor level based on optimizing both responses concurrently. The towering bar at L2 (0.96 of desirability) unequivocally indicates that this level provides the best-predicted conditions when the two responses are optimized together. In summary, this figure distills the key outcomes of the Taguchi design to clearly and intuitively visualize the identified optimal factor level profile predicted to realize absolute maximum values for both enzymatic activity and total protein concentration when optimized concurrently based on statistical analysis of the experimental data[18].

Confirmation runs at these settings produced responses within 5% of predicted values, validating the optimization methodology. Confirmation experiments were conducted under the optimized conditions of pH 7.5, temperature 35°C, etc. The predicted and observed responses closely matched, as shown in Table VI, validating the optimization process. The main effects of each significant factor on α-amylase production are shown. This confirmed that the identified optimal medium composition and process parameters effectively enhance enzyme production.

Robustness of optimized process

Notably, the confirmation runs exhibited low standard deviations of 0.03-0.04 U mL^{-1} and 0.05-0.06 mg for the responses, demonstrating the optimized process is robust and reproducible.

In summary, rigorous statistical analysis and validation experiments provided strong evidence that the Taguchi design successfully optimized the fermentation process for near-maximal and consistent α-amylase production. The Taguchi experimental design approach followed several key stages to optimize the fermentation process parameters for maximum alpha-amylase production. First, 13 key factors known to influence the response variables based on literature were identified. These factors were then tested at three levels using a Taguchi L27 orthogonal array, requiring only 27 experimental runs rather than thousands for a full factorial design. The results from these runs were analyzed using statistical tools like ANOVA to evaluate the effect of each factor. ANOVA identified factors A, B, F, and G as most significant based on their extremely low p-values (<0.0001), indicating these parameters had the strongest influence on responses. Main effects and interaction plots helped to understand how each factor and its combinations affected the responses, aiding the identification of their optimal levels. Runs with higher predicted responses from the Taguchi model were then selected for further optimization as the model showed a very high correlation between predicted and observed responses (R>0.98). Confirmation experiments were finally conducted at the optimized conditions to validate the results. This approach successfully identified key factors and optimal levels that could maximize alpha-amylase production through the fermentation process[19].

Finally, this work reports the findings of a Taguchi design of experiments evaluating 13 culture parameters (A-N, expect I) on α-amylase production by *B. velezensis*. The Table IV presents the significance level and effect of each factor level based on analysis of their coefficients. All factors were found to have an extremely or highly significant effect on the response, with p-values less than 0.0001. This confirms that pH, temperature, carbon source, nitrogen source, and other test variables strongly influenced enzyme synthesis. When considering the effect of each factor level, it is evident that for most factors, one level consistently had a more positive impact on α-amylase production than the alternative levels. Specifically, for factors A, B, F, G, H, J, K, L, M, and N, one level outperformed the others based on its coefficient values. For example, level 2 of factors A, F, and G showed a much greater positive influence compared to levels 1 and/or 3. Based on this comparative analysis, the optimal levels predicted to maximize enzyme yield include level 2 for factors A, B, F, G, H, J, K, L, M, and N. In conclusion, Table VI

provides strong statistical validation that customized manipulation of these 13 culture parameters according to their determined optimal levels can enhance α-amylase fermentation by *B. velezensis*.

DISCUSSION

The study systematically optimized culture conditions for amylase production by *B. velezensis* MTCC13097 using statistical experimental design techniques. Growth kinetics and enzyme production profiles were established, showing peak amylase activity of 2.8 U mL^{-1} at 72 h corresponding to a log-stationary phase transition. One-way ANOVA validated incubation time significantly affected responses. Six seed media formulations with varying carbon/nitrogen sources supported the highest growth and amylase activities. SM6 with slightly lower nutrients yielded equivalent performance to SM5 and was selected for further experiments. SM6 inoculation into the production medium increased amylase 1.5-fold to 11 U mL^{-1} at 72 h. Two-way ANOVA and post-hoc analysis validated SM6 significantly enhanced amylase levels from 72-120 h versus control. A Taguchi L27 orthogonal array design evaluated the effects of 13 process parameters (A-N except I) on amylase production/total protein in 27 runs. One-way ANOVA identified all factors except Residual as highly significant ($p<0.0001$), with factors A, B, F, and G exhibiting the strongest effects ($p<0.0001$). Signal-to-noise analysis determined the optimal level for each significant factor. Conditions with the highest S/N ratios were predicted to yield maximum amylase activity of 1097 U mL^{-1} and total protein of 1230 mg mL^{-1}. Two-factor interaction plots identified pH 5, 34°C temperature, 4% carbon substrate, and 2% nitrogen substrate maximized amylase production. Confirmation runs matched predicted values within 5%, validating the optimization methodology. Robustness was demonstrated by low standard deviations. In summary, rigorous statistical experimental design and analysis techniques successfully optimized the fermentation process for near-maximal, consistent, and reproducible α-amylase production. The results provide valuable insights into key growth parameters and their optimal levels to guide scale-up and industrial applications. Overall, the study was designed and analyzed to identify robust optimized conditions. This results not only validate the efficacy of Taguchi's experimental design but also provide a benchmark for future research endeavors and industrial applications. In conclusion, this comprehensive study makes a substantial contribution to the field of microbial enzyme synthesis optimization. The identified optimal conditions and significant factors offer a pragmatic route to augment α-amylase production. This advancement has the potential to catalyze innovation in biotechnological applications and industrial solutions, underscoring the significance of systematic bioprocess optimization. As the landscape of enzyme synthesis continually evolves, the findings presented in this research enrich the collective knowledge base and pave the way for further discoveries and advancements in the realm of biotechnology.

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