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Optimisation of experimental variables for extracellular amylase production by *Bacillus cereus* AS2

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Abstract: Amylases are one of the biotechnologically important enzymes that have multiple applications in the food, pharmaceutical, cosmetics, textile, detergent, paper and pulp, bioremediation and nano-biotechnology industries. Amylases can be isolated from animals, plants and microbial regions, but nowadays enzymes from prokaryotic species have gained more importance. Among the microbes, amylases from *Bacillus cereus* have gained considerable demand in various industrial sectors. Growing industrial demand for enzymes compels the availability of enzymes in large quantities that can only be achieved by employing efficient fermentation techniques. Therefore, the current study is aimed at the statistical optimisation of the production conditions for extracellular amylase production from *Bacillus* strain AS2. In a recent study, the optimum amylase producing AS2 strain was identified on a molecular level, and it was found that it has close relation with the already reported strains of *Bacillus cereus*. The further enzyme production was optimised by using a statistical optimisation tool. A full-factorial central composite design (CCD) consisting of 53 experiments was designed using six significant variables (incubation period, pH, temperature, carbon and nitrogen source and metal ion). The analysis revealed that the optimal media concentrations were 54.34 g·L^{-1} starch, 0.63 g·L^{-1} $CaCl_2$, 1 g·L^{-1} glycine, pH 7.0, 76 h, and 40 °C, respectively. A 1.23-fold increase in the enzyme yield (1 050 IU·mL^{-1}·min^{-1}) was noticed as compared to the original production level. The statistical optimisation approach gives the exact variables that influence the enzyme production and, hence, offers the best way to optimise the bioprocess. The optimised enzyme can be used in industries for various purposes such as de-sizing, de-inking, hydrolysing starch residues, etc.

Keywords: amylases; statistical optimisation; response surface methodology; central composite design; bioprocess

Nowadays, microorganisms are the most significant source for many industrially important enzymes. Among these industrially important enzymes, amylases account for nearly 30% of the world's commercial enzyme production (Gupta et al. 2003). Amylases break down large polysaccharides into simpler sugars such as maltose or glucose, and have extensive utilisation in the baking, beverage, leather, textile, paper, detergent and pharma-

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ceutical industries (Gangadharan et al. 2008). Amylases are broadly classified into four groups; endoamylases carry out the cleavage of α-1,4-glycosidic bonds, isoamylase act on both α-1,4 and α-1,6 linkages, transferases cleave α-1,4 linkages and carry out the transfer of groups within the molecule and debranching enzymes break α-1,6 glycosidic linkages (Jujjavarapu and Dhagat 2019).

Amylases can be isolated from animals, plants, and microorganisms. Microorganisms are the most preferred source for industrially important enzymes because products with the desired characteristics can be obtained from microbial sources. Industrially important amylase-producing microbial species include *Saccharomyces cerevisiae*, *Penicillium fellutanum*, *Penicillium chrysogeneum*, *Aspergillus oryzae*, *Pseudomonas aeruginosa*, *Streptomyces diastaticus*, *Bacillus amyloliquifaciens*, *Bacillus licheniformis*, and *Bacillus cereus* (Bessler et al. 2003).

Members of the genus *Bacillus* are found ubiquitously in the environment. A wide range of physiological characteristics and the production of industrially important enzymes makes the genus *Bacillus* suitable for various industrial applications. Although a genomic study of the various strains of *Bacillus* revealed that the genome has a size of around 5.35 Mbp, most of the genes code for industrially important enzymes (Lee et al. 2015). Enzyme production from *Bacillus* can be improved by mutagenesis using N-methyl-N'-nitro-N-nitrosoguanidine (Nanmori et al. 1987). Among all the *Bacillus* species, *Bacillus cereus* has been widely reported for amylase enzyme production (Raplong et al. 2014; Raul et al. 2014).

The remarkable importance of amylase in various industrial sectors places demands on the process optimisation for a bulk enzyme yield at a low-cost level. Genetic manipulation and media composition are two important strategies for enzyme overproduction. Strain improvement via mutation may not be stable for consistent enzyme production, therefore media manipulation is considered a better strategy (Dey et al. 2001). The conventional technique for media engineering involves changing one independent parameter at a time and keeping the others constant (Tanyildizi et al. 2005). This classical method is not only tedious and time-consuming, but also fails to depict the true optimum values as it does not study the combined effects of multiple factors at a time. The drawbacks of the one-variable-at-a-time (OVAT) approach can be overcome by employing statistical optimisation techniques. Response surface methodology (RSM) is an effective statistical and mathematical tool for constructing model designs and exploring significant relationships between independent variables and responses. Therefore, the present study aimed to statistically optimise the fermentation medium for the enhanced production with the help of full-factorial composite design using RSM.

MATERIAL AND METHODS

Selection and molecular identification of the strain. *Bacillus* strain AS2 was used for the optimisation of the production conditions for the extracellular amylase. Strain AS2 was isolated earlier and showed the highest amylase activity (Rehman and Saaed 2015).

The selected strain was subjected to molecular identification by amplification of the 16s rRNA gene sequence. The DNA was extracted through a Promega DNA extraction kit. A polymerase chain reaction (PCR) mixture consisting of the template DNA, Hot start master mix, forward primers (5'-AGA GTT TGA TCI TGG CTC AG-3') and reverse primers (5'-ACG GIT ACC TTG TTA CGA CTT-3') was subjected to amplification by maintaining the PCR conditions (denaturation at 94 °C for 1 min followed by 35 cycles of denaturation (94 °C for 30 s), annealing (54 °C for 30 s) and extension (72 °C for 1 min) and final elongation at 72 °C for 10 min) in 1% agarose gel. After purification, the amplified region was sequenced. The obtained data were searched through the BLAST analysis in GenBank. The sequences were aligned by CLUSTAL W2 and the neighbour-joining tree was constructed in MEGA software (version 11) by the Kimura-2-parameter (Cosa et al. 2011).

Amylase assay. The fermentation for the amylase production was carried out in 250 mL conical flasks containing 100 mL of Luria broth (LB). The production medium was supplemented with an overnight seed culture (8%) and incubated in an orbital shaker at 150 rpm (revolutions per minute) at 37 °C for 24 h. After fermentation, the culture broth was centrifuged in a benchtop centrifuge at 13 000 g for 15 min and the total amylase activity was determined according to the method reported by Bernfeld (1955). The enzyme production, in terms of international units, was calculated by applying the following formula (Haq et al. 2003):

$$\textit{Enzyme production}\ (\mathrm{IU{\cdot}mL^{-1}{\cdot}min^{-1}}) = \frac{\textit{enzyme activity}\left(\mathrm{U{\cdot}mL^{-1}}\right) \times 1\,000}{\textit{molecular weight of maltose} \times \textit{incubation time}\left(\mathrm{min}\right)} \quad (1)$$

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Table 1. Levels of independent variables in regression model

Factors	Name	Level				
		–2	–1	0	+1	+2
Y_1	pH	5	6	7	8	9
Y_2	temperature (°C)	35	40	45	50	55
Y_3	incubation period (h)	24	48	72	96	120
Y_4	substrate concentration ($g \cdot L^{-1}$)	20	30	40	50	60
Y_5	glycine ($g \cdot L^{-1}$)	1	3	5	7	9
Y_6	$CaCl_2$ ($g \cdot L^{-1}$)	0.1	0.3	0.5	0.7	0.9

Optimisation of the amylase production. The optimal level of the selected variables was studied by using a statistical optimisation design. The experimental design in the study consists of 53 trials and the dependent response (Y) was the mean of three replications. Significant independent variables were assessed at levels: –1, –2 (low); 0 (middle); and +1, +2 (high); as shown in Table 1. To predict the optimal point, a second-order polynomial function was used to find the correlation between the independent variables (y) and response (Z). The equation for the six variables is as follows:

$$Z = \alpha_0 + \acute{\epsilon}\alpha_i \times y_i \times \acute{\epsilon}\alpha_{ii} \times y_{ii}^2 \times \acute{\epsilon}\alpha_{ij} \times y_{ij} \quad (2)$$

where: Z – predicted response (amylase production); α_0 – intercept; α_i and y_i – linear coefficients; α_{ii} and y_{ii} – quadratic coefficients; α_{ij} and y_{ij} – cross-product coefficients; y_{ii}^2 – independent variable; $\acute{\epsilon}$ – random error term.

Minitab (version 17) was used for the regression analysis of the obtained experimental data. The significance of the polynomial model equation and coefficient terms was evaluated through Fisher's test (for overall model significance). The fitness of the second order polynomial model equation was expressed as R^2 (coefficient of determination) and adjusted R^2. For illustrating a relationship between the response and the optimal level of each variable, 3D surface plots and contour plots were also generated using the Minitab software (Mohammad et al. 2021).

Statistical analysis. All the designed experiments were performed in triplicate and the final results were analysed as the mean ± statistical deviation (SD). The response prediction was analysed by using multiple linear regression. Moreover, Student's t-test was used to analyse and calculate the significant coefficient parameters. The P-value was calculated by applying a one-way analysis of variance (ANOVA) at a 95% confidence interval.

RESULTS AND DISCUSSION

Amylases are one of the most industrially important enzymes and have been reported to have useful applications in various industrial sectors. Therefore, it is an important factor to optimise the production conditions that can minimise the time and production cost on a commercial level. In the current study, the AS2 strain was isolated, characterised and identified at the molecular level by amplification of the 16s RNA gene. The isolate was identified as the *Bacillus cereus* AS2 strain (AS2 SUB5324671 seq MK640654) (Figure 1).

Microbial strains differ in specific requirements and conditions for their maximum enzyme yield. Moreover, the metabolic activities of microbes are highly affected by changes in the temperature and pH of the medium which ultimately affects the enzyme production. The effect of the physiochemical parameters on the enzyme production has been extensively studied for the genus *Bacillus* (Osman et al. 2020). In the current work, the optimisation of enzyme production was carried out by using statistical approach, i.e. RSM. The screening of significant factors for the statistical optimisation technique was conducted by OVAT (Rehman et al. 2019b). These variables include the pH, incubation period, temperature, carbon and nitrogen sources, and metal ions. Significant parameters (with a confidence level above 95%) were determined through Student's t-test and were used for further optimisation techniques (Table 2).

To determine the optimal production level of each independent variable, the study was conducted at three levels (low, middle, high) for each variable (Table 1). The results of the study are presented as an average of each trial performed in triplicate. The maximum amylase activity was observed in run order 39 (1 051.543 $IU \cdot mL^{-1} \cdot min^{-1}$), whereas the minimum enzyme units were observed (170.67 $IU \cdot mL^{-1} \cdot min^{-1}$) in experiment 20 (Table 3).

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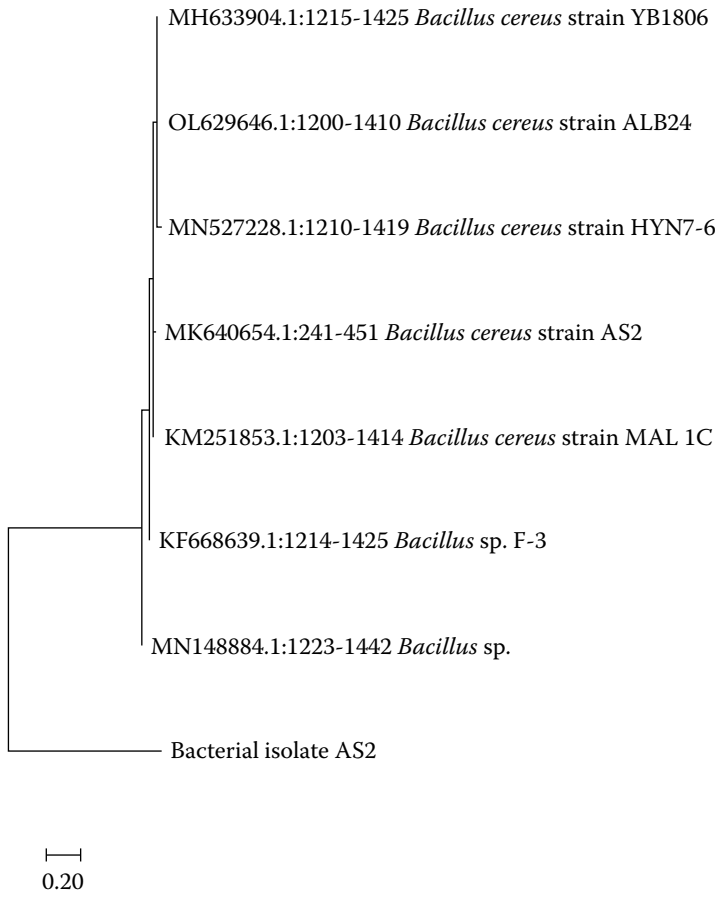

Figure 1. Neighbor-joining tree by using multiple CLUSTAL W2 alignment software of MEGA (version 11)

Fisher's *F*-test was used to test the ANOVA, which shows the significance of the quadratic regression model. The *F*-value of the model was 10.02 and, for the lack of fit, it was 2.64 (Table 4). The accuracy of the model was explained by the high *F*-values along with an insignificant lack of fit. Moreover, the fitness of the obtained experimental data/values to the model is also suggested by the *P*-value ($P < 0.0001$) for the model and the lack of fit (0.082). Previously, a reported RSM-based statistical model for enzyme optimisation was found to be significant based on an insignificant lack of fit (1.52) and *F*-value (65.59) (Reddy et al. 2008). Moreover, the applicability and reliability of the RSM model depends on the R^2-value (coefficient of determination). An R^2-value closer to 1 shows a better prediction accuracy (correlation between the predicted and observed values) (Gangadharan et al. 2008). The designed model in the current study was observed to be highly significant after a comparative analysis among the R^2-values (91.54%) to the adjusted R^2-values (82.41%). Other studies also reported significant RSM-based optimisation models after a comparative analysis of the R^2 and adjusted R^2-values (Ponraj et al. 2011).

Student's *t*-test was used to identify the significant regression coefficients (Table 5). The significant linear term includes the temperature, whereas the incubation period and the temperature were found as significant square terms. Among the interaction terms, no signifi-

Table 2. Factors for one-variable-at-a-time (OVAT) approach

Factors	Description
Temperature (°C)	35–55
pH	3.0–11.0
Incubation period (h)	24–120
Carbon source	sucrose, sorbitol, starch, fructose, xylose, maltose, lactose, arabinose, maltose, galactose, mannose
Nitrogen source	casein, tryptone, yeast extract, glycine, malt extract, gelatin, peptone, urea
Metal ions	$MgSO_4$, NaCl, $FeCl_3$, $HgCl_2$, $ZnSO_4$, $CUSO_4$, and $CaCl_2$

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Table 3. The randomised design used in response surface methodology (RSM) with actual and predicted amylase activity

Run	Coded values						Amylase activity (IU·mL^{-1}·min^{-1})	
	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6	actual	predicted
1	–1	1	–1	1	–1	–1	971.2957	854.35
2	–1	1	1	1	1	–1	617.5920	625.77
3	0	0	0	0	0	–2	742.9006	745.15
4	–1	–1	–1	1	–1	1	648.9685	805.81
5	0	0	0	0	0	0	841.6660	809.04
6	1	–1	–1	–1	–1	1	279.3204	354.49
7	–1	–1	1	–1	–1	1	213.8883	264.92
8	–1	1	1	–1	–1	–1	248.4562	274.80
9	1	–1	–1	1	1	1	662.6537	705.38
10	0	0	0	2	0	0	297.2216	292.76
11	–1	–1	–1	–1	1	1	333.0241	328.87
12	–1	–1	–1	1	1	–1	737.3451	760.21
13	1	–1	1	–1	1	1	771.9130	787.58
14	–1	–1	1	1	1	1	883.0241	761.12
15	–1	–1	–1	–1	–1	–1	264.5056	293.99
16	0	0	2	0	0	0	308.3327	383.37
17	0	2	0	0	0	0	273.7648	409.12
18	1	–1	1	1	–1	1	272.5302	288.69
19	0	0	0	0	2	0	817.5920	813.49
20	0	0	0	0	0	0	170.6784	60.00
21	1	–1	1	1	1	–1	817.5920	840.15
22	0	0	0	0	0	0	727.4685	843.20
23	0	0	0	0	0	0	706.4809	699.60
24	0	0	0	0	–2	0	921.2957	813.49
25	1	1	1	–1	1	–1	257.7154	270.85
26	1	1	–1	–1	–1	–1	765.7401	721.57
27	–1	1	1	–1	1	1	852.7772	813.49
28	1	1	–1	–1	1	1	633.0241	681.25
29	1	1	1	1	–1	–1	860.8019	813.49
30	0	0	–2	0	0	0	751.8512	813.49
31	0	0	0	0	0	0	824.9994	708.59
32	0	0	0	0	0	0	739.1969	716.98
33	1	–1	–1	1	–1	–1	717.5920	813.49
34	1	1	1	–1	–1	1	795.9870	813.49
35	–1	1	–1	–1	–1	1	742.9006	813.49
36	0	0	0	–2	0	0	754.6290	759.91
37	–1	1	1	1	–1	1	449.0735	420.01
38	0	0	0	0	0	0	732.4068	680.94
39	1	1	–1	1	–1	1	1 051.5430	862.18
40	0	0	0	0	0	2	274.9994	285.21
41	–2	0	0	0	0	0	300.9253	326.21
42	–1	1	–1	1	1	1	278.7031	280.54
43	–1	–1	1	1	–1	–1	256.5216	259.27

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Table 3. To be continued

Run	Coded values						Amylase activity ($IU \cdot mL^{-1} \cdot min^{-1}$)	
	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6	actual	predicted
44	1	1	−1	1	1	−1	297.8389	316.71
45	0	0	0	0	0	0	246.6043	309.18
46	−1	−1	1	−1	1	−1	758.3327	701.70
47	0	0	0	0	0	0	812.6537	755.89
48	2	0	0	0	0	0	662.6537	651.57
49	0	−2	0	0	0	0	681.1722	697.75
50	−1	1	−1	−1	1	−1	716.9747	709.04
51	1	−1	1	−1	−1	−1	373.7648	329.15
52	1	1	1	1	1	1	745.3698	744.91
53	1	−1	−1	−1	1	−1	713.2710	813.49

Y_1 – pH; Y_2 – temperature; Y_3 – incubation period; Y_4 – substrate concentration; Y_5 – glycine; Y_6 – $CaCl_2$

cant value was found. Our findings are in accordance with the results of Shaktimay and co-workers who reported the temperature and incubation period as the significant linear and square terms (Shaktimay et al. 2010).

The polynomial equation from the regression analysis of the data is as follows:

$$\begin{aligned} Z = & -9\,279 + 364.4\,Y_1 + 324\,Y_2 + 40.2\,Y_3 + 13.0\,Y_4 + \\ & + 31\,Y_5 - 345\,Y_6 - 4.405\,Y_1^2 - 28.6\,Y_2^2 + \\ & - 0.1905\,Y_3{}^2 - 0.231\,Y_4{}^2 + 2.45\,Y_5{}^2 - 666\,Y_6{}^2 + \\ & + 0.31\,Y_1Y_2 - 0.171\,Y_1Y_3 - 0.032\,Y_1Y_4 + \\ & - 0.59\,Y_1Y_5 + 6.3\,Y_1Y_6 - 0.314\,Y_2Y_3 + 1.09\,Y_2Y_4 + \\ & + 1.31\,Y_2Y_5 + 52.1\,Y_2Y_6 - 0.0296\,Y_3Y_4 + \\ & - 0.105\,Y_3Y_5 - 0.38\,Y_3Y_6 - 0.557\,Y_4Y_5 + \\ & + 12.33\,Y_4Y_6 - 20.1\,Y_5Y_6 \end{aligned} \quad (3)$$

where: Y_1 – temperature; Y_2 – pH; Y_3 – incubation period; Y_4 – starch; Y_5 – glycine; Y_6 – $CaCl_2$.

For a better understanding of the optimal level of each variable for the maximum amylase yield, the predicted model was also presented in the form of 3D response surface graphs and 2D contour plots. In the contour plots, two variables are tested for multiple combinations at a time while the other tested variables are kept at their respective zero level. The area confined in the smallest ellipse of the contour diagram demonstrates the maximum predicted value. The minimum and maximum profile of the ellipses in the contour plots are further explained by the 3D surface plot. The optimum interaction between two independent variables is noticed in the form of elliptical contour plots (Muralidhar et al. 2001). In the current study, the amylase production increased by increasing the value of the temperature and pH up to optimum level, i.e. 40 °C and pH 7.0, whereas any further increase in these values resulted in a sharp decline in the enzyme yield. The temperature and pH, as the significant independent variables in the RSM optimised model, were also reported in earlier studies (Stergiou et al. 2014).

Table 4. One-way analysis of variance (ANOVA) for regression model

Source of variation	Degree of freedom	Sum of squares	Mean of squares	*F*-value	*P*-value
Linear	6	1 914 157	319 026	29.16	0.000*
Square	6	993 028	165 505	15.13	0.000*
Regression	27	2 960 523	109 649	10.02	0.000*
Interaction	15	53 337	3 556	0.32	0.986
Lack-of-fit	17	232 202	13 659	2.64	0.082
Residual error	25	273 540	10 942	–	–
Pure error	8	41 337	5 167	–	–
Total	52	3 234 062	–	–	–

*Statically significant at $\alpha = 0.01$; *F* – Fisher's test

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Table 5. Regression coefficients significant for amylase activity

Term	Effect	Coefficients	*t*-test	*P*-value
Constant	–	813.5	24.77	0.000*
pH	–18.0	–9.0	–0.54	0.591
Temperature	–432.8	–216.4	–13.09	0.000*
Starch	39.9	20.0	1.21	0.239
Incubation period	45.4	22.7	1.37	0.182
$CaCl_2$	1.6	0.8	0.05	0.962
Glycine	–9.5	–4.8	–0.29	0.776
pH × pH	–57.3	–28.6	–1.58	0.126
Temperature × temperature	–220.3	–110.1	–6.09	0.000*
Starch × starch	–46.2	–23.1	–1.28	0.214
Incubation period × incubation period	–219.5	–109.7	–6.06	0.000*
$CaCl_2$ × $CaCl_2$	–53.2	–26.6	–1.47	0.154
Glycine × glycine	19.6	9.8	0.54	0.593
pH × starch	21.8	10.9	0.59	0.560
pH × incubation period	–15.0	–7.5	–0.41	0.688
pH × $CaCl_2$	20.8	10.4	0.56	0.578
pH × glycine	5.2	2.6	0.14	0.888
Temperature × pH	3.1	1.5	0.08	0.934
Temperature × starch	–3.2	–1.6	–0.09	0.932
Temperature × incubation period	–41.0	–20.5	–1.11	0.278
Temperature × $CaCl_2$	12.7	6.3	0.34	0.735
Temperature × glycine	–11.9	–5.9	–0.32	0.751
Starch × $CaCl_2$	49.3	24.7	1.33	0.194
Starch × glycine	–22.3	–11.1	–0.60	0.552
Incubation period × starch	–14.2	–7.1	–0.38	0.704
Incubation period × $CaCl_2$	–3.6	–1.8	–0.10	0.923
Incubation period × glycine	–10.1	–5.1	–0.27	0.787
Glycine × $CaCl_2$	–16.0	–8.0	–0.43	0.668

*Statically significant at α = 0.01; R^2 – coefficient of determination, R^2 = 91.54%; R^2(adj) – adjusted R^2, R^2(adj) = 82.41%; *t*-test – Student's *t*-test

In the designed model, the surface and contour plots of the starch and glycine depicts the maximum amylase yield at 0.68 g·L^{-1} of $CaCl_2$, 54.34 g·L^{-1} of starch, and 1 g·L^{-1} of glycine (Figure 2A–D). Starch was reported by Tanyildizi and co-workers (Tanyildizi et al. 2005) as being a significant variable for enhancing amylase production. It was also reported that the maximum enzyme yield obtained at 0.68 g·L^{-1} of $CaCl_2$ and amylase production could not be increased by further increasing the concentration of these variables. A two-fold increase in the amylase production in a statistically optimised medium (containing 0.02% $CaCl_2$) was reported in another previously reported study (Abdel-Fattah et al. 2013).

Bacillus AS2-derived amylases can be utilised in many industrial processes. The enzyme's ability to break down starch efficiently makes it valuable asset in a number of applications.

Bacillus AS2-derived amylases are also reported to be useful in the baking industry by breaking down starches into fermentable sugars, which are then used by the yeast for fermentation (Rehman et al. 2019a). Also, *Bacillus* AS2-derived amylase has been reported as being useful in the textile industry for desizing, which involves removing starch-containing substances from fabrics. This process improves the quality of the fabric and facilitates further processing (Rehman et al. 2023). Overall, the significant benefits and uses of amylase en-

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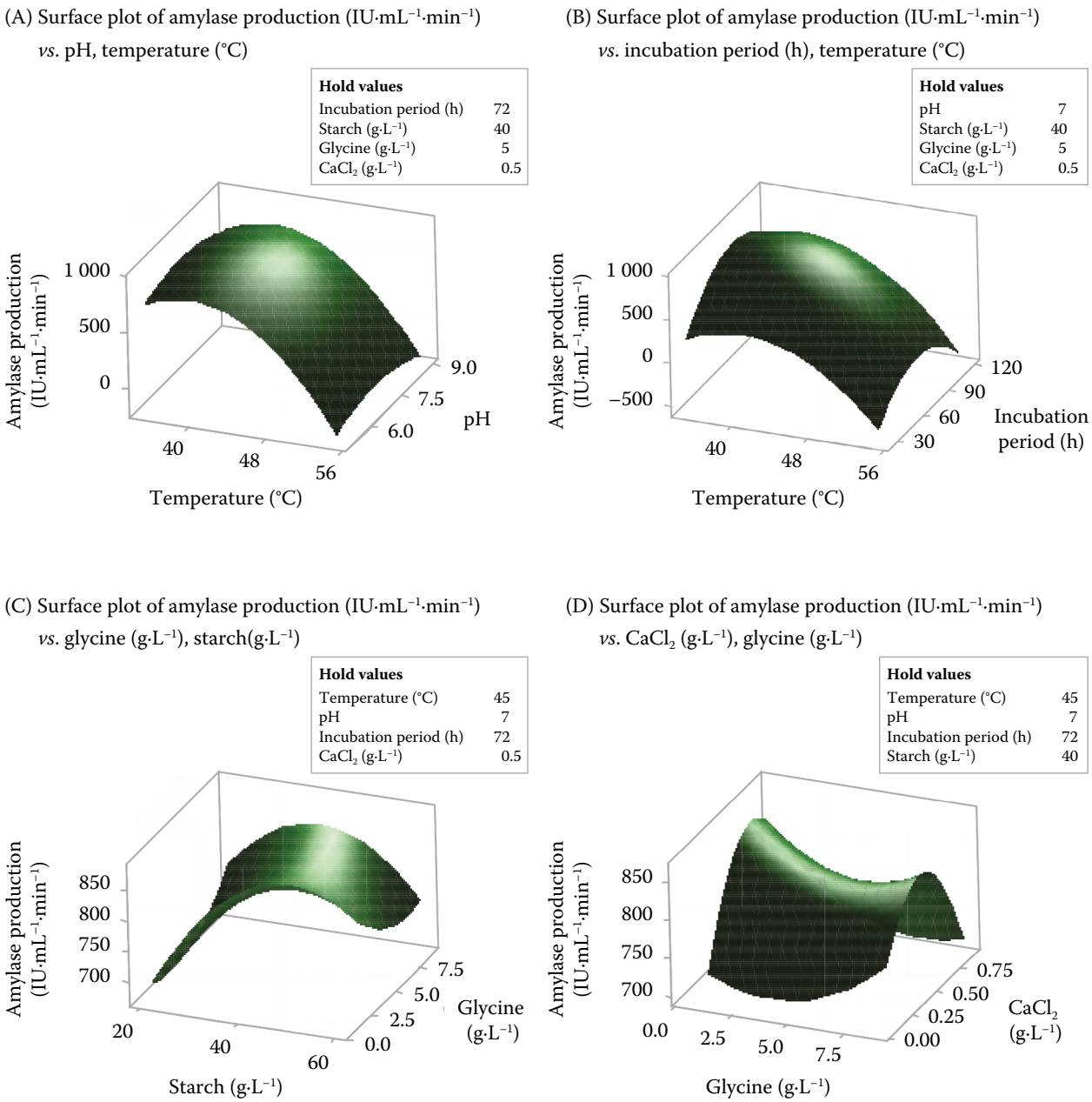

Figure 2. Graphical representation of optimised conditions

zymes in various industrial processes, along with their applications in food, beverage and medical industries, make them valuable assets in many applications.

CONCLUSION

Amylases are the main enzymes involve in starch saccharification and account for 30% of all the enzymes used commercially. Strain AS2 with its maximum amylase producing potential was isolated from the soil and optimised for the optimum enzyme yield. The present study showed that the statistical design offers a better optimisation of the cultural conditions for the maximum amylase yield. The enzyme production was significantly enhanced at pH 7.0, 40 °C after 76 h in the presence of 54.34 g·L^{-1} of starch, 0.68 g·L^{-1} of $CaCl_2$, and 1 g·L^{-1} of glycine. The statistical optimisation resulted in a 1.23-fold increase in the amylase yield than optimisation through the OVAT approach. The chosen method not only resulted in an increased enzyme production, but also had a significant effect on the overall cost of the optimisation studies, as the desired enzyme units can be achieved in a limited number of experiments. The current optimisation approach is simple,

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time-saving, efficient, and inexpensive so it provides a basis for further large-scale fermentation.

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