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Full Length Article

Response surface methodology as an approach for optimization of alpha amylase production by using bacterial consortium under submerged fermentation

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ABSTRACT

The present study aims to isolate amylolytic bacteria and later the formation of a bacterial consortium for alpha-amylase production. The potent amylolytic strains in a selected consortium were identified as *B. subtilis* and *B. licheniformis* on the basis of 16S rRNA sequencing. Initially cultural conditions for alpha amylase were optimized in Plackett–Burman design, followed by the central composite design. Analysis of Variance (ANOVA) of the optimization study indicated pH 7, 0.05 %$CaCl_2$ and 24 h incubation time were the significant variables that mainly effect the alpha amylase production. After the process of statistical optimization, the enzyme was then partially purified by means of ammonium sulfate precipitation at a 60% saturation level, resulting in a 2-fold purification with 1.4 U/mg specific activity. Enzyme kinetics studies showed that the maximum velocity of the reaction (Vmax) and Michaelis constant (Km) were 47.4 mg/ml and 13.2 mM, respectively. Current results suggest the newly isolated bacterial consortium could be considered as a promising candidate for industrial applications.

1. Introduction

α- amylase is a common enzyme found in nature and is also known as 1,4-α-D-glucan glucanohydrolase (EC 3.2.1.1). It breaks down 1, 4-glucosidic bonds in amylose, amylopectin, and glycogen through an endo-acting process (Cotarleț and Bahrim, 2012). A variety of organisms, including plants, animals, bacteria, and fungi, can produce amylases. However, microbial sources are preferred due to their easy growth, simple production, and low cost (Saha and Mazumdar, 2019). Among all the microbes, bacterial strains, including, *Bacillus licheniformis*, *Bacillus stearothermophilus, Bacillus subtilis, and Bacillus amyloliquefaciens*, are effective producers of alpha-amylase and have numerous applications in pharmaceuticals, food, baking, textile, leather, detergent, paper, and pulp industries (Kar et al., 2010). However, it is observed that demand for novel alpha amylase enzymes increases considerably if they are isolated from distinct natural habitats, possess unique physiological properties, metabolic pathways, and utilize diverse chemical nutrients (Bahri et al., 2022).

In the last few years, microbial consortia have gained more attention than single species. The idea of combining species with complementing traits is beneficial because consortium members might complement each other's activities to create a greater impact. Nutritional and physicochemical factors like incubation temperature, pH, nitrogen, and carbon sources affect amylase production. Hence, optimizing fermentation media and physiological growth conditions enhances the product yield. The traditional method of using single-variable optimization is expensive, requires more time, is laborious, and neglects the combined impact of the physicochemical parameters. On the contrary, response surface methodology (RSM) helps researchers to design experiments and evaluate factor-response interactions throughout the project. This method combines experimental design to minimize labor, time and reduce process costs (Nor et al., 2017). Response surface methodology is a robust and powerful approach that is routinely used in the optimization of the production of different primary and secondary metabolites of microbial origin, such as alpha amylase (Matrawy et al., 2022), xylanase (Matrawy et al., 2021), protease (Matrawy et al., 2023), chitooligosaccharides (Embaby et al., 2018). Additionally, in the current study, kinetics was also studied, which provides the detailed mechanisms of many chemical and biological reactions of enzymes. Therefore, this work focused on the screening of amylolytic bacterial consortium, optimization using a statistical full factorial design, and the partial purification of alpha-amylase.

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Table 1
Composition of different fermentation media (g/l).

Fermentation Media	Composition of media (g/l)
Medium 1	Starch 15.0, Peptone 5.0 g, Yeast extract 1, $MgSO_4.7H_2O$ 0.5, NaCl 0.5, $CaCl_2$ 0.002, 1000 ml distilled water (Riaz et al., 2009).
Medium 2	Starch 10.0, Tryptone 3.0, Yeast extract 3.0, $(NH_4)_2SO_4$ 3.0, NaCl 1.0, $Mg.SO_4.7H_2O$ 0.2, K_2HPO_4 1.0, 1000 ml distilled water (Riaz et al., 2009).
Medium 3	Starch 15.0, Peptone 5.0, Yeast extract 10.0, Glucose 30, $NH_4(SO_4)_2$ 15, NaCl 0.5, $CaCl_2$ 0.002, 1000 ml distilled water (Riaz et al., 2009)
Medium 4	Basal medium, Starch 5, Peptone 10, Beef extract 5, KH_2PO_4 3, $MgSO_4$ 0.5, $CaCl_2$ 0.02, 1000 ml distilled water (Samanta et al., 2014).
Medium 5	Starch 20, Peptone 10, Yeast extract 4, $MgSO_4$ 0.5, $CaCl_2$ 0.2; 1000 ml distilled water (Kanimozhi et al., 2014).

2. Materials and methods

2.1. Isolation and screening of amylolytic bacteria

Different soil samples were collected from several localities in Punjab. Bacterial isolation was carried out in accordance to Adiguzel et al. (2009) on Czepek-dox starch agar plates, and at 37°C, the plates were incubated for 24 h. After the incubation process, iodine solution was flooded onto the plates. Bacterial colonies surrounded by a clear zone indicated positive starch hydrolysis, while blue-black coloration indicated a negative test. Positive isolates were further used for investigation (Pranay et al., 2019).

2.2. Compatibility test

Bacterial compatibility test was performed using techniques followed by Santiago et al. (2017) on the selected amylolytic bacterial isolates. According to this test, only those bacterial strains were selected that favored the growth of other bacterial strains. The bacterial strains that inhibited the growth of the other bacterial strain are said to be non-compatible with each other. The test was performed by inoculating two different bacterial strains on nutrient agar plates. After inoculation, Petri plates were kept in an incubator at 37°C for 24 h. Those strains were selected which did not inhibit the growth of each other.

2.3. Vegetative inoculum

A small amount of pure bacterial colony was added to 20 ml of sterilized nutrient broth. For 24 h, all the flasks were placed at 37°C in a rotatory shaker (Ahmed et al., 2017). Different media were screened for alpha amylase production (Table 1).

2.4. Submerged fermentation

1.0 mL of vegetative inoculum (0.5 mL from each bacterial strain) was added to 50 mL of fermentation media. The flasks were then kept in an incubator shaker at 37°C and 160 rpm for 24 h. The supernatant obtained from the centrifugation of the fermentation broth at 5000 rpm for 20 min was used to estimate the enzyme (Riaz et al., 2009).

2.5. Enzyme assay

According to Haq et al. (2010), the enzyme assay was carried out. For assay, 1 mL of crude enzyme was added to 1 mL of 1.0 % solution of soluble starch and incubated at 40°C for 10 min. After the process of incubation, 1.0 mL of Dinitrosalicylic acid (DNS) reagent was added, and the reaction mixture was boiled for 5 min. With the addition of distilled water, the total volume of each tube was raised to 10 mL. The absorbance was measured by means of spectrophotometer at 546 nm. One unit of amylase activity is defined as the amount of enzyme that releases 1 μmol of glucose from 1% starch as a substrate per minute under the assay-stated conditions.

Table 2
PCR conditions for DNA amplification.

Steps	Conditions	
Initiation	94 °C	4 min
Denaturation	94 °C	30 s
Annealing	47 °C	30 s
Extension	72 °C	30 s
Primers	Forward primer (5'-AAACTYAAAKGAATTGACGG-3')	
	Reverse primer (5'-ACGGGCGGTGTGTRC-3')	

Table 3
Screening of media components for alpha amylase production using Plackett–Burman design.

Variables	Units	Low	High
Incubation Time	(h)	24	48
Inoculum size	(ml)	0.5	1.5
pH	–	7	9
Starch	(%)	1	2.5
Peptone	(%)	1	3
Yeast extract	(%)	0.2	1
Mg.SO4.7H2O	(%)	0.05	0.1
NaCl	(%)	0.05	0.1
$CaCl_2$	(%)	0.05	0.1

2.6. Molecular characterization

The genomic DNA of bacterial isolates was isolated, according to Green and Sambrook (2012). The isolated DNA was further amplified for 16S rRNA by polymerase chain reaction under optimized conditions (Table 2). The polymerase chain reaction (PCR) product after gene clean was sent to 1st base Singapore for commercial sequencing.

2.7. Response surface methodology (RSM)

Response surface methodology (RSM) was employed to evaluate the experimental data. It is a combination of mathematical and statistical tools that are used in designing experiments, developing models, and evaluating the effects of independent parameters by reducing error. The most significant model was designed by computing, a lack-of-fit test, an F test, and other precision measurements.

2.7.1. Plackett Burman design

In the present investigation, the Plackett Burman design was used to determine the most influential factors affecting alpha amylase production. Different experimental combinations were executed to minimize unrelated variables that affect alpha amylase production. Seven different variables were examined: incubation time (h), inoculum size (ml), pH, starch (%), peptone (%), yeast extract (%), $MgSO_4.7H_2O$ (%), NaCl (%), and $CaCl_2$ (%). Each variable depicted two levels, maximum ('high, +') and minimum ('low, -') levels of the range covered by each variable and the response (Table 3). The software used for Plackett Burman design was Design expert version 11. The screening in the Plackett–Burman design was determined by the following equation of a first-order polynomial.

$$Y = Bo + \Sigma Bi\ Xi \quad \text{Eq. 1}$$

Y denotes response of α-amylase activity, B_0 denote the model intercept, Bi and Xi shows linear coefficient, and value of independent factor, respectively.

Table 4
Screening of selected media components for alpha amylase production through Central composite design.

Name	Units	Low	High
Incubation time	H	24	72
pH		7	11
$CaCl_2$	%	0.05	0.1

2.7.2. Central composite design (CCD)

The independent variables that exhibited a significant effect on amylase activity by Plackett-Burman design were again analyzed in CCD (Table 4). The software used for CCD was Design expert version 11. In CCD, the optimum values and the combined effect of selected variables on alpha amylase production were analyzed by plotting 3-D response curves against any two independent parameters while keeping the third independent factor at constant or at '0' level. An analysis of variance (ANOVA) was performed to evaluate the significance of the model and regression coefficients. The quality of the second-order polynomial equation was investigated through a regression equation (Bhardwaj et al., 2021).

$$Y = \beta 0 + \Sigma \beta i\ Xi + \Sigma \beta i\ Xi\ \beta ij + \Sigma Xi\ Xj \quad \text{Eq. 2}$$

Provided that Y is the predicted response. β_0, bi, and b_{ij} are model constant regression coefficients.

2.8. Partial purification

After the process of optimization, the crude enzyme extract was then partially purified with ammonium sulfate precipitation to get (0–70%) saturation level. The pellet was recovered by centrifugation at 10,000 rpm for 15 min at 4 °C and dissolved in (0.2 M) phosphate buffer (pH 7). The 10,000 (molecular weight (MW) cutoff dialysis bag was used for the overnight dialysis against the same buffer with three-four changes (Kizhakedathil, 2021). The molecular weight of the partially purified enzyme was determined using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS PAGE).

2.9. Estimation of protein content

The protein content was determined by following the Bradford (1976) method. In accordance with that, 0.1 ml of supernatant and 5 ml of Bradford reagent were taken and vortexed well in a separate test tube. A blank was also run parallel to it, containing 0.1 ml of distilled water and 5 ml of Bradford reagent. After 15 min, absorbance was taken at 595 nm on a spectrophotometer.

2.10. Kinetic properties of alpha amylase

Enzyme characterization was carried out. A few essential parameters, such as substrate concentration, were optimized. Whereas Km and Vmax were calculated using a double reciprocal Line weaver–Burk plot. This plot was drawn using Microsoft Excel (Rasmey, 2018). The Lineweaver Burk plot is a graphical representation of enzyme kinetics. The x-axis is the reciprocal of the substrate concentration, or 1/[S], and the y-axis is the reciprocal of the reaction velocity, or 1/V. In this way, the Lineweaver Burk plot is often also called a double reciprocal plot.

3. Results and discussion

3.1. Isolation of amylolytic bacteria

Proper evaluation of strain combinations is a crucial step for improved enzyme production. Studies have shown that bacterial consortia can produce enzymes in higher yields with improved stability as they are resistant to environmental changes, so, they are more suitable for industrial applications (Far et al., 2020). Qualitative screening was carried out on the basis of zone of hydrolysis that was formed on the starch agar plates. Among all the 16 bacterial strains, five highest amylase producing bacterial strains (Fig. 1) were subjected to a compatibility test. In between 15 successful consortium tests, consortium No. 6 showed a synergistic effect and gave the highest titers of alpha amylase production through submerged fermentation. The selected bacterial consortium was identified using 16S ribosomal RNA (16S rRNA) sequencing. For this purpose, the PCR product was sent to Ist base Singapore for commercial sequencing. The obtained nucleotide sequences were analyzed using Chromas Lite version 2.1. The retrieved sequences were aligned using CLUSTAL W, and then compared with GenBank using basic local alignment search tool (BLAST). The phylogenetic tree was constructed for evolutionary analysis using MEGA 7. Results indicated the newly isolated strains were closely related to *Bacillus subtilis and Bacillus licheniformis* with a 100% similarity index and

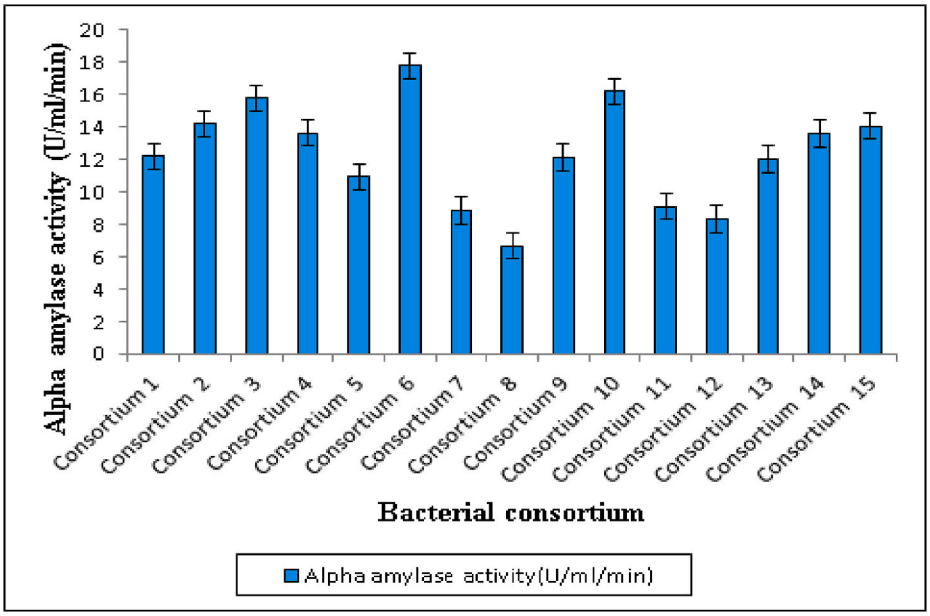

Fig. 1. Screening of bacterial consortium for alpha-amylase production.

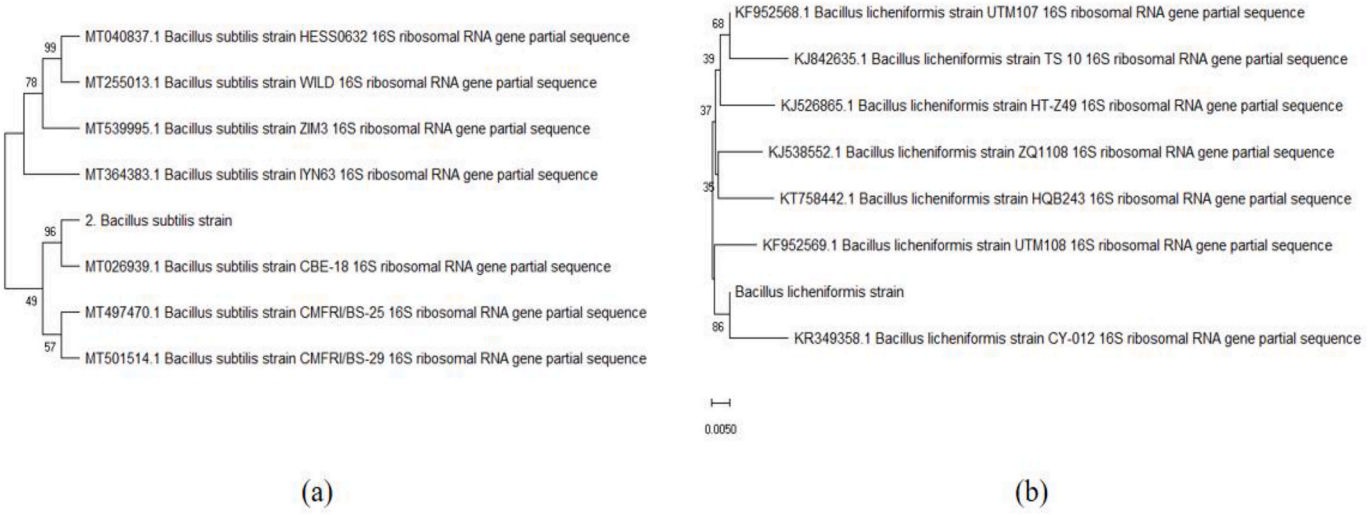

Fig. 2. Phylogenetic construction of 16S rRNA sequences by neighbor-joining method with a bootstrap value of 1000 (a) *Bacillus subtilis* (b) *Bacillus licheniformis*.

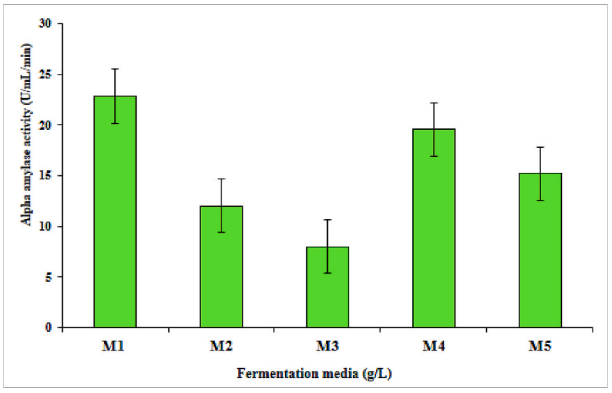

Fig. 3. Effect of fermentation media for the production of alpha-amylase by bacterial consortium.

were identified as *Bacillus subtilis* (accession no.OR294221) and *Bacillus licheniformis* (accession no.OR294227) respectively (Fig. 2). Traditionally isolated samples required morphological identification which is a laborious and time-consuming process. On the contrary, 16S rRNA sequence analysis is an accurate and rapid tool and commonly possess phylogenetic marker that is used for identification purposes (Parmar and Trivedi, 2021).

Table 6
The ANOVA table for a linear model for alpha amylase production.

	Sum of Squares	df	Mean Square	p-value	F-value	
Model	616.66	3	205.55	<0.0001	63.43	significant
A-Incubation Time	503.11	1	503.11	<0.0001	155.24	
C-pH	46.81	1	46.81	0.0052	14.44	
J-$CaCl_2$	66.74	1	66.74	0.0019	20.59	
Residual	25.93	8	3.24			
Cor Total	642.58	11				

P-value is significant at the level of $P \leq 0.05$.

3.2. Screening of fermentation media

Selection of appropriate fermentation media plays a significant role in microbial enzyme production. The current findings revealed that, out of all the five fermentation media, Medium 1 containing starch 15.0, peptone 5.0, NH_4 $(SO_4)_2$ 15, yeast extract 10.0, glucose 30, NaCl 0.5, $CaCl_2$ 0.002 produced the highest alpha-amylase activity (Fig. 3). This may be due to the supplementation of polysaccharides, nitrogen sources i.e. peptone and yeast extract and the Ca^{++} ions stimulate the amylase production (Cotarleț and Bahrim, 2012).

Table 5
Screening of some physicochemical determinants affecting the amylase production by Plackett Burman design.

Run	A:Incubation Time (h)	B: Inoculum size (ml)	C: pH	D: Starch (w/v)	E: Peptone (w/v)	F: Yeast extract (w/v)	G: $Mg.SO_4.7H2O$ (w/v)	H: NaCl (w/v)	J: $CaCl_2$ (w/v)	Alpha amylase activity (U/mL/min)
1	48	0.5	9	2.5	3	0.2	0.05	0.05	0.1	25
2	24	0.5	9	1	3	1	0.05	0.1	0.1	14
3	48	1.5	9	1	1	0.2	0.1	0.05	0.1	30
4	48	0.5	7	1	3	0.2	0.1	0.1	0.05	20
5	48	1.5	7	2.5	3	1	0.05	0.05	0.05	19
6	24	0.5	7	1	1	0.2	0.05	0.05	0.05	4
7	24	1.5	7	2.5	3	0.2	0.1	0.1	0.1	9.3
8	24	0.5	7	2.5	1	1	0.1	0.05	0.1	12
9	24	1.5	9	2.5	1	0.2	0.05	0.1	0.05	10
10	24	1.5	9	1	3	1	0.1	0.05	0.05	11
11	48	0.5	9	2.5	1	1	0.1	0.1	0.05	21
12	48	1.5	7	1	1	1	0.05	0.1	0.1	23

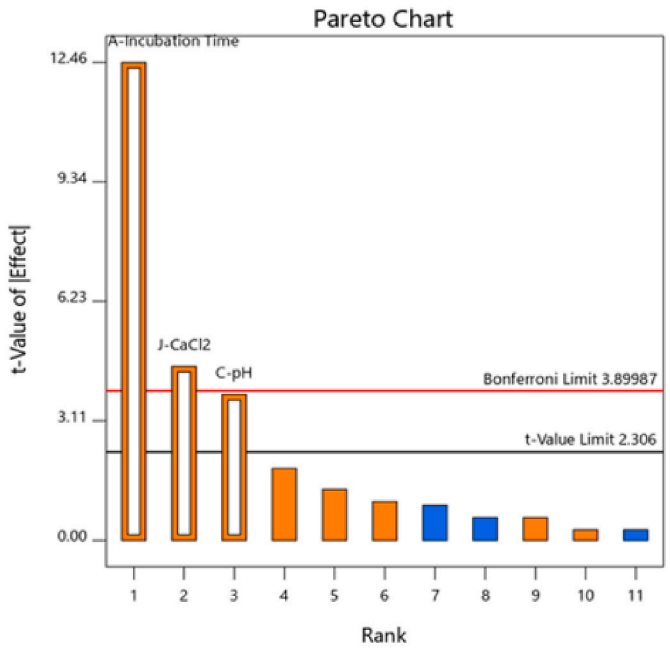

Fig. 4. Effect of seven physicochemical parameters in Pareto chart by Plackett–Burman.

Table 7
Effect of independent variables for amylase production by CCD.

Run	A: Incubation time h	B: pH	C: $CaCl_2$	Alpha amylase U/ml/min
1	7.63697	9	0.075	36
2	48	9	0.075	34
3	24	11	0.1	42
4	88.363	9	0.075	02
5	48	9	0.0329552	36
6	72	7	0.05	25
7	24	7	0.1	42
8	48	9	0.117045	36
9	24	11	0.05	41
10	48	5.63641	0.075	32
11	72	11	0.05	22
12	24	7	0.05	44
13	48	9	0.075	30
14	72	11	0.1	21
15	72	7	0.1	24
16	48	12.3636	0.075	37
17	48	9	0.075	39

Table 9
Model statistical summary for alpha amylase production.

Std. Dev.	3.69	R^2	0.9466
Mean	31.94	Adjusted R^2	0.8779
C.V. %	11.57	Predicted R^2	0.7157
		Adeq. Precision	13.2815

3.3. Screening of significant factors using Plackett-Burman design

In the present study, seven variables of media components, including incubation time (h), inoculum volume (ml), pH, starch (%), peptone (%), yeast extract (%), $MgSO_4.7H_2O$ (%), NaCl (%), and $CaCl_2$ (%), were evaluated using a Plackett–Burman design with twelve different trials.

Among all the trials, the combination in run three was found to be significant and gave optimum response value whereas the less production was obtained by trial number 6 (Table 5). The F-value in ANOVA table for factorial model implies that the model is significant. Whereas P-values less than 0.0500 indicated that the model terms were significant. In this case, A = incubation time (h), C = pH, and J = $CaCl_2$ are considered significant model terms (Table 6). The components of the fermentation media had both positive and negative effects on alpha-amylase production. As shown in Fig. 4, the impact of media components was also examined graphically using a Pareto chart. The t values in the chart indicate a positive effect of A = Incubation time (h), C = pH, and J = $CaCl_2$ on alpha amylase production. By analyzing the data in a linear equation the effect of significant parameters were determined.

$$\text{Alpha amylase} = +16.53 + 6.48A + 1.97C + 2.36J \quad \text{Eq. 3}$$

3.4. Optimization of the levels of physicochemical factors using central composite design

The final medium optimization and interaction between the screened factors were further studied using CCD (Table 7). The analysis of variance ANOVA of second-order polynomial model on alpha amylase production was observed in Table 8. It showed the significant effect of A = incubation time (h), C = pH and J = $CaCl_2$ on alpha amylase production. In addition to this predicted R^2 of 0.7157 was in reasonable agreement with the adjusted R^2 of 0.8779; i.e. the difference is less than 0.2 which indicate the significance of the model (Table 9). By putting the values in second polynomial equation, effect of significant parameters was analyzed.

$$\text{Alpha amylase} = +34.14–9.83A-0.0433B-0.2197C-0.3750AB-0.1250 AC+0.3750\ BCE\ -4.74\ A^2\ +0.7393\ 85\ B^2\ +1.27\ C^2 \quad \text{Eq. 4}$$

The interaction among the selected fermentation conditions and

Table 8
Analysis of variance of second-order polynomial model on alpha amylase production.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1693.37	9	188.15	13.78	0.0011	significant
A-Incubation time	1318.35	1	1318.35	96.56	<0.0001	
B-pH	0.0256	1	0.0256	0.0019	0.9667	
C–CaCl2	0.6590	1	0.6590	0.0483	0.8324	
AB	1.12	1	1.12	0.0824	0.7824	
AC	0.1250	1	0.1250	0.0092	0.9265	
BC	1.13	1	1.13	0.0824	0.7824	
A^2	253.37	1	253.37	18.56	0.0035	
B^2	6.16	1	6.16	0.4513	0.5233	
C^2	18.17	1	18.17	1.33	0.2865	
Residual	95.57	7	13.65			
Lack of Fit	54.90	5	10.98	0.5400	0.7499	not significant
Pure Error	40.67	2	20.33			
Cor Total	1788.94	16				

P-value is significant at the level of $P \leq 0.05$.

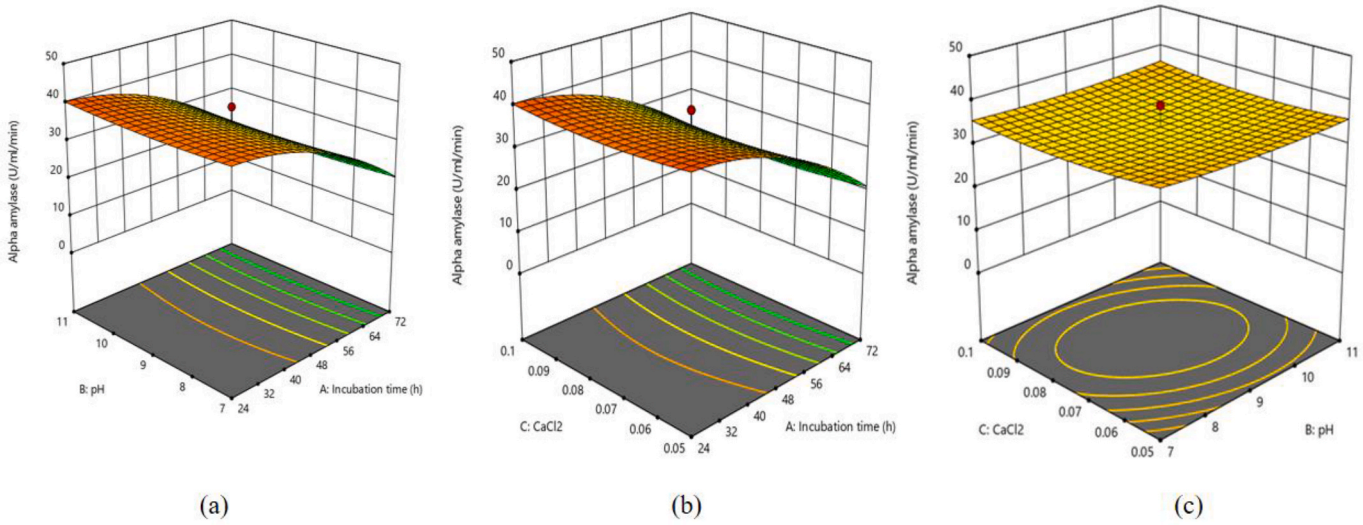

Fig. 5. Effect of significant parameters by 3-D response surface plot (a) pH and incubation time (b) $CaCl_2$ and incubation time(c) pH and $CaCl_2$.

Table 10
Partial purification of alpha amylase.

Purification Steps for	Total enzyme activity (IU)	Specific activity (U/mg)	Total protein content (mg/ml)	Purification fold	% Yield	Volume
Crude	46.3	0.70687	65.5	1	100	100
Ammonium sulfate precipitation	69.6	1.474576	47.2	2.086064	150.324	21.8

their optimum levels for alpha amylase production were studied through the contour plots along with the 3D response surface. Fig. 5a exhibited the interaction between pH and incubation time for alpha amylase production. The higher titers of alpha amylase were recorded at pH 7 after 24 h of incubation. However, enzyme activity decreases with further increases in incubation time and pH. The alteration in pH above or below the optimal value may inhibit the microbial growth, which eventually drops enzyme activity. Additionally, changes in pH affects the stability and can reduce enzyme secretion. The present work lines up with Viswanathan et al. (2014), who mentioned pH 7 for *Bacillus* spp. However, low enzyme production was observed after 24 h. It might be due to the nature of the organism, media status, or the environmental conditions. Current findings contradict Deb et al. (2013), who reported 48 h of incubation for *B. subtilis*.

Fig. 5b demonstrated the effect of $CaCl_2$ concentration and time of incubation on alpha amylase production. It can be observed that maximum alpha amylase production was obtained at 0.05% of $CaCl_2$ concentration after 24 h. The current study suggests that level of alpha amylase depends on the effect of incubation time. An increase in incubation time had a negative impact on alpha amylase production, which was possibly due to the denaturation of the enzyme. The findings do not support Simair et al. (2017), who mentioned 36 h for alpha amylase production. However, the presence of Ca^{2+} ions as $CaCl_2$ in culture media induces the alpha-amylase production. On the other hand, an increase in the concentration of Ca^{++} ions decrease enzyme production. This might be due to the fact that at higher concentrations of Ca^{++} ions bind to all Ca^{2+} binding sites, which overlap with the catalytic site. As a result, the enzyme is no longer able to act on the substrate, and as a result the enzyme activity decreases (Kizhakedathil, 2021). The current work is in accordance with the previous studies reported by Far et al. (2020) who mentioned the positive effect of Ca^{++} ions on alpha-amylase production with *Bacillus amyloliquefaciens*. Fig. 5c demonstrates the positive effect of pH and $CaCl_2$ on enzyme production by keeping the incubation time at a constant level. Maximum amount of alpha amylase production was achieved by 0.05% addition of $CaCl_2$ at pH 7.

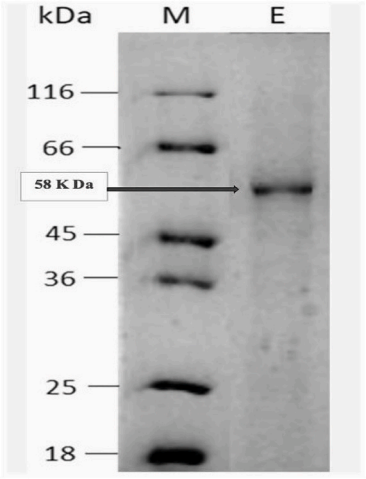

Fig. 6. Partial purification of alpha amylase obtained from *Bacillus* consortium Lane1 Marker Lane 2: Partially purified enzyme.

3.5. Partial purification

After the process of statistical optimization, the fermentation media was partially purified. The crude extract yielded about 69.6 IU of enzyme. The 2-fold increase with 1.4 U/mg specific activity was obtained at 60% saturation level (Table 10). Present work contradicts Ozdemir et al. (2011), who reported 65% of saturation level and agreed Tallapragada et al. (2017) who reported 60% of saturation for alpha amylase. The molecular weight of partially purified enzyme was found to be 58 kDa (Fig. 6).

3.6. Kinetic properties of alpha amylase

The effect of temperature (40°C–80°C) and pH (7–12) on alpha

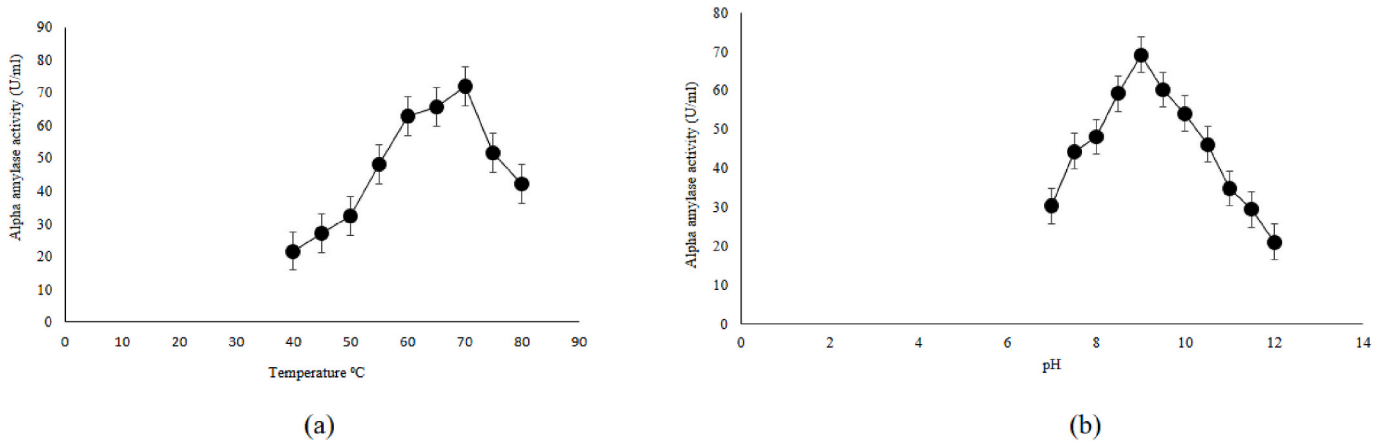

Fig. 7. Effect of different factors on amylase activity (a) Temperature (b) pH.

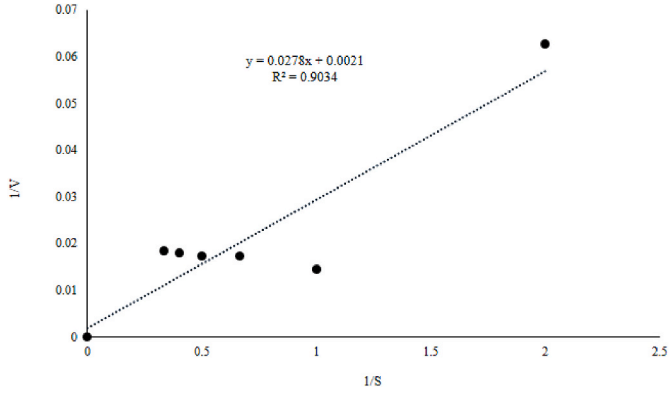

Fig. 8. Lineweaver-Burk plot calculating km and Vmax.

amylase activity were also determined. Optimum enzyme activity was achieved at 70°C and lowest enzyme activity was obtained at 40°C (Fig. 7a). The present work agreed with Xie et al. (2014) who reported similar amylase activity temperature. On the other hand, optimal enzyme activity was witnessed at pH 9 and less activity was achieved at pH 12. Present studies were comparable with Yang et al. (2011) who mentioned pH 9 for alpha amylase (Fig. 7b). The kinetic parameters such as Michaelis constant (Km) and maximum velocity (Vmax) were determined through the Line weaver Burk plot. Km and Vmax values were evaluated to be 13.2 mM and 47.4 mg/ml, respectively (Fig. 8).

4. Conclusion

The present study was performed to screen the most promising bacterial consortium for alpha amylase production. Bacterial strains were the main choice over fungal species due to their characteristics and kinetic properties. The molecular characterization of the consortium indicates it consists of *Bacillus subtilis* and *Bacillus licheniformis*. From an economic point of view, the current study revealed that this bacterial consortium has a good ability to produce alpha amylase. A statistical tool such as RSM was confirmed to be a powerful method for media component optimization for alpha amylase production. 3-D response surface plots were generated which evaluated the effect of significant factors such as pH, incubation time, and $CaCl_2$ concentration on the yield of alpha amylase. Interestingly, remarkable similarities were found between the predicted and experimental results. According to the findings of response surface plots, neutral pH and 1 day incubation period gave optimal alpha amylase production. 0.05% $CaCl_2$ concentration along with 24 h incubation period also induced alpha amylase production. Shorter incubation time is a novel and promising factor for the utilization of this bacterial combination in industry for large scale production of alpha amylase, as it reduces the cost of the fermentation process. Moreover, partial purification followed by enzyme characterization was carried out. The smaller Km value indicated greater affinity between the enzyme and substrate, whereas Vmax value demonstrated that maximum product was generated. The outcome of this investigation suggested that this indigenously isolated bacterial consortium could be a promising candidate for industrial applications. In context of the current world scenario, a cost effective and sustainable approach for large scale and commercial production of alpha amylase is needed. In addition to novel approaches like use of bacterial consortiums and RSM, agricultural waste can also be utilized to add the nutrients. In addition to this, different purification techniques can also be used to fully purify the alpha amylase. So that it can be used in a large number of industries.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Adiguzel, A., Ozkan, H., Baris, O., Inan, K., Gulluce, M., Sahin, F., 2009. Identification and characterization of thermophilic bacteria isolated from hot springs in Turkey. J. Microbiol. Methods 79 (3), 321–328. https://doi.org/10.1016/j.mimet.2009.09.026.

Ahmed, S.A., Mostafa, F.A., Helmy, W.A., Abdel-Naby, M.A., 2017. Improvement of bacterial α-amylase production and application using two steps statistical factorial design. Biocatal. Agric. Biotechnol. 10 (17), 224–233. https://doi.org/10.1016/j.bcab.2017.03.004.

Bahri, S., Homaei, A., Mosaddegh, E., 2022. Zinc sulfide chitosan hybrid nanoparticles as a robust surface for immobilization of *Sillago sihama* α-amylase. Colloids Surf. B Biointerfaces 218 (1), 112754. https://doi.org/10.1016/j.colsurfb.2022.112754.

Bhardwaj, K.K., Kumar, R., Bhagta, S., Gupta, R., 2021. Optimization of culture conditions by response surface methodology for production of extracellular esterase from *Serratia sp*. EST-4. J. King Saud Univ. Sci. 33 (8), 1–7. https://doi.org/10.1016/j.jksus.2021.101603.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Cotarlet, M., Bahrim, G.E., 2012. Application of Plackett-Burman experimental design to optimize the cold-active alpha amylase biosynthesis by psychrotrophic *Streptomyces* 4 alga. The Annals of the University Dunarea de Jos of Galati. Fascicle VI-Food Technology 36 (2), 9–19.

Deb, P., Talukdar, S.A., Mohsina, K., Sarker, P.K., Sayem, S.A., 2013. Production and partial characterization of extracellular amylase enzyme from *Bacillus amyloliquefaciens* P-001. SpringerPlus 154 (2), 1–12. https://doi.org/10.1186/2193-1801-2-154.

Embaby, A.M., Melika, R.R., Hussein, A., El-Kamel, A.H., Marey, H.S., 2018. Biosynthesis of chitosan-Oligosaccharides (COS) by non-aflatoxigenic *Aspergillus* sp. strain EGY1 DSM 101520: a robust biotechnological approach. Process Biochem. 64 (1), 16–30. https://doi.org/10.1016/j.procbio.2017.09.030.

Far, B.E., Ahmadi, Y., Khosroshahi, A.Y., Dilmaghani, A., 2020. Microbial alpha-amylase production: progress, challenges and perspectives. Adv. Pharmaceut. Bull. 10 (3), 350–358. https://doi.org/10.34172/apb.2020.043.

Green, M.R., Sambrook, J., 2012. In: Moleculer Cloning: a Laboratory Manual, fourth ed. Cold Spring Harbor Laboratory Press, pp. 127–129.

Haq, I., Ali, S., Javed, M.M., Hameed, U., Saleem, A., Adnan, F., Qadeer, M.A., 2010. Production of alpha amylase from a randomly induced mutant strain of *Bacillus amyloliquefaciens* and its application as a desizer in textile industry. Pakistan J. Bot. 42 (1), 473–484.

Kanimozhi, M., Johny, M., Gayathri, N., Subashkumar, R., 2014. Optimization and production of α -amylase from halophilic *Bacillus* species isolated from mangrove soil sources. Journal of Applied and Environmental Microbiology 2 (3), 70–73. https://doi.org/10.12691/jaem-2-3-2.

Kar, S.D., Kumar, D.T., Chandra, R.R., 2010. Optimization of thermostable α-amylase production by *Streptomyces erumpens* MTCC 7317 in solid-state fermentation using cassava fibrous residue. Braz. Arch. Biol. Technol. 53 (2), 301–309. https://doi.org/10.1590/S1516-89132010000200008.

Kizhakedathil, M.P.J., 2021. Acid stable α-amylase from *Pseudomonas balearica* VITPS19—production, purification and characterization. Biotechnology Reports 30 (1), 1–8. https://doi.org/10.1016/j.btre.2021.e00603.

Matrawy, A.A., Khalil, A.I., Embaby, A.M., 2022. Bioconversion of bread waste by marine psychrotolerant *Glutamicibacter soli* strain AM6 to a value-added product: cold-adapted, salt-tolerant, and detergent-stable α-amylase (CA-AM21). Biomass Conversion and Biorefinery 13 (1), 12125–12142. https://doi.org/10.1007/s13399-022-02325-3.

Matrawy, A.A., Khalil, A.I., Marey, H.S., 2021. Biovalorization of the raw agro-industrial waste rice husk through directed production of xylanase by *Thermomyces lanuginosus* strain A3-1 DSM 105773: a statistical sequential model. Biomass Conversion and Biorefinery 11 (5), 2177–2189. https://doi.org/10.1007/s13399-020-00824-9.

Matrawy, A.A., Marey, H.S., Embaby, A.M., 2023. The agro-industrial byproduct wheat bran as an Inducer for alkaline protease (ALK-PR23) production by pschyrotolerant *Lysinibacillus sphaericus* strain AA6 EMCCN3080. Waste and Biomass Valorization 1 (1), 1–16. https://doi.org/10.1007/s12649-023-02283-5.

Nor, N.M., Mohamed, M.S., Loh, T.C., Foo, H.L., Rahim, R.A., Tan, J.S., Mohamad, R., 2017. Comparative analyses on medium optimization using one-factor-at-a-time, response surface methodology, and artificial neural network for lysine–methionine biosynthesis by *Pediococcus pentosaceus* RF-1. Biotechnol. Biotechnol. Equip. 31 (5), 935–947. https://doi.org/10.1080/13102818.2017.1335177.

Ozdemir, S., Matpan, F., Guven, K., Baysal, Z., 2011. Production and characterization of partially purified extracellular thermostable α-amylase by *Bacillus subtilis* in submerged fermentation (SmF). Prep. Biochem. Biotechnol. 41 (4), 365–381. https://doi.org/10.1080/10826068.2011.552142.

Parmar, B., Trivedi, S., 2021. Production of microbial keratinase using newly isolated strain of *Stenotrophomonas maltophilia* its characterization and applications. Kuwait Journal of Science 48 (4), 1–4. https://doi.org/10.48129/kjs.v48i4.9973.

Pranay, K., Padmadeo, S.R., Jha, V., Prasad, B., 2019. Screening and identification of amylase producing strains of *Bacillus*. J. Appl. Biol. Biotechnol. 7 (1), 57–62. https://doi.org/10.7324/JABB.2019.70409.

Rasmey, A.H.M., 2018. Kinetic Properties of α-amylase produced by *Bacillus megaterium* RAS103 under optimum conditions in submerged fermentation. Egypt. J. Microbiol. 53 (1), 219–230. https://doi.org/10.21608/EJM.2018.5628.1075.

Riaz, A., Qadar, S., Anwar, A., Iqbal, S., Bano, S., 2009. Production and characterization of thermostable α-amylase from a newly isolated strain of *Bacillus subtilis* KIBGE-HAR. Internet J. Microbiol. 6 (1), 1–8.

Saha, S.P., Mazumdar, D., 2019. Optimization of process parameter for alpha-amylase produced by *Bacillus cereus* AMY3 using one factor at a time (OFAT) and central composite rotatable (CCRD) design-based response surface methodology (RSM). Biocatal. Agric. Biotechnol. 19 (1), 1–11. https://doi.org/10.1016/j.bcab.2019.101168.

Samanta, S., Das, A., Halder, S.K., Jana, A., 2014. Thermodynamic and kinetic characteristics of an α-amylase from SKB4. Acta Biol. Szeged. 58 (2), 147–156.

Santiago, C.D., Yagi, S., Ijima, M., Nashimoto, T., Sawada, M., Ikeda, S., Asano, K., Orikasa, Y., Ohwada, T., 2017. Bacterial compatibility in combined inoculations enhances the growth of potato seedlings. Microb. Environ. 32 (1), 14–23. https://doi.org/10.1264/jsme2.ME16127.

Simair, A.A., Khushk, I., Qureshi, A.S., Bhutto, M.A., Chaudhry, H.A., Ansari, K.A., Lu, C., 2017. Amylase production from thermophilic *Bacillus sp*. BCC 021-50 isolated from a marine environment. Fermentation 3 (1), 1–25. https://doi.org/10.1264/jsme2.ME16127.

Tallapragada, P., Dikshit, R., Jadhav, A., Sarah, U., 2017. Partial purification and characterization of amylase enzyme under solid state fermentation from *Monascus sanguineus*. J. Genet. Eng. Biotechnol. 15 (1), 95–101. https://doi.org/10.1016/j.jgeb.2017.02.003.

Viswanathan, S., Rohini, S., Rajesh, R., Poomari, K., 2014. Production and medium optimization of amylase by *Bacillus spp* using submerged fermentation method. World J. Chem. 9 (1), 1–6. https://doi.org/10.5829/idosi.wjc.2014.9.1.76128.

Xie, F., Quan, S., Liu, D., Ma, H., Li, F., Zhou, F., Chen, G., 2014. Purification and characterization of a novel α-amylase from a newly isolated *Bacillus methylotrophicus* strain P11-2. Process Biochem. 49 (1), 47–53. https://doi.org/10.1016/j.procbio.2013.09.025.

Yang, H., Liu, L., Li, J., Du, G., Chen, J., 2011. Heterologous expression, biochemical characterization and overproduction of alkaline α-amylase from *Bacillus alcalophilus* and *Bacillus subtilis*. Microb. Cell Factories 10 (17), 1–9. https://doi.org/10.1186/1475-2859-10-77.