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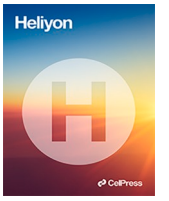

Research article

Optimization of amylase production using response surface methodology from newly isolated thermophilic bacteria

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ABSTRACT

Present study was aimed at screening and characterizing thermostable amylase-producing bacteria from water and sediment samples of unexplored hot spring of Tatta Pani Kotli Azad Kashmir. Four thermophilic isolates were characterized on morphological, biochemical, physiological basis and were authenticated by molecular analysis. By 16S rDNA sequencing, isolates were identified as *Anoxybacillus mongoliensis* (MBT001), *Anoxybacillus flavithermus* (MBT002), *Bacillus* (MBT004). Among all identified strains, MBT003 showed maximum homology with both *Anoxybacillus mongoliensis* and *Anoxybacillus flavithermus*. Amylase activity was analyzed qualitatively in starch agar and quantitatively by DNS method. The optimal enzyme production was observed and authenticated by Response Surface Methodology at 7 pH, 70 °C, 1.25% substrate concentration, 300 μL of inocula volume after 48 h of incubation. Optimum amylase activity (4.4 U/mL) and stability (3.3 U/mL) was observed with 1.5% soluble starch at 70 °C. Maximum activity (3.7 U/mL) and stability (1.5 U/mL) was found at pH 8. Enzyme activity was increased in the presence of $MgSO_4$ and $CaCl_2$. Amylase was stable with surfactants and commercial detergents for 30 min. Supplementation of the enzyme with commercial detergent improved the washing ability of the detergent. This investigation has revealed that these thermostable bacteria are excellent source of amylase which can be used commercially for generating economic activity on sustainable basis.

1. Introduction

Hot water springs were formed as a result of geothermic central heating due to which ground water oozes out from the earth crust [1]. Alkaline/freshwater thermal springs are categorized as extreme hot environments which are niche of diversity of thermophilic bacteria [2]. Main habitats of thermophilic microorganisms are geothermal areas [3]. They usually grow at temperatures of 50 °C or higher. Thermophilic microorganisms are very important from economical point of view because they are source of many thermophilic enzymes, such as protease, lipase, amylases, glucoamylases, pullulanase, cellulases, xylanases, chitinases, DNA polymerases [3].

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Enzymes from thermophiles are capable to tolerate proteolysis and harsh environments like the occurrence of organic solvents, denaturing agents and high salinity [4]. Hot springs are the best source of industrially significant thermophiles like *Thermus aquatics*, from which very important enzyme Taq polymerase was isolated [5].

Among commercially important enzymes, amylases are most widely used in the starch industry. Amylases are contributing 25–30% of worldwide market of catalyst [6]. Many of the industrial processes are carried out at high temperature, but due to poor stability of amylase its applications are limited. Discovery of the thermostable amylases not only resolves the stability problem but also enhances the catalytic activity and rate of reaction. Hence forth, the use of thermostable amylase lessens the possible microbial contaminations and reduces the viscosity of medium, which promotes the starch processing industries at extreme temperatures [7].

Amylases are starch hydrolyzing enzymes that degrade the internal alpha 1–4 glycosidic linkage in polysaccharides with the maintenance of alpha anomeric conformation in the products [8]. At high temperatures (100–110 °C) enzymatic liquefaction and saccharification of starch are carried out. Currently thermo active amylases have been explored to enhance the economic practices of starch hydrolysis. These are highly applicable in manufacturing of valued foodstuffs like crystalline dextrose, dextrose syrup, maltodextrins, glucose and maltose [9]. Thermostable α-amylase have gained importance in pharmaceutical, brewing, detergent and textile industries [10]. Characterization of microbes with high α-amylase activity, high thermostability, optimum conditions of pH and temperature could, therefore assist in the discovery of more promising α-amylase for industrial uses [9].

Tatta Pani hot spring located in Azad Kashmir (33.60557° N/73.94,814° E), is a repository of variety of thermophilic bacteria. The study area is part of the Sub-Himalayas and is positioned on the northern tip of Tatta Pani anticline. It is positioned on the bank of river Poonch at the altitude of 2237 feet [11]. This area of Pakistan has not been explored yet for the microbiological diversity. No significant research has been reported with comprehensive analysis, isolation, purification and commercial application of the thermostable enzymes from Tatta pani hot spring. The aim of current study was isolation and characterization of thermostable amylase yielding bacteria from geothermal spring of Tatta Pani Kotli AJ&K and statistical optimization of the conditions for maximum enzyme production. Furthermore, its potential application in laundry was investigated.

2. Materials and methods

2.1. Isolation and characterization of thermophilic bacteria

One sediment sample (10 g of sediment soil) and two water samples (10 ml each) were collected from different sites of Tatta Pani hot spring of Kotli AJK in sterile poly bags and bottles. The samples were immediately brought into the laboratory. Two water samples of about 100 μl were spreaded on LB agar plates, whereas, sediment sample was spreaded on LB agar by serial dilution method. After 24 h of incubation at 70 °C bacterial strains, presenting clear morphological difference, were purified by further streaking on LB agar media. Cell and colony morphology of purified isolates were examined. For physiological characterization, isolates were grown in LB broth at different pHs, temperature, incubation periods and inocula volumes. By API strips (bioMérieux) biochemical analysis was done by following the procedure of manufacturer.

2.2. Molecular identification

For molecular identification of bacterial isolates, genomic DNA was isolated by using the method of Cheng and Jiang et al. [12] with slight modification. For extraction of DNA, cell suspension of 1 ml was centrifuged at 8000 rpm for 2 min. The obtained pellet was rinsed with 300 μl of STE buffer and centrifuged at 8000 rpm for 2 min. TE buffer (200 μl) and tris-saturated phenol (100 μl) was suspended in pellet and centrifuged at 13,000 rpm for 5 min. In next step, supernatant (160 μl), TE buffer (40 μl) and chloroform (100 μl) was transferred to Eppendorf tube and mixed gently by inverting which give rise a white interface. Mixture was again centrifuged for 5 min at 13,000 rpm and process was repeated until no interface left. Finally, upper layer of DNA was transferred to a new tube. 16S rDNA amplification was carried out by polymerase chain reaction by means of forward primer 27F (AGAGTTTGATCCTGGCTCAG) and reverse primer 1492R (TACGGCTACCTTGTTACGACTT). The PCR products were sent to Macrogen Inc., South Korea for sequencing. After sequencing, obtained sequences were evaluated with BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and submitted to NCBI GenBank with the accession numbers OM918280, OM918281, OM918282 and OM918283. In order to find the evolutionary links among bacteria in the community, phylogenetic trees of strains were constructed by using MEGA6 [13].

2.3. Screening of amylase producing bacterial isolates

The amylase producing potential of the thermotolerant bacterial strains was investigated by growing the bacteria on LB agar plates containing 1% (w/v) soluble starch. After incubation of one day at 70 °C, plates were flooded with iodine solution to detect the presence of clear halos around bacterial colonies [14]. Bacteria with positive amylase results were used for further studies.

2.4. Amylase production

For enzyme production, about 100 μl suspension of bacteria was transferred in test tubes having sterile LB broth (10 ml) medium supplemented with soluble starch (1.5%). Production medium was incubated for 48 h at 70 °C. After incubation, the culture broths were centrifuged at 8000 rpm for 10 min. After centrifugation, the supernatant was used as the crude enzyme for enzyme assay. For further assay, reaction mixture was prepared by taking 100 μl of crude enzyme and 1 ml of 0.05 M Sodium Phosphate buffer pH 6.5. It

was placed at 70 °C for 10 min. Then, about 2 ml of DNS was added in reaction mixture to stop the reaction by placing in water bath for 10 min at 90 °C. The release of reducing sugar was recorded at 450 nm by using the spectrophotometer (Model: AE-S90-MD, UK). Glucose was utilized as calibration standard (1–15 μg ml-1) [14,15].

2.5. Effect of different conditions on enzyme production

One variable at a time approach was used to study the effect of different factors on enzyme production under laboratory conditions. Effect of various conditions i.e. incubation periods (24, 48 and 72 h), substrate concentrations (0.5–2%), temperatures (60–80 °C), inocula volumes (100–500 μl) and pH ranges (5–9) was checked by using DNS method.

2.6. Statistical optimization of enzyme production by response surface methodology

To validate the optimum enzyme production at different conditions, Central Composite Design (CCD) was applied using the statistical software Design Expert 13 (State-Ease). In CCD, five factors, i.e. incubation periods, substrate concentrations, temperatures, inocula volumes and pH were optimized for enzyme production. Response surface methodology (RSM) was applied to the experimental data. In order to acquire regression equation linear and first order monomial was used. By the computation of successive F test, lack-of-fit test and other accuracy measurements, best model was designated [16].

2.7. Determination of amylase activity

To study enzyme activity, crude enzyme (500 μl) from freshly grown bacterial culture was mixed with 0.05 M sodium phosphate buffer (1 mL) having 1.5% soluble starch. The reaction mixture was incubated at 70 °C for 10 min. Further enzyme assay was accomplished by DNS procedure. The reduction in color was recorded at 450 nm by spectrophotometer [15].

2.8. Effect of different conditions on enzyme activity

Effects of different conditions such as different pH (5–9) of 1 mL 0.05 M sodium phosphate buffer, inocula volumes (500 μl–1000 μl) and incubation time (10–60 min) on enzyme activity was also evaluated. Likewise, effects of various metal ions i.e. $MgSO_4{\cdot}7H_2O$, $CaCl2{\cdot}7H_2O$, $CuSO_4{\cdot}7H_2O$ and $K_2SO_4.7H_2O$ (5 mM concentration) on enzyme activity was also observed using DNS method described above [17].

2.9. Stability of amylase enzyme in different conditions

Stability of amylase enzyme in different conditions such as temperature, pH, in commercially available detergents and surfactants was measured. The thermo stability of catalyst was evaluated by incubating 500 μL of crude amylase at temperatures between 50 and 100 °C for 30 min. Further assay was performed by DNS method. For pH stability, crude amylase (500 μL) was transferred separately in sodium phosphate buffer (1 ml) of different pH values (5, 6, 7,8 and 9). Mixture was pre-incubated at 70 °C temperature for 30 min. By DNS protocol, further assay was done. Additionally, crude enzyme (500 μl) was mixed with commercially available detergents (1% of liquid surf excel and ariel) and with surfactants (1% of Tween 80 and Tween 20). Mixture was incubated at 70 °C for 30 min. Further amylase activity was determined by DNS method [18].

2.10. Partial purification of amylase

For purification of enzyme, broth culture (50 ml) was centrifuged at 12,000 rpm for 15 min on 4 °C. Crude enzyme in sample was saturated up to 60–80% with ammonium sulphate. After centrifugation, the obtained pellet was suspended in 50 mM Sodium Phosphate buffer and dialyzed for 24 h against the same buffer at 4 °C. The buffer was continuously stirred and changed three times during the process in order to get proper partial purification [19].

2.11. Protein estimation of partially purified and crude amylase

Protein contents of crude enzyme and partially purified proteins was evaluated by following Bradford [20] method. For protein estimation, partially purified and crude amylase (100 μl) was added separately in glass tubes and 5 ml of Bradford was placed in it. Under dark conditions, vortexed the mixture and left for 10 min at room temperature. Optical density was recorded at 595 nm. About 100 μl of BPS comprising of Bradford reagent (5 ml) was used as control sample. Bovine serum albumin (0.1 mg ml^{-1}) was used as standard [21].

2.12. Applications of crude amylase in laundry

Cleaning of potato curry and chocolate stains from cotton cloth pieces (2 × 2 inches) was checked by means of commercially available detergent (Surf excel) and thermozymes from all isolates. The criteria for selection of detergent (Surf excel) were that it is most commonly used commercial detergent in Pakistan. White cloth pieces stained with potato curry and chocolate (0.5%). After that,

fabrics were placed at 70 °C for 60 min for strong binding of stains. Stained cloth pieces of both potato curry and chocolate were dipped in four different treatments sets separately. 1)-Flask having 50 ml of distilled water, 2)- Flask containing commercial detergent (7 mg/ml) + 50 ml of distilled water, 3)-Distilled water (50 ml) + crude amylase (1 ml), 4)-Distilled water (50 ml) + detergent (Surf excel) (7 mg/ml) + amylase. Control sample was without enzyme and detergent. All sets were placed for 1 h at 40 °C in water bath at 80 rpm. After that the cloths were observed visually [22].

2.13. Statistical analysis

All the experimentations were performed in replicates. Results are signified as the mean ± standard error.

3. Results

3.1. Isolation and screening of amylase producing bacteria

A total of eight distinct colonies were isolated from sediment and water samples of Tatta Pani Kotli, AJK on LB agar plates. Four colonies named as MBT001, MBT002, MBT003 and MBT004 showed clear zones with 1% starch agar and therefore selected for further evaluation.

3.2. Characterization of enzyme producing bacteria

Colony morphology revealed that colonies of MBT001 and MBT002 were circular while MBT003 and MBT004 were irregular in shape. All isolated bacterial strains, possessed smooth margins, with colony size of 0.2–3 mm. All isolates were Gram-positive and oxidase positive. MBT001 and MBT003 showed negative results on catalase test, while rest of the isolates showed positive results on catalase test. The current experiments revealed that all bacterial isolates were thermophilic because they were able to grow between 50 and 80 °C temperatures and maximum growth of all strains was obtained at 70 °C. Bacterial strains were found very stable, as all isolates were able to grow at different pH ranges i.e. 6–9, inocula volumes of 100–500 μL and different incubation period i.e 24, 48 and 72 h pH 7 was observed as an optimum pH for maximum growth of studied bacterial isolates. Highest growth was obtained with 400 μL

Table 1
Phenotypic/physiological and biochemical characters of amylase producing thermophilic bacteria.

Characters	MBT001	MBT002	MBT003	MBT004
Colonies shape	Round	Round	Irregular	Irregular
Colonies pigmentation	Golden brown	Brown	Pale yellow	Brown
Cell shape	Rod	Rod	Rod	Rod
Gram reaction	+ve	+ve	+ve	+ve
Temperature range (°C)	50–80	50–80	50–80	50–80
Temperature optimum	70 °C	70 °C	70 °C	70 °C
Incubation period range (hours)	24–72	24–72 h	24–72 h	24–72 h
Optimum incubation period	24	24	24	24
pH range	5–9	5–9	5–9	5–9
pH optimum	7	7	7	7
Inocula volumes (μL)	100–500	100–500	100–500	100–500
Optimum inocula size	400	400	400	400
O-nitrophenyl-βD galactopyranosidase	0	0	0	0
Arginine dihydrolase	0	0	0	0
Lysine decarboxylase	0	0	0	0
Ornithine decarboxylase	0	0	0	0
Citrate utilization	0	0	0	1
H2S production	0	0	0	0
Urease	0	0	0	0
Tryptophan deaminase	1	1	1	1
Indole production	0	0	0	0
Voges Proskauer	1	1	0	1
Gelatinase	1	1	1	1
Glucose	0	0	0	0
Mannitol	0	0	0	0
Inositol	0	0	0	0
Sorbitol	0	0	0	0
Rhamnose	0	0	0	1
Saccharose	0	0	0	0
Melibiose	1	0	0	1
Amygdalin	1	0	0	1
Arabinose	1	0	0	1
Catalase	0	1	0	1
Oxidase	1	1	1	1

of inocula volume and optimum incubation period was 24 h (Table 1).

Biochemical tests showed that all bacterial strains were incapable to use various sugars, though, they efficiently produced tryptophan deaminase and arginase activities. Furthermore, strain MBT004 also produce arabinose, melibiose, amygdalin and also showed citrate utilization (Table 1). Phylogenetic examination based on 16S rRNA sequences represented that strain MBT001 was highly similar to *Anoxybacillus mongoliensis* (99% identity). Isolates MBT002 was similar to *Anoxybacillus flavithermus* (98%) and MBT003 was closest to both *Anoxybacillus mongoliensis* and *Anoxybacillus flavithermus* (99% identity). Isolate MBT004 showed maximum homology with *Bacillus* (97%) (Fig. 1(a–d)).

3.3. Optimization of enzyme production

Optimum levels of significant factors for enzyme production such as temperatures, incubation periods, inocula volumes, pH and substrate concentration was assessed by using Central Composite Design (CCD). A set of 20 experiments was conducted for four isolates (Table 2). The optimal levels of particular variables were attained by solving the regression equation and by evaluating the 3D surface plots. A brief description of statistics of the factors used for optimization of enzyme production is provided in supplementary (Table S1). The findings of the linear response surface model for MBT001, MBT002, MBT003 and MBT004 were attained in the ANOVA form are provided in supplementary (Table S2–S5). The precision of model was specified by the determination of coefficient (R2 = 0.5593), which explicated 55% of the response variation in terms of enzyme obtained from isolate MBT001. Determination coefficient of (R2 = 0.3371), showed 33% of the response dissimilarity in case of MBT002. Determination coefficient of R2 = 0.5647, revealed 56% of response variability in case of MBT003 and R2 = 0.5960, showed 59% of response variation in terms of MBT004. A first-order monomial function was sufficient to the enzyme production in laboratory by all bacterial isolates and following regression equations are obtained:

Regression equation for MBT001

Unit activity = 3.45908 + −0.015154*temperature + −0.00701065*incubation time + 0.00395676*inocula volumes + 0.0581732*pH + −0.120439*substrate concentrations

Regression equation for MBT002

Unit activity = +4.64835 – 0.053658*substrate concentrations − 0.013931*incubation periods − 0.027180 *temperatures + 0.003035*inocula volumes + 0.065308*pH

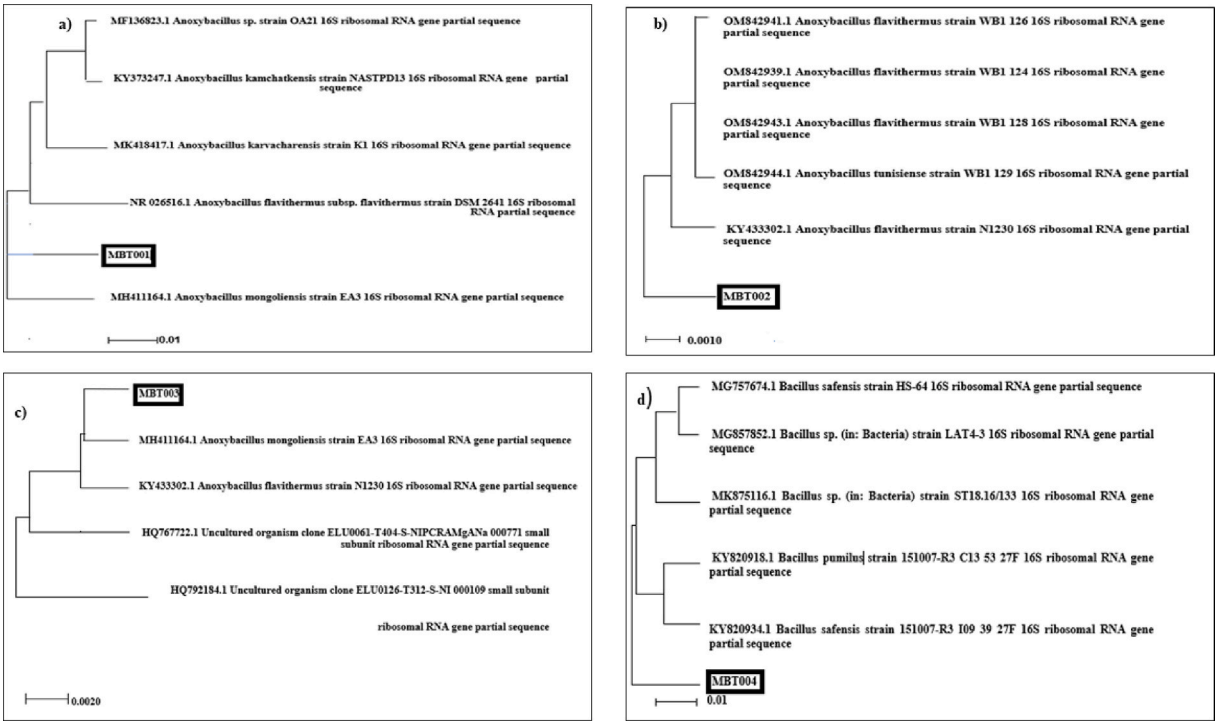

Fig. 1. Phylogenetic analysis of thermophilic bacteria based on 16S rDNA gene sequences. a) Phylogenetic analysis of MBT001, b) Phylogenetic analysis of MBT002, c) Phylogenetic analysis of MBT003, d) Phylogenetic analysis of MBT004.

Table 2
Central composite design (CCD) matrix for amylase derived from selected thermophilic bacteria.

Experimental variables						Response			
Runs	Temperature	Incubation periods	Inocula volumes	pH	Substrate con.	Unit activity (MBT001)	Unit activity (MBT002)	Unit activity (MBT003)	Unit activity (MBT004)
1	70	48	100	5	1.5	2.32496	2.00785	2.20044	2.23785
2	70	48	100	6	1.5	2.89402	2.8832	2.53142	2.71325
3	70	48	100	7	1.5	3.23041	3.01973	3.01156	3.20973
4	70	48	100	8	1.5	3.6209	4.00355	4.00013	4.0135
5	70	48	100	9	1.5	2.25239	2.16921	2.316	2.20692
6	70	48	100	7	0.5	2.57429	2.70734	2.5593	2.51584
7	70	48	100	7	1	2.75164	2.77329	2.59344	2.68003
8	70	48	100	7	1.5	3.05555	3.15797	3.0414	2.99913
9	70	48	100	7	2	2.17087	2.51251	2.35655	2.48228
10	70	48	100	7	1.5	3.05555	3.15797	3.0414	2.56513
11	70	48	200	7	1.5	3.06804	3.15963	3.08886	3.04473
12	70	48	300	7	1.5	3.08719	3.1155	3.15797	3.17629
13	70	48	400	7	1.5	4.10884	4.1 2525	3.29119	3.90818
14	70	48	500	7	1.5	3.95536	3.95289	4.62508	4.54182
15	70	24	100	7	1.5	2.55264	2.8757	2.6792	2.54515
16	70	48	100	7	1.5	3.05555	3.15797	3.0414	2.99913
17	70	72	100	7	1.5	2.21613	2.20704	2.00459	2.05703
18	60	48	100	7	1.5	2.35031	3.03141	2.58012	2.54681
19	70	48	100	7	1.5	3.05555	3.15797	3.0414	2.85129
20	80	48	100	7	1.5	2.04723	2.3578	2.52097	2.21517

Regression equation for MBT003

Unit activity = +2.18202 + 0.009146*substrate concentrations − 0.014054*incubation periods 0.002958*temperatures + 0.003621*inocula volumes + 0.145033*pH

Regression equation for MBT004

Unit activity = +2.96876 + 0.034003*substrate concentrations-0.010169*incubation periods + 0.004211*inocula volumes-0.016582*temperatures + 0.124821*pH

The F test analysis of variance revealed that the obtained regression is very important for the optimization of enzyme production. The Model F-value of 3.55 for MBT001, Model F-value of 3.44 for MBT002, Model F-value of 3.63 for MBT003 and Model F-value of 4.13 for MBT004 suggested that the model is significant. The P-values were lesser than 0.0500, hence, terms of the model were significant. The 3D surface plots characterized the optimal values of the factors in such a way that the response was maximum (Fig. 2 (a–d)). Response surface plots elucidated high enzyme yield at, 70 °C, pH 7, 300 μL inocula volumes, 48 h incubation period and 1.25% substrate concentration.

Similar results with slight differences were obtained when influence of different conditions on production of amylase was measured in laboratory by using one variable at a time approach. Maximum enzyme yield was attained at 70 °C, 48 h of incubation and 1.5% starch concentration, 400–500 μL inocula volumes and 8 pH, on tentative basis (Fig. S1(a–d)).

3.4. Effects of different physiological conditions on thermozymes activity

Effect of various environmental conditions i.e., substrate concentration, inocula volumes, pH, reaction times and metals ions on activity of amylase was estimated. Crude amylase was active in different substrate concentrations. Maximum activity was obtained with 1.5% of soluble starch at 48 h of incubation period and at 70 °C i.e. 4.4 U/mL, 4.1 U/mL, 4.3 U/mL, 4 U/mL for MBT001, MBT002, MBT003 and MBT004, respectively.

It was observed that maximum enzyme activity was obtained with inocula volume of 900 μL such as 2.9 U/mL, 3.2 U/mL, 3.3 U/mL and 3.2 U/mL for MBT001, MBT002, MBT003 and MBT004, respectively. After that activity was gradually decreased. All isolates exhibited maximum amylase activity at pH 8. On the other hand, enzyme was active at different incubation time i.e. 10, 20, 30, 40, 50 and 60 min. Activity of catalyst was gradually lessened by increasing incubation period. Good amylase activity was observed within 10 min of reaction time i.e. 4.2 U/mL, 4.4 U/mL, 4.3 U/mL, 4.4 U/mL for MB001, MB002, MB003 and MB004, respectively. In case of activity with metals, isolates MBT001 and MBT002 gave maximum activity with magnesium sulphate (3.4 U/mL, 3.5 U/mL). Bacterial isolates MBT003 and MBT004 gave high activity with calcium chloride (3.8 U/mL, 3.7 U/mL). However, very low activity was obtained with Cu_2SO_4 in the range of 1 U/mL (Fig. 3(a–e)).

3.5. Stability of amylase in different conditions

Stability of enzyme was recorded under various environmental conditions such as temperature (50–100 °C) and pH (5–9) and also

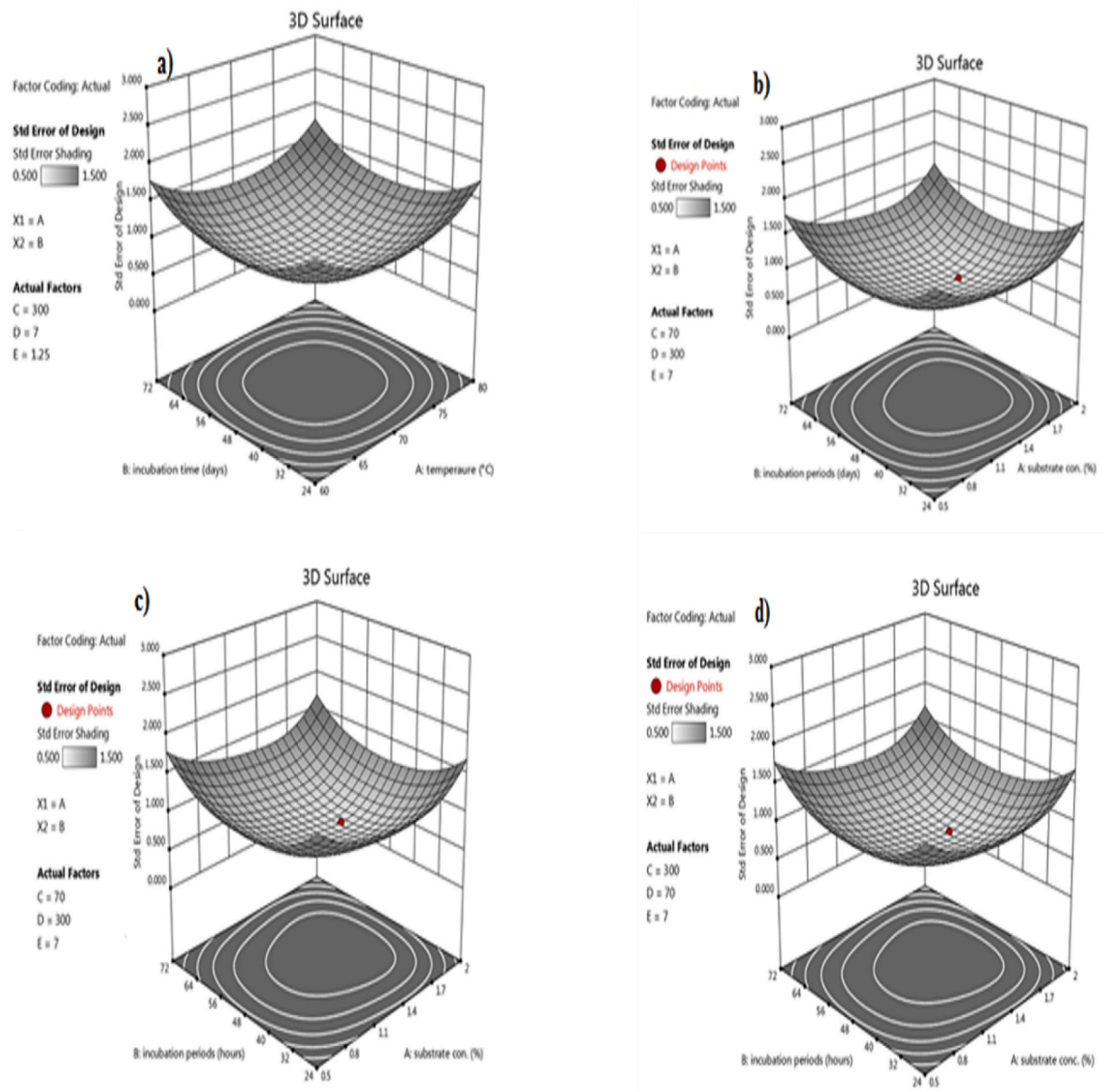

Fig. 2. 3D surface plots showing the effects of the medium components on enzyme production. Such as incubation period, temperature and substrate concentrations. a) Showing effects of temperature and incubation period on enzyme production of MBT001, b) Displaying effects of substrate concentrations and incubation period on enzyme production from MBT002, c) Exhibiting effects of incubation period and substrate concentration on enzyme production from MBT003, d) Displaying the effects of incubation period and substrate concentration on enzyme production of MBT004.

observed with commercially available detergents and surfactants. Findings presented that enzyme was greatly stable at 70 °C with unit activity of 3.8 U/mL, 5.1 U/mL, 3.9 U/mL and 3.8 U/mL for MBT001, MBT002, MBT003 and MBT004, respectively. The stability was decreased gradually at 80 °C i.e. 2.6 U/mL, 2.8 U/mL, 2.5 U/mL and 2.7 U/mL for MBT001, MBT002, MBT003 and MBT004, respectively. At 90 °C and 100 °C, the stability was decreased in the range of 1.0–1.1 U/mL for all isolates.

In terms of pH, crude enzyme from all isolates was stable in range of 7–8 pH at 70 °C temperature for 30 min. In case of stability in various detergents and surfactants, crude enzyme from MBT004 showed high stability in the presence of surf excel (1.7 U/mL). However, amylase from MBT002 and MBT003 was highly stable with Tween80 i.e. 1.4 U/mL and 1.7 U/mL, respectively. Crude amylase from MBT001 showed good stability with Tween 20 (1.8 U/mL) (Fig. 4(a–c)).

3.6. Protein estimation

Protein estimation of purified and crude enzyme revealed high protein contents i.e. 2.0 μg/mL, 2.2 μg/mL, 2.0 μg/mL and 2.15 μg/

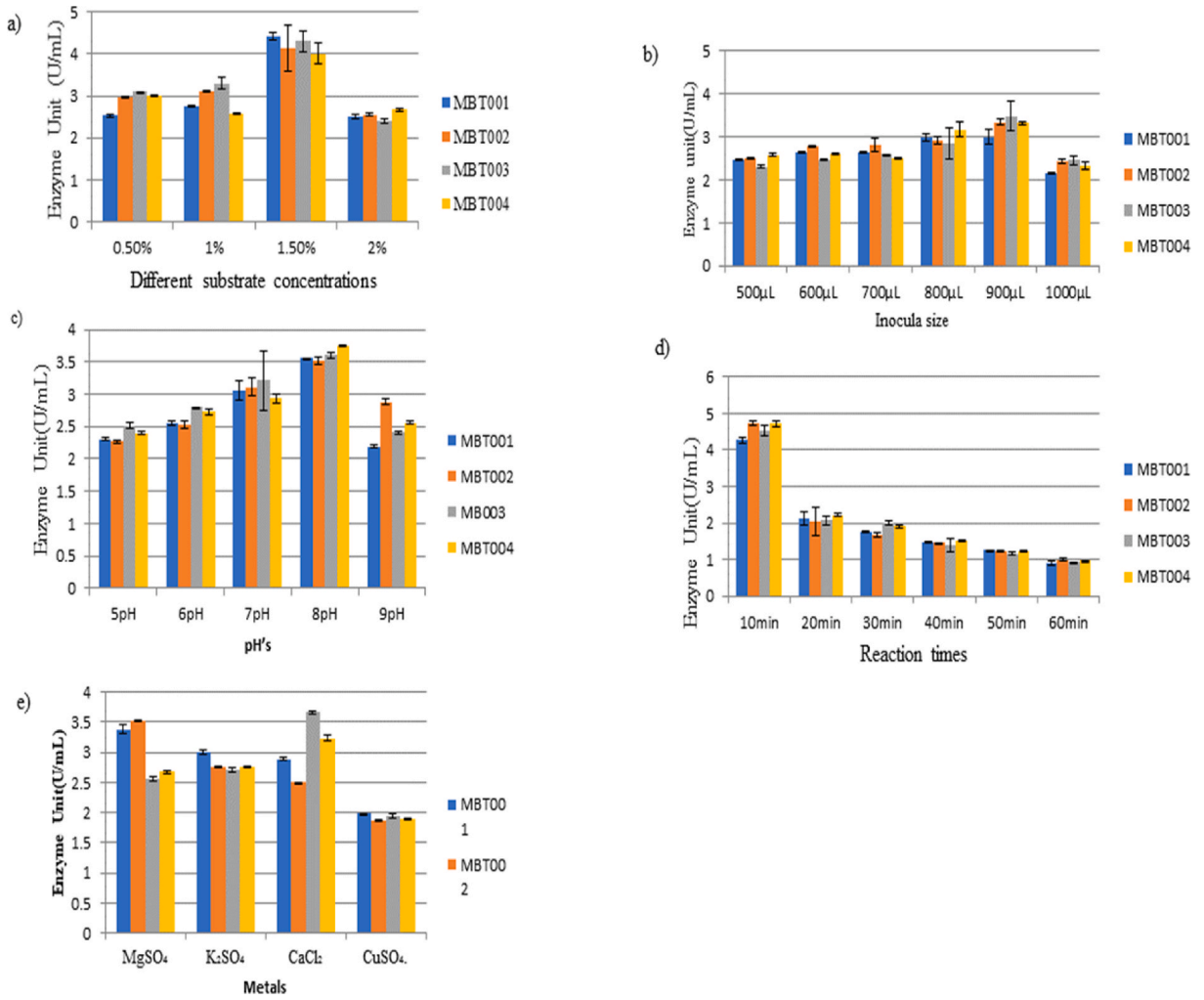

Fig. 3. Effect of different conditions on enzyme activity and thermal stability of enzyme. a) Different substrate conc. at 48 h s, b) Inocula volumes of crude enzyme, c) Activity in different pH ranges, d) Enzyme activity in different reaction times, e) Activity in the presence of different metals ions.

mL in purified protein as compared to crude enzyme i.e. 1.5 µg/mL, 1.6 µg/mL, 1.8 µg/mL and 1.7 µg/mL for MBT001, MBT002, MBT003 and MBT004, respectively (Fig. 5).

3.7. Evaluation of washing performance

Detergent only or crude amylase alone was not enough for eliminating the potato curry and chocolate stains from the selected fabrics. It was observed that when crude amylase combined to detergent, stains were highly faded from all the fabrics. However, removal of stain with only distilled water and detergent was found very low. It was visually observed that the stain was more faded after incubation of 50 min. This showed an improvement in washing ability of detergent with crude enzyme.

4. Discussions

Current study is the first report on production of thermostable amylase from thermophilic bacteria of Tatta Pani hot spring of Kotli, AJ & K, Pakistan. In previous studies Amin et al. [8] reported about the wide distribution and diversity of thermostable bacterial isolate in hot springs of Pakistan. Amylase enzyme obtained from thermophilic bacteria are highly thermostable and have great significance in industry. Moreover, these thermostable enzymes showed low chances of medium contamination and catalytic potentials. All these characteristics empower them to be applied in multiple industrial areas for the manufacture of variety of products as compared to mesophilic amylase.

Selected isolates in this study were identified as *Anoxybacillus mongoliensis* (MBT001), *Anoxybacillus flavithermus* (MBT002) and *Bacillus* (MBT004). MBT003 showed maximum homology with both *Anoxybacillus mongoliensis* and *Anoxybacillus flavithermus*. All the

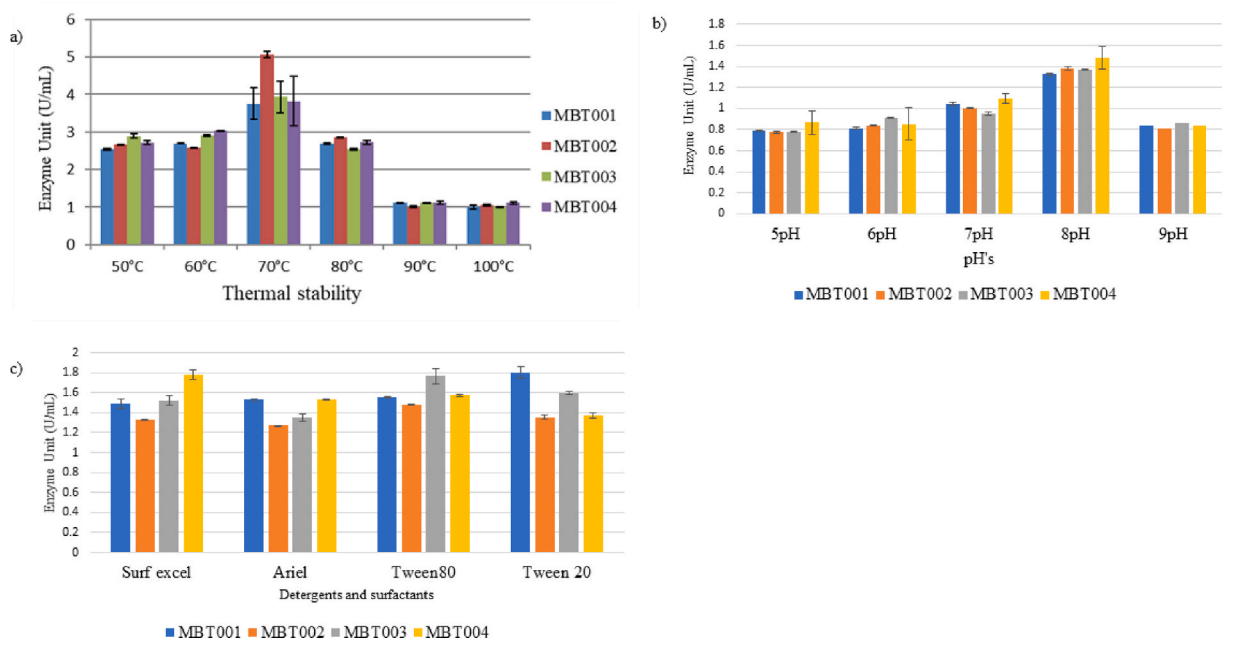

Fig. 4. Stability of amylase enzyme in different conditions. a) Thermal stability of crude enzymes at various temperatures, b) pH stability of enzymes, c) Enzyme stability in different commercially available detergents and in various surfactants. Enzyme unit activity was studied by using glucose as standard and in terms of absorbance measured at 450 nm on UV spectrophotometer.

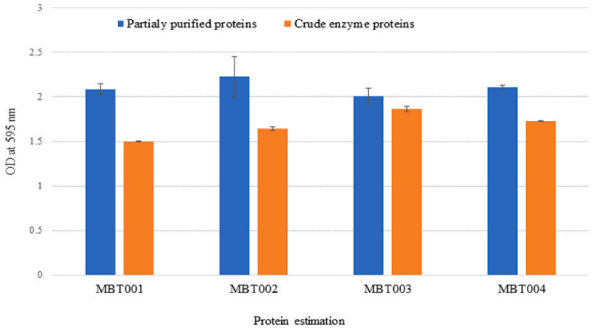

Fig. 5. Estimation of protein contents in partially purified proteins and in crude enzyme. Protein estimation was studied by using Bovine serum albumin as standard and in terms of absorbance measured at 595 nm on UV spectrophotometer.

bacterial strains isolated were able to grow and produce amylase enzyme at 70 °C. It was reported that large number of *Anoxybacillus* species are moderately thermostable which grow between 30 and 75 °C temperature range [23]. Similarly, Özdemir et al. [24] documented the isolation and production of thermoactive α-amylase from *Anoxybacillus* sp. nov. under solid-state fermentation (SSF) at 70 °C from geothermal water spring of Gazlıgöl, Afyonkarahisar in Turkey. However, in previous studies Bouacem and Darenfed [25] reported the isolation and characterization of *Bacillus* and *Anoxybacillus* as producers of amylase from Hammam Righa hot spring at 60 °C temperature on 1% soluble starch agar. The closest relative *A. flavithermus* was originally isolated from hot springs of northern island of New Zealand and it was originally named *Bacillus flavothermus* [26]. The name *B. flavothermus* was revised by Pikuta et al. [27] to *A. flavithermus*. Accordingly, Seyda et al. [28] documented the isolation of amylase producing Anoxybacillus mongoliensis (EA3), from sediment and water samples of different hot springs positioned in different sections of Turkey. Nearly all genera of *Bacillus* produce the α-amylase enzyme [29].

Different environmental factors affect the production of amylase from thermophilic bacteria. Characterization of an enzyme in different conditions leads to the determination of optimum fermentation condition for enzyme. In our study maximum enzyme production was obtained in starch broth at 70 °C temperature, 1.5% soluble starch concentration and 8 pH. In this study, enzyme gave very low production at 5 and 9 pH. Previous studies revealed that a slight change in optimal pH lower the enzyme production due to protein denaturation [30,31]. Similarly, in study of Kikani et al. [32] the best enzyme production from *Anoxybacillus* FMB1 was found with 1.5% soluble starch. A previous study described optimal production of amylase from *Anoxybacillus bepuensis* TSSC-1 in starch broth at 55 °C temperature, 1% soluble starch concentration and 7 pH [33]. The slight variations in the conditions for enzyme

production in our study and previously reported literature might be due to different sources of bacterial isolations and types of the strains.

Statistical method such as response surface methodology (RSM) have been employed in many studies for optimization of conditions for enzyme production. Statistical optimization enables fast screening of huge experimental domain and describes precisely the role of each tested variable. The optimal conditions attained in our RSM model were highly similar to the typical conditions used for enzyme production in laboratory. However, a small difference was recorded on comparison of influence of various experimentally tested environmental conditions in laboratory with predicted environmental conditions using RSM, on enzyme yield. Surface plots obtained from Central Composite Design presented highest enzyme production by MBT001, MBT002 and MBT004 at pH 7, 70 °C, 1.25% soluble starch concentration, 300 μL and 48 h incubation period. Optimum conditions elucidated by RSM for MBT003 were same as for other isolates with the exception of optimum inocula volume which was observed as 100 μL. Abdel-Fattah et al. [34] also applied RSM for conditions optimization of thermostable amylase production in *Bacillus licheniformis* isolate AI20. Saeed et al. [35] studied statistical factorial designs for optimum production of thermostable α-amylase by the bacterium *Parageobacillus thermoglucosidasius*. The optimization of conditions for, thermostable amylase production through RSM, can improve the product quality.

In terms of activity, in our study (Fig. 3) maximum amylase activity was obtained with 1.5% soluble starch, at 70 °C and optimum pH of 8. With accordance to our results, α-amylase reported from *Bacillus* sp. *Ferdowsicous* showed good activity at 70 °C [36]. In another report, *Bacillus stearothermophilus* isolated from potato processing industry, had a thermoactive α-amylase and optimal temperature for the activity of that catalyst was 70 °C [37]. Similarly, maximum amylase activity from thermostable *Bacillus* was obtained at 8 pH [38]. In current study maximum enzyme activity was obtained with 1.5% soluble starch and enzyme activity was decreased below and above the mentioned concentration. This may be depending on metabolic ability of strain against different starch concentrations. However, various studies have reported different concentrations of substrate for amylase production and activity. Yassin et al. [38] reported that activity of amylase from *Bacillus* improved with the increasing concentration of starch (0.5–4%) and it reduced after that. Previous research has indicated that a large amount of carbohydrate might be suppressing the enzyme yield. Therefore, carbon sources can be added with continuous intervals during fermentation to complement the exhausted constituent [39].

Thermostable amylases now utilized by the starch industry needs cofactor such as Ca^{2+} for activity and stability [40]. Metals might act as co-factor which is vital to upsurge the enzyme activity [41]. In our study, crude enzyme was also found highly active in the presence of some metals such as Mg^{2+}, K^{+}, Ca^{2+} and very slight activity was observed with Cu^{2+} (Fig. 3). Similarly, Acer et al. [17] documented maximum activity of thermostable amylase from *Anoxybacillus*-AH1 in the presence of Mg^{2+}, Ca^{2+}, whereas, Cu^{2+} showed inhibitory effect on amylase activity from *Anoxybacillus*-AH1.

In our study, amylase was highly stable at 70 °C, though at 80 °C and 90 °C the stability of enzyme was decreased (Fig. 4). It was also observed that enzyme activity was completely lost at 100 °C. The α-amylase from *Anoxybacillus flavithermus* showed good stability at 60 °C, 70 °C and 80 °C after 2 h of incubation, which is similar to our findings [42]. Temperature above the optimum range, started to change the shape of the active site, which will diminish enzyme activity or stop its function. Thus, it is important to study appropriate temperature range for the enzyme for its large-scale application. In present study it was observed that enzyme was highly stable between 7 and 8 pH, whereas, previous study report that the *α*-amylases of *Anoxybacillus* sp. DT3-1 and *Anoxybacillus* sp. SK3-4 were stable in 6.0–9.0 pH ranges [43]. The usage of α-amylases in detergent formulations is restricted because it depends on their activity and stability. Thus, it is necessary that enzymes should be stable against detergents [44]. In our results, crude amylase enzyme was found stable in laundry detergents and in surfactants such as Tween 20 and Tween 80. Similarly, Negi and Banerjee et al. [45] studied that α-amylase gave significant stability towards Tween 80 and Tween 20 that is similar to our current findings. Acer et al. [44] reported that surfactants increase the interaction in catalyst, which is essential for stability of structure of protein and hence enhance activity of catalyst.

Enzyme purification is a vital process of getting contents of pure catalyst from an impure crude enzyme extracted from various available sources. In current study, for partial purification of enzyme, ammonium sulphate precipitation (60–80%) and dialyzing technique was performed. Dialysis is the most frequently used desalting procedure, dependent on the buffers used to dissolve the pellet obtained after ammonium sulphate precipitation [46]. Different salt concentrations ranging from 33 to 90% were used for the precipitation and purification of α-amylases but the most common used concentration was 80% [46]. Previous studies documented that amylase from *Anoxybacillus flavithermus* and *Anoxybacillus* sp YIM342 salt was purified by 70% salts [47].

In previous literature, applications of crude and partially purified enzyme in different fields are well documented. They are used for the production of glucose syrups, crystalline glucose, high fructose corn syrups, maltose syrups [48]. Previous study documented that partially purified enzyme from B. licheniformis HULUB1 and B. subtilis SUNGB2 has played a vital role in degradation of waste food material at 65 °C [49]. Soy et al. [15] reported about application of crude amylase from *Geobacillus icigianus*. Enzyme was highly efficient in starch hydrolysis and showed 34.5% hydrolysis of corn starch slurry. Also, thermostable amylase showed antibiofilm activity. Current findings verified α-amylase as a very effective component in eliminating starch stains. Similarly, it is also observed that combination of detergent, water and crude amylase improved the ability of reducing starchy stain from cotton cloth pieces. Correa et al. [50] similarly reported that the addition of Campeiro® (detergent) with the enzyme enhanced its laundry performance for eliminating stains of tomato sauce and egg yolk.

5. Conclusions

Present study was the first investigation of thermostable amylase production and purification from *Anoxybacillus mongoliensis* (MBT001), *Anoxybacillus flavithermus* (MBT002), *Bacillus* (MBT004), *Anoxybacillus flavithermus* and *Anoxybacillus mongoliensis* (MBT003) from geothermal spring of Tatta Pani Kotli AJK. It also demonstrated that the Central Composite Design is an effective

arithmetical model for optimization of enzyme production at different environmental conditions. In addition, crude amylase enzymes from thermophilic isolates revealed increased washing ability of detergents. In general, the studied properties of crude amylase enzyme make it a potential candidate for wide range of industrial applications on sustainable basis.

Author contribution statement

Sobia Sharif: Performed the experiments and wrote the paper.
Asad Hussain Shah: Conceived and designed the experiments.
Anila Fariq: Analyzed and interpreted the data.
Sammyia Jannat: contributed reagents, materials, analysis tools.
Sajida Rasheed: Analyzed and interpreted the data.
Azra Yasmin: Contributed reagents, materials and analysis of data.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

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.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.heliyon.2023.e12901.

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