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Application of one –factor- at-a-time and statistical designs to enhance α-amylase production by a newly isolate *Bacillus subtilis* strain-MK1

Samia A. Ahmed [a,*], Mohamed A.A. Abdella [a], Gamal M. El-Sherbiny [b], Atef M. Ibrahim [c], Aliaa R. El-Shamy [a], Sherien M.M. Atalla [a]

[a] Chemistry of Natural and Microbial Products Department, National Research Centre, Dokki, Cairo, Egypt
[b] Department of Botany and Microbiology, Faculty of Science (Boys), Al-Azhar University, Cairo, Egypt
[c] Microbial Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Menoufia, Egypt

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ABSTRACT

α-amylase is a starch hydrolyzing enzyme which has many industrial applications. In the present study, α-amylase producers were isolated from farm soil in Egypt (Sadat City). Screening was done by iodine test, based on the clear zone around the sample in starch agar plates. The most potent isolate MK1 was identified (morphologically and biochemically) and confirmed by 16S rRNA gene sequence method. This sequence was deposited to the GenBank under accession number, *B. subtilis* strain-MK1 (MF614924). α-Amylase production parameters were optimized using one-factor-at-a-time (OFAT) and Response Surface methodology (RSM). The result obtained from OFAT showed 72.4U/ml of amylase activity which was 1.8- fold higher as compared to unoptimized conditions (40.3 U/ml). The most significant factors were identified and its values were optimized using Plackett-Burman (PB) and Central Composite (CC) design, respectively. Among thirteen independent variables tested in PB design, beef extract, $MgSO_4{\cdot}7H_2O$, K_2HPO_4 and incubation time were the most significant on α-amylase production and showed positive effect. Whereas $MnCl_2{\cdot}4H_2O$, soluble starch and culture pH showed negative effect on α-amylase production. The model validation was clear up on comparing the statistical predicted yield (140.14 U/ml) with the actual experimental yield (145.4 U/ml) of α-amylase which was closely related. The model showed higher amylase activity of 3.6 and 7.5-fold as compared to the basal and the initial media, respectively. Optimized medium by OFAT and RSM enhanced enzyme production by 7.5-folds confirming the need to optimize the production parameters to achieve maximum yield.

1. Introduction

Enzymes have been used preferable over chemical catalysts in many industrial processes due to the increasing knowledge of the technical and economical advantages together with the need for environmentally safe technology (Cherry et al., 2004).

α-Amylase (EC 3.2.1.1) is starch degrading enzyme that produces branched and linear oligosaccharides of different chain length. α-Amylase enzyme has different applications in a wide variety of industries such as textile, food, detergent, paper, sugar industries and pharmaceutical (Pandey et al., 2017; Asrat and Girma, 2018). Currently, amylase production has reached up to 65% of enzyme market in the world and is continuously increasing (Simair et al., 2017). Microbial amylases have a wide range of industrial applications due to their broad biochemical diversity, feasibility of mass culture, high enzymatic stability under extreme conditions and ease of genetic manipulation (Vijayalakshmi et al., 2012; Sreena and Sebastian, 2018).

Due to the importance of amylases, isolation of new microbial producers capable of producing amylase provides potential new sources of enzyme (Aullybux and Puchooa, 2013). The comparative analysis of DNA sequences using phylogenetic methods become more significant with the rapid accumulation of molecular sequence detail. Gene sequencing and phylogenetic analysis are helpful to detect the nature and extent of selective forces that shape the evolution of genes and species (Huma et al., 2014).

Alkaliphiles have been reported to grow in extreme alkaline environments. They can be categorized into two main groups (1) alkaliphillic microorganisms which require an alkaline pHs for optimal growth (2) alkali-tolerant microorganisms which can survive at high alkaline pHs, but optimum growth takes place at medium with near neutral pH.

* Corresponding author.
E-mail address: dr_sa_ahmed@yahoo.com (S.A. Ahmed).

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Alkaliphillic and alkali-tolerant bacteria are considered a rich source for active enzymes that have numerous industrial applications and suitable for harsh condition compared to their counterparts (Mahapatra and Mishra, 2014).

The cost of enzymes is one major factor determining the economics of a biocatalytic reaction and it can be reduced by isolation of hyper-producers and finding the optimum conditions for their production (Sreena and Sebastian, 2018). Microbial growth and enzymes production mainly depends on the nutrient content of culture medium and production conditions. Moreover, for maximizing enzyme synthesis and minimizing the production cost, optimization of medium contents and process parameters play vital roles (Saha and Mazumdar, 2019). Optimization of production process by only one-factor-at-a-time (OFAT) method is time- consuming, expensive and often impractical when great number of variables needs to be investigated (Saha and Mazumdar, 2019). Response Surface methodology (RSM) is statistical models that optimize the fermentation process by combining all the factors included in the experimental analysis for improving the enzyme yield. Also, RSM help to select optimum conditions, identify the effective variables, and quantify the relationships between one or more measured responses (Khusro et al., 2017). Moreover, RSM minimize the number of individual experiments required that enhances the obtained information in addition to knowledge of the interactions between different variables (Abdel Wahab and Ahmed, 2018).

This work aims to isolate and identify bacteria efficient in producing α-amylase enzyme via the morphological, physiological and molecular characteristics. Increase the enzyme production initially by using OFAT method. Finally, optimize the significant variables using Response Surface methodology (PBD followed by CCD) and the model was verified.

2. Materials and methods

2.1. Isolation of alkali-tolerant bacteria

The sample was collected in winter from rhizospheric soil of a farm located in Sadat City (30.446369 N, 30.624044 E), Menoufia governorate, Egypt in sterile plastic bags. Sample (1 gm) was suspended in 10 ml of sterile saline solution (NaCl 0.9%) and serial dilutions were plated by spreading method on modified Luria Bertani (LB) agar medium contains (g % w/v): tryptone (1), yeast extract (0.5), NaCl (0.5) and agar (1.5) according to Mouafi et al. (2016). The initial pH was adjusted to 9 and 10 before sterilization at 121 °C for 20 min. After inoculation, the plates were incubated at 35 °C for 24 h in inverted position. The bacterial isolates were further sub-cultured to obtain pure isolates which were transferred to nutrient agar slants and kept at 4 °C for further studies.

2.2. Qualitative screening for maximum α-amylase producing isolates

The isolated colonies were further purified and screened for their ability to produce α-amylase enzyme. The screening was done by streaking the isolated colonies on starch-agar plate containing (g/l): starch (20), peptone (20) and agar (20) at pH 9 and 10 (Simair et al., 2017). The inoculated plates were incubated at 35 °C for 48 h. Positive bacterial isolates show the clearance area around colony representing starch decomposition. The amylolytic bacterial colonies were further screened for maximum α-amylase activity on the basis of the diameter of the halo zone around the colonies at a dark blue background when staining with iodine solution.

2.3. Quantitative screening for maximum α-amylase producing isolates

The three positive amylolytic bacterial isolates (MK1, MK9 and MK10) were cultivated on initial medium M4 (Thebti et al., 2016) containing (g/l): soluble starch (10), yeast extract (5), peptone (10), NaCl (10) at pH 9. The culture was kept at 35 °C for 48 h in shaking incubator (150 rpm) and further centrifuged at 8000×*g* and 4 °C for 15 min to collect the supernatant. The quantitative screening (enzyme assay) was done according to Sajjad and Choudhry (2012) with some modifications by adding 0.5 ml of enzyme solution to 0.5 ml of starch solution (1% w/v) prepared in 0.1 M sodium phosphate buffer (pH 7). The mixture was incubated at 40 °C for 30 min and the released reducing sugars were determined according to Miller (1959) using dinitrosalicylic acid reagent (DNS). All the cultures were triplicates and the results are the mean. One unit of enzyme activity is defined as the amount of enzyme that liberated one μ mole of reducing sugar (as glucose) per min under assay conditions.

2.4. Phenotypic and biochemical characterizations

The highest producing α-amylase isolate was characterized on the basis of different biochemical and morphological parameters to tentatively identify it to the genus level based on Bergey's Manual of Determinative Bacteriology (Holt et al., 1994; Mouafi et al., 2016). The parameters used were test for Gram stain reaction, Catalase test, Gelatin hydrolysis and Oxidase test, etc. Microscopic examination and colony feature characterization were also employed (Silpa et al., 2018).

2.5. Molecular identification of potent bacterial isolate

Genomic DNA of the most potent isolate was extracted from cells pregrown on nutrient broth media according to the protocol for bacterial DNA extraction with the GeneJET Genomic DNA Purification Kit (#K0721) provided by Thermo Scientific.

2.6. PCR of 16S rRNA gene of amylolytic bacterial isolate

The 16S rRNA gene of the most potent isolate was amplified by thermocycler (Biometra Thermocycler, Germany) using two universal oligonucleotide primers, 27F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The PCR amplification condition is consisted of an initial denaturation at 94 °C for 2 min, followed by 35 cycles (94 °C denaturation for 30 s, 55 °C annealing for 30 s, 72 °C extension for 1min) and final elongation at 72 °C for 5 min.

2.7. 16S rRNA gene sequencing

Nucleotide sequencing of 16S rRNA gene was carried out on applied Biosystems model 373 A (Lincoln, Nebr) model 4000L automated DNA sequencer with appropriate dye primers (Macrogen Inc.,Souel, South Korea). The unknown bacterial isolate was identified using the BLAST software at (NCBI) (Kenji et al., 1996).

2.8. Analysis of 16S rRNA gene sequence

The sequence of 16S rRNA was analyzed by Mac GENETYX v9 computer software (Software Development Co. ltd., Tokyo, Japan) and compared to the GenBank nucleotide data library. Similarity search was done for the 16S rRNA sequence using online website (http://www.ncbi.nlm.nih.gov/blast/) in order to determine its closest phylogenetic relatives.

2.9. Enzyme production by submerged fermentation of bacterial isolate-MK1

2.9.1. Inoculum preparation

Nutrient broth (20 ml and pH 9) was prepared in a conical flask and kept for cooling after sterilization. A loopful of bacterial isolate was inoculated into the medium under sterile conditions. Finally, the culture was incubated overnight at 35 °C with agitation speed of 150 rpm.

Table 1
PB experimental design for screening and evaluating factors influencing α-amylase production from *B. subtilis* strain-MK1.

Run	X_1: Lactose [%]	X_2 Starch [%]	X_3: Beef extract [%]	X_4: Yeast extract [%]	X_5: K_2HPO_4 [%]	X_6: $MgSO_4{\cdot}7H_2O$ [%]	X_7: $FeSO_4{\cdot}7H_2O$ [%]	X_8: $MnCl_2{\cdot}4H_2O$ [%]	X_9: $CaCl_2$ [%]	X_{10}: Culture pH	X_{11}: Incubation time [h]	X_{12}: Inoculum size [ml]	X_{13}: Agitation [rpm]	A activity [U/ml]	Predicted activity [U/ml]
1	+1 (1)	−1 (0)	+1 (0.5)	−1 (0)	−1 (0)	−1 (0)	−1 (0)	+1 (0.05)	+1 (0.05)	−1 (7)	−1 (48)	+1 (2)	−1 (150)	44.9	39.56
2	−1 (0.2)	−1 (0)	−1 (0.1)	−1 (0)	+1 (0.1)	+1 (0.025)	−1 (0)	−1 (0)	+1 (0.05)	−1 (7)	−1 (48)	+1 (2)	+1 (180)	75.5	74.33
3	−1 (0.2)	−1 (0)	−1 (0.1)	+1 (0.5)	+1 (0.1)	−1 (0)	−1 (0)	+1 (0.05)	−1 (0)	−1 (7)	+1 (72)	+1 (2)	+1 (180)	33.65	34.4
4	+1 (1)	+1 (0.5)	−1 (0.1)	+1 (0.5)	−1 (0)	+1 (0.025)	−1 (0)	−1 (0)	−1 (0)	−1 (7)	+1 (72)	+1 (2)	−1 (150)	56.86	61.24
5	−1 (0.2)	+1 (0.5)	−1 (0.1)	−1 (0)	+1 (0.1)	+1 (0.025)	+1 (0.025)	+1 (0.05)	−1 (0)	+1 (9)	−1 (48)	+1 (2)	−1 (150)	43.24	44.98
6	−1 (0.2)	+1 (0.5)	+1 (0.5)	+1 (0.5)	+1 (0.1)	−1 (0)	+1 (0.025)	−1 (0)	+1 (0.05)	−1 (7)	−1 (48)	−1 (1)	−1 (150)	64.25	69.1
7	+1 (1)	−1 (0)	−1 (0.1)	+1 (0.5)	−1 (0)	+1 (0.025)	+1 (0.025)	−1 (0)	−1 (0)	+1 (9)	−1 (48)	−1 (1)	+1 (180)	62.95	62.21
8	−1 (0.2)	+1 (0.5)	−1 (0.1)	−1 (0)	−1 (0)	−1 (0)	+1 (0.025)	+1 (0.05)	−1 (0)	−1 (7)	+1 (72)	−1 (1)	−1 (150)	40.5	33.74
9	−1 (1)	+1 (0.5)	+1 (0.5)	−1 (0)	+1 (0.1)	−1 (0)	+1 (0.025)	−1 (0)	−1 (0)	−1 (7)	−1 (48)	+1 (2)	+1 (180)	72.31	72.46
10	+1 (1)	−1 (0)	−1 (0.1)	+1 (0.5)	−1 (0)	−1 (0)	+1 (0.025)	+1 (0.05)	+1 (0.05)	+1 (9)	−1 (48)	+1 (2)	−1 (150)	11.96	17.29
11	−1 (0.2)	−1 (0)	+1 (0.5)	−1 (0)	−1 (0)	+1 (0.025)	+1 (0.025)	+1 (0.05)	+1 (0.05)	−1 (7)	+1 (72)	−1 (1)	+1 (180)	71.4	77.27
12	+1 (1)	+1 (0.5)	+1 (0.5)	+1 (0.5)	−1 (0)	+1 (0.025)	−1 (0)	+1 (0.05)	−1 (0)	−1 (7)	−1 (48)	−1 (1)	+1 (180)	35.17	36.37
13	+1 (1)	+1 (0.5)	−1 (0.1)	−1 (0)	+1 (0.1)	−1 (0)	−1 (0)	+1 (0.05)	+1 (0.05)	+1 (9)	+1 (72)	−1 (1)	+1 (180)	16.45	21.59
14	+1 (1)	−1 (0)	−1 (0.1)	+1 (0.5)	+1 (0.1)	+1 (0.025)	+1 (0.025)	−1 (0)	+1 (0.05)	−1 (7)	+1 (72)	−1 (1)	−1 (150)	98.3	94.32
15	+1 (1)	−1 (0)	+1 (0.5)	−1 (0)	+1 (0.1)	−1 (0)	−1 (0)	−1 (0)	−1 (0)	+1 (9)	+1 (72)	−1 (1)	−1 (150)	75.81	76.25
16	−1 (0.2)	+1 (0.5)	−1 (0.1)	+1 (0.5)	−1 (0)	−1 (0)	−1 (0)	−1 (0)	+1 (0.05)	+1 (9)	−1 (48)	−1 (1)	+1 (180)	19.34	14.6
17	−1 (0.2)	−1 (0)	+1 (0.5)	+1 (0.5)	−1 (0)	−1 (0)	+1 (0.025)	−1 (0)	−1 (0)	+1 (9)	+1 (72)	+1 (2)	+1 (180)	65.31	65.44
18	−1 (0.2)	+1 (0.5)	+1 (0.5)	−1 (0)	−1 (0)	+1 (0.025)	−1 (0)	−1 (0)	+1 (0.05)	+1 (9)	+1 (72)	+1 (2)	−1 (150)	75.28	75.9
19	−1 (0.2)	−1 (0)	+1 (0.5)	+1 (0.5)	+1 (0.1)	+1 (0.025)	−1 (0)	+1 (0.05)	−1 (0)	+1 (9)	−1 (48)	−1 (1)	−1 (150)	53.74	52.4
20	+1 (1)	+1 (0.5)	+1 (0.5)	+1 (0.5)	+1 (0.1)	+1 (0.025)	+1 (0.025)	+1 (0.05)	+1 (0.05)	+1 (9)	+1 (72)	+1 (2)	+1 (180)	70.9	64.26

Table 2
CCD with 25 experimental and predicted values of independent variables.

Run	(A) Beef extract [%]	(B) $MgSO_4·7H_2O$ [%]	(C) K_2HPO_4 [%]	(D) Incubation time [h]	α-amylase activity [U/ml]	Predicted activity [U/ml]
1	+1 (2)	+1 (0.5)	+1 (1)	−1 (72)	120.4	119.93
2	+1 (2)	−1 (0.03)	−1 (0.1)	+1 (120)	81.14	86
3	0 (1.25)	+1 (0.5)	0 (0.55)	0 (96)	109.3	107.57
4	−1 (0.5)	+1 (0.5)	+1 (1)	+1 (120)	111.58	114.64
5	+1 (2)	+1 (0.5)	+1 (1)	+1 (120)	77.34	78
6	+1 (2)	−1 (0.03)	+1 (1)	−1 (72)	55.7	57.77
7	0 (1.25)	0 (0.265)	+1 (1)	0 (96)	79.62	85.1
8	−1 (0.5)	+1 (0.5)	−1 (0.1)	−1 (72)	83.1	86.6
9	+1 (2)	0 (0.265)	0 (0.55)	0 (96)	86.32	82.1
10	−1 (0.5)	0 (0.265)	0 (0.55)	0 (96)	97.43	101.1
11	0 (1.25)	0 (0.265)	0 (0.55)	−1 (72)	100.78	99.21
12	+1 (2)	−1 (0.03)	−1 (0.1)	−1 (72)	56.79	54.22
13	0 (1.25)	0 (0.265)	−1 (0.1)	0 (96)	86.16	80.16
14	+1 (2)	+1 (0.5)	−1 (0.1)	+1 (120)	110.06	108.97
15	0 (1.25)	0 (0.265)	0 (0.55)	+1 (120)	101.38	102.38
16	−1 (0.5)	−1 (0.03)	+1 (1)	−1 (72)	94.79	96.37
17	−1 (0.5)	−1 (0.03)	−1 (0.1)	+1 (120)	102.9	103.87
18	0 (1.25)	0 (0.265)	0 (0.55)	0 (96)	89.97	91.68
19	−1 (0.5)	−1 (0.03)	+1 (1)	+1 (120)	101.4	97.32
20	+1 (2)	+1 (0.5)	−1 (0.1)	−1 (72)	99.86	103.59
21	−1 (0.5)	+1 (0.5)	+1 (1)	−1 (72)	145.4	140.14
22	0 (1.25)	−1 (0.03)	0 (0.55)	0 (96)	73.07	74.23
23	−1 (0.5)	−1 (0.03)	−1 (0.1)	−1 (72)	56.64	55.62
24	+1 (2)	−1 (0.03)	+1 (1)	+1 (120)	45.29	42.29
25	−1 (0.5)	+1 (0.5)	−1 (0.1)	+1 (120)	110.82	108.4

2.9.2. Screening of different media for α-amylase production

Different pre-optimized production media (M) were initially tested for α-amylase production by the potent strain with the following composition (g/l):

M1 (Simair et al., 2017): glucose (20), yeast extract (10), $MgSO_4·7H_2O$ (1) and KH_2PO_4 (2).

M2 (Khusro et al., 2017): soluble starch (10), peptone (6), $MgSO_4$ (0.5) and KCl (0.5).

M3 (Singh et al., 2016): soluble starch (20), yeast extract (5), $MgSO_4$ (1), $CaCl_2·2H_2O$ (0.2) and NaCl (1).

M4 (Thebti et al., 2016) is the initial medium: soluble starch (10), yeast extract (5), peptone (10) and NaCl (10).

M5 (Blanco et al., 2016): soluble starch (10), peptone (2.5), $KH_2PO_4·3H_2O$ (1.5), Na_2SO_4 (1.5), $MgSO_4·7H_2O$ (0.15), $FeSO_4·7H_2O$ (0.03), $MnCl_2·4H_2O$ (0.1) and $CaCl_2·2H_2O$ (0.45).

M6 (Ramezani and Asoodeh, 2016) is the basal media: soluble starch (10), peptone (10), K_2HPO_4 (1), $MgSO_4·7H_2O$ (0.25), $FeSO_4·7H_2O$ (0.25) and $MnCl_2·4H_2O$ (0.5).

The media pH was initially adjusted to 7 before autoclaving and α-amylase activities were measured after 48h of cultivation at 35 °C with agitation speed of 150 rpm.

2.10. Optimization of enzyme production by one-factor-at-a-time (OFAT)

2.10.1. Effect of different carbon sources on α-amylase production

The production of α-amylase was performed by growing the bacterial cells into basal medium (M6) containing various carbon sources (C-sources) such as soluble starch, glucose, lactose, wheat bran, maize starch, wheat flour, broken rice, mango peel and banana peel. Fermentation media with different C-sources (1% w/v) were inoculated and incubated at 35 °C for 48 h with agitation speed of 150 rpm. The culture filtrate was used for the quantitative assay of extracellular α-amylase (Khusro et al., 2017).

2.10.2. Effect of different nitrogen sources on α-amylase production

Different organic and inorganic N-sources such as peptone, yeast extract, beef extract, soybean meal, casein, baker's yeast, ammonium chloride, potassium nitrate and ammonium sulphate were added individually at a concentration of 1% (w/v). The conical flasks containing the fermentation media were incubated at 35 °C for 48 h with agitation speed of 150 rpm and the enzyme assay was investigated (Khusro et al., 2017).

2.10.3. Effect of fermentation temperature on α-amylase production

Optimum temperature for enzyme production was checked in the range of 30°C–50 °C for 48h and 150 rpm.

2.11. Optimization of α-amylase production by multi-factorial experiments

The optimization protocol was conducted on three major steps. First: evaluating the relative importance of various nutrients and conditions for α-amylase production according to PBD (Plackett and Burman, 1946). Second: the most significant factors were selected for further estimation of their optimal levels using central composite design (CCD). Lastly: applying a computational analysis to verify the quality of the model fitness as expressed by the coefficient of determination, R^2.

2.11.1. Plackett-Burman design (PBD)

In this design we investigated the effect of thirteen variables on the production of α-amylase including: lactose, soluble starch, beef extract, yeast extract, K_2HPO_4, $MgSO_4·7H_2O$, $FeSO_4·7H_2O$, $MnCl_2·4H_2O$, $CaCl_2$, culture pH, incubation time, inoculum size and agitation speed. Each of these factors was represented at two levels, low value (−1) and high value (+1) as shown in Table 1. Also, in the experimental design, each row represented an experiment, and each column represented an independent variable (Table 1). All trials were done in Erlenmeyer flasks (250 ml) containing 50 ml of the medium. PB screening design depends on the first order model:

$$Y = \beta_0 + \Sigma\beta_i X_i$$

In this model Y representing the response α-amylase activity, β_0 is the model intercept, β_i is the variable estimate (linear coefficient) and X_i represents the level of independent variable. The Pareto Plot best demonstrates results of PBD that illustrates the absolute relative significance of variables independent on their nature. The statistical significance was determined by F-value, and the proportion of variance explained by the model obtained was given by the multiple coefficient of

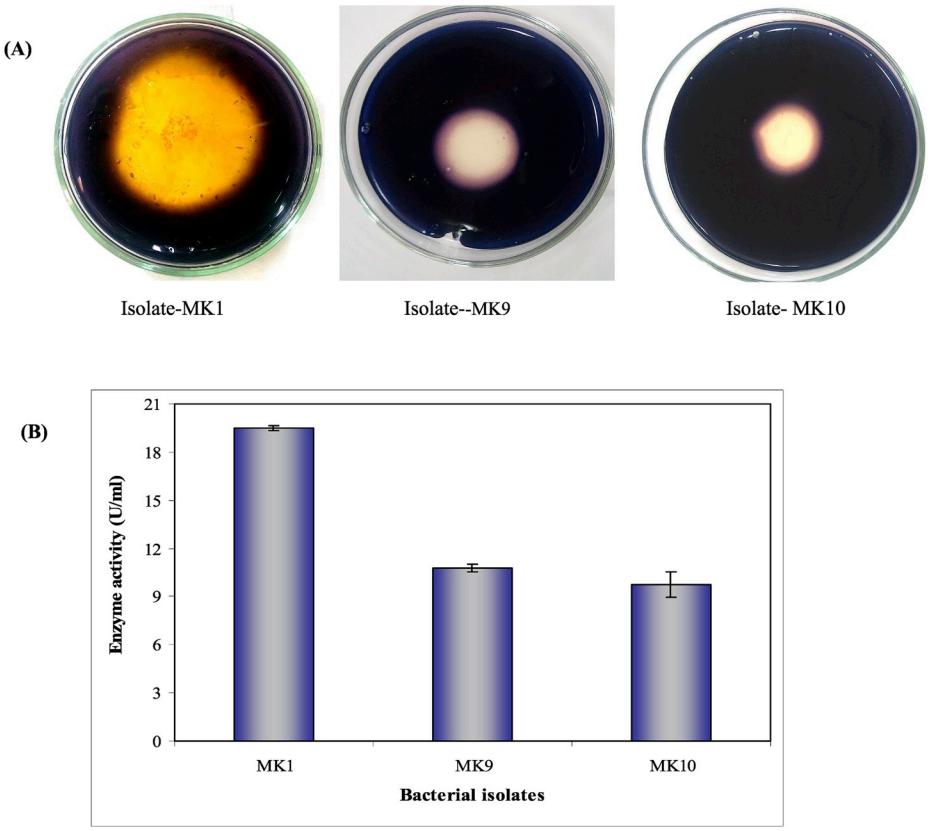

Fig. 1. (A) Qualitative and (B) quantitative screening of bacterial isolates for α-amylase activity.

determination, R^2. On the basis of regression analysis, the variables that showed a significant effect (95% confidence level, Prob > F ≤ 0.05) on α-amylase production were evaluated in further optimization experiments.

2.11.2. Central composite design (CCD)

Central Composite design (CCD) of RSM was used to optimize the variables level for enhancing α-amylase production. According to the CCD, the total number of experimental combinations is $2^k + 2k + n_0$ where k is the number of independent variables and n_0 is the number of repetitions of the experiments at the center point. After determination of the most significant variables which affect α-amylase production by PB design, four of them were chosen for CCD. The effects of these variables to the response were analyzed by using a second-order polynomial equation:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^{\ 2} + \Sigma\beta_{ij} X_i X_j$$

Where, Y = predicted response, β_0 = intercept term, β_i = linear effect, β_{ii} = squared effect, β_{ij} = interaction effect, X_i, X_j = independent variables. For α-amylase production, the four significant variables elucidated through PB experimental design were: Beef extract (A), $MgSO_4 \cdot 7H_2O$ (B), K_2HPO_4 (C) and incubation time (D). The three levels of each variable were designated as, low (−1), central (0) and high (+1), respectively. The coded and actual values of α-amylase variables are given in Table 2. The CCD matrix of the four factors leads to twenty five combinations, with respect to mean of enzymes activity (U/ml) as response (Table 2). Other media components and fermentation conditions were chosen at the significant level concentrations from the PBD.

2.12. Model validation

The mathematical model generated during RSM implementation was validated by conducting check point studies. The experimentally obtained data (actual) were compared with the predicted values and the prediction error was calculated.

2.13. Statistical analysis of data

All the experiments were performed in triplicate and the results were expressed as mean values. The independent variables of the experimental design were optimized and interpreted using (JMP) statistical software. Statistical analysis of the model was carried out according to the analysis of variance (ANOVA). The quality of the fit of the polynomial model equation was assessed by determining the R^2 coefficient and the adjusted R^2 coefficient; its statistical and regression coefficient significance were checked with F-test and P-value, respectively. Three-dimensional (3D) surface plot and corresponding contour plots were drawn to illustrate the effect of the independent variables on the responses (α-amylase activity).

2.14. Comparison of growth curve/production kinetics between unoptimized and optimized medium

This experiment was undertaken to investigate the fermentation course for the production of α-amylase enzyme using unoptimized and optimized medium at different incubation periods (6–72h).

3. Results and discussion

3.1. Screening for maximum α-amylase producing bacteria

Sixteen pure colonies were isolated and qualitative tested for α-amylase production by flooding iodine reagent on starch-agar plate. The results showed that 3 isolates (MK1, MK9 and MK10) were positive by the formation of clear zone around colonies due to starch hydrolysis at a dark blue background (Fig. 1A). The largest clear zone was around a

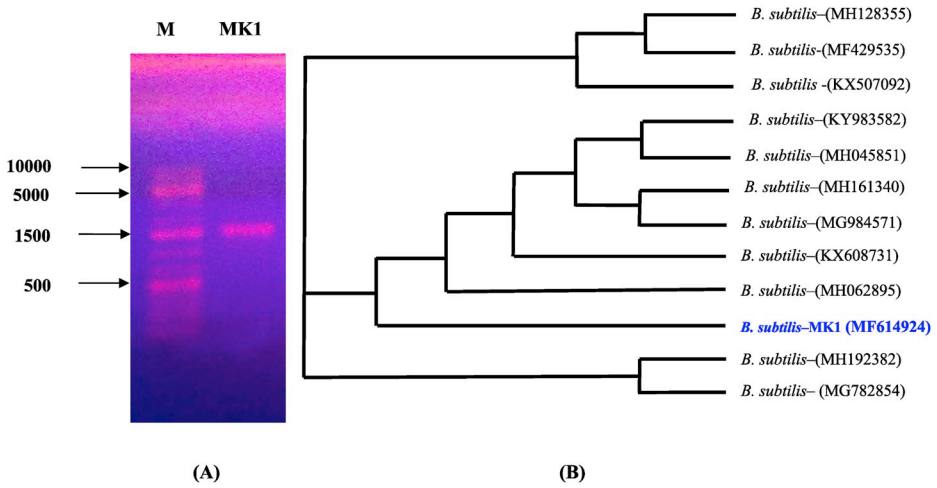

Fig. 2. Agarose gel electrophoresis shows the PCR products of 16s rRNA of *B. subtilis* strain-MK1, M, Ladder DNA (**A**) and Phylogenetic tree of 16s rRNA from *B. subtilis* strain-MK1 (MF614924), with some of its closest phylogenetic relatives in the NCBI using the CLUSTAL W2 multiple sequence alignment software (**B**).

colony of MK1 isolate at pH 9 and 10 (approximately the same zone) confirming that it is alkali-tolerant.

Quantitative screening of the potent amylolytic isolates using initial medium (M4) at pH 9 was performed by assaying the enzyme activity (Fig. 1B). The highest activity was possessed by isolate- MK1 (19.5 U/ml), followed by MK9 and MK10 (10.8 and 9.75 U/ml), respectively which confirms the previous qualitative assessment. Consequently, MK1 isolate was selected for the following studies.

3.2. Morphological and biochemical characteristics of the amylolytic bacterial isolate

The most potent amylolytic bacterial isolate MK1 was characterized morphologically, physiologically and biochemically according to Bergey's Manual of Determinative Bacteriology methods. The reference data showed that, the isolate MK1 was belong to the genus of *Bacillus*, gram (+), methyl red (−), indole test (−), catalase (+), urease activity (−), casein hydrolysis (+), gelatin hydrolysis (+), glucose fermentation (+), lactose Fermentation (+), nitrate reductase (+), oxidase activity (+), CMC hydrolysis (+), LBG hydrolysis (−), motile, short rod-shaped, alkali-tolerant at 8% NaCl, spore forming and aerobic organism.

3.3. Molecular identification of the amylolytic bacterial isolate

The genome DNA of MK1 isolate was extracted. The PCR product of the isolate was about 1.5 kbp (Fig. 2A), excised from the agarose gel, purified and shipped to Macrogen Inc., Souel, South Korea for sequencing. The obtained sequences of the strain was assembled, analyzed and submitted in the GenBank under accession numbers MF614924.

Sequence of *Bacillus subtilis* strain-MK1was submitted to NCBI database and compared with already existing sequence. Partial 16S rRNA gene sequence was quire to NCBI BLAST (**Error! Hyperlink reference not valid.** www.ncbi.nlm.nih.gov/Blast) and confirmed the closest neighbor of the isolated strain.

Phylogenetic tree based on a comparison of the 16s ribosomal DNA sequences of α-amylase producing bacterial strain and some of nearest phylogenetic relatives in the NCBI, GenBank was analyzed by the CLUSTAL W2 multiple sequence alignment software. It was created from evolutionary distances using the neighbor-joining method of Mega 6 program (**Error! Hyperlink reference not valid.** www.megasoftware.net/) (Tamura et al., 2013). The resulted phylogenetic tree of the 16s rRNA gene from the isolated strain *Bacillus subtilis* strain-MK1 (MF614924) showed 99% identity with their closest phylogenetic relatives in the NCBI (Fig. 2B).

3.4. Screening of different media for α-amylase production by B. subtilis strain-MK1

The selected strain *B. subtilis* strain-MK1 was grown on different media for α-amylase enzyme production. The result illustrated in Fig. 3A showed that maximum production (40.3 U/ml) was obtained when grown in M6 which is 2.1-time higher than that obtained by the initial medium (M4). In addition, enzyme production by M6 is 2.5 and 3.7-time higher than that obtained by medium M5 and M3, respectively. Higher production might be related to the presence of some metal ions which essential for bacterial growth and enzyme production as shown in M6 (K_2HPO_4, $MgSO_4$, $MnCl_2$). On the other side, other metals (Na_2SO_4, KCl and $CaCl_2$) inhibited microbial growth and enzyme production as shown in M5, M3 and M2 (Duque et al., 2016). These results have been confirmed after the statistical analysis.

3.5. Optimization of α-amylase production by one-factor- at-a- time (OFAT)

3.5.1. Effect of different carbon sources on α-amylase production

Various synthetic C-sources, agricultural and industrial residues were evaluated for maximum α-amylase production in SmF using the basal media (M6). The result in Fig. 3B showed that, the highest α-amylase production (57.5 U/ml) was obtained in presence of lactose followed by soluble starch (39.6 U/ml). On the other side, glucose reduced enzyme production by 85.4%. This suggested that glucose was repressor of α-amylase enzyme which is similar to the observed by Ali et al. (2018). In contrast to our findings, Abel-Nabey and Farag (2016) obtained maximum production of α-amylase from *B. lichineformis* AH214 in presence of maltose followed by glucose while lactose gave the lowest amylase enzyme activity. Sreekanth et al. (2013) suggested that the ability to use the C-source to produce enzymes varies with the type of the organism.

3.5.2. Effect of different nitrogen sources on α-amylase production

For the organisms N-sources play an important role in the growth and enzyme production. Beef extract and yeast extract enhanced enzyme production to 72.4 and 57.3 U/ml. Abou Dobara et al. (2011) confirmed that organic nitrogen sources support maximum α-amylase yields. On the other side, ammonium chloride and ammonium sulphate reduced the production of α-amylase by 3.7 and 1.9-fold as shown in Fig. 3C. Our results are consistent with Simair et al. (2017) who suggested that beef

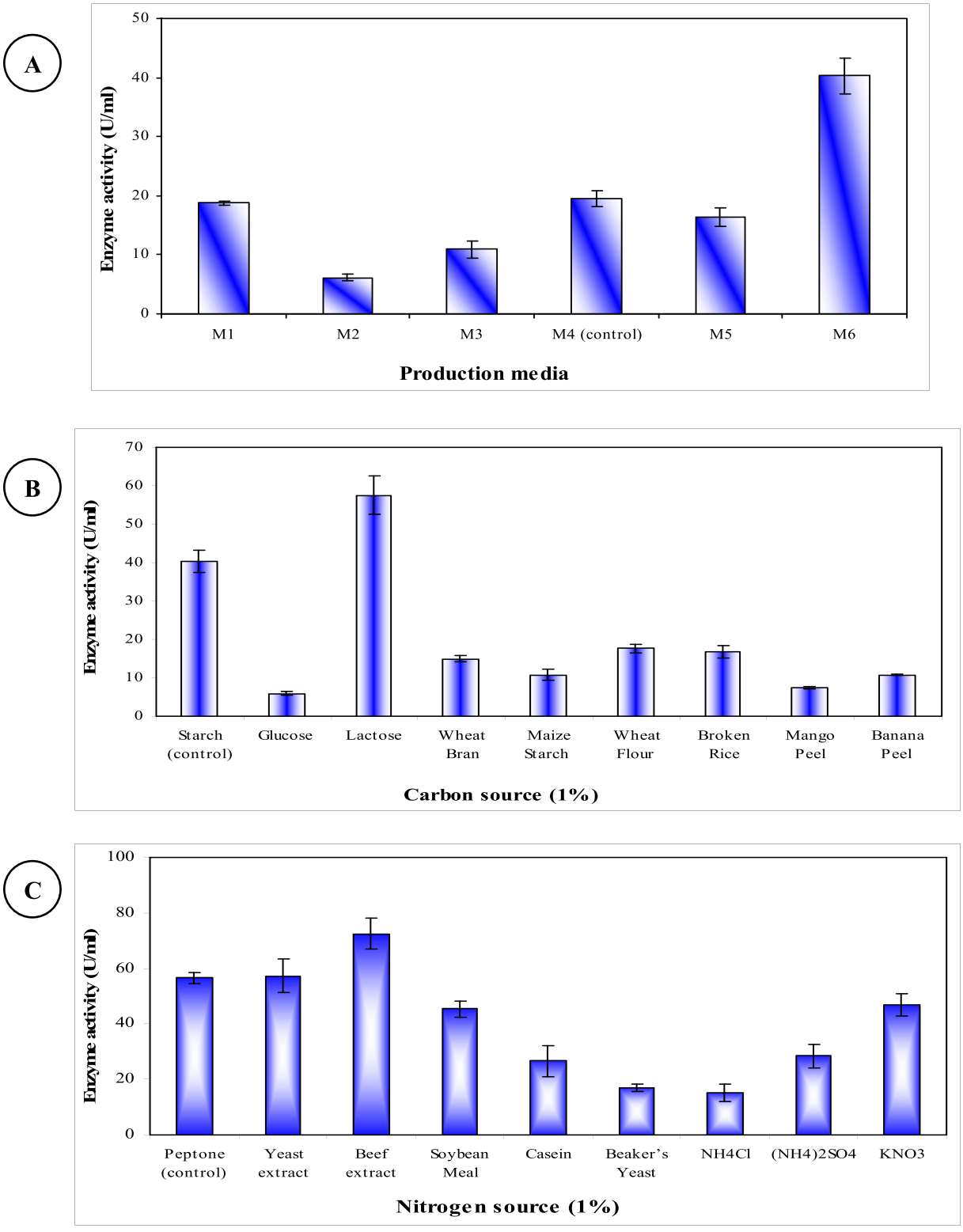

Fig. 3. Effect of (**A**) different media (**B**) carbon sources, (**C**) nitrogen sources on α-amylase. production from *B. subtilis* strain-MK1.

extract was the best N- source for α-amylase production by *Bacillus* sp. BCC 01–50. Kumar et al. (2014) reported that each organism or strain has its own special conditions for maximum enzyme production.

3.5.3. *Effect of fermentation temperature on α-amylase production*

Optimized production temperature did not enhance enzyme production and the highest production (72.4 U/ml) was obtained at 35 °C (data not shown). In addition, higher (50 °C) and lower (30 °C) temperatures reduced the enzyme production by 1.3 and 1.2-fold, respectively. These results are due to the effect of temperature on the metabolism of the microorganism and consequently, enzyme synthesis. The increase in temperature over its optimum value inhibited amylase

Table 3
Analysis of variance (ANOVA) for PBD.

Source	Sum of squares	Df	Mean square	F- Value	p-Value Prob > F	
Model	9825.494	13	755.807	15.22	0.0016*	Significant
X_1- Lactose	0.5780	1	0.578	0.0116	0.9176	
X_2-Soluble Starch	492.2304	1	492.230	9.9151	0.0198*	Significant
X_3-Beef extract	1450.4451	1	1450.445	29.2167	0.0017*	Significant
X_4-Yeast extract	237.0850	1	237.085	4.7757	0.0715	
X_5-K_2HPO_4	725.7715	1	725.772	14.6194	0.0087*	Significant
X_6-$MgSO_4.7H_2O$	1977.2650	1	1977.265	39.8286	0.0007*	Significant
X_7-$FeSO_4.7H_2O$	654.5968	1	654.597	13.1857	0.0109*	Significant
X_8-$MnCl_2.4H_2O$	2976.8000	1	2976.800	59.9625	0.0002*	Significant
X_9-$CaCl_2$	3.8194	1	3.819	0.0769	0.7908	
X_{10}-Culture pH	478.8290	1	478.829	9.6452	0.0210*	Significant
X_{11}-Incubation time	733.2605	1	733.261	14.7703	0.0085*	Significant
X_{12}-Inoculum size	7.2000	1	7.200	0.1450	0.7164	
X_{13}-Agitation speed	87.6130	1	87.613	1.7648	0.2323	
Error	297.866	6				
C. Total	10123.360	19				

$R^2 = 0.9705$, Adj $R^2 = 0.9068$, Root Mean Square Error (RMSE) = 7.045, Mean of Response = 54.391, CV = 12.95%.
Df (degree of freedom), Significant ($p \leq 0.05$), Non-significant ($p > 0.05$).

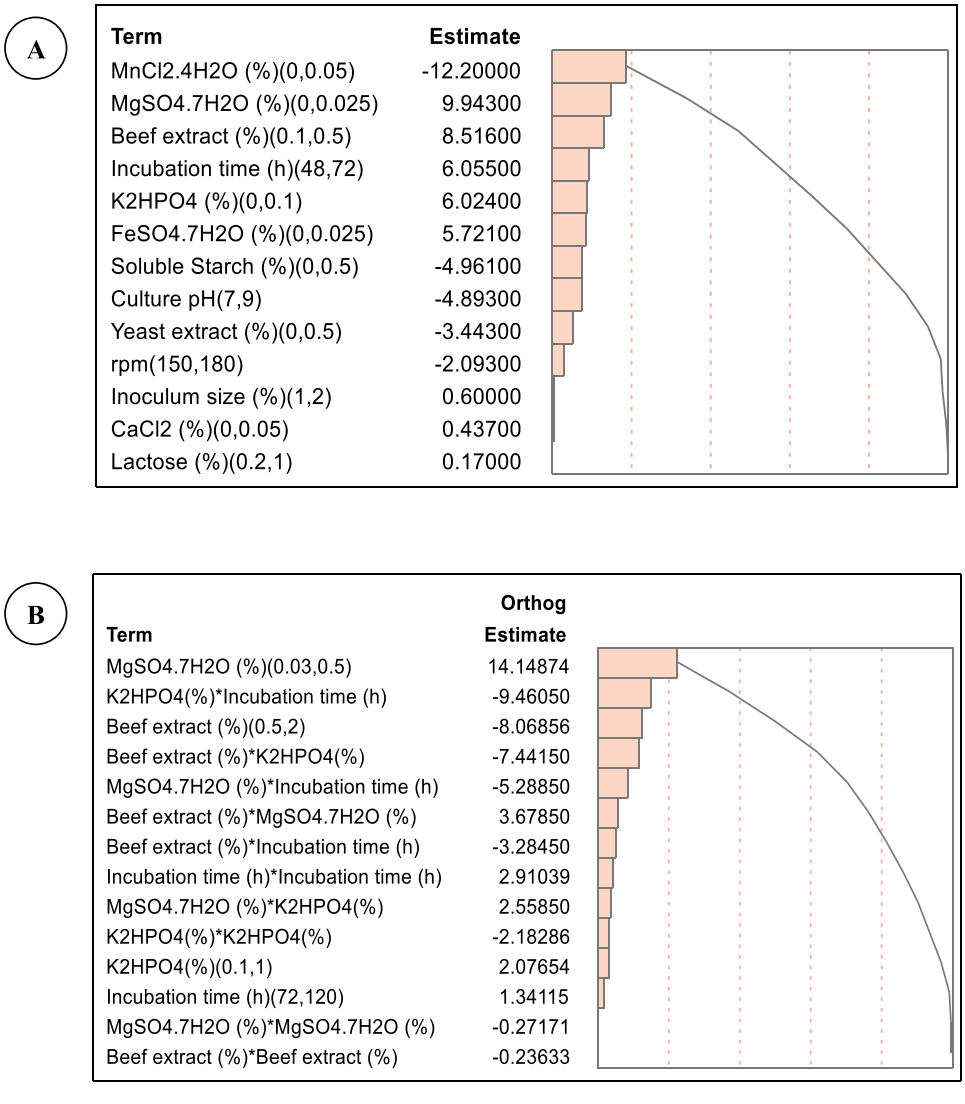

Fig. 4. Pareto chart of PBD (A) and of CCD (B) for the most significant variables on α-amylase activity.

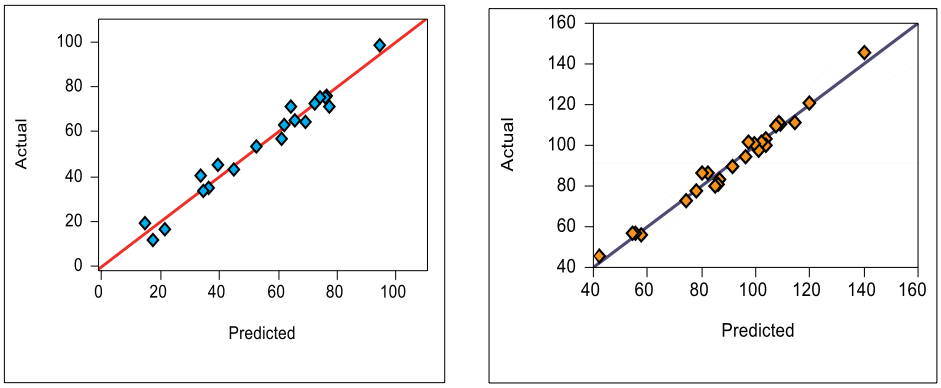

Fig. 5. The relation between predicted and actual α-amylase activity using PBD (A) and CCD (B).

Table 4
Analysis of variance (ANOVA) for CCD.

Source	Sum of squares	Df	Mean square	F- Value	p-Value Prob > F	
Model	12211.859	14	872.276	35.807	<.0001*	Significant
A-Beef extract	1627.5414	1	1627.541	66.8106	<.0001*	Significant
B–$MgSO_4.7H_2O$	5004.6678	1	5004.668	205.4418	<.0001*	Significant
C–K_2HPO_4	107.8001	1	107.800	4.4252	0.0617	
D-Incubation time	44.9668	1	44.967	1.8459	0.2041	
AB	338.2841	1	338.284	13.8866	0.0039*	Significant
AC	1384.3981	1	1384.398	56.8296	<.0001*	Significant
AD	269.6985	1	269.699	11.0711	0.0077*	Significant
BC	163.6481	1	163.648	6.7178	0.0269*	Significant
BD	699.2058	1	699.206	28.7024	0.0003*	Significant
CD	2237.5265	1	2237.527	91.8505	<.0001*	Significant
A^2	0.0190	1	0.019	0.0008	0.9783	
B^2	1.5353	1	1.535	0.0630	0.8069	
C^2	209.5757	1	209.576	8.6031	0.0150*	Significant
D^2	211.7585	1	211.759	8.6927	0.0146*	Significant
Error	243.605	10				
C. Total	12455.464	24				

$R^2 = 0.0.9804$, Adj $R^2 = 0.0.9530$, Root Mean Square Error (RMSE) = 4.935, Mean of Response = 91.089, CV = 5.42%.
Df (degree of freedom), Significant ($p \leq 0.05$), Non-significant ($p > 0.05$).

Table 5
Statistical analysis of CCD showing coefficients, *t*-values, *P*-values and standard errors.

Term	Coefficient estimate	Std Error	t-Ratio	p-Value Prob > F	VIF
Intercept	91.676949	2.131148	43.02	<.0001*	1
A-Beef extract	−9.508889	1.163341	−8.17	<.0001*	1
B–$MgSO_4.7H_2O$	16.674444	1.163341	14.33	<.0001*	1
C–K_2HPO_4	2.4472222	1.163341	2.10	0.0617	1
D-Incubation time	1.5805556	1.163341	1.36	0.2041	1
AB	4.598125	1.233909	3.73	0.0039*	1
AC	−9.301875	1.233909	−7.54	<.0001*	1
AD	−4.105625	1.233909	−3.33	0.0077*	1
BC	3.198125	1.233909	2.59	0.0269*	1
BD	−6.610625	1.233909	−5.36	0.0003*	1
CD	−11.82563	1.233909	−9.58	<.0001*	1
A^2	−0.086441	3.09278	−0.03	0.9783	1.9789831
B^2	−0.776441	3.09278	−0.25	0.8069	1.9789831
C^2	−9.071441	3.09278	−2.93	0.0150*	1.9789831
D^2	9.1185593	3.09278	2.95	0.0146*	1.9789831

enzyme formation, probably by suppression of cell viability and enzymatic inactivation. In contrast, low temperature values may slow down the metabolism of the microorganism and consequently, enzyme synthesis (Blanco et al., 2016). Vijayaraghavan et al. (2015) obtained maximum amylase secretion from *Bacillus cereus* at 45 °C.

The result obtained from OFAT showed 72.4 U/ml of amylase activity which was 1.8- fold higher as compared to unoptimized conditions. Saha and Mazumdar (2019) reported that OFAT showed activity 3.9- fold higher as compared to unoptimized conditions.

3.6. Multi-factorial designs to improve α-amylase production

Current study presents an ideal model for gradual optimization of α-amylase production depending on the factorial designs (Response Surface Methodology) to save effort, time and cost.

3.6.1. Plackett-Burman design (PBD)

The PB design was used to screen important variables (Plackett and Burman, 1946) affecting α-amylase production. It evaluates thirteen independent variables that tested at two levels, low level (−1) and high level (+1). α-Amylase activity (the average) for the trials (U/ml) was calculated as presented in Table 1. Enzyme activity showed a wide variation (from 11.96 to 98.3 U/ml). Run 14 showed the maximum enzyme production which is 2.5-fold higher than that obtained from

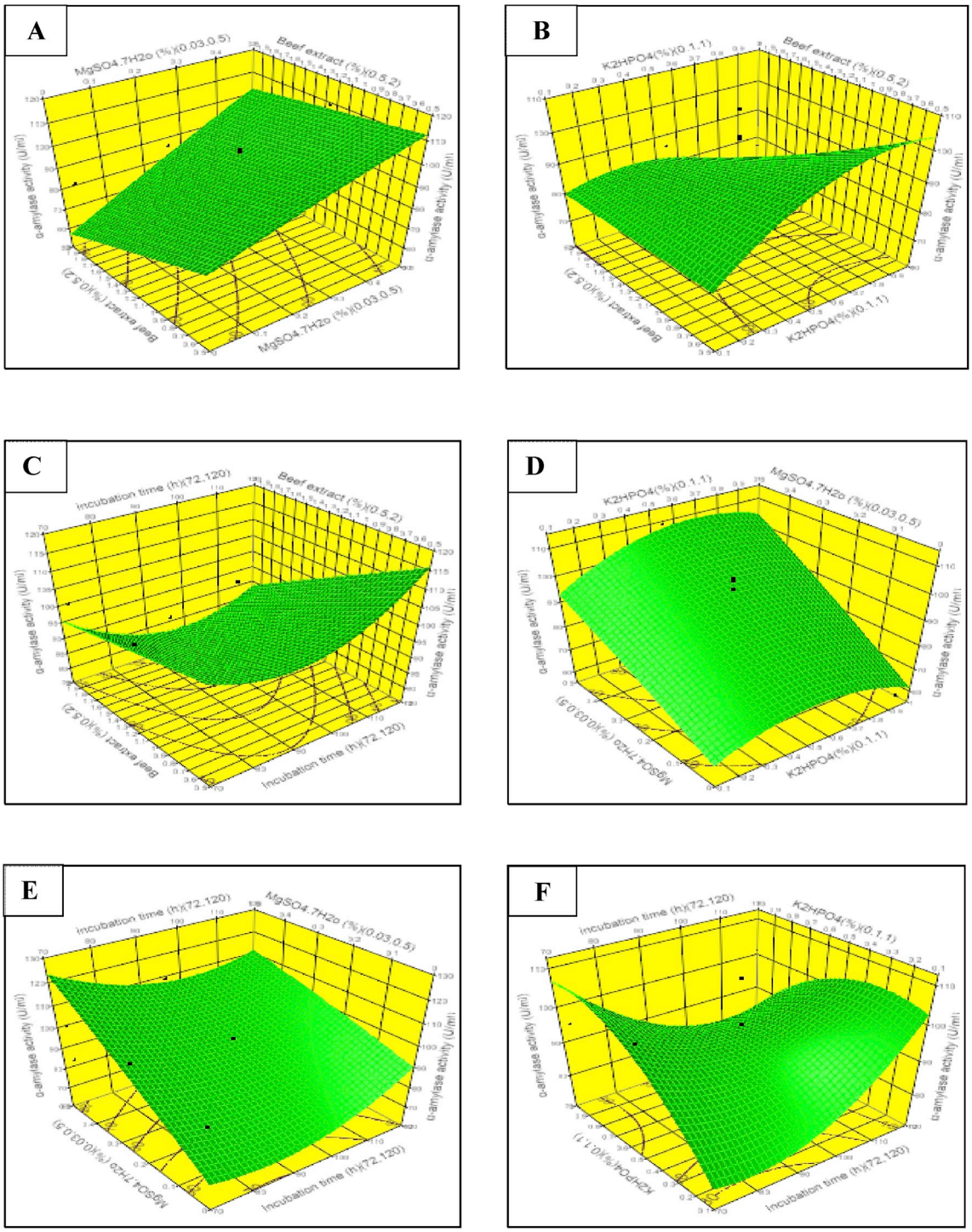

Fig. 6. Response surface 3D contour plots representing interaction between variables affecting α-amylase production (**A**) Beef extract and $MgSO_4{\cdot}7H_2O$ (**B**) Beef extract and K_2HPO_4 (**C**) Beef extract and Incubation time (**D**) $MgSO_4{\cdot}7H_2O$ and K_2HPO_4 (**E**) $MgSO_4{\cdot}7H_2O$ and Incubation time (**F**) K_2HPO_4 and Incubation time. Other variables were kept constant.

basal media (M6). Multi-regression analysis on the experimental data, established first-order polynomial equation to explain the production of α-amylase as following:

$$Y\ (U/ml) = 54.391 + 0.17X_1 - 4.961X_2 + 8.516X_3 - 3.443X_4 + 6.024X_5 + 9.943X_6 + 5.721X_7 - 12.2X_8 + 0.437X_9 - 4.893X_{10} + 6.055X_{11} + 0.6X_{12} - 2.093X_{13}$$

Where: Y, α-amylase activity; X_1, lactose; X_2, soluble starch; X_3, beef extract; X_4, yeast extract; X_5, K_2HPO_4; X_6, $MgSO_4{\cdot}7H_2O$; X_7, $FeSO_4{\cdot}7H_2O$; X_8, $MnCl_2{\cdot}4H_2O$; X_9, $CaCl_2$; X_{10}, culture pH; X_{11}, incubation time; X_{12}, inoculum size; X_{13}, agitation speed. The significance of the model is indicated by analysis of variance of the PB design (ANOVA) for α-amylase production as represented in Table 3. The model F value of 15.22 indicates the model is significant. There is only a 0.16% chance that an F-value this large might be due to noise. Values of "Prob > F" is < 0.05 implies that the model terms are significant. In the current

Table 6
Comparison of growth curve/production kinetics between unoptimized and optimized medium.

Incubation time (h)	Enzyme activity (U/ml)		Protein content of (mg/ml)		Specific activity (U/mg protein)		$O.D_{660}$		Final pH	
	U	O	U	O	U	O	U	O	U	O
6	9.75	12.50	2.10	3.21	4.65	3.89	0.17	0.29	6.7	6.7
12	14.21	22.54	3.25	5.45	4.37	4.14	0.45	0.66	6.5	6.6
24	24.37	88.13	6.6	14.6	3.69	6.04	0.61	0.90	6.4	6.5
36	29.40	117.37	10.7	18.95	2.75	6.18	0.70	1.16	6.5	6.8
48	37.63	125.01	18.6	20.09	2.02	6.22	0.99	1.28	7.5	7.0
60	23.23	131.12	13.0	20.51	1.79	6.39	0.72	1.40	8.5	7.1
72	18.31	145.8	11.1	20.42	1.65	7.14	0.54	1.58	8.6	7.1

Where: U is the unoptimized medium (M6) and O is the optimized medium.

case, eight variables including soluble starch, beef extract, K_2HPO_4, $MgSO_4{\cdot}7H_2O$, $FeSO_4{\cdot}7H_2O$, $MnCl_2{\cdot}4H_2O$, culture pH and incubation time are significant model terms. The other variables with values > 0.05 pointed to their insignificant effect on enzyme production. The determination of the squared regression coefficient (R^2) for α-amylase production was 0.9705 indicating that the data variability could be interpreted very well by the models.

The Pareto chart (Fig. 4A) for experimental data analysis showed that, $MgSO_4{\cdot}7H_2O$, beef extract, incubation time and K_2HPO_4 were the most influencing factors followed by $FeSO_4{\cdot}7H_2O$ and showed positive effect on α-amylase production. On the other hand, factors including $MnCl_2{\cdot}4H_2O$, soluble starch and culture pH showed negative effect on α-amylase production. The closure between predicted and actual values of enzyme activity (Fig. 5A) refers to the high significance of the design. In the current investigation, lactose was reported as a robust C-source for of α-amylase production under SmF. It was documented that supplementation of starch at low level as an additional C-source was found to have an inductive effect and also it has notable efficiency in the of α-amylase production. Similarly, Prajapati et al. (2015) found that, starch and lactose were used as inducers for production of α-amylase by *B. amyloliquefaciens* KCP2. In accordance with our findings, Duque et al. (2016) notified that beef extract as N-source showed significant effect on α-amylase production from *Enterococcus faecium* DMF78. The current study demonstrated that, $MgSO_4{\cdot}7H_2O$ was the most significant variable affecting positively the α-amylase production. On the other hand, $CaCl_2$ showed non-significant effect on the production of α-amylase agreeing with Duque et al. (2016). In contrast to this result most α-amylases known as metallo-enzymes need Ca^{+2} ions for preserving the final enzyme structure (Prajapati et al., 2015; Ahmed et al., 2017).

3.6.2. Central composite design (CCD)

Based on the results of PBD (first order model), four variables that showed maximum effect on α-amylase production (beef extract, $MgSO_4{\cdot}7H_2O$, K_2HPO_4 and incubation time) were further optimized by the second order model Central Composite design (CCD) to determine their optimal levels. The design model and the corresponding experimental data of the four independent variables are presented in Table 2. By employing a multi-regression analysis for the experimental data, the predicted response Y for α-amylase activity could be obtained using the following second-order polynomial equation:

$$Y\ (U/ml) = 91.677 - 9.509A + 16.674B + 2.447C + 1.581D + 4.598AB - 9.302AC - 4.106AD - 3.198BCE - 6.611BD - 11.826CD - 0.086A^2 - 0.776B^2 - 9.071C^2 + 9.118D^2$$

Where, Y is the predicted α-amylase activity (U/ml), A, B, C and D are the code values (beef extract, $MgSO_4{\cdot}7H_2O$, K_2HPO_4 and incubation time), respectively.

The ANOVA which carried out to test the significance of the fit of the second-order polynomial equation for the enzyme activity was illustrated in Table 4. ANOVA was used to estimate the statistical significant of the variables and its interactions. A model F-value (35.807) and a low probability value (Prob > F) < 0.05) mean the model was significant and fit of the model was investigated. There is 0.01% chance that an F-value this large may occur due to noise. The coefficient of variation (CV) refers the degree of accuracy with which the treatments were compared (Cao et al., 2009). The low CV value (5.42%) clearly indicates a high degree of accuracy and a great deal of reliability for the experimental values. The quality of the fit of the quadratic regression model equation was estimated by R^2 (the coefficient of determination). The value of R^2 (0.9804) meaning that 98.04% of the variability in the response could be expound by the statistical model. The model is strong and the predicted response better as the R^2 value becomes closer to 1.0. A regression model, with R^2 value ˃ 0.9000, was considered to have very high correlation (Jaya et al., 2010). Moreover, the value of the adjusted R^2 was (0.953), which was also high. These results show that the regression model presents a good fit to the data.

The coefficients, *t*-test values, *P*-values and standard errors on all the variables of linear (A, B, C, D), quadratic (A^2, B^2, C^2, D^2) and interactions (AB, AC, AD, BC, BD, CD) terms were determined and are shown in Table 5. Also, "Prob > F" Value < 0.05 means that the model terms were significant. Whereas the value ˃0.100 means that model terms were not significant (Diler and Ipek, 2012). In this case, linear terms (A, B), quadratic terms (C^2, D^2) and two-way interaction terms (AB, AC, AD, BC, BD and CD) were significant model terms (P < 0.05) whereas linear terms (C, D) and quadratic terms (A^2, B^2) were not significant model terms (P > 0.05). This suggests that beef extract and $MgSO_4{\cdot}7H_2O$ have a significant effect on α-amylase activity as shown in the Pareto chart (Fig. 4B). The model validation was clear up on comparing the statistical predicted yield (140.14 U/ml) with the experimental (actual) yield of α-amylase (145.4 U/ml) which is closely related (Fig. 5B). Three dimensional (3D) response surface graphs (Fig. 6A–F) provide a visual interpretation of the average interaction between two independent variables while keeping the other variables at their constant level (zero level). The response surface plots were created by plotting the enzyme activity on the z-axis versus any two independent variables. α-amylase production differed significantly when independent factors levels were changed. In this study, all the 3D plots exhibited significant response on α-amylase activity. It was observed that the interaction terms AB and BC showed positive effect while, AC, AD, BD and CD showed negative effect on α-amylase production.

The highest enzyme production (145.4 U/ml) is 3.6 and 1.5-fold increase compared to the basal medium (M6) and Plackett-Burman design medium, respectively. This result is higher than that reported by Kumar et al. (2013) and Blanco et al. (2016) on the production of α-amylase using RSM (4.84 and 9.26 U/ml, respectively). In addition, Poddar et al. (2014) mentioned that optimized medium by statistical methods increase amylase production from *B. subtilis* DJ5 by 1.5-times.

Optimized medium by OFAT and RSM enhanced enzyme production by 7.5-folds confirming the need to optimize the production parameters to achieve maximum yield. This result is higher than that obtained by Saha and Mazumdar (2019) by 2-fold.

The optimized medium components (g/l) were soluble starch (2), lactose (10), beef extract (5), K_2HPO_4 (10.0), $MgSO_4{\cdot}7H_2O$ (5), $FeSO_4{\cdot}7H_2O$ (0.25), $CaCl_2$ (0.5), pH 7 and incubation time 72h.

3.7. Comparison of growth curve/production kinetics between unoptimized and optimized medium

The results in Table 6 showed that the highest enzyme yield (37.63 and 145.8 U/ml) was reached at 47 and 72 h, respectively for unoptimized and optimized medium. Thereafter, the enzyme production decreased using unoptimized and optimized medium. Unakal et al. (2012) attributed the decline in enzyme synthesis to a possible denaturation and/or decomposition of the enzyme due to interactions with other compounds in the fermented medium.

4. Conclusion

Alkali-tolerant bacterial isolates were evaluated for their ability to produce α-amylase enzyme. The most potent isolate was characterized (morphologically and biochemically) and was identified on the basis of 16S rRNA gene homology. This sequence was deposited to the GenBank under accession numbers *Bacillus subtilis* strain-MK1 (MF614924). The goal of this study was the optimization of physico-chemical factors affecting α-amylase production from *B. subtilis* strain- MK1 under SmF by employing OFAT and statistical analysis (RSM). Plackett-Burman design (PBD) identifies the most significant factors and then the optimum levels of the screened factors and their interactions were identified by Central Composite design (CCD). Analysis of variance (ANOVA) of CCD revealed high value of R-square (0.9804) and adjusted R-square (0.9530) at significant level ($p \leq 0.05$) providing a good model to fit the data obtained. The α-amylase production was increased by 3.6 and 7.5-fold using statistically optimized medium compared with the basal media and initial medium, respectively.

Declaration of competing interest

There is no conflict of interests regarding the publication of this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101397.

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