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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)

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ABSTRACT

Alpha amylase is a starch hydrolyzing important enzyme which has numerous industrial applications. In the present research an alpha amylase producing *Bacillus cereus* amy3 was isolated from garden soil of University of North Bengal, India. We have optimized the enzyme production parameters using cheep agro-residues as carbon source through one factor at a time (OFAT) approach. The result obtained from OFAT study indicates that the following medium composition and culture conditions, mosambi peel 1.75% (w/v), medium pH 7, incubation temperature 37 °C, incubation time 48 h, maltose 1% (w/v), Sodium nitrate 0.09% (w/v), tyrosine 0.09% (w/v), inoculum size 3% (v/v) [3×10^7CFU/ml] showed 3122 ± 56 IU/ml of amylase activity which was 3.9 fold higher as compared to unoptimized conditions (800 ± 43 IU/ml). Further optimization was done using central composite rotatable design (CCRD) based response surface methodology (RSM). RSM model was found to have a predicted R^2 and Adjusted R^2 value of 0.9998 and 0.9997, respectively and can significantly ($p < 0.0001$) predicted the response variables. Within the model interaction effect between the model variables such as mosambi peel (A), medium pH (B) and incubation temperature were found to be significant and the model showed 4386.09 ± 21 IU/ml of amylase activity which was approximately 5.48 and 1.40 fold higher as compared to unoptimized and OFAT optimized culture conditions, respectively.

1. Introduction

In the present day biotechnological research α-amylase is considered as one of the most valuable enzymes as it has importance and application of many fields such as in clinical, medicinal pharmaceutical and analytical chemistry (Agger et al., 2001). The α-amylase (endo-1,4-α-D-glucan glucanohydrolase (E.C.3.2.1.1) use starch as its substrates and cleave α-1,4-glycosidic bonds of starch which in turn liberates glucose, maltose and malto-oligosaccharide mixtures (Akcan, 2011; Bakri et al., 2012). Due to this microbial amylase have a numerous application in starch dependent industries, especially, textile, detergent, food, paper, leather and pharmaceutical industries (Kaur et al., 2012; Abd-Elhalem et al., 2015). Different type of organism like plants, animals and microbes are the important sources of α-amylase. Among these different sources of amylase microbial amylases have wide range of advantages such as its easy production optimization process, time and space effective as well as its cost effectiveness (Burhan et al., 2003). The α-amylase enzyme that were used commercially obtained mostly from *Bacillus* genus (Pandey et al., 2000). As a heterogenous organism and its ability to adapt versatile environment different *Bacillus* species such as *Bacillus licheniformis* (Morgan and Priest, 1981), *Bacillus stearothermophilus* (Wind et al., 1994), *Bacillus flavothermus* (Kelly et al., 1997) and *Bacillus megaterium* (Jana et al., 1997) were reported as theromosatble alpha-amylase producer.

Metabolite synthesis and microbial growth mainly depends on the nutrient content of culture medium and its cultivation condition (Prescott et al., 2002; Nayak et al., 2013). The production cost of microbial metabolite production can be reduced by the use of cheep agro residues as carbon sources in the microbial growth medium (Lakshmipathy and Nanda, 2013; Saha and Ghosh, 2014). Moreover, for maximizing the microbial metabolite synthesis and minimizing the production cost, optimization of medium components and process parameters play vital roles (Bezbaruah et al., 1994). The variables responsible for enzyme production by microorganisms were generally optimized using one factor at a time (OFAT) approach, but it involves more experiment and it is time consuming also (Saha and Ghosh, 2014).

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The application of statistical experimental design for optimization of enzyme production was reported in several scientific reports in recent years (Tanyildizi et al., 2005; Kunamneni and Singh, 2005). In the present study the optimization of α-amylase production by a newly isolated *Bacillus cereus* amy3 under submerged fermentation (SmF) had been performed using one factor at a time (OFAT) and central composite rotatable design (CCRD) based response surface methodology (RSM) approach.

2. Materials and method

2.1. Isolation and screening of amylase producing microorganism

Samples collection was conducted according to the method described by Zarina and Nanda (2014) and Majeed et al. (2014), with slight modification. Soil samples were collected from the site where cooked food wastes were regularly deposited in the locality near University of North Bengal, India (Latitude 26°42′41.29″ N, Longitude 88°21′03.02″ E). 10 g soil sample was serially diluted in the range of 10^{-1} to 10^{-10} using 90 ml sterilized saline water. Serially diluted samples were spread plated in the amylase agar medium(AAM, pH7) containing(g/l): peptone, 0.90; $(NH_4)_2HPO_4$, 0.40; KCl, 0.10; $MgSO_4,7H_2O$, 0.10; starch soluble, 10 and 2% (w/v) agar-agar and the plates were incubated at 37 °C for 24 h for propagation of amylase producer. Microbial colonies observed after 24 h of growth were then isolated depending on the distinctive colony morphology. Individual colonies were then purified using successive streaking on AAM plates. Isolated bacterial strains were then evaluated qualitatively for amylase production. To do this each of the individual colonies were streaked in the middle of AAM plates and the plates were incubated at 37 °C for 24 h. After incubation the plates were flooded with iodine solution followed by 30 min of incubation. Plates were then washed with double distilled water and observed for starch hydrolysis zone of clearance around the colony growth. Microbial colonies showing the highest zone of clearance was selected as amylase producer. Isolates showing starch hydrolysis zone were further screened for quantitative amylase production ability. Pure culture of the isolated microorganisms were inoculated into the nutrient agar slant and stored in refrigerator at 4 °C for further studies (Nayak et al., 2014).

2.2. Amylase production

Strain amy3 was grown in 50 ml of Luria berteni (LB) broth and incubated at 37 °C for 24 h at static condition. After completion of incubation 2% (v/v) of LB broth culture having 2×10^7CFU/ml of bacteria were inoculated into 100 ml of amylase production media (APM = AAM-Agar) and incubated for 24–144 h with at 37 °C. Samples were withdrawn at an interval of 24 h and centrifuged at 8000 RPM at 4 °C for 10 min. According to the slight modification of the method described by Majeed et al. (2016), the supernatants were separated from the bacterial cell pellet and filter through 0.2 μm membrane filter and were used as cell free crude enzyme extract (CFCEX). These CFCEX were used for analysis of amylase activity.

2.3. Amylase assay

Amylase was assayed based on the determination of reducing sugar liberated from starch using 3, 5-dinitrosalicylic acid (DNS) reagent as proposed by Miller (1959). The reaction mixture containing 0.25 ml of CFCEX and 0.25 ml of 1% (w/v) starch soluble dissolved in 0.1 M phosphate buffer at pH 7, was incubated at 37 °C for 30 min. After incubation 0.50 ml of DNS reagent was added to the mixture followed by incubation of 10 min in boiling water bath. The mixture was then cooled and absorbance was recorded at 540 nm. The amount of reducing sugar was measured from the standard curve generated using maltose (10–100 μg/ml). Tube without incubation between enzyme and substrate mixture was served as blank. One international unit (IU) of amylase activity is defined as the amount of the enzyme required to release 1 μmol of maltose equivalent per min under standard condition which were maintained during the assay.

2.4. Identification of bacterial strain amy3

Colony morphology, growth pattern, motility, spore formation and gram staining were performed to identify the isolated most potent amylase producing bacterial strain. Aerobic/anaerobic nature of the isolates were determined by growing it on APM under aerobic or anaerobic condition, sulphide indole motility agar medium was used for its motility test, malachite green staining was used to determine its spore formation capability and finally to classify the strain in specific bacterial genera gram staining was conducted. Among the biochemical test catalase test, Voges - Proskaur test, methyl red test, urease test, nitrate reduction test, oxidase test, citrate utilization, indole test, starch hydrolysis test, casein hydrolysis, gelatine liquefaction test, lipid hydrolysis, fermentation of sugars (glucose, fructose, mannitol, lactose, sucrose, maltose, starch, xylose, sorbitol, mannose) were performed according to the method described by Smibert and Krieg (1994) to identify the bacterial strain.

Further the identification of the bacterial strain amy3 was done by PCR amplification of 16S rDNA using the universal primer 27F (5′ AGAGTTTGATCCTGGCTCAG3′) and 1492R (5′TACGGTTACCTTGTTA CGACTT3′) followed by sequencing. The resultant nucleotide sequence has been submitted to NCBI gene bank data base (Accession No. **MK106155.1**). The true identity of the isolated strain was confirmed by phylogenetic analysis of 16S rRNA gene using software Mega-6 (Tamura et al., 2007).

2.5. Optimization of amylase production by one factor at a time (OFAT) approach

Amylase production by *Bacillus cereus* amy3 was optimized through OFAT by using various agro residues. The medium parameter optimized was incorporated in the succeeding steps of optimization process.

2.5.1. Effect of agro residues on amylase production

The various kind of agro residues such as potato peel, sugarcane bagasse, mosambi peel, orange peel, maize bran, banana peel, coconut husk, water hyacinth, rice bran and wheat bran collected from the house hold waste and local market near University of North Bengal, were washed with distilled water, sundried until they get constant weight and sliced into small pieces for use as carbon source in the APM (pH7) instead of starch at a concentration of 1.0% (w/v). The 100 ml medium was inoculated with 2% (v/v) (2×10^7 CFU/ml) overnight grown culture of *Bacillus cereus* amy3 and incubated for 24 h at 37 °C. After incubation the amylase activity and total extracellular proteins were assayed for all the tubes. Effect of different concentration of best agro-residues (mosambi peel) showing the optimum amylase production was also determined by varying its concentration ranging from 0.125% to 2% (w/v) in the APM.

2.5.2. Effect of pH and temperature on amylase production

Amylase production was determined at pH values ranging from 3 to 9. The media (APM) of different pH (100 mM citrate buffer pH 3, 4 and 5, 100 mM phosphate buffer pH 6, 7 and 100 mM Tris–HCl buffer 9) were inoculated with 2% (v/v) (2×10^7 CFU/ml) overnight grown culture of *Bacillus cereus* amy3 and incubated at 37 °C for 24 h. Thereafter, CFCEX was assayed for amylase activity and total extracellular proteins.

The effect of incubation temperature in the production medium (pH7) containing 1.75% (w/v) of mosambi peel was evaluated by growing the organism at 4, 25, 30, 37, 50 and 60 °C for 24 h. After incubation the amylase activity and total extracellular proteins were assayed for all the tubes.

2.5.3. Time course of amylase production

To determine the time course of amylase production, *Bacillus cereus* amy3 was grown in optimized APM. Samples were withdrawn at 24 h intervals for a period of 144 h and amylase activity in the CFCEX was monitored.

2.5.4. Effect of additional carbohydrates on amylase production

Once the optimum agro-residue, medium pH, incubation temperature and incubation time for amylase production were obtained, the optimized APM was supplemented with various additional carbohydrates such as fructose, maltose, sucrose, dextrose and lactose at 1% (w/v) each. The resulting medium was inoculated with the bacterial culture and incubated at 37 °C for 48 h. Thereafter, CFCEX was assayed for amylase activity and total extracellular proteins.

2.5.5. Effect of nitrogen sources on amylase production

In order to find out the effect of different nitrogen sources viz. Ammonium sulphate, di-ammonium hydrogen phosphate, sodium nitrate, peptone, yeast extract and ammonium nitrate on amylase production, the bacterial culture was inoculated in the APM medium optimized so far. N_2 source at a concentration of 0.09% (w/v) were added into the medium. Fermentation was allowed to continue for 48 h at 37 °C and CFCEX was used for amylase activity and total extracellular protein assay.

2.5.6. Effect of amino acids on amylase production

To determine the effect of various amino acids on amylase production cystein, proline, tyrosine, glutamine and proline were added into the optimized APM at a concentration of 0.09% (w/v). Medium was inoculated with 2% (v/v) of bacterial culture and incubated at 37 °C for 48 h. The CFCEX obtained after fermentation was evaluated for amylase activity and protein assay.

2.5.7. Effect of inoculam size on amylase production

To determine the effect of bacterial inoculam size on amylase production, inoculam was prepared by inoculating *B. cereus* amy3 into 50 ml Luria Berteni (LB) broth and incubated at 37 °C for 24 h at static condition. To study the effect of inoculam size on amylase production, the 100 ml APM optimized so far were inoculated with 1.0, 2.0, 3.0, 4.0 and 5.0% (v/v) of 24 h old *B. cereus* amy3 culture (10^7 CFU/ml). The fermentation medium was then incubated at 37 °C for 48 h. The culture filtrate was centrifuged and the obtained CFCEX was evaluated for amylase and total extracellular protein assay.

2.6. Optimization of amylase production by Central Composite Rotatable Design (CCRD) based Response Surface Methodology (RSM)

The three effective parameters i.e. mosambi peel concentration, medium pH and incubation temperature, selected from OFAT method were further employed by CCRD based RSM for optimization of amylase production by *B. cereus* amy3 under submerged fermentation condition. Due to the presence of a centre point and axial points in CCRD the curvature of the established model is possible which interns allow the flexibility in the design. The centre point is separated from each of the factorial point by ± 1 and the distance between axial points and centre of the design space is $\pm \alpha$, where $\alpha = (2^k)^{1/4}$ {k = number of independent variables}. The three independent variables mosambi peel (A), medium pH (B) and incubation temperature (C) were used in five different coded levels ($-\alpha$,-1, 0, +1, $+\alpha$) to established the model. For mosambi peel (A) the actual value of the coded levels $-\alpha$, −-1, 0, +1, and $+\alpha$ are, 0.66, 1, 1.50, 2 and 2.34% (w/v), respectively. For medium pH (B) the actual value of the coded levels $-\alpha$, -−1, 0, +1, $+\alpha$ are 2.64, 4, 6, 8 and 9.36, respectively. For incubation temperature the actual value of the coded levels $-\alpha$, -1, 0, +1, $+\alpha$ are 23.18, 30, 40, 50 and 56.82 °C, respectively. The coded value and actual value of each of the independent variables used in the study are related with the following Eq. (1).

$$X_a = (Z_a - Z_0)/\Delta z \tag{1}$$

Where, X_a is a coded value of the independent factor, Z_a is the actual value; Z_0 is the centre point actual value of the same and ΔZ the step change value of the variable. Total number of experimental run (R) in the CCRD based RSM is determined by following Eq. (2).

$$R = 2^k + 2k + n_0. \tag{2}$$

Where k is the number of independent variables used to construct the model and n_0 is the number of repeated experiments in the centre point. In this present study 20 experimental runs were created with 8 factorial, 6 axial and 6 centre point runs. A second order quadratic polynomial regression equation [Eq. (3)] was generated to analyse the amylase produced (activity IU/ml) in the experiments.

$$Y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_{11}x_{12} + a_{22}{x_2}^2 + a_{33}{x_3}^2 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{23}x_2x_3 \ldots \tag{3}$$

Where, Y represents the predicted amylase activity, a_0 represent the model intercept terms, x_i is the independent variables and a_i is the model coefficient parameters. To get the better response in amylase activity all the independent variables were optimized through Eq. (3).

2.7. Protein estimation

Protein was estimated by the method described by Bradford (1976), using BSA as standard. Reaction mixture contains 3.0 ml of Bradford reagent and 100 µl of proteins. The resulting mixture was incubated at room temperature for 10 min followed by recording absorbance at 595 nm.

2.8. Statistical analysis

All the data were recorded as triplicate of mean along with standard deviation. Homogeneity of mean was evaluated by one way analysis of variance followed by Duncan Multiple Range Tests (DMRT) at a level of significance $p < 0.05$ using software package SPSS version 16. CCRD based RSM was conducted using Design Expert software (Version 8.0.7.1, State-Ease, Minneapolis, MN, USA).

3. Results

3.1. Screening and identification of amylase producing microorganism

Microorganisms having the capability of amylase production were isolated from soil by serial dilution technique using AAM plates. Six microorganisms showing the growth on AAM plates were selected preliminary by their formation of zone of clearance on AAM plates which were detected by iodine staining. These six strains were inoculated into the APM media to check their ability for amylase production. The amylase activity of 321 ± 12, 432 ± 21, 800 ± 18, 543 ± 15, 587 ± 14 and 645 ± 16 IU/ml, were recorded for strain amy1, amy2, amy3, amy4, amy5 and amy6, respectively. Highest production of amylase was found for strain amy3 and was selected for further studies.

The identity of the strain was further revealed by morphological and biochemical analysis as shown in Table 1. The strain amy3 was morphologically characterised as gram positive, rod shaped, non motile, non spore former bacteria. The colonies of this bacteria were white, opaque, matte, entire margin and convex in nutrient agar plates. The bacterium was characterised biochemically as negative in lipase and Voges-Proskauer tests whereas found positive in methyl red, nitrate reduction test, citrate utilization (Table 1). Analysis of the entire test

Table 1
Morphological and biochemical properties of amylase producing strain amy3.

Sl. No.	Traits	amy3
1.	Gram staining	+
2.	Shape of vegetative cell	Rod
3.	Diameter of colony (mm)	2.7
4.	Colony morphology	White, Opaque, Matte, Entire margin convex
5.	Spore formation	–
6.	Motility	–
7.	Growth on usual media (Aerobic)	+
8.	Growth on NaCl	+
9.	Starch hydrolysis	+
10.	Casein hydrolysis	+
11.	Gelatin hydrolysis	+
12.	Lysine decarboxylase	+
13.	Urease test	+
14.	Catalase test	+
15.	Oxidase test	+
16.	Lipase test	–
17.	Citrate utilization	+
18	Voges-Proskauer test	–
19	Methyl Red test	+
20	Nitrate reduction test	+
21	Fermentation	
21.1	Starch	G
21.2	Lactose	G
21.3	Glycerol	A + G
21.4	Mannose	A + G
21.5	Glucose	A + G
21.6	Fructose	G
21.7	Mannitol	A + G
21.8	Xylose	A + G
21.9	Arabinose	G
21.10	Cellobiose	G
21.11	Sucrose	G
21.12	Maltose	G
21.13	sorbitol	G

'+'positive for the test, '–' negative for the test, 'A + G' production of both acid and gas, 'G' production of gas only.

revealed that this organism belongs to the genus *Bacillus*. The bacterial strain amy3 was further identified by phylogenetic analysis (Fig. 1) of 16S rDNA sequences and found 99% similarity with the *Bacillus cereus* ATCC 14579 strain and hence named as *Bacillus cereus* amy3.

3.2. Optimization of amylase production by one factor at a time (OFAT) approach

Amylase production by *B. cereus* amy3 was carried out in presence of different agro residues such as potato peel, sugarcane bagassae, mosambi peel, orange peel, maize bran, banana peel, coconut husk, water hyacinth, rice bran and wheat bran [1.0% (w/v) each] as primary carbon source in amylase production medium at 37 °C for 24 h. As shown in the Fig. 2a, mosambi peel showed the highest amylase yield of 1321 ± 43 IU/ml as compared to that of expensive commercial starch 800 ± 18 IU/ml. Further, when the effect of different concentration of mosambi peel was evaluated for amylase production, highest amylase activity (1482 ± 54 IU/ml) was recorded at concentration of 1.75% (w/v) (Fig. 2b). For determination of optimum initial medium pH for amylase production, *B. cereus* amy3 was grown in APM containing 1.75% (w/v) mosambi peel at different pH ranging from 3 to 9, keeping all the other growth variables constant. Although *B. cereus* amy3 produced considerable amount of amylase at pH 6 but the maximum amylase yield (1476 ± 66 IU/ml) was recorded at pH 7 (Fig. 2c). The optimum incubation temperature of amylase production by the strain amy3 in the above optimized culture medium was determined by incubating the medium at various temperature 4, 25, 30, 37, 50 and 60 °C followed by determination of extracellular enzymatic activity. Amylase production was recorded maximum (1487 ± 23 IU/ml) at 37 °C (Fig. 2d). To determine the optimum incubation time for amylase production, *B. cereus* amy3 was inoculated in the above optimized APM and the resulting fermentation medium was incubated at 37 °C for various time interval such as 24, 48, 72, 96, 120 and 144 h. Maximum amylase activity (1875 ± 25 IU/ml) was achieved after 48 h of incubation with a protein yield and specific activity of 1.4 ± 0.1 mg/ml and 1339.28 ± 75 IU/mg, respectively (Fig. 3a). Amylase production medium prepared in 0.1 M phosphate buffer at pH 7, containing 1.75% (w/v) mosambi peel, supplemented with either of the sugars [1% (w/v)] such as, fructose, maltose, sucrose, dextrose, lactose and starch was

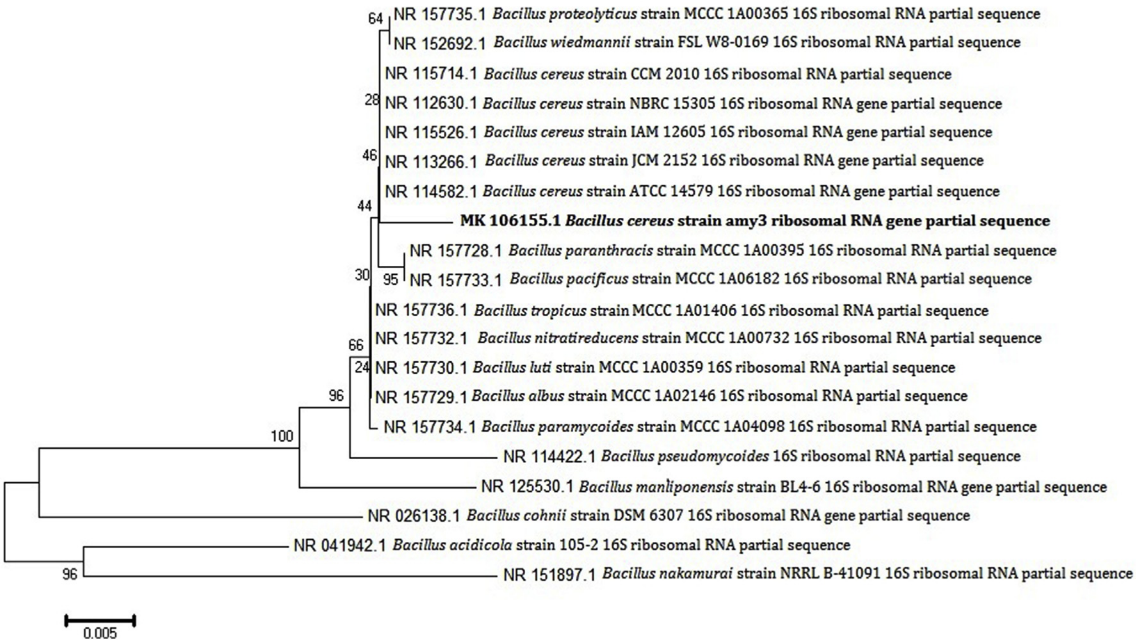

Fig. 1. Phylogenetic tree constructed using 16S rDNA gene sequences reflecting the position of the strain amy3 with other *Bacillus* species. Number at nodes represents bootstrap values. Accession numbers are given extreme left of each strain.

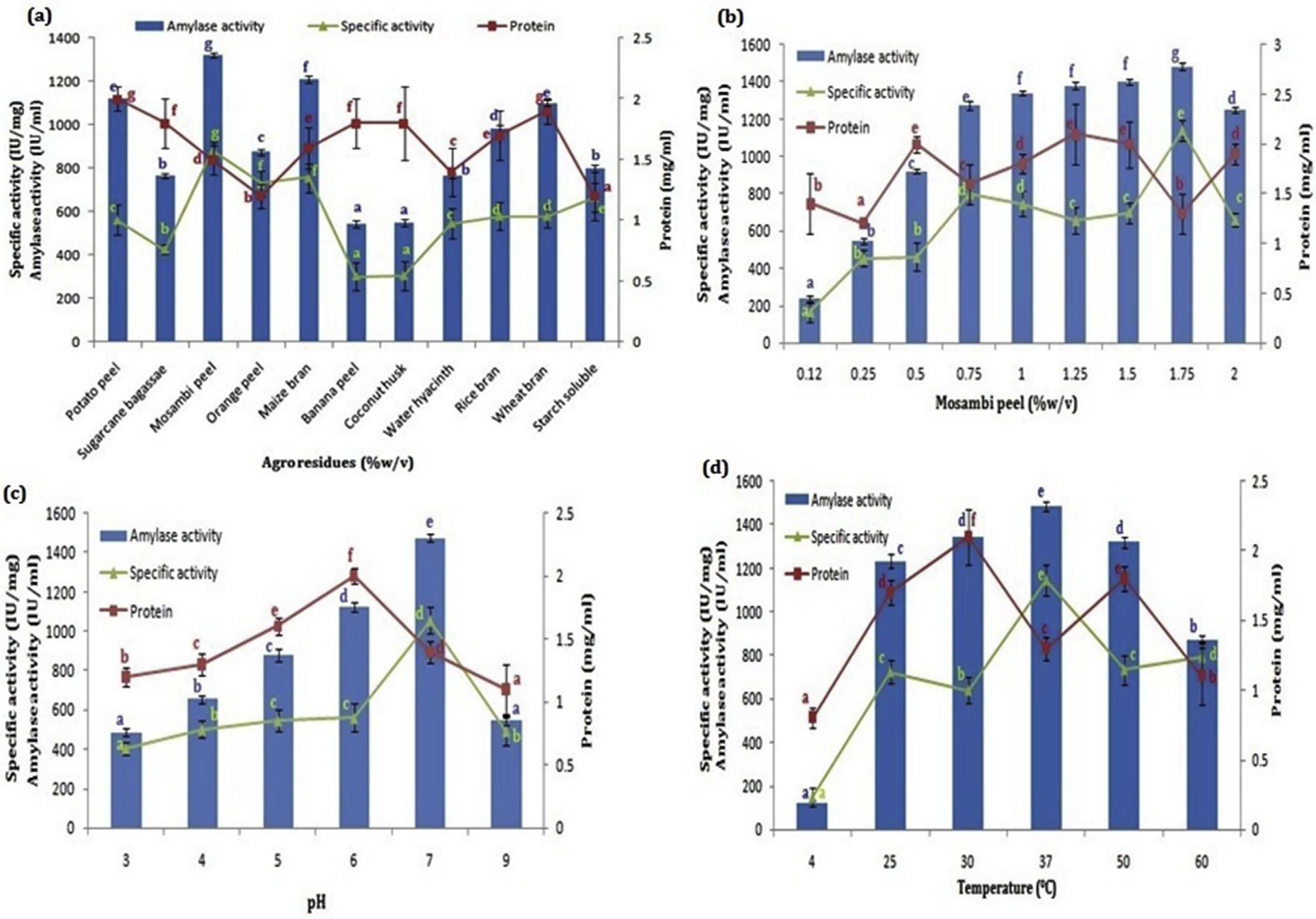

Fig. 2. Optimization of α-amylase production by *B. cereus* amy3 by OFAT approach. Effect of (a) agro-residues [1% (w/v)] (b) Mosambi peel concentration [% (w/v)] (c) medium pH (d) incubation temperature [°C], on α-amylase production under submerged fermentation [SmF]. The parameter optimized was incorporated in subsequent experiment. Each bar/line marked with different alphabets is significantly ($p < 0.01$) different.

inoculated with *B. cereus* amy3 and the medium was incubated at 37 °C for 48 h. Amylase production increased significantly to 2165 ± 22 IU/ml on supplementation of maltose (Fig. 3b). However, the maximum declined in amylase activity was recorded in presence of dextrose (456 ± 31 IU/ml) and fructose (765 ± 21 IU/ml) as compared to control (1886 ± 30 IU/ml). Nitrogen source in the media is vital parameters that influence the microbial growth and enzyme production. To determine the effect of Nitrogen source on amylase production by *B. cereus* amy3, the APM was supplemented with 0.09% (w/v) of various N_2 sources viz., ammonium sulphate, di-ammonium hydrogen phosphate, sodium nitrate, ammonium nitrate, peptone and yeast extract individually. As shown in the Fig. 3c, sodium nitrate causes maximum amylase activity of 2456 ± 39 IU/ml, followed by ammonium nitrate (2235 ± 21 IU/ml). In addition to nitrogen sources, amylase production was also evaluated in presence of different amino acids [0.09% (w/v)] in the APM. Amylase production was found to be further enhanced in presence of tyrosine and showed maximum activity of 2876 ± 43 IU/ml (Fig. 3d). Finally, the various inoculum sizes [1–5% (v/v)] were evaluated for enhanced production of amylase by *B. cereus* amy3. Enzyme production was found to be minimum when inoculum size is low [1% (v/v)], as the size of inoculums gradually increased the amylase production was also gradually increased and found optimum at 3% (v/v) [(3×10^7 CFU/ml)] with an activity of 3122 ± 56 IU/ml (Fig. 4a). Moreover, as shown in the Fig. 4a, the specific activity of the amylase in this condition was recorded as 1643.15 ± 45 IU/mg protein.

3.2.1. Optimization of amylase production using Central Composite Rotatable Design (CCRD) based Response Surface Methodology (RSM)

Amylase production by *B. cereus* amy3 was investigated through CCRD based RSM using the different range of three independent variables, Mosambi peel (A), medium pH (B) and incubation temperature (C). All the other variables of APM were kept constant in their optimum level as they obtained in OFAT experiment. According to the CCRD twenty experimental runs were designed using different combination of variables. The medium components used for RSM are present in the material and method section where as the experimental run along with actual and model predicted amylase activity were displayed in Table 2. The significance of the current model was evaluated by analysis of variance (ANOVA). The ANOVA results showed that the model terms was highly significant with a level of significance of $p < 0.0001$ and can better predict the response variable (amylase activity). Within the model B (medium pH), C (incubation temperature), AB (mosambi peel X medium pH), AC (mosambi peel X incubation temperature), BC (medium pH X incubation temperature), A^2 (mosambi peel^2), B^2 (medium pH^2) and C^2 (incubation temperature^2) are the significant ($p < 0.0001$) model parameters. A second order polynomial quadratic regression equation was established for amylase activity using the results of the design which are presented in equations (4) and (5) in coded and actual terms respectively.

$$\text{Amylase Activity (IU/ml)} = +4131.97 + 4.36 \text{ X } A + 419.99 \text{ X } B - 795.67 \text{ X } C + 72.38 \text{ X } A \text{ X } B - 38.87 \text{ X } A \text{ X } C - 290.87 \text{ X } B \text{ X } C - 300.55 \text{ X } A^2 - 857.57 \text{ X } B^2 - 905.48 \text{ X } C^2.. \quad (4)$$

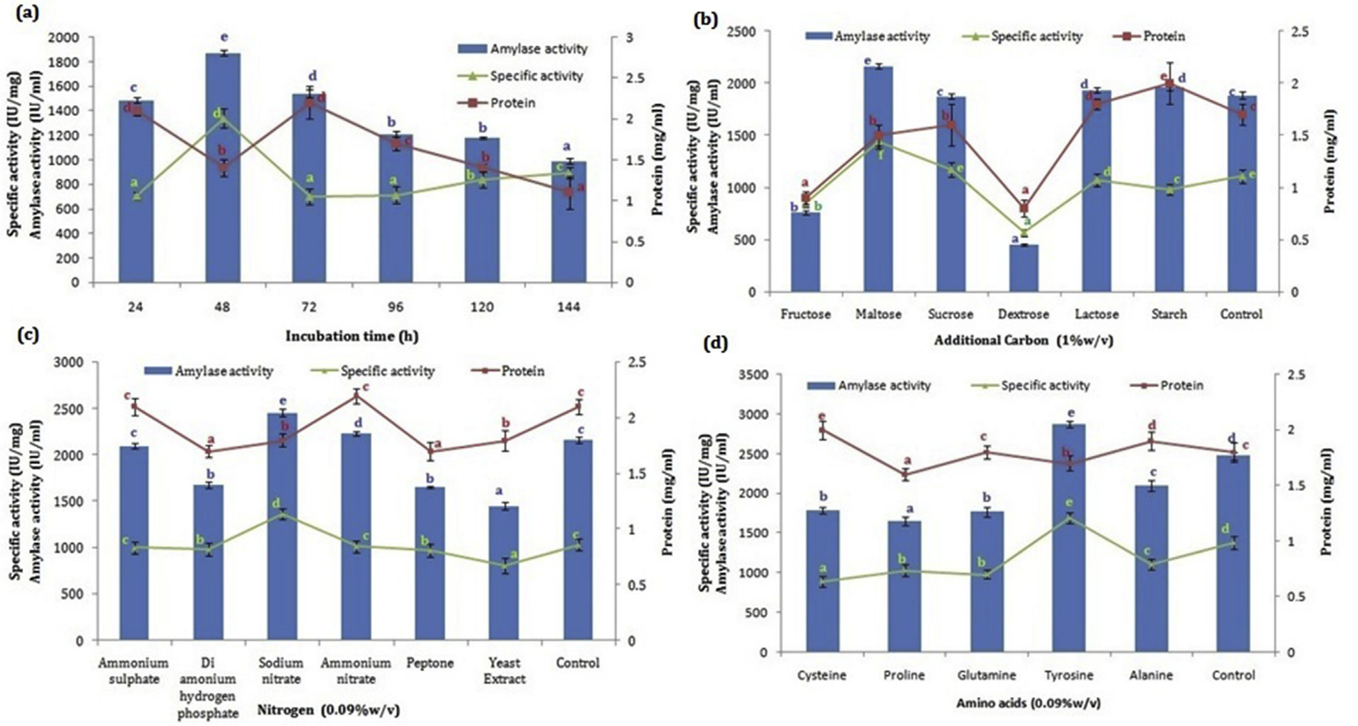

Fig. 3. Optimization of α-amylase production by *B. cereus* amy3 by OFAT approach. Effect of (a) incubation time [h] (b) additional carbon sources [1% (w/v) along with mosambi peel concentration 1.75% (w/v)] (c) nitrogen sources [0.09% (w/v)] (d) amino acids [0.09% (w/v)] on α-amylase production. The parameter optimized was incorporated in subsequent experiment. Each bar/line marked with different alphabets is significantly ($p < 0.01$) different.

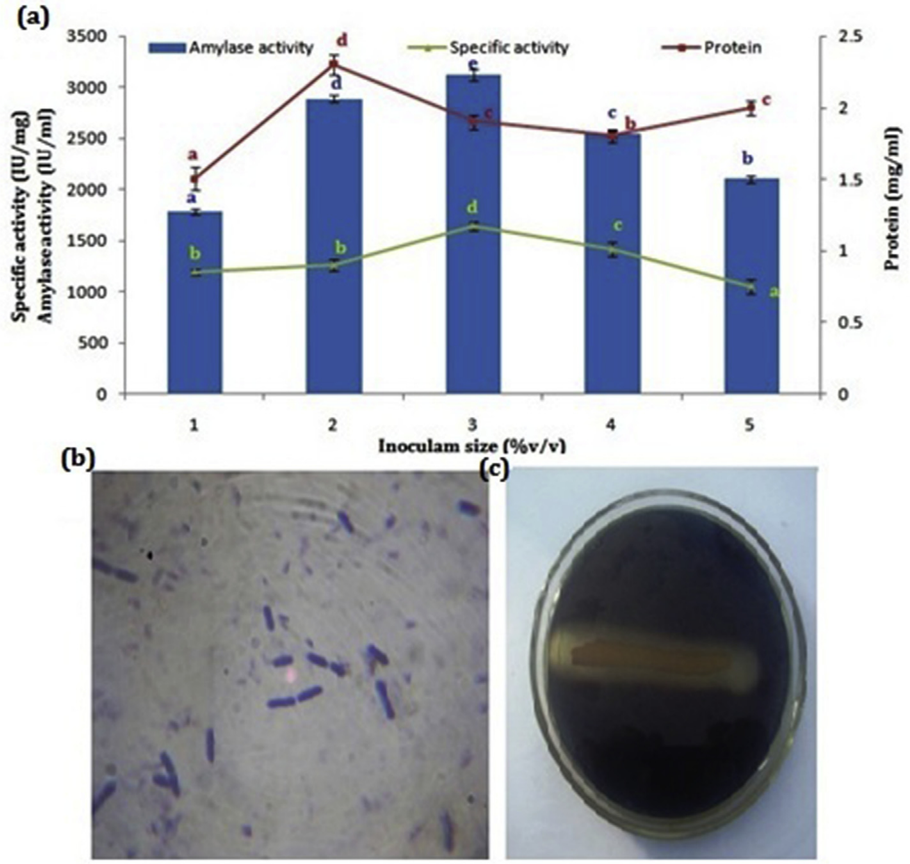

Fig. 4. Effect of (a) inoculums size [1% (v/v) = 10^7 CFU/ml of viable bacterial count] of *B. cereus* amy3 on α-amylase production (b) gram nature of the amylase producing strain amy3 (c) clear zone of starch hydrolysis by *B. cereus* amy3 on starch agar medium plate.

Table 2
Actual and predicted amylase activity with the experimental run obtained through CCD based RSM design.

Run No.	Std. Order	Type	A	B	C	$X_{Predicted}$	X_{Actual}
1	5	Factorial	1.00	4.00	50.00	1250.47	1252
2	6	Factorial	2.00	4.00	50.00	1036.69	1040
3	1	Factorial	1.00	4.00	30.00	2182.31	2209
4	14	Axial	1.50	6.00	56.82	232.73	253
5	4	Factorial	2.00	8.00	30.00	3690.01	3698
6	7	Factorial	1.00	8.00	50.00	1363.94	1350
7	12	Axial	1.50	9.36	40.00	2412.71	2428
8	8	Factorial	2.00	8.00	50.00	1439.67	1422
9	9	Axial	0.66	6.00	40.00	3274.54	3267
10	16	Center	1.50	6.00	40.00	4131.97	4123
11	13	Axial	1.50	6.00	23.18	2909.03	2876
12	2	Factorial	2.00	4.00	30.00	2124.03	2147
13	20	Center	1.50	6.00	40.00	4131.97	4122
14	17	Center	1.50	6.00	40.00	4131.97	4165
15	15	Center	1.50	6.00	40.00	4131.97	4126
16	18	Center	1.50	6.00	40.00	4131.97	4132
17	3	Factorial	1.00	8.00	30.00	3459.28	3465
18	10	Axial	2.34	6.00	40.00	3289.22	3284
19	19	Center	1.50	6.00	40.00	4131.97	4126
20	11	Axial	1.50	2.64	40.00	1000.05	972

[A] Mosambi peel (%w/v).
[B] pH.
[C] Incubation Temperature (°C).
[X] Amylase activity (IU/ml).

Amylase Activity (IU/ml) = −22174.84 + 3492.08 X mosambi peel + 3255.90 X pH + 743.74 X incubation temperature + 72.37 X mosambi peel X pH – 7.77 X mosambi peel X incubation temperature −14.54 X pH X incubation temperature – 1202.20 X mosambi peel^2 -214.39 X pH^2 -9.05X incubation temperature^2.. (5)

The fitness of the model was justified using various criteria (Table 3). In the current model, coefficient of determinant (R^2), adjusted R^2, predicted R^2, adequate precision and “lack of fit” were evaluated. The model R^2 value of 0.9998 indicates that the model could explain 99.99% of the variability of the amylase activity predicted response. Predicted R^2 value of 0.9989 was found to be in reasonable agreement with model adjusted R^2 value of 0.9997. Adequate precision which measure the signal to noise ratio was found to be 229.020 and indicates an adequate signal with less noise in the experimental design. The model “lack of fit” F- value of 3.32 implies the “lack of fit” is not significant (p = 0.1068) relative to the pure error. Non significant “lack of fit” is good to fit the model for its better estimation of predicted response. Moreover the normal probability plot (Fig. 5a) indicates the residual follows a normal distribution as the points follows a straight line. Residuals verses ascending predicted response plots (Fig. 5b), which test the assumption of constant variance found to be in random scatter (constant range of residuals across the graph) which indicates that further transformation in the data series is not required. Fig. 5c, indicates the plot of the residuals versus the experimental run order. It allows checking for lurking variables that may have influenced the response during the experiment. As the plot (Fig. 5c) showed a random scatter it indicates the fitness of the model. A graph of the actual response values versus the predicted response values was shown in the Fig. 5d. It helps to detect a value, or group of values, that are not easily predicted by the model. As the data points were split evenly by the 45° line it signifies that the predicted response is in reasonable agreement with the observed ones. So from the above observation it can be concluded that the model can be efficiently used to navigate the design space.

3.2.2. *Interpretation of interaction effects between the independent factors and localization of optimum condition for xylanase production*

The response surface plots (Fig. 6) and their contour plots (data not shown) were generated using the second order quadratic polynomial regression equation was used to investigate the interaction effect between the media variables for amylase production. Fig. 6a represents the response surface plot of amylase production in presence of two media variables mosambi peel (A) and medium pH (B). From the result in the Fig. 6a, it is evident that the amylase production by *B. cereus* amy3 was significantly affected by the interaction effect between mosambi peel (A) and medium pH (B). Amylase production was found to be gradually enhanced from 2647.24 IU/ml to 3363.84 IU/ml as the medium pH was increased from 4.00 to 8.00 keeping the mosambi peel concentration fixed at concentration 1.00% (w/v). Whereas changing the concentration of mosambi peel from 1.00 to 2.00% (w/v) at a fixed pH, enzyme production did not changed significantly which was also supported by the ANOVA result, where mosambi peel alone have a non significant p value of 0.0581. The interaction between these two variable significantly ($p < 0.001$) affect the amylase production which was supported by the fact that when pH was changed in the same mosambi peel concentration or vice-versa amylase production fluctuated greatly. Highest amylase production (4182 IU/ml) was predicted at a medium pH 6.56 and mosambi peel concentration of 1.50% (w/v).

The interaction between mosambi peel concentration (A) and incubation temperature (C) for amylase production is shown in Fig. 6b. The results suggested that the maximum amylase activity of 4303.27 IU/ml was recorded in presence of wheat bran (A) 1.5% (w/v) and incubation temperature of 35.04 °C. The interaction between these two variables also affect amylase production significantly (p = 0.001).

Fig. 6c represents the interaction between medium pH (B) and

Table 3
ANOVA for amylase activity yield generated by response surface quadratic model.

Source	Sum of Squares	df	Mean Squares	F value	p-value Prob > F	Remarks
Model	3.247E+7	9	3.608E+6	6223.34	< 0.0001	Significant
A-Mosambi peel	260.02	1	260.02	0.45	0.5182	
B-pH	2.409E+6	1	2.409E+6	4155.05	< 0.0001	
C-Temperature	8.646E+6	1	8.646E+6	14913.23	< 0.0001	
AB	41905.13	1	41905.13	72.28	< 0.0001	
AC	12090.13	1	12090.13	20.85	0.0010	
BC	6.769E+5	1	6.769E+5	1167.50	< 0.0001	
A^2	1.302E+6	1	1.302E+6	2245.41	< 0.0001	
B^2	1.060E+7	1	1.060E+7	18281.09	< 0.0001	
C^2	1.182E+7	1	1.182E+7	20380.61	< 0.0001	
Residual	5797.55	10	579.75			
Lack of fit	4456.21	5	891.24	3.32	0.1068	Not significant
Pure Error	1341.33	5	268.27			
Cor total	3.248E+7	19				

R^2 = 0.9998 Adj-R^2 = 0.9997 Pred- R^2 = 0.9989 Adequate precision = 229.020.

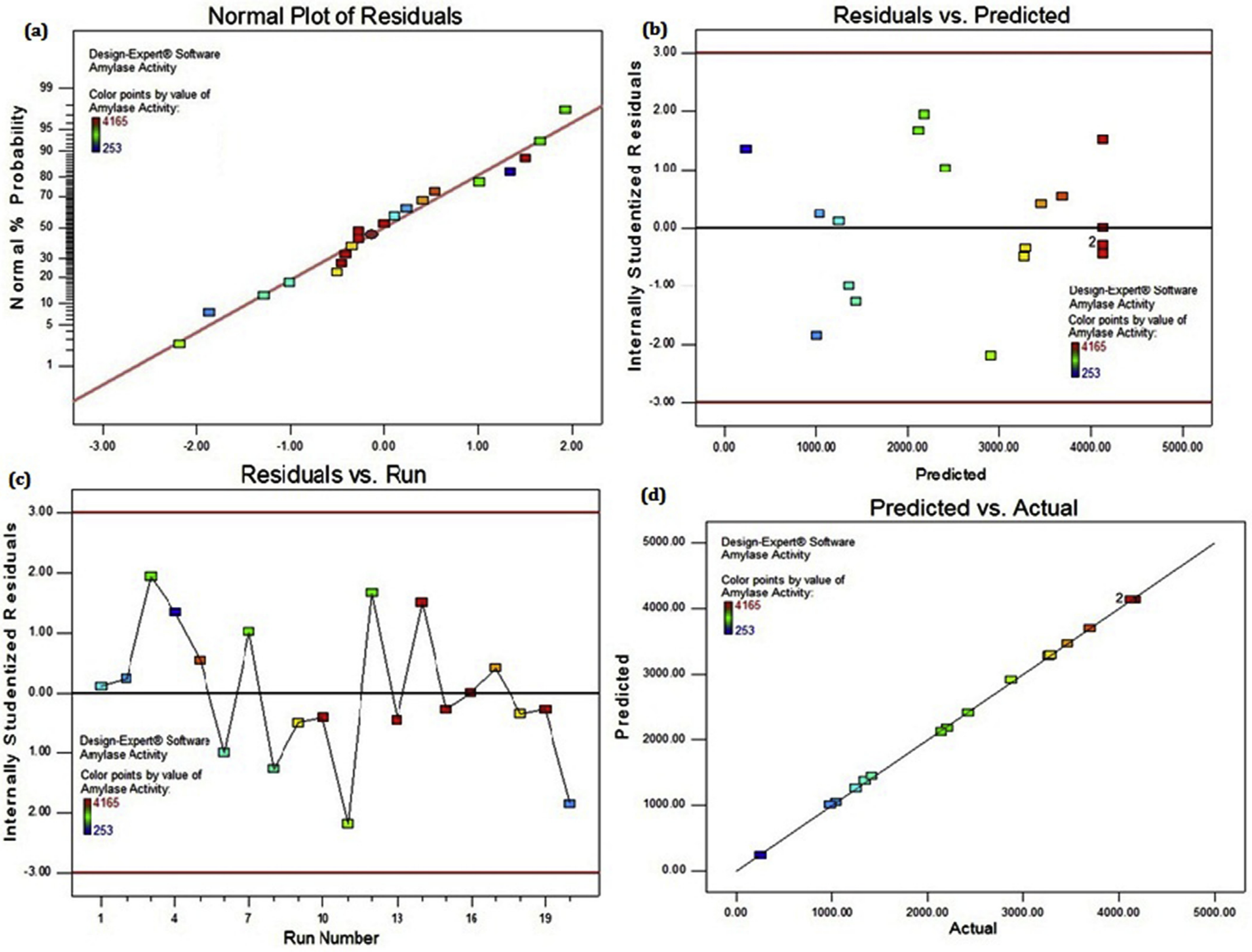

Fig. 5. Validity of the CCRD based RSM model is evaluated using the (a) normal plot of residuals (b) residual versus predicted activity plot (c) internally studentized residuals versus run number plot (d) model predicted versus actual α-amylase activity plot.

incubation temperature (C). Significant increment of amylase activity was recorded from 2480 to 4169 IU/ml when cultivation temperature was reduced from 50 to 30 °C, keeping the medium pH fixed at 6.28.

The Fig. 6d, represent the perturbation plot of the model terms. From the plot it was clearly observed that all the model parameters significantly affect the amylase production from their coded level (−1 to +1). Among the variables incubation temperature (C) marked with red, show significant variation in amylase production.

3.2.3. *Validation of the model*

The validity of the model was tested by performing ten experimental run generated using the numerical optimization tool in the model (Table 4). Among the ten experimental run the predicted and actual amylase activity was not significantly different ($p > 0.05$) and hence it can be concluded that the current model could better predicted the amylase activity. Hence the value of these three variables were found as mosambi peel concentration (A) 1.57% (w/v), medium pH (B) 6.44 and incubation temperature (C) 38 °C, which showed the maximum predicted amylase activity of 4338.31 IU/ml which was found to be closed to the actual activity of 4386.09 ± 21 IU/ml. All the other media parameters were kept constant in their optimum level as they were obtained in the OFAT experiment. Hence it can be concluded that the CCRD based RSM can significantly enhanced the amylase production and showed an amylase activity of 4386.09 ± 21 IU/ml. The specific activity of alpha amylase on RSM optimized condition was recorded as 2088.61 ± 32 IU/mg protein.

4. Discussion

In this present study an amylase producing *B. cereus* amy3 had been isolated from garden soil samples. There are several reports that indicate the isolation of amylase producing microbial strain from various environmental samples such as, water (Sudan et al., 2018), honey (Du et al., 2018), press mud soil of sugar industry (Kumar et al., 2013), Atlantic forest soil (Pereira et al., 2017), Vegetable wastes (Paul et al., 2017), hot springs (Sen et al., 2014) etc. Current study showed that amylase production by *B. cereus* amy3 was significantly enhanced under submerged fermentation using mosambi peel as the agro residue in comparison to the available commercial starch. Various type of agro residues such as citrus and orange wastes, rice husks, wheat bran and sugar cane baggassae had been reported to be used as a sole carbon source by microbial strain for the production of various extracellular enzymes including amylase (Imen and Mahmoud, 2015; Demir et al., 2012; Mohamed et al., 2010). As like that of our finding amylase production by *Bacillus* strain had been cited in various scientific literature such as, *Bacillus amyloliquefaciens* BH072 (Du et al., 2018), *Bacillus amyloliquefaciens* P-001 (Deb et al., 2013), *Bacillus laterosporus* (Kumar et al., 2013), *Bacillus* sp. MB6 (Paul et al., 2017), *Bacillus licheniformis*

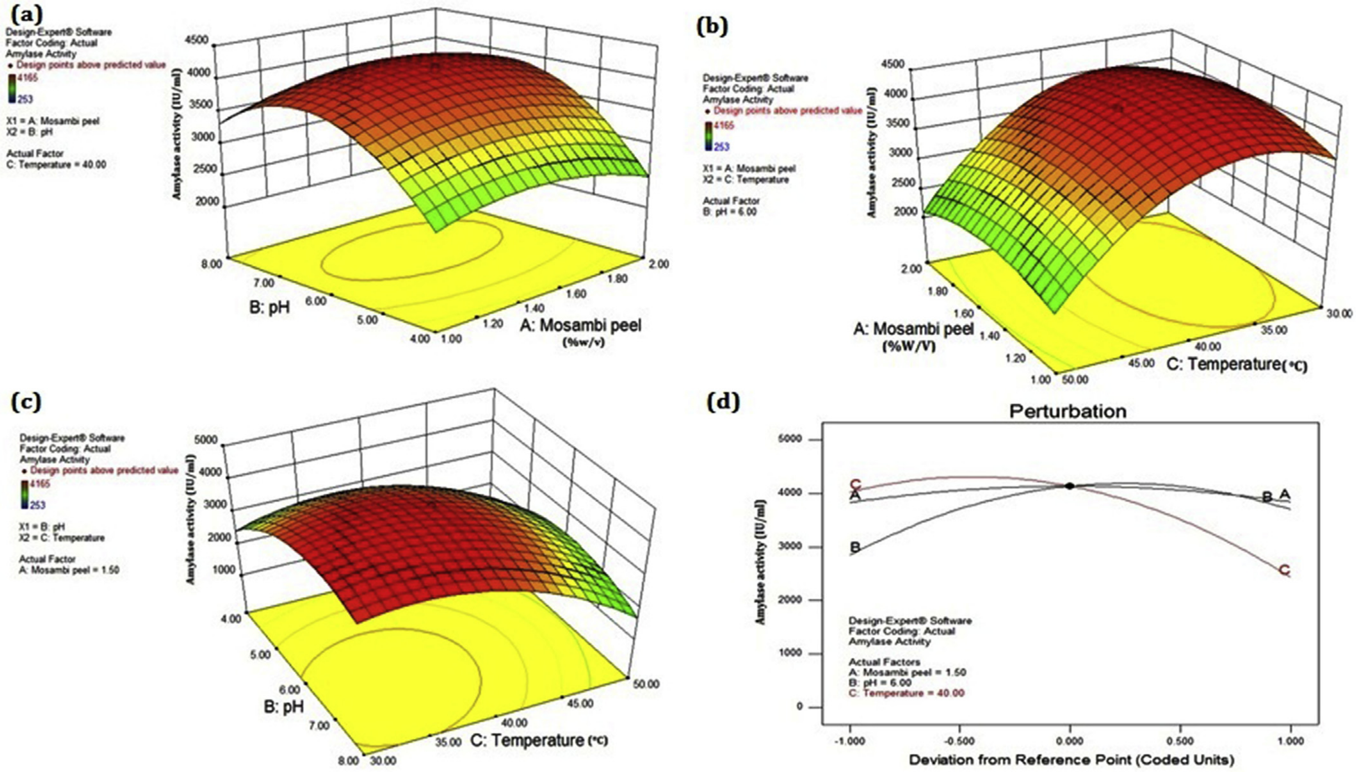

Fig. 6. Response surface curves of α-amylase production from B. cereus amy3, showing interaction between (a) mosambi peel concentration and medium pH (b) mosambi peel concentration and incubation temperature (c) medium pH and incubation temperature after 48 h of submerged fermentation. (d) Perturbation plot of the model identifying the most significant variables. [Unit of amylase activity was recorded in IU/ml].

Table 4
Validation of the response surface quadratic model for optimization of amylase production by *B. cereus* amy3.

Exp. No.	A	B	C	X $_{\text{Predicted}}$	X $_{\text{Actual}}$
1.	1.50	7.32	37	4267.07	4254.08 ± 28
2.	**1.57**	**6.44**	**38**	**4338.31**	**4386.09 ± 26**
3.	1.60	7.18	37	4268.98	4251.08 ± 31
4.	1.73	5.98	34	4224.51	4237.09 ± 21
5.	1.51	6.74	38	4326.85	4211.07 ± 12
6.	1.26	6.44	34	4284.93	4297.02 ± 15
7.	1.28	7.35	36	4196.02	4200.60 ± 18
8.	1.72	7.35	36	4257.03	4267.08 ± 19
9.	1.51	5.85	34	4234.50	4212.43 ± 20
10.	1.29	6.45	33	4257.68	4254.54 ± 25

^{A} Mosambi peel (%w/v), ^{B} pH, ^{C} Incubation Temperature (°C), ^{X} Amylase activity (IU/ml).

ATCC 9945a (Slavic et al., 2015), *Bacillus subtilis* (Yan and Wu, 2017) etc. However report for optimization of amylase production by using agro residues and bacterial strain are few. In this present study, optimization of amylase production by *B. cereus* amy3 using mosambi peel as agro residues had been optimized through OFAT and CCRD based response surface methodology. Optimization of amylase production using OFAT approach used in this research showed that the enzyme production was enhanced up to 3122 ± 56 IU/ml as compared to unoptimized conditions (800 ± 43 IU/ml) when *B. cereus* amy3 was cultivated in the APM having the following medium variables and cultivation condition, mosambi peel 1.75% (w/v), medium pH 7, incubation temperature 37 °C, incubation time 48 h, Maltose 1% (w/v), Sodium nitrate 0.09% (w/v), tyrosine 0.09% (w/v), inoculam size 3% (v/v) [3 × 10^{7}CFU/ml]. Similar to our result 230.45 U/ml of amylase production by *Bacillus* sp. MB6 had been reported to produced at medium pH 6, incubation temperature 37 °C and in incubation time of 48 h (Paul et al., 2017). The optimization of amylase production was also carried out by other researcher. Bakri et al. (2012), found that the pH range between 4 and 6 and incubation temperature of 37 °C was optimum for amylase yield by *Bacillus* sp. which also similar to our findings. Alkaline pH 8 and incubation temperature of 40 °C enhanced the amylase production by *Bacillus* sp. when cultivated for 24 h was reported by Anupama and Jayaraman (2011). Similar optimum temperature of 37 °C and incubation time of 21 h was reported by Bozic et al. (2011).

In this work, the application of CCRD based RSM techniques resulted in approximately 5.48 fold increased in amylase activity as compared to unoptimized culture media conditions (800 IU/ml). The level of amylase yield was higher as compared to the other reports available for production by *Bacillus* sp. The amount of α-amylase produced by *Bacillus laterosporus* using RSM technique was found to be 4.838 U/ml (Kumar et al., 2013). Bashir et al. (2014), observed that when *Bacillus licheniformis* was grown using banana wastes as carbon source 27 IU/ml of amylase activity was recorded whereas using sweet potato as substrate *Aspergillus niger* showed 0.89 U/ml of amylase activity (Ubalua, 2014). The specific activity of the alpha amylase produced by *B. cereus* amy3 in OFAT and RSM optimized condition was 1643.15 ± 45 IU/mg and 2088.61 ± 32 IU/mg, respectively, which is comparable with several other amylases produced by different strains cited in various scientific literature. Amylase specific activity of 987.34 ± 23.34 U/mg of amylase production was recorded for *B. amyloliquefacience* (Du et al., 2018), *Bacillus* sp.MB6 was found to produce 230.45 U/ml of amylase but when immobilized in calcium alginate beads showed 270.59 ± U/mg of specific amylase activity (Paul et al., 2017). Hernandez et al. (2006) also reported for 70.29 U/ml of amylase production by *A. niger* using brewer's spent grain. *Thermomyces lanuginosus* was reported to produce 414 U/g amylase using peptone, 356 U/g amylase using tryptone, and 338 U/g amylase using meat extract as nitrogen source (Kim et al., 1995). *Bacillus cereus* MTCC

1305 was reported to produce 0.04 g/g amylase activity using glucose as carbon source (Sivaramakrishnan et al., 2007), *B. amyloliquefaciens* have been shown that when wheat bran and groundnut oil cake in a ratio of 1:1 was used as carbon source amylase yield became maximum 1671 U/g (Moreira et al., 1999). To meet the increasing demand of amylase for industrial application cost effective production strategies using cheep agro residues are highly required (Saxena and Singh, 2014). Considering the above features *Bacillus cereus* amy3 isolated from soil, may be used as promising candidate for amylase production owing to its ability to produce high extracellular amylase using mosambi peel in a cost effective manner.

5. Conclusion

The present study investigate the production and optimization of α-amylase by *Bacillus cereus* amy3 using OFAT and CCRD based RSM technique. OFAT methods showed that mosambi peel was the most suitable cheep agro residues for amylase production. This method also showed approximately 3.9 fold increased amylase production as compared to unoptimized media conditions. Moreover, the use of CCRD based RSM showed approximately 5.48 and 1.40 fold increased in enzyme activity as compared to unoptimized and OFAT optimized culture conditions, respectively. The production of 4386.09 ± 21 IU/ml of *B. cereus* amy3 amylase enzyme under submerged fermentation of mosambi peel using CCRD based RSM technique was significantly higher as compared to the previous amylase activity cited in scientific literature and due to its low cost of production can be proven usefull for industrial application.

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