ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of
DEVELOPMENT RESEARCH

International Journal of Development Research
Vol. 5, Issue, 09, pp. 5417-5424, September, 2015

Full Length Research Article

ISOLATION AND IDENTIFICATION OF A NOVEL THERMOALKALOPHILIC ISOLATE *BACILLUS AERIUS* GC6 FOR AMYLASE PRODUCTION UNDER SUBMERGED FERMENTATION

*Gitanjali Vyas and Nivedita Sharma

Department of Basic Sciences, Dr. YS Parmar University of Horticulture and Forestry Nauni, Solan, HP, India- 173230

ARTICLE INFO

Article History:
Received 03rd June, 2015
Received in revised form
08th July, 2015
Accepted 22nd August, 2015
Published online 30th September, 2015

Key words:
Amylase,
Submerged Fermentation,
RSM,
Bacillus aerius,
Starch hydrolysis

ABSTRACT

A potential amylase producing bacterial strain was isolated from mushroom compost and identified as *Bacillus aerius* GC6 using 16S rRNA gene technique and deposited in NCBI gene bank vide accession number |KJ775810.1|. The aim of this study was to enhance amylase production by optimizing various medium components and process parameters viz. substrate concentration, pH, temperature, inoculums size, Ca^{2+} and Mg^{2+} concentrations through the statistical approach under submerged fermentation from this isolate. The optimum levels of these six parameters and their interaction effects were determined employing the response surface central composite design (CCD). A maximum amylase activity of 84.645 IU/ml had been obtained at 47.5 °C and 9.5 pH @ 8.75% inoculum using 1.75 % substrate with 0.5% Ca^{2+} and Mg^{2+} concentrations. An overall increase of 13.11% has been observed after the factors were optimized by using RSM.

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INTRODUCTION

α- amylase (E.C 3.2.1.1) catalyses the hydrolysis of α-D-(1,4) glycosidic linkages in starch components and related carbohydrates. They can specifically cleave the *O*-glycosidic bonds in starch. Starch depolymerization by amylases is the basis for several industrial processes such as preparation of glucose syrups, bread making and brewing. Thus, it is a key enzyme in the production of starch derivatives and also used in desizing fabrics, in pharmaceuticals and detergents. Submerged fermentation (SmF) has been traditionally used for the production of industrially important enzymes because of the ease of handling and greater control of environmental factors such as temperature and pH (Ramachandran *et al*., 2010).

Thermostable amylases which have been isolated mainly from thermophilic organisms have found a number of commercial applications because of their overall inherent stability (Demirijan *et al*., 2001). The ability of microorganisms to grow at high temperatures is associated with thermally stable macromolecules.

Corresponding author: Gitanjali Vyas
Department of Basic Sciences, Dr. YS Parmar University of Horticulture and Forestry Nauni, Solan, HP, India- 173230

As a consequence of growth at high temperature and unique macromolecular properties, thermophilic bacteria can possess physically and chemically stable enzymes. With the availability of thermostable enzymes, a number of new possibilities for industrial processes have emerged. Even though several microorganisms produce amylases, it remains still as a challenging task to obtain the strains, which are capable of producing commercially acceptable yields and this selection of suitable strain is the most significant factor. Thus, recent research with thermostable α-amylase has been concentrated on the screening of thermophilic micro-organisms with thermostable α- amylase that can, facilitate the discovery of novel α- amylase producing strain suitable for new industrial applications (Gupta *et al*., 2003). To meet the growing demands in the industry it is necessary to improve the performance of the system and thus increase the yield without increasing the cost of production. The growth and enzyme production of the organism are strongly influenced by medium composition thus optimization of media components and cultural parameters is the primary task in a biological process (Djekrif-Dakhmouche 2013). The main strategy used is media engineering for which the optimal operating condition of a parameter is optimized by changing one parameter at a time and keeping the others at a constant level (Liu and Tzeng 2008).

The optimization studies do not consider the interaction effects among the variables as any process is influenced by several variables (Silva and Roberto 2012). Limitations of the single factor optimization can be eliminated by employing response surface methodology (RSM) which is used to explain the combined effects of all the factors in a fermentation process (Elibol 2014). Single variable optimization methods are not only tedious, but also can lead to misinterpretation of results, especially because the interaction effects between different factors are overlooked (Wenster-Botz 2013). Response surface methodology may be summarized as a collection of experimental strategies, mathematical methods and statistical inference for constructing and exploring an approximate functional relationship between a response variable and a set of design variables. So, in this study along with optimizing the individual factors we have examined the interactive effects between physico-chemical parameters using response surface methodology to enhance the enzyme production.

MATERIALS AND METHODS

Collection and Enrichment of Mushroom Compost

Mushroom compost samples were collected in sterile containers from different sites of Solan district of Himachal Pradesh. The collected compost samples were pooled together. To 50 g of compost sample in each of the petriplates, 1g of starch was added and water was sprinkled and mixed thoroughly to have adequate moisture. The petriplates containing above mixture were incubated at 50 ±2 °C for 3 days to enrich the samples.

Isolation of Alkalothermophilic Amylolytic Microorganisms

Isolations were done by serially diluting the samples and incubating at 50 ±2 °C for 24 h in starch agar medium (peptic digest of animal tissue-5.0 g, yeast extract-1.5 g, beef extract-1.5 g, starch soluble -2.0 g, Sodium chloride-5.0 g, agar-15.0 g, distilled water -1000 ml, pH -9.0 ±0.1). These pure line cultures were maintained on starch agar slants and preserved in refrigerator at 4 °C and subcultured once in a month.

Screening of Hyperamylolytic Alkalothermophilic Microorganisms

Isolated microorganisms were screened on the basis of their amylase production efficiency. Following qualitative and quantitative tests were used for the screening of the thermoalkalophillic amylolytic bacteria isolated from mushroom compost.

Qualitative Screening of Hyperamylolytic Alkalothermophilic Isolates

Starch hydrolysis test was performed on isolated strains to screen amylolytic strains (Shaw *et al*. 1995). The starch agar plates were spot inoculated with the isolated strains and were incubated at 50 °C for 72 h. The growth then obtained was flooded with 5-10 ml of iodine solution for about 20 min. Area of hydrolysis appeared as a clear zone among the blue stained unhydrolysed starch. The diameter of the clear zone formed indicated the extent of amylolytic activity of the strain.

Quantitative Screening of Hyperamylolytic Alkalothermophilic Isolates

Starch-iodine assay based on the chromogen formation by binding of iodine with starch is the basis of quantitative screening. Standard curve was prepared by using soluble starch as a standard (Xiao *et al*. 2006). One International Unit (IU) of amylase activity is defined as the disappearance of an average of 1μmol of iodine binding starch material per minute in the assay reaction.

Morphological and Physiological Characteristics of Alkalothermophilic Amylase Producing Isolate

Morphological, cultural and biochemical characterization of screened hyperamylolytic bacterial isolate was done by applying standard techniques for bacteria (Aneja 2003).

Genotypic Identification of Hyperamylolytic Bacterial Isolates Using 16S rRNA

Isolation of Genomic DNA (Genei DNA Isolation Kit)

The pure culture of selected bacterial isolate was inoculated in 10 ml of nutrient broth and grown at 50 ±2 ºC for 18 h. Isolation of total genomic DNA from the culture was carried out by following the standard procedure.

PCR Amplification of 16S rRNA Region

PCR amplification was done to confirm the identity of the bacterial strain, the small subunit 16S rRNA genes were amplified from the genomic DNA with 16SF (5'AGAGTTTGATCCTGGTCAG3') and 16SR (5'TACCTTGTTACGACTT3') primers to get an amplicon size of 1500 bp. Amplification was carried out in 20 µl reaction volume consisting of 10 X buffer, 2.0 µl; 2mM dNTPs, 2.0 µl; 3 U/µl Taq DNA polymerase, 0.2 µl; 100 ng/µl of each primer, 1 µl; template DNA, 1µl and sterilized distilled water 12.8 µl in a ASTEC thermalcycler using the PCR conditions 92 ºC for 1min (denaturation), 50 ºC for 1 min (annealing) and 72 ºC for 1 min (extension). The total number of cycles was 36 with the final extension at 72 ºC for 1 min.

The amplified product (20 µl) was size separated on 1% agarose gel prepared in 1% TAE buffer containing 0.5µg/ml ethidium bromide and photographed with the gel documentation system (alpha Imager 2200). A 100 bp DNA ladder (Genei) was used as molecular weight size markers. The PCR product (1500 bp) was purified from contaminating products by electroelution of the gel slice containing the excised, desired fragment with Qiaquick gel extraction kit (Qiagen, USR). The elution was carried out in 30 µl of nuclease free water.

Nucleotide Sequencing

The PCR amplicon obtained by amplifying PCR product was diluted in Tris buffer (10 mM, pH 8.5). Dilution used was 1:1000 in order to obtain the DNA concentration required for sequencing (30 ng/ µl, the sequencing required 8 µl DNA. The primer used in sequencing reaction was

BITS-1 (5'AGAGTTTGATCCTGG) and BITS-4 (5'-TACCTTGTTACGACTT) at a concentration of 3 µM. PCR products were got sequenced by the services provided by Bioserve, India. Pvt. Ltd. to confirm the results.

Blastn Analysis

Translated nucleotide sequence was then analyzed for similarities by BLASTN tool (www.ncbi.nlm.nih.gov:80/BLAST).

Optimization of Media Components and Process Parameters for Enhanced Amylase Production

Medium employed for amylase production was fortified with respect to certain physical and chemical conditions in order to get higher enzyme yields. For that purpose, the selected process parameters and medium components viz. substrate concentration, pH, temperature, inoculum size, Ca^{2+} and Mg^{2+} concentrations were standardized as below:

The levels of the significant parameters and their interaction effects between various medium components which influence amylase production significantly were analyzed and optimized by using Response Surface Methodology (RSM). The central composite design (CCD) with six factors at four levels was employed to investigate the first and higher-order main effects of each factor and interactions among them.

The design involved 8 centre points with an alpha value being ± 2. The five coded levels of alpha, studied in the present study were -2, -1, 0, +1 and +2. The statistical software package Design Expert® version 7.0 (Stat Ease, Inc, Minneapolis, USA) was used to generate polynomials and the contour plots. All experiments were carried out in triplicates.

Analysis of Variance (ANOVA)

A second-order polynomial equation was established based on analysis of variance and the optimum ratio of the medium components was found using the Design-Expert 7.0 software optimization toolbox. Standard deviation, PRESS, r^2 values were also analyzed.

Model Validation

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

Effect of Surfactants and Metal Ions on Amylase Production

The media thus optimized by using RSM was supplemented with surfactants such as Triton X 100, tween 20, tween 80, SDS, glycerol at a concentration of 5 mM and metal ions viz. Mn^{2+}, Fe^{2+}, Ni^{2+}, Co^{2+}, Ba^{2+}, Hg^{2+}, Zn^{2+} and Cu^{2+} at a concentration of 5mM and tested for amylase production by following the standard procedure.

RESULTS AND DISCUSSION

Isolation

In total, 13 bacterial isolates were obtained from pooled samples of compost in Starch Agar medium having pH 9.0 and temperature 50 °C. Among all, isolate GC6 (Fig. 1) showing 74.829 IU/ml of amylolytic activity was chosen for further studies based upon its hyperamylolytic and thermoalkalophilic nature.

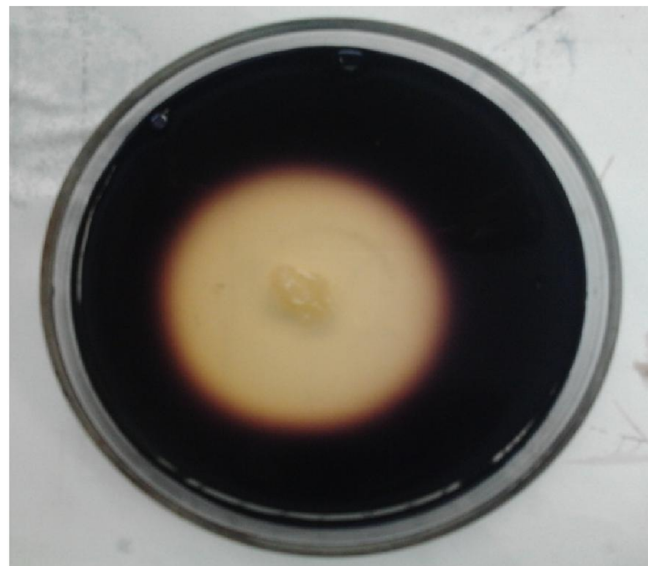

Fig.1. GC6 isolate showing hyperamylolytic activity

Identification of Screened Hyperamylolytic Bacterial Isolate

This strain was identified at genomic level by using 16S rRNA gene technique. Genomic DNA of isolate was extracted by using DNA purification kit (Banglore, Genei). The DNA was quantified by using standard protocol (Sambrook and Rusell 1989). The genomic DNA obtained from GC6 isolate had a concentration of 41 ng/ µl. The isolated genomic DNA was subjected to PCR to amplify small subunit of 16S rRNA using universal primers having product size of approximately, 1500 bp. The PCR product so obtained after amplification was purified and sequenced.

Nucleotide Sequencing

>0913_378_01_F1_27F-A01.ab1

TGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGA
TGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAAC
CTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGG
GCTAATACCGGATGCTTGATTGAACCGCATGGTTCAA
TTATAAAAGGTGGCTTTTAGCTACCACTTACAGATGG
ACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGC
TCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGG
TGATCGGCCACACTGGGACTGAGACACGGCCCAAAC
TCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAAT
GGACAAAAGTCTGACGGAGCAACGCCGCGTGAGTGA

Table 1. Experimental design for CCD of RSM

Substrate Conc. (%)	pH	Temperature (°C)	Inoculum size (%)	Calcium ions (%)	Magnesium ions (%)	Enzyme activity (IU/ml/min)
	3.00	7.00	30.00	2.50	0.01	1.00
38.648						
0.50	12.00	65.00	15.00	0.01	1.00	5.443
3.00	12.00	65.00	2.50	1.00	1.00	0.996
0.50	7.00	30.00	15.00	0.01	0.01	57.165
1.75	9.50	47.50	8.75	0.50	0.50	84.448
0.50	12.00	65.00	15.00	1.00	0.01	0.545
3.00	12.00	30.00	15.00	1.00	1.00	4.949
3.00	7.00	30.00	2.50	1.00	0.01	46.499
0.50	7.00	65.00	2.50	1.00	1.00	0.893
3.00	12.00	30.00	15.00	0.01	0.01	2.178
3.00	7.00	65.00	15.00	1.00	0.01	1.488
3.00	12.00	65.00	2.50	0.01	0.01	0.483
0.50	12.00	30.00	2.50	0.01	1.00	0.511
0.50	12.00	30.00	2.50	1.00	0.01	5.596
0.50	7.00	65.00	2.50	0.01	0.01	1.779
1.75	9.50	47.50	8.75	0.50	0.50	84.645
3.00	7.00	65.00	15.00	0.01	1.00	8.499
0.50	7.00	30.00	15.00	1.00	1.00	42.992
3.00	7.00	65.00	2.50	0.01	0.01	16.601
0.50	12.00	65.00	2.50	1.00	1.00	10.511
3.00	7.00	65.00	2.50	1.00	1.00	1.488
0.50	12.00	30.00	15.00	1.00	1.00	4.996
3.00	7.00	30.00	15.00	0.01	0.01	56.367
0.50	7.00	30.00	2.50	0.01	1.00	51.065
0.50	7.00	30.00	2.50	1.00	0.01	52.703
3.00	12.00	30.00	2.50	1.00	0.01	14.58
3.00	12.00	65.00	15.00	1.00	0.01	10.22
0.50	12.00	65.00	2.50	0.01	0.01	14.068
1.75	9.50	47.50	8.75	0.50	0.50	83.399
1.75	9.50	47.50	8.75	0.50	0.50	84.211
0.50	12.00	30.00	15.00	0.01	0.01	11.22
3.00	12.00	30.00	2.50	0.01	1.00	7.699
3.00	12.00	65.00	15.00	0.01	1.00	17.499
0.50	7.00	65.00	15.00	0.01	1.00	11.553
3.00	7.00	30.00	15.00	1.00	1.00	59.448
0.50	7.00	65.00	15.00	1.00	0.01	1.089
0.50	12.00	30.00	2.50	0.01	0.01	3.998
0.50	7.00	65.00	2.50	1.00	0.01	0
0.50	12.00	30.00	2.50	1.00	1.00	1.72
1.75	9.50	47.50	8.75	0.50	0.50	83.499
3.00	12.00	30.00	15.00	0.01	1.00	2.25
0.50	12.00	65.00	15.00	1.00	1.00	1.499
3.00	7.00	65.00	15.00	1.00	1.00	1.112
3.00	12.00	65.00	2.50	1.00	0.01	6.899
0.50	7.00	30.00	15.00	1.00	0.01	41.942
3.00	12.00	65.00	2.50	0.01	1.00	7.559
0.50	7.00	65.00	2.50	0.01	1.00	1.399
3.00	7.00	30.00	2.50	0.01	0.01	49.409
3.00	7.00	30.00	2.50	1.00	1.00	45.288
3.00	7.00	65.00	15.00	0.01	0.01	7.58
0.50	7.00	30.00	15.00	0.01	1.00	51.719
1.75	9.50	47.50	8.75	0.50	0.50	83.396
3.00	12.00	30.00	15.00	1.00	0.01	1.477
0.50	12.00	65.00	15.00	0.01	0.01	0.499
3.00	12.00	65.00	15.00	1.00	1.00	0.0499
3.00	12.00	65.00	15.00	0.01	0.01	0.131
3.00	12.00	30.00	2.50	0.01	0.01	4.94
0.50	12.00	30.00	15.00	0.01	1.00	0
3.00	7.00	30.00	15.00	0.01	1.00	42.446
1.75	9.50	47.50	8.75	0.50	0.50	82.02
3.00	7.00	65.00	2.50	1.00	0.01	0.499
0.50	7.00	30.00	2.50	1.00	1.00	39.711
0.50	7.00	30.00	2.50	0.01	0.01	46.259
0.50	12.00	65.00	2.50	0.01	1.00	1.106
3.00	7.00	65.00	2.50	0.01	1.00	0.499
0.50	12.00	30.00	15.00	1.00	0.01	1.616
1.75	9.50	47.50	8.75	0.50	0.50	65.616
0.50	7.00	65.00	15.00	1.00	1.00	1.5811
3.00	12.00	30.00	2.50	1.00	1.00	1.976
0.50	12.00	65.00	2.50	1.00	0.01	0.284
0.50	7.00	65.00	15.00	0.01	0.01	1.443
3.00	7.00	30.00	15.00	1.00	0.01	45.72

Continue……………..

0.50	12.00	65.00	2.50	1.00	0.01	0.284
0.50	7.00	65.00	15.00	0.01	0.01	1.443
3.00	7.00	30.00	15.00	1.00	0.01	45.72
1.75	9.50	47.50	8.75	-0.90	0.50	47.479
1.75	9.50	47.50	8.75	0.50	0.50	83.499
1.75	9.50	97.00	8.75	0.50	0.50	0
1.75	9.50	47.50	8.75	0.50	0.50	83.299
1.75	16.57	47.50	8.75	0.50	0.50	0
1.75	9.50	47.50	8.75	0.50	1.91	34.57
1.75	9.50	47.50	8.75	0.50	0.50	84.599
-1.79	9.50	47.50	8.75	0.50	0.50	25.425
1.75	2.43	47.50	8.75	0.50	0.50	0
1.75	9.50	47.50	8.75	0.50	0.50	82.499
1.75	9.50	47.50	8.75	0.50	0.50	84.565
1.75	9.50	-2.00	8.75	0.50	0.50	12.234
1.75	9.50	47.50	26.43	0.50	0.50	0
1.75	9.50	47.50	-8.93	0.50	0.50	0
1.75	9.50	47.50	8.75	0.50	0.50	84.464
1.75	9.50	47.50	8.75	0.50	-0.90	44.979
5.29	9.50	47.50	8.75	0.50	0.50	64.468
1.75	9.50	47.50	8.75	1.91	0.50	32.191

TGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGA
AGAACAAGTACCGTTCGAATAGGGCGGTACCTTGAC
GGTACCTAACCAAAAACCCACGGCTAACTACGTGCC
ACCAGCCGCGGTAAGACGTAGGTGGCAAGCGTTGTC
CGGAATTATGGGGCGTAAAGCGCGCGCAGGCGGTTT
CTTAACTCTGATGTGAAAGCCCGCGGCTCAAC

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GGCTCCAAAGGTTACCTCACCGACTTCGGGTGTTACA
AACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCC
CGGGAACGTATTCACCGCGGCATGCTGATCCGCGATT
ACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGAC
TGCGATCCGAACTGAGAACAGATTTGTGGGATTGGC
TTAGCCTCGCGGCTTCGCTGCCCTTTGTTCTGCCCATT
GTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGA
TGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCAC
CGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGC
AACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTA
ACCCAACATCTCACGACACGAGCTGACGACAACCAT
GCACCACCTGTCACTCTGCCCCCGAAGGGGAAGCCC
TATCTCTAGGGTTGTCAGAGGATGTCAAGACCTGGTA
AGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTC
CACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTT
TCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTA
ATGCGTTTGCTGCAGCACTAAAGGGCGGAAACCCTC
TAACACTTAGCACTCATCGTTTACGGCGTGGACTACC
AGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCG
CCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGC
CACTGGTGTTCCTCCACATCTCTACGCATTTCACCGC
TACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAG
TTCCCCAGTTTCCAATGACCCTCCCCGGGTTGAGCCG
GGGGCTTTCACATCAGACTTAAGAAAACCGCCTGGC
GCGCGCTTTACGCCCAATAAT

The isolate GC6 was identified as *Bacillus aerius* using BLASTN analysis and has been registered under the accession number |KJ775810| in National Centre for Biotechnology Information (NCBI). This strain has been reported for the very first time to be an amylase producer and produces very high IU/ml of amylase. The newly isolated strain offers interesting hydrolytic properties, being active upto pH 10. Besides being alkalophilic, the most striking feature of the strain is its thermostability. The significant thermostability of the isolate makes it eligible for use in industrial applications which are being carried out at very high temperatures.

Optimization of process parameters for enhanced amylase production

In order to explore the maximum potential of *Bacillus aerius* GC6 to synthesize amylase production for biotechnological applications, determination of cumulative effect of multiple parameters regulating the rate of enzyme production have become warranted. For optimization of process parameters, RSM is more satisfactory than classical methods such as one-variable-at-a-time (OVAT) or mathematical models because it can study many variables simultaneously with low number of observations, saving time and costs (Bezerra *et al*. 2008; Box *et al*., 1978). Response surface methodology may be summarized as a collection of statistical tools and techniques for constructing and exploring an appropriate functional relationship between a response variable and a set of design variables. Central composite design (CCD) is one of the response surface methodologies usually utilized to obtain data that fits a full second-order polynomial equation.

Response surface methodology

Central composite design (CCD) of RSM was employed to determine the interactions among the significant media components and process parameters and also to determine their optimal levels. The full experiment plan as per the experimental design along with the experimental and predicted values is given in Table 1. From multiple regression analysis we found that the second order polynomial equation can explain amylase production regardless of the significance of coefficients. If Y is the response value than the fitted response surface model is:

$$\text{Enzyme Activity (Y)} = 77.08+1.96 * A-9.64 * B-9.72 * C+0.14 * D-1.14 * E-0.97 * F-7.95 *A^2-11.98 * B^2-11.98 * C^2-11.98 * D^2-7.00 * E^2-7.01 * F^2+0.34 * A * B+0.11* A * C+0.090 * A * D+0.21 * A * E +0.014 * A * F+10.78* B * C -0.25* B * D+0.32 * B * E+0.50 * B * F-0.17 * C * D-0.44 * C * E+1.10 * C * F0.73*D*E+1.11*D*F+0.41*E*F$$

where Y is enzyme activity (IU/ ml), A is substrate concentration, B is pH, C is temperature (^{0}C), D is inoculum size (%), E is Ca^{2+} concentration (%) and F is Mg^{2+} concentration (%). By optimizing the above parameters by using RSM, maximum enzyme activity obtained was 84.645 IU/ml at 47.5 °C temperature and 9.5 pH @ 8.75% inoculum using 1.75% substrate with 0.5% Ca^{2+} and Mg^{2+} concentration. The coefficient of determination (R^2) was calculated as 0.9311 for enhanced amylase production, indicating that the statistical model can explain 93.11% of variability in the response. The R^2 value is always between 0 and 1. The closer the R^2 is to 1.0, the stronger the model and the better it predicts the response (Haaland 1989). An adequate precision of 21.646 for amylase production was recorded. The predicted R^2 of 0.7508 is in reasonable agreement with the adjusted R^2 of 0.8991. This indicated good agreement between the experimental and predicted values for amylase production. The adjusted R^2 corrects the R^2 value for the sample size and for the number of terms in the model. If there are many terms in the model and the sample size is not very large, the adjusted R^2 may be noticeably smaller than the R^2.The model F-value of 29.04 and values of prob > F (<0.05) indicated that the model terms are significant. For amylase production B, C, A^2, AE and DE are significant model terms. Values of "Prob > F" less than 0.0500 indicate model terms are significant. The coefficient of variation (CV) indicates the degree of precision with which the treatments were compared. Usually the higher value of CV(4.089) indicated a better precision and reliability of the experiment (Gangadharan *et al*. 2008).

The application of statistical design CCD of Response Surface Methodology for optimization of process parameters and medium components for an enhanced amylase production allows the quick identification of the important and significant factors and interactions among them. The major objective of RSM is to determine the optimum region of the factor space in which operating specifications are satisfied. An over all increase of 13.11% (Fig. 2) has been observed in amylase production after the factors were optimized by using RSM. Similarly, a significant increase in the enzyme production have been reported by Gangadharan *et al.,* (2008); Kiran and Chandra (2008) and Tanyildizi *et al*., (2005).

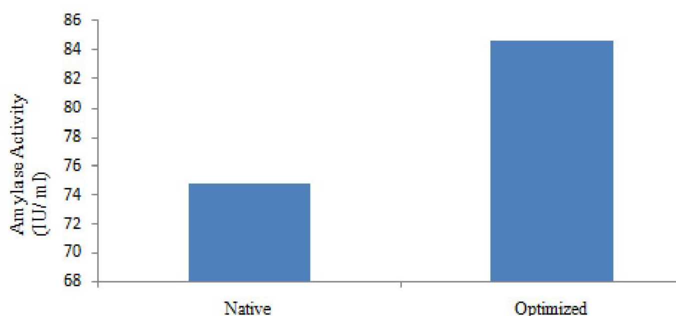

Fig.2. Amylase activity of *Bacillus aerius* GC6 before and after optimization

Among the variables screened, substrate concentration, incubation time, temperature and Ca^{2+} concentration were identified as most significant variables influencing α-amylase production.

The interaction effects and optimal levels of the variables were determined by plotting the response surface curves. The response surface curves are represented in fig.3(a-d). Fig 3a represents the interaction betweeen pH and temperature. The shape of the contour graph shows a positive interaction between the two variables. The enzyme yield was found to increase with simultaneous increase in both the factors. The response surface curve of inoculum size and substrate concentration is represented in fig. 3b. Lower and higher values of both the variables did not result in higher enzyme yields. The shape of the response surface curve showed a moderate interaction between these tested variables. Fig. 3c and 3d represent the interaction of the inoculum size with temperature and pH respectively, which showed a positive interaction among themselves.

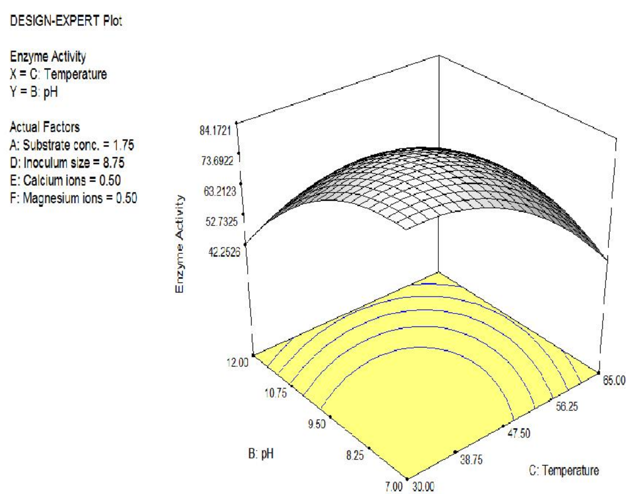

Fig. 3a Response surface curve representing interaction betweeen pH and temperature

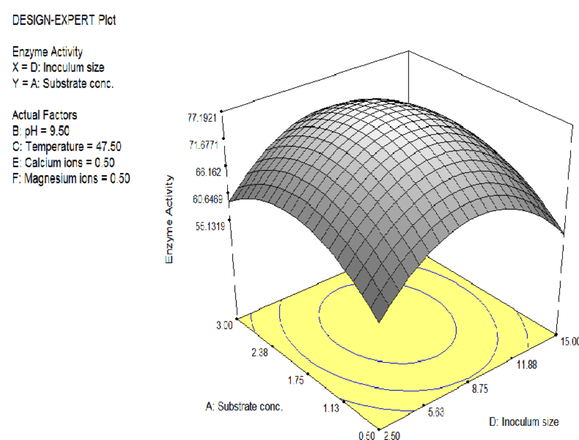

Fig. 3b Response surface curve representing inoculum size and substrate concentration

The supplementation of medium with metal ions has been reported to provide good growth and also influence higher enzyme production (Sivaramakrishnan *et al*. 2006). Most of the α- amylases are metalloenzymes and in most cases Ca^{2+} ions are required for maintaining the spatial conformation of the enzyme and increase its thermal stability, thus play an important role in enzyme stability (Bano *et al*. 2009).

It proves the significant effect of these vital components for higher synthesis of amylase thus, rendering them an important constituents of production medium.

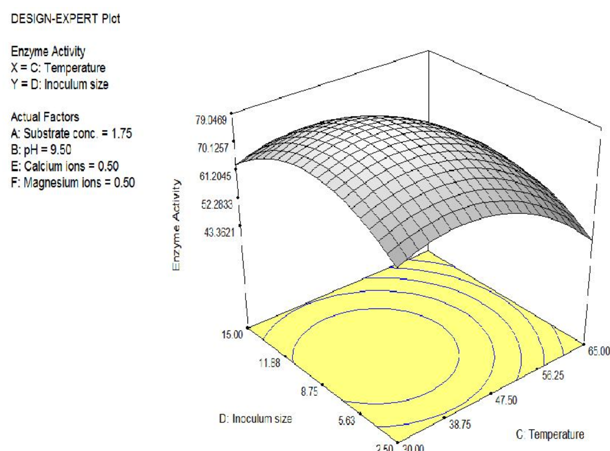

Fig. 3c Response surface curve representing inoculum size and temperature

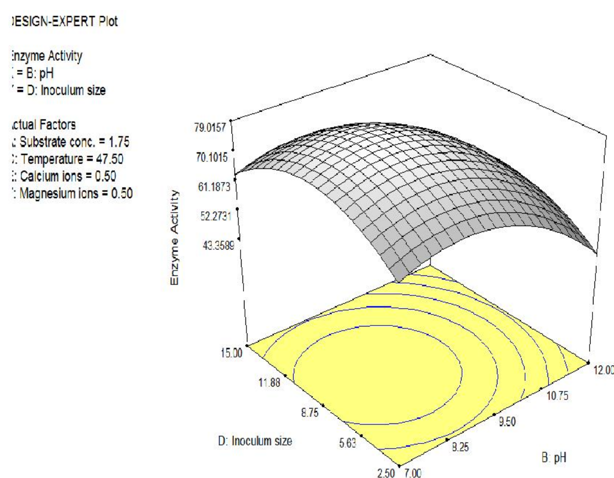

Fig. 3d Response surface curve representing inoculum size and pH

Effect of surfactants and metal ions on amylase production

Medium supplementation with surfactants showed retention of 90% of enzyme activity with SDS, and almost similar results were observed with other medium additives viz. tween 20, tween 80 and triton X 100, making it a potential candidate for use in detergent industry. Being a metalloenzyme, medium supplemented with metal divalent ions stabilized the enzyme. However, a significant decrease in the enzyme activity was observed with Co^{2+}, Zn^{2+}, Ba^{2+}, Hg^{2+} and Cu^{2+}. The inactivation by these metals may be due to their binding to catalytic residues in the active site of the enzyme.

Conclusion

The statistical approach RSM showed significant results for optimizing the process parameters for maximal amylase production under SmF from a newly isolated hyperamylase producing thermoalkalophilic strain *Bacillus aerius* GC6 |KJ775810| isolated from mushroom compost and allowed rapid screening of large number of variables simultaneously. The present study identified the effects of various process parameters on the enzyme yield and the production was found to be significantly influenced by concentrations of substrate, inoculum, $CaCl_2$ and $MgSO_4$. This is the first report on surfactant and SDS stable amylase production from *Bacillus aerius* GC6. The significant thermostability of the isolate makes it eligible for use in industrial applications which are being carried out at very high temperatures.

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