Research Paper

Production and Optimization of α -Amylase from a Novel Thermoalkalophilic *Bacillus sonorensis* GV2 Isolated from Mushroom Compost

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Amylase producing thermophilic, alkali-tolerant bacterial strain was isolated from mushroom compost and identified as *Bacillus sonorensis* GV2 using 16S rRNA gene technique and deposited in NCBI gene bank vide accession number [KJ775811]. Medium components and process parameters for an enhanced amylase production were optimized with respect to media types, substrate concentration, pH, temperature, inoculum size, divalent ion concentrations, carbon and nitrogen sources, surfactants etc. through one variable at a time (OVAT) approach followed by a statistical approach under submerged fermentation from this isolate. Optimum levels of six significant parameters obtained after OVAT and their interaction effects were determined employing the response surface central composite design (CCD). A maximum amylase activity of 82.78 IU had been obtained at 50°C and 9.5 pH @ 8.75% inoculum using 0.5% substrate with 0.5% Ca²⁺ and Mg²⁺ concentrations. An overall increase of 21.30% has been observed after the factors were optimized by using Response Surface Methodology.

Keywords: Amylase; Submerged Fermentation; RSM; *Bacillus sonorensis*; Starch Hydrolysis

Introduction

Extremophilic microorganisms are adapted to survive in ecological niches such as at high temperatures, extremes of pH, high salt concentrations and high pressure. These microorganisms produce unique biocatalysts that function under extreme conditions comparable to those prevailing in various industrial processes. Extremozymes have a great economic potential in many industrial processes, including agricultural, chemical and pharmaceutical applications. Consequently, unique properties of these biocatalysts have resulted in several novel applications of enzymes in industrial processes such as protease, amylase etc.

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 basic mechanism 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).

Driven by increasing industrial demands for biocatalysts that can cope with industrial process

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conditions, considerable efforts have been devoted to the search for such enzymes. However, it still remains 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, 2006). The main strategy used is media engineering for which the optimal operating condition of a parameter is standardized by changing one parameter at a time and keeping the others at a constant level (Liu and Tzeng, 1998). The optimization studies do not consider the interaction effects among the variables, as any process is influenced by several variables (Silva and Roberto, 2006). 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 conventional fermentation process (Elibol, 2004). 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. In this study, α -amylase production from *Bacillus sonorensis* GV2 has been studied. Optimization of the individual factors has been conducted along with their interactive effects between physico-chemical

parameters using response surface methodology to enhance the enzyme production.

Materials and Methods

Collection and Isolation of Alkalothermophilic Amyolytic Microorganisms

Mushroom compost samples were collected in sterile containers from different sites of Solan District of Himachal Pradesh. 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 the above mixture were incubated at $50 \pm 2^\circ\text{C}$ for 3 days to enrich the compost samples. Isolations were done by applying the enrichment technique. Enriched compost samples were serially diluted and incubated at $50 \pm 2^\circ\text{C}$ for 24h 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). Pure line cultures obtained were maintained on starch agar slants and preserved in the 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 thermoalkalophilic amylogenic bacteria isolated from mushroom compost.

Qualitative Screening of Hyperamylolytic Alkalothermophilic Isolates

Starch hydrolysis test was performed on isolated strains to screen amyolytic strains (Shaw *et al.*, 1995). The starch agar plates were spot inoculated with the isolated strains and incubated at 50°C for 72 h. The growth thus obtained was flooded with 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 a clear zone formed indicated the extent of amyolytic activity of the strain.

Quantitative Screening of Hyperamylolytic Alkalothermophilic Amylase Producing Isolates

Isolated bacterial strains were grown in 100 ml of starch broth at 50±2°C for 24 h. Standard enzyme production medium (Kwan *et al.*, 1993), -which comprised of Soluble starch- 4.0 g, (NH₄)₂HPO₄-0.5 g, Yeast extract- 0.5 g, Sodium citrate- 0.2 g, MgSO₄·7H₂O-0.05 g, CaCl₂·2H₂O- 0.008 g, Distilled water- 100 mL at pH- 9.0± 0.2 in 250 ml of Erlenmeyer flask was used for extracellular enzyme production. An uninoculated culture medium was kept as a control. The inoculated and uninoculated enzyme production media were then incubated for 3 days at 50±2°C at 120 rpm. After incubation, the culture contents were centrifuged at 12,000 rpm for 20 min (4°C). The supernatant thus obtained was used as crude enzyme for quantifying extracellular amylolytic activity.

Amylase activity was determined by measuring the amount of hydrolyzed starch using the method of Xiao *et al.*, (2006). 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. The enzyme solution with 0.2% starch was incubated at 37°C for 30 min. The reaction was then stopped by addition of 1N acetic acid. Iodine solution was used as a chromogen and the preparation was read spectrophotometrically at 580 nm.

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 Isolates

Morphological, cultural and metabolic fingerprinting of screened hyperamylolytic bacterial isolates was done by applying standard techniques for bacteria (Aneja, 2003).

Genotypic Identification of Hyperamylolytic Bacterial Isolates using 16S rRNA

Bacterial isolate GV2 giving highest titres of amylase

was selected for further studies. It was identified by using 16S rRNA technique.

Isolation of Genomic DNA (Genei DNA Isolation Kit)

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

PCR Amplification of 16S rRNA Region

PCR amplification was done to confirm the identity of bacterial strain, small subunit 16S rRNA genes were amplified from the genomic DNA with 16SF (5'AGAGTTTGATCCTGGCTCAG3') 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 thermocycler, using the PCR conditions 92°C for 1 min (denaturation), 50°C for 1 min (annealing) and 72°C for 1 min (extension). The total number of cycles was 36 with the final extension at 72°C 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 electro-elution of the gel slice containing the excised, desired fragment with Qiaquick gel extraction kit (Qiagen, USA). 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'AGAGTTTGAT

CCTGG) and BITS-4 (5'-TACCTTGTTACGACTT) at a concentration of 3 μ M.

PCR products were further sequenced by the services provided by Bioserve, India. Pvt. Ltd. to confirm the results.

BLASTN Analysis

All the sequences were compared with 16S rRNA gene sequences available in the GenBank databases by BLASTn search (www.ncbi.nlm.nih.gov:80/BLAST). Multiple sequence alignments of partial 16S rRNA gene sequences were aligned using CLUSTAL Omega, version 1.8. Tree generated was analyzed with the TREEVIEW program. Sequences obtained were submitted to NCBI for accession number.

Optimization of Media Components and Process Parameters for Enhanced Enzyme Production

Amylase production from selected strain GV2 was optimized first using conventional One Variable At a Time (OVAT) approach by varying certain media components and process parameters one by one. Significant variables thus obtained were chosen for further optimization studies by using a statistical approach Central Composite Design (CCD) of Response Surface Methodology (RSM).

Effect of Media, Inoculum Size and Incubation Period

Various inoculum sizes ranging from 2.5%, 5.0%..., 15% were tested to enhance amylase production on different media viz. Babu and Satyanarayana, (1993), Kwan *et al.* (1993), Starch Agar medium (Saxena *et al.*, 2007), Modified starch medium (Jyoti *et al.*, 2011), Modified medium (Fooladi and Sajjadian, 2010) for an incubation period ranging from 24, 48..., 144h.

Effect of Temperature and pH on Enzyme Production

The effect of different temperature (30, 40..., 70°C) and pH (7.0, 8.0..., 12.0) on amylase production was studied by subjecting the organism to variable temperatures and pH for enzyme production and

observing enzyme production potential of the organism at particular temperature and pH.

Effect of Carbon Sources and Their Concentration

Various carbon sources viz. starch, mannose, arabinose, dextrose, maltose and sucrose were used for exploring their effect on amylase production at a concentration ranging from 0.5% to 3.0%.

Effect of Nitrogen Sources, Divalent Ions and Media Additives

Various nitrogen sources viz. gelatin, tryptone, $(\text{NH}_4)_2\text{SO}_4$, NH_4Cl , NH_4NO_3 and NaNO_3 at a concentration of 0.1%, divalent ions viz. Ca^{2+} , Mg^{2+} , Cu^{2+} , Ba^{2+} , Fe^{2+} , Mn^{2+} , Co^{2+} , Zn^{2+} , Ni^{2+} at a concentration of 5mM and media additives viz. SDS, EDTA, CTAB, Tween 20, Tween 80, Triton X 100 and glycerol at a concentration of 5mM were tested for their effects on amylase production.

Optimization by Using Central Composite Design (CCD) of RSM

Using the One Variable At a Time (OVAT) approach, the effects of media types, incubation time, temperature, pH, inoculum size, carbon sources, substrate concentration, nitrogen sources, media additives and divalent ions etc. were studied. Based on these optimization results, six independent variables were chosen for further optimization studies by employing Central Composite Design (CCD) of Response Surface Methodology (RSM). The dependent variable was the enzyme activity, and, the independent variables chosen were substrate concentration (X_1), pH (X_2), temperature (X_3), inoculum size (X_4), Ca^{2+} concentration (X_5) and Mg^{2+} concentration (X_6). Central Composite Design (CCD) with six factors at five levels was employed to investigate the first and higher order main effects of each factor and the interactions among them. The experiment divided into 5 blocks contained 90 runs. The design involved 14 centre points with an alpha value being 2.82843. The minimum and maximum ranges of independent variables are given in Table 1. Experimental design included 90 flasks with three replicates at their central coded values. Mathematical

Table 1. Range of values for independent variables used in Central Composite Design (CCD) of the RSM

Independent variables	Units	Low	High
Substrate concentration (X_1)	%	0.5	3.0
pH (X_2)	-	7	12
Temperature (X_3)	°C	30	65
Inoculum size (X_4)	%	2.5	15
Ca ²⁺ concentration (X_5)	%	0.2	1.0
Mg ²⁺ concentration (X_6)	%	0.2	1.0

relationship of response (enzyme production, protein, specific activity) and variables i.e. X_1 , X_2 , X_3 , X_4 , X_5 and X_6 was approximated by a quadratic model equation. The response value in each case is the average of triplicate experiments. 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.

Statistical Analysis

Statistical software package Design Expert® version 7.0 (Stat Ease, Inc, Minneapolis, USA) was used for regression analysis of the experimental data to obtain working parameters and to generate polynomials and the contour plots. A second-order polynomial equation was established based on analysis of variance and the optimum ratio of medium components was found using Design-Expert 7.1 software optimization toolbox. Standard deviation, PRESS, R^2 values were also analyzed.

Analysis of Variance (ANOVA)

A second-order polynomial equation was established based on analysis of variance and the optimum ratio of 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. Experimentally obtained data were compared with the predicted one and the prediction error was calculated.

Results and Discussion

Isolation and Identification of Screened Hyperamylolytic Bacterial Isolate

In total, 13 bacterial isolates were obtained from pooled samples of compost in Starch Agar medium, having pH 9.0 and temperature 50°C. All the isolates were preliminarily identified by conducting their morphological and metabolic fingerprinting studies (Table 2) and tentatively identified using ABIS-online software (http://www.tgw1916.net/bacteria_abis.html). On the basis of similarity, the isolates were 70% homologous to *Bacillus* sp.

Highest amylase activity after qualitative starch-agar zone assay and quantitative starch-iodine assay was observed in GV2 i.e. 68.24 IU while the least amylase activity was that of GC4 i.e. 7.37 IU. As it is clear from Fig. 1, strain GV2 is entirely different from the cluster showing the amylogenic potential of all the screened isolates. Thus, among all the screened amylolytic isolates, isolate GV2 (Fig. 1) exhibiting highest amylolytic activity of 68.24 IU, was chosen for further studies based on its hyperamylolytic and thermoalkalophilic nature. 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). DNA was quantified by using the standard protocol (Sambrook and Russell, 1989). The genomic DNA obtained from GV2 isolate

Fig. 1: Dendrogram showing the similarity and differences among the strains in terms of their amylogenic potential

Table 2. Biochemical and metabolic characteristics of screened alkalothermophilic amylolytic bacteria isolated from mushroom compost

Characteristics	Screened Amylolytic Isolates												
	GC1	GC2	GC3	GC4	GC5	GC6	GC7	GC8	GV1	GV2	GV3	GV4	GV5
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate utilization	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	+	-	-	+	-	-	-	+	-	+	-	-	-
H ₂ S	+	-	+	+	-	+	-	-	+	-	+	+	-
Methyl Red	-	-	+	+	+	+	-	+	+	-	-	+	+
Voges Proskeur	-	-	-	-	-	-	-	-	-	-	-	-	-
DNase activity	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	-	-	-	-	+	+	-	+	-	+	+	+
Cellulolytic	-	-	-	-	-	-	-	-	-	-	-	-	-
Sugar fermentation													
• Glycerol	A	A	A	AG	A	A	AG	A	A	AG	A	AG	A
• Sorbitol	-	-	AG	-	A	AG	-	-	-	A	-	-	-
• Dextrose	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
• Galactose	AG	A	A	A	AG	A	A	AG	A	A	AG	A	A
• Xylose	A	-	-	A	-	-	-	-	A	-	-	-	-
• Mannose	A	A	A	A	AG	A	A	AG	A	A	AG	A	A
• Sucrose	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
• Arabinose	-	-	A	-	A	-	AG	-	-	AG	A	-	-
• Lactose	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
• Raffinose	A	A	A	A	A	AG	A	A	A	A	A	A	A
• Inulin	A	A	A	A	-	-	-	-	-	-	A	-	A
• Maltose	AG	AG	AG	AG	A	A	AG	AG	AG	A	AG	AG	AG
• Sucrose	A	A	A	A	AG	-	A	-	A	-	-	-	-
Tentative identification	<i>Bacillus</i> sp.	<i>Bacillus</i> sp.	<i>Bacillus</i> sp.	<i>Bacillus</i> sp.	<i>Bacillus</i> sp.	<i>Bacillus</i> sp.	<i>Bacillus</i> sp.	<i>Bacillus</i> sp.	<i>Bacillus</i> sp.	<i>Bacillus</i> sp.	<i>Bacillus</i> sp.	<i>Bacillus</i> sp.	<i>Bacillus</i> sp.

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

>0114_305_001_1_27F-C07.ab1

TAAGACTGGGATAACTCCGGGAAACCGGGACTAATA
CCGGATGCTTGATTGAACGCATGGTTCAATTCTAAAA
GGTGGCTTTTAACCTTCACTTACAGATGGACCCGCGG
CGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAA
G GCGACGATGCGTAGCCGACCTGAGGAGGGTGA
TCGGCCACACTGGGACTGAGACACGGCAACAAACT
CATACGGGAGGCAGCTGTAGGGAACTTCCGCAATG

GACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGA
TGAAGGTTTTTCGGATCGTAAACTCTGTTGTTAGGG
AAGAACAAGTTCCGTTTCGAATAGGGCGGTACCTTGACG
GTTCTTAACCAGAAAGCCACGGCTAACACAGTGCCA
GCAGCCGCGGTCATACGTATGTGGCAAGCGTTGTCCGGTA
TTATTGGGCGTAAAGCGCGCGCAGGCTGTTTCTTTAGTC
TGATGTGAAAGCCCCGGCTCAAGCGGGGAGGGTCA
TTGG

>0114_305_002_1_1492R-D07.ab1

TGTCAGAGGATGTCAAGACCTGGTAA
GGTTCTTCGCGTTGCTTCGAATTAAA
CCACATGCTCGACCGCATGTGCGGGCCC
CCGTTTATTCTTTGAGTTTCAAACCTT
GCCACCGTACTCGGGGAGGCGGAGT
GCTTAATGCGTTTGCTGCAGCGCTAAA

GAGGGGGGAAACGCTCTAACACTTAG
CACTCATCGTTTACAGCGTGGACTACGAA
GGTATCTAATCCTGTTCACTCCCCACGCTTT
CGCGCCTCAGGGGAGTTAAAGACC
AGAGAGTCGCCTTGGCTACTGGCG
TTGGTCCACATCTCTA

The isolate GV2 was identified as *Bacillus sonorensis* using BLASTN analysis and has been registered under the accession number |KJ775811| in National Centre for Biotechnology Information (NCBI). Phylogenetic tree of *Bacillus sonorensis* GV2, with respect to other *Bacillus* sp. as inferred by neighbor joining method which has been shown in Fig. 2.

Optimization of Process Parameters for Enhanced Amylase Production by *Bacillus sonorensis* GV2 by Using one Variable at a Time (OVAT) Approach

In order to explore the maximum potential of *Bacillus sonorensis* GV2 to synthesize amylase for biotechnological applications, determination of cumulative effect of multiple parameters regulating the rate of enzyme production have become warranted.

Effect of Media

Bacillus sonorensis GV2 showed maximum growth in Starch Agar medium containing Soluble starch- 1.0

Fig. 2: Neighbour joining tree based on 16S rRNA gene sequence showing the phylogenetic relationship of isolate GV2 with the analysed sequences

g, Yeast extract- 0.4 g, KH_2PO_4 -0.1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -0.15 g, dH_2O -1000 ml, pH- 9.0. Enhanced enzyme production using defined medium may be due to the presence of nitrogen, carbohydrate and other compounds in appropriate concentration, as per the physiological requirement of the organism that would have resulted in enhanced ability of the cells to synthesize most of the extra cellular amylase (Barsar *et al.*, 2010).

Effect of Incubation Period

Enzyme activity was measured at regular intervals from 24h to a period of 144h and maximum amylase production was noticed at 72nd h i.e. 68.36 IU, with a specific activity of 3.27 $\mu\text{mol}/\text{min}/\text{mg}$ (Fig. 3A). Similar findings have been reported by Thippeswamy *et al.* (2006) and Vidyalakshmi *et al.* (2009). After 72h, there was a decrease in the amylolytic activity. A decrease was observed due to depletion of nutrients available causing a stressed microbial physiology resulting in an inactivation of enzyme (Flores *et al.*, 1997). Another reason could be the catabolite repression i.e. the increase in production of reducing sugars, which after a certain period of growth could exhibit inhibitory effect on enzyme production, since α -amylase is an inducible enzyme (Kiran and Chandra, 2008; Premila, 2013).

Effect of Different Carbon Sources and Substrate Concentration

Carbon source in the form of either monosaccharides or polysaccharides influence the production of amylase. *Bacillus sonorensis* GV2 exhibited highest amylase activity of 69.13 IU (Fig. 3B) and specific activity of 3.27 $\mu\text{mol}/\text{min}/\text{mg}$ with supplementation of starch among all other carbon sources used for enzyme production. Starch has also been found to increase enzyme production in *B. subtilis* IMG22, *Bacillus* sp. PS-7 and *Bacillus* sp. I-3 as reported by Tanyildizi and Elibol, (2005); Sodhi *et al.* (2005) and Goyal *et al.* (2005) respectively. The α -amylase production is also subjected to catabolite repression by glucose and other sugars, like most other inducible enzymes. Synthesis of amylases in most species of the genus *Bacillus* is repressed by readily metabolizable substrates such as glucose and fructose

as reported by Haseltine *et al.* (1996); Lin *et al.* (2007); Kiran and Chandra, (2008); Premila, (2013). A reduced enzyme activity of 50.57 IU is in consonance with these reports.

Varying concentrations of the best yielding carbon source as substrate ranging from 0.5 to 3.0% were explored for an increase in the enzyme yield. At a concentration of 0.5% starch yielded a maximum enzyme activity of 70.12 IU with 3.31 $\mu\text{mol}/\text{min}/\text{mg}$ specific activity. There were statistically significant variations among the different substrate concentrations used for amylase production ranging from 0.5-3.0%. Optimum substrate concentration normally results in an increase in the yield and reaction rate of hydrolysis (Regina *et al.*, 2008). However, high substrate concentration can cause substrate inhibition, which substantially lowers enzyme

production (Liu and Yang, 2007 and Singhania *et al.*, 2007).

Effect of Inoculum Size

Different inoculum sizes studied for enhanced enzyme production were: 2.5, 5.0..., 15.0 % (v/v). Highest amylase production from *B. sonorensis* GV2 i.e. 71.82 IU was obtained at 7.5% of inoculum size, having statistically significant differences over the range of variable inoculum sizes. Specific activity of 3.40 $\mu\text{mol}/\text{min}/\text{mg}$ was observed (Fig. 3C). There was a steady increase in the enzyme production from 2.5 to 7.5%. However there was a steep decrease in the enzyme activity beyond this inoculum size because of an overload of the cells in the production medium resulting in nutrient starvation. Maximal enzyme activity at optimal level was observed because at this point, there

Fig. 3: Effect of (A) Incubation period (days), (B) Inoculum size (%), (C) pH, (D) Temperature (°C) on amylase production from *Bacillus sonorensis* (GV2)

is equilibrium between the number of cells and availability of nutrients for growth and substrate to act upon, while the decline in enzyme yield at larger inoculum size might be due to formation of thick suspensions and improper mixing of substrates in shake flasks.

Effect of pH

The pH of the growth medium plays an important role in terms of inducing enzyme production and morphological changes in the microbes (Pederson and Nielson, 2000; Kathiresan and Manivannan, 2006). The highest amylase titres 71.89 IU were observed at alkaline pH 10.0 with specific activity 3.41 $\mu\text{mol}/\text{min}/\text{mg}$ (Fig. 3D). Similar results have been reported by Haq *et al.* (2002); Mukherjee *et al.* (2009); Erdal and Taskin, (2010) and Pathak and Narula, (2013).

Effect of Temperature

Incubation temperature is the most important physical factor which affects enzyme production. *B. sonorensis* GV2 exhibited maximum amylase activity of 72.76 IU at an optimum temperature of 50°C with a specific activity of 4.02 $\mu\text{mol}/\text{min}/\text{mg}$ (Fig. 4), which is significantly higher than others. Similarly, optimum incubation temperature of 50°C for an enhanced amylase production has been reported in studies by Kunamneni *et al.* (2005), Hernández, (2006), Asgher *et al.* (2007); Aygan *et al.* (2008); Zohra and Ahmad, (2012) and Singh *et al.* (2014). A decrease in enzyme activity i.e. 18.13 IU with an increase in incubation temperature to 65°C of *B. sonorensis* GV2 is evident from the findings of Vidyalakshmi *et al.* (2012) and

Andualem, (2014). The reduction in enzyme activity at higher temperature could be due to deformed enzyme structures. Maximum enzyme activity at optimum temperature may be due to an increased metabolic activity of the cells resulting in an increased extracellular enzyme production in culture supernatant. At very low temperatures, membranes solidify and high temperatures damage microorganisms by denaturing enzymes, transport carriers and other proteins, thus lowering the enzyme activity (Willey *et al.*, 2008).

Effect of Various Nitrogen Sources

Nitrogen sources are the most important secondary energy compounds for the growth and metabolism of microorganisms. The nature of these compounds and the concentration used may stimulate or down regulate the production of enzymes (Sharma *et al.*, 2012). *Bacillus sonorensis* GV2 enzyme production medium supplemented with ammonium sulphate showed an increased enzyme activity of 71.72 IU with a specific activity of 3.86 $\mu\text{mol}/\text{min}/\text{mg}$ (Fig. 5). Rest of the other nitrogen sources used viz. gelatin, tryptone, NH_4Cl , NH_4NO_3 , NaNO_3 however showed a decline in enzyme activity. It has been observed that inorganic sources gave comparatively higher enzyme yields than organic nitrogen sources (Sankaralingam *et al.*, 2012).

However, presence of more than one nitrogen source in the medium gave better results which are evident from the results of the experiment. Among the nitrogen sources tested, yeast extract amended media showed the maximum production of α -amylase probably due to its high content in minerals, vitamins,

Fig. 4: Effect of different carbon sources on amylase production from *Bacillus sonorensis* (GV2)

Fig. 5: Effect of different nitrogen sources on amylase production from *Bacillus sonorensis* (GV2)

coenzymes and nitrogen components (Asgher *et al.*, 2007; Ashwini *et al.*, 2011; Vijayabaskar *et al.*, 2012). In consonance with these findings, starch agar medium used for enzyme production in the present study having yeast extract, when supplemented with an inorganic nitrogen source ammonium sulphate, resulted in an elevated enzyme yield.

Effect of Divalent Ions

Metal ions accept or donate electrons and act as electrophiles, mask nucleophiles to prevent unwanted side reactions, bind enzyme and substrate by coordinate bonds, hold the reacting groups in the required 3D orientation, and simply stabilize a catalytically active conformation of the enzyme (Palmer, 2001). Most of the enzymes are metallo-enzymes exhibiting an increased activity in the presence of metal ions. However, the enzyme action is specific to the presence or absence of a particular metal ion. Likewise, *Bacillus sonorensis* GV2 exhibited quite a variable behaviour towards extracellular α -amylase secretion in the presence of various metal ions in the production medium. Ca^{2+} and Mg^{2+} showed a positive effect on enzyme production giving 73.44 and 70.43 IU respectively. Ca^{2+} and Mg^{2+} are reported to enhance enzyme activity by Goyal *et al.* (2005); Ashger *et al.* (2007) and Kiran and Chandra, (2008). In accordance to the findings of Burhan *et al.* (2003) and Singh *et al.* (2014), Ni^{2+} and Zn^{2+} almost inhibited the enzyme production while in the medium. Ba^{2+} , Fe^{2+} and Co^{2+} showed a negative effect on enzyme production lowering the titres considerably. Mn^{2+} and Cu^{2+} however did not affect the enzyme production to a greater extent as reported by Rao and Satyanarayana, (2007).

Effect of Surfactants

Surfactants in the fermentation medium are known to increase the secretion of proteins by increasing cell membrane permeability. Therefore, addition of these surfactants is used for the production of extracellular enzymes (Sivaramakrishnan *et al.*, 2006). α -amylase production by *Bacillus sonorensis* GV2 was increased with the addition of surfactants such as Tween 80. With Tween 80 in the medium, enzyme activity was observed as 69.46 IU with a specific activity of 3.26 $\mu\text{mol}/\text{min}/\text{mg}$ protein. Addition of an

anionic detergent SDS in the medium resulted in an increased enzyme activity up to 72.90 IU, with a specific activity of 3.64 IU/mg protein. Similar increase in enzyme activity by addition on surfactants in the medium has been reported by Rao and Satyanarayana, (2003) and Sodhi *et al.* (2005). However, the addition of EDTA and CTAB in the medium resulted in inhibition of activity proving it to be α -amylase. Tween 20, Triton X100 and glycerol also failed to increase the enzyme activity considerably, thus were not added in the medium.

Classical approach, i.e., One Variable At a Time (OVAT) used for optimization of *Bacillus sonorensis* GV2 α -amylase has resulted in statistically significant increase in the production of amylase proving the direct utility of this technique in increasing enzyme titres. The significant variables chosen from one variable at a time optimization were further taken up for precise standardization by using a statistical tool of Response Surface Methodology.

Response Surface Methodology

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.

In this method, prior knowledge obtained from the previous experiment i.e. OVAT was necessary for achieving a more realistic model. Table 1 shows the minimum and maximum levels of variables chosen for trials in the CCD. 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 values is given in Table 3. 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, then the fitted response surface model is:

$$\begin{aligned} \text{Enzyme Activity (Y)} = & 74.25 + 4.17 * X_1 - 10.32 * X_2 - 7.38 * X_3 + 0.53 * X_4 - 2.20 * X_5 - 0.90 * X_6 - 8.77 * A^2 - 12.33 * B^2 - 11.68 * C^2 - 12.28 * D^2 - 5.23 * E^2 - 0.61 * F^2 - 5.94 * X_1 * X_2 + 2.87 * X_1 * X_3 + 4.21 * X_1 * X_4 + 1.68 * X_1 * X_5 - 0.055 * X_1 * X_6 + 5.40 * X_2 * X_3 - 2.10 * X_2 * X_4 + 1.25 * X_2 * X_5 - 0.52 * X_2 * X_6 + 4.17 * X_3 * X_4 + 3.35 * X_3 * X_5 + 1.46 * X_3 * X_6 + 1.01 * X_4 * X_5 + 1.14 * X_4 * X_6 + 1.72 * X_5 * X_6 \end{aligned}$$

Where, Y is enzyme activity (IU/ml), X_1 is substrate concentration, X_2 is pH, X_3 is temperature ($^{\circ}\text{C}$), X_4 is inoculum size (%), X_5 is Ca^{2+} concentration (%) and X_6 is Mg^{2+} concentration (%). By optimizing the above parameters using RSM, maximum enzyme activity obtained was 82.78 IU/ml at 50°C temperature and 9.5 pH @ 8.75% inoculum using 0.5% substrate with 0.5% Ca^{2+} and Mg^{2+} concentration.

The statistical significance was checked by F-Test and the analysis of variance (ANOVA) for the response surface quadratic model. It is evident, that the model was highly significant, as suggested by the model F-value and a low probability value [$P \text{ model} > F] = 0.0001$). The coefficient of determination (R^2) was calculated as 0.8844 for enhanced amylase production, indicating that the statistical model can explain 88.44% 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 17.422 for amylase production was recorded. The predicted R^2 of 0.6349 was in reasonable agreement with the adjusted R^2 of 0.8305. This indicated a 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 16.43 and values of prob > F (< 0.05) indicated that the model terms are significant.

Table 3. Full experimental design and results of Central Composite Design of RSM

Run	Substrate concentration	pH	Temp.	Inoculum size	Ca^{2+} ions	Mg^{2+} ions	Amylase activity (IU)
1	1	1	-1	1	1	1	10.98
2	0	-1	1	-1	-1	-1	3.76
3	0	-1	-1	1	1	1	24.78
4	0	1	-1	-1	1	-1	12.56
5	1	1	1	-1	1	1	1.142
6	-1	1	-1	-1	-1	1	1.77
7	1	-1	1	1	-1	1	42.146
8	-1	1	1	1	1	-1	0.783
9	-1	1	1	1	-1	1	0.04
10	1	-1	-1	-1	1	-1	49.73
11	1	-1	1	1	1	-1	47.9
12	1	1	1	-1	-1	-1	1.218
13	1	-1	-1	-1	-1	1	45.61
14	1	1	-1	1	-1	-1	1.44
15	0	0	0	0	0	0	63.475
16	-1	-1	1	-1	1	1	3.26
17	-1	-1	-1	1	-1	-1	44.65
18	0	0	0	0	0	0	54.55
19	1	-1	-1	1	1	1	57.74
20	1	1	1	1	-1	1	1.48
21	1	1	1	1	1	-1	11.09
22	1	-1	-1	1	-1	-1	55.56
23	1	1	-1	-1	-1	1	12.218
24	1	-1	1	-1	1	1	32.926
25	-1	1	1	-1	1	1	0.87
26	-1	-1	-1	-1	-1	1	57.33
27	0	0	0	0	0	0	80.053
28	-1	1	1	-1	-1	-1	0.179
29	1	1	-1	-1	1	-1	9.21
30	-1	1	-1	1	1	1	2.12
31	1	-1	1	-1	-1	-1	44.48
32	0	-1	-1	-1	1	1	37.086
33	-1	-1	1	1	-1	1	16.79
34	-1	1	-1	1	-1	-1	10.14
35	0	0	0	0	0	0	79.75
36	-1	-1	1	1	1	-1	6.44
37	-1	-1	-1	1	1	-1	17.74
38	1	-1	-1	-1	1	1	32.94
39	0	-1	1	-1	-1	1	1.74
40	0	1	-1	-1	1	1	11.09
41	1	1	-1	1	1	-1	2.47
42	-1	1	1	1	-1	-1	0.49
43	1	1	1	-1	-1	1	0.04
44	0	0	0	0	0	0	68.74
45	0	0	0	0	0	0	69.55
46	-1	-1	-1	1	-1	1	32.49
47	-1	1	-1	-1	-1	-1	49.39
48	-1	1	1	1	1	1	0.39
49	1	1	1	-1	1	-1	1.74
50	1	-1	1	1	1	1	61.67
51	1	-1	1	1	1	1	47.24
52	-1	-1	1	-1	1	-1	1.47
53	1	1	-1	1	-1	1	1.76
54	1	-1	1	1	-1	-1	47.07
55	-1	1	-1	1	1	-1	1.02
56	1	-1	1	-1	-1	1	1.49
57	-1	1	-1	1	-1	1	1.37
58	1	1	1	1	-1	-1	0.49
59	0	0	0	0	0	0	81.33
60	1	1	-1	-1	-1	-1	0.66
61	1	1	-1	-1	1	1	0.50
62	0	0	0	0	0	0	81.31
63	-1	-1	-1	-1	1	1	39.71
64	-1	-1	-1	-1	-1	-1	59.97
65	1	-1	-1	1	1	-1	59.711

66	1	1	1	1	1	1	0.04
67	0	-1	1	1	-1	-1	7.47
68	0	1	1	-1	-1	1	0.39
69	0	1	1	-1	1	-1	0.04
70	1	-1	1	-1	1	-1	0.14
71	-1	-1	1	1	1	1	5.71
72	1	-1	-1	1	1	-1	52.74
73	0	0	0	0	0	0	0
74	0	0	0	0	0	0	82.47
75	0	0	0	0	0	0	11.46
76	0	0	0	0	0	0	49.47
77	0	0	0	0	0	0	0.779
78	0	0	0	0	0	0	0
79	-1	0	0	0	0	0	82.77
80	0	0	0	0	0	0	32.24
81	0	0	0	0	0	0	0
82	0	0	0	0	0	0	12.87
83	-1	0	0	0	0	0	79.44
84	-1	0	0	0	0	0	81.36
85	-1	0	0	0	0	0	81.34
86	-1	0	0	0	0	0	81.39
87	0	0	0	0	0	0	0
88	-1	0	0	0	0	0	82.77
89	-1	0	0	0	0	0	55.47
90	-1	1	-1	-1	1	1	82.47

For amylase production $X_1, X_2, X_3, X_1^2, X_2^2, X_3^2, X_4^2, X_5^2, X_1X_2, X_1X_4, X_2X_3, X_3X_4, X_3X_5$ are significant model terms. Values of “Prob > F” less than 0.05 indicate model terms are significant. The coefficient of variation (CV) indicates the degree of precision with which the experiments are compared. The lower

reliability of any such experiment is usually indicated by high value of CV. In the present case a low CV (4.33) denotes that the experiments performed are highly reliable (Gangadharan *et al.*, 2008). Fig. 6 depicts an increase in amylase activity after optimization of process parameters.

The application of statistical design for screening and optimization of α -amylase allows quick identification of the important factors and interactions among them. The major objective of RSM is to determine optimum region of the factor space, in which operating specifications are satisfied. An over-all increase of 21.30% has been observed in α -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; Tanyildizi *et al.*, 2005).

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

Fig. 6: 3D-response surface curves showing interaction between various factors

interaction effects and optimal levels of the variables were determined by plotting the response surface curves. The response surface curves are represented in Fig. 7(a-d). Fig. 7a represents the interaction between pH and temperature. The shape of contour graph shows a positive interaction between the two variables. The enzyme yield was found to increase with simultaneous increase in both the factors. Response surface curve of inoculum size and substrate concentration is represented in Fig. 7b. 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. 7c represent the interaction of the inoculum size with temperature and Fig. 7d represents the interaction of temperature and calcium ions, which showed a positive interaction among themselves towards an increase in enzyme activity.

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 metallo-enzymes, and, in most cases Ca^{2+} ions are required for maintaining the spatial conformation of enzyme and to increase its thermal stability, thus play an important role in enzyme stability (Bano *et al.*, 2009). It proves the significant effects of these vital components for higher synthesis of amylase, thus, rendering them important constituents of the production medium.

On the basis of the literature available on amylase production, we report this strain for the very first time as an amylogenic strain yielding very high titers of amylase. The newly isolated strain offers interesting hydrolytic properties, being active upto pH

Fig. 7: Amylase activity of *Bacillus sonorensis* GV2 before and after optimization

11. Besides being alkalophilic, the most striking feature of this strain is its thermostable nature. The significant thermostability of the isolate makes it eligible for use in industrial applications which are carried out at very high temperatures.

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 sonorensis* GV2 [KJ775811] isolated from mushroom compost and allowed rapid screening of large number of variables simultaneously. Present study has 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 sonorensis* GV2. Significant thermostability of the isolate makes it eligible for use in the industrial applications which are normally carried out at very high temperatures.

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