Biological Forum – An International Journal **8**(2): 186-195(2016)

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Statistical Studies on Exploring and Enhancing the Catalytic Potential of Nitrilase from Thermo-tolerant *Geobacillus subterraneus* from Hot Water Springs of Manikaran

Mamta Sharma, Chandrika Attri and Amit Seth
Faculty of Applied Sciences and Biotechnology,
Shoolini University, Solan-173212, (Himachal Pradesh), INDIA

(Corresponding author: Amit Seth)
(Received 26 August 2016, Accepted 01 October, 2016)
(Published by Research Trend, Website: www.researchtrend.net)

ABSTRACT: The importance of thermostable biomolecules has led to an upsurge of research into organisms from thermophilic sites. The present study was aimed to isolate mandelonitrile degrading microbes from hot water springs of Manikaran using indicator plate assay method. Extensive screening resulted in obtaining nitrilase producing thermophile. On the basis of biochemical tests and 16s rDNA gene sequencing it was designated as *Geobacillus subterraneus* MAC VI. Further, statistical optimization of culture conditions and process parameters was performed. The nitrilase enzyme of this isolate is inducible in nature which is activated in presence of mandelonitrile. The enzyme activity was increased manifold by giving multiple feedings of inducer. The highest nitrilase activity was recorded at 90°C. The enzyme was highly thermostable as it showed activity (0.098 U/mgdcw) at 100°C even after 5h of incubation. This is the first report on a thermophilic *Geobacillus subterraneus* mandelonitrile degrading bacterium from Manikaran hot springs of Himachal Pradesh.

Keywords: Thermophiles, Manikaran, *Geobacillus subterraneus,* hyperinduction, mandelonitrile, RSM

INTRODUCTION

Thermophiles are heat-loving organisms flourishing at an optimum growth temperature above 70°C. They are considered as a key to evolution of living organisms in the primordial earth (Kimura *et al*. 2006) and have envisaged a great interest as they contain metabolites functioning efficiently at high temperatures. Hyperthermophiles are those which require a very high temperature ranging from 80°C-105°C for their growth (Hamilton-Brehm *et al*. 2005). The recent interest in biotechnology has encouraged studies on utilization of thermophiles and their enzymes for industrial purposes (Kanokratana *et al*. 2004). The application of thermophilic enzymes as biocatalyst has added advantages of increased substrate solubility, enhanced diffusion rate and mass transfer effect due to reduced viscosity as well as reduction in contamination (Li and Zhang 2005).

The state of Himachal Pradesh, situated between 30”22’ and 30”12’ north latitude and between 75”47’ and 79”4’ east longitude has been blessed with a number of hot water springs *viz.* Vashisht, Manikaran, Kasol, Tattapani, Khirganga etc. which are probably the richest repository of thermophilic organisms and needs to be explored as thermophiles represent the largest reservoir of unexplored biodiversity (Nichols *et al*. 2002). The temperature of these hot springs vary from 45-70°C but Manikaran is hottest amongst them with maximum temperature of 98-99°C.

Nitriles are organo-cyanides (RC-N) which are intermediates, products, byproducts and waste products of agriculture, chemical and pharmaceutical industries (Martinkova *et al* 2009). Nitriles are hydrolyzed by nitrilases to corresponding acids and ammonia without formation of an amide intermediate and this catalytic property can be exploited for production of carboxylic acids which are utilized for drug synthesis. This bioconversion is not only economic but also environment friendly. Nitrilases have been recognized as prominent biocatalysts not only in nitrile degradation (Gong *et al.* 2012) but also for the development of sustainable green technologies for the production of selective ingredients in pharmaceuticals and agrochemicals.

A major disadvantage of using nitrilase in industrial application is its relative poor stability (Nagasawa *et al.* 1993) as the temperature requirement for product synthesis is comparatively higher as compared biotransformation at a small scale. In order to meet the current requirement there is a need to find and develop an ideal catalyst which fits into category constrained by a set of parameters. The present study was carried out to identify and characterize a thermostable isolate capable of hydrolyzing mandelonitrile.

MATERIALS AND METHODS

A. Chemicals

All the chemicals used were of analytical grade and procured from Merck and Hi-Media, India.

Isolation and Primary Screening of nitrile hydrolyzing bacteria. Soil samples were collected from hot water springs of Manikaran (1760m) in Kullu district of Himachal Pradesh, India in the year 2013 and stored in the laboratory at Shoolini University, Solan. Enrichment culture technique was used for the isolation of nitrile degrading microbes where nutrient broth supplemented with 0.4% isobutyronitrile as the sole source of nitrogen and carbon was used for enrichment. Primary screening of nitrile utilizing bacteria was done by plate assay method with nutrient agar plate augmented with 0.1% phenol red followed by overnight incubation at 70°C (Santoshkumar *et al*. 2010). An isolate having maximum nitrilase activity was selected after secondary screening for further optimization experiments.

Enzyme Assay. The reaction mixture (1ml) consisting of phosphate buffer, resting cells and mandelonitrile was incubated at 80°C for 15 minutes followed by addition of 1ml of 0.1N HCl to stop the reaction. The amount of ammonia released was estimated according to Fawcett and Scott method (1960). One unit of nitrilase enzyme activity is defined as the amount of enzyme that catalyzes release of one micromole ammonia in one minute from the prospective substrate under the standard assay conditions.

Molecular characterization of bacterial isolate. The identification of the strain was based on molecular characterization. The sequences obtained were complied and compared with sequences available in the GenBank databases using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

Optimization of culture conditions for production of enzyme. The selection of the media, pH, temperature, carbon and nitrogen sources, growth factors and inducers were studied through traditional 'One-variable-at-a-time' approach and tested to evaluate their effect on nitrilase production.

B. Experimental design and optimization by RSM

Ammonium acetate, Yeast extract, Peptone and K_2HPO_4 are essential media components which influence growth of microbial isolate. Response surface methodology using a four factor, three level central composite design was used to study the response of these four variables. A 2^4 factorial design was generated in order to study the effects of these factors. The optimum levels of the variables were obtained by graphical and numerical analysis using Design Expert program.

Optimization of reaction conditions for enhancing nitrilase activity. The reaction conditions were initially optimized by using one variable at a time approach. Different variables which were optimized included buffer systems with pH ranging from 4.0-10.0, reaction temperature (50-100°C), substrate concentration, incubation time and thermostability of the enzyme at temperatures ranging from 60°C-100°C for 5h. Further optimization was performed by RSM approach.

Thermal and Operational stability. Thermostability is the index of biocatalyst stability which was determined by pre incubating the wet cells of *G. subterraneus* MAC VI for 5h at 50°C -100°C. In order to check the thermal inactivation of the enzyme, the cells were withdrawn after every 1h and the residual activity was assayed.

C. Statistical analysis

The statistical software package Design-Expert 8.0.4 (StatEase, Minneapolis, MN) was used for regression analysis of experimental data to obtain working parameters and to generate response surface graphs. ANOVA was used to estimate statistical parameters.

RESULTS AND DISCUSSION

Thermophiles are least explored reservoir of biodiversity, however they are referred to as 'Universal Ancestors' as they were among the first living organisms which evolved during the primordial birth days of earth when the temperatures were quite high (Doolittle 1999). Majority of the studies on thermophiles have been conducted on low elevation habitats (Reigstad *et al*. 2010). Manikaran, situated in District Kullu (Himachal Pradesh) represents a high altitude thermophilic site located at an elevation of 1760m along the banks of Parvati river (Cinti *et al*. 2009). A number of theromophilic microorganisms producing thermostable enzymes like hydrolytic enzymes (Verma *et al*. 2005), xylanases (Chauhan *et al*. 2015), α-amylases (Sodhi *et al*. 2005) have been isolated from Manikaran hot water springs.

A thermostable nitrilase producing microorganism identified as *Geobacillus subterrneus* has been isolated from hot water springs of Manikaran by enrichment culture technique followed by plate assay method with phenol red as indicator. The colonies showing a change in colour indicated presence of nitrile hydrolyzing bacteria which was in accordance with the method developed by Santoshkumar *et al*. (2010). Similarly other indicators like bromothymol blue (Banerjee *et al*. 2003a), $CoCl_2$ (Yazbeck *et al*. 2006) have been used for high-throughput colorimetric assay. Banerjee *et al*. 2003b also reported a fluorometric assay method for the determination of nitrilase activity. The partial 16S r DNA sequence of the isolate was determined by using universal primers and the sequence has been deposited in NCBI with Accession No. KU644138.1. The strain had 100% similarity to *Geobacillus subterraneus* and was named as *Geobacillus subterraneus* MAC VI.

Optimum temperature for the growth of the isolate was 60°C which clearly display its thermophilic characteristic. Medium with composition (g/l): Ammonium acetate-10, Yeast extract-5, Peptone-5, K_2HPO_4-5, $MgSO_4.7H_2O$-0.2, $FeSO_4.7H_2O$-0.3 and NaCl-1 was most appropriate medium for growth as well as nitrilase activity of the strain. Banerjee *et al.* (2003a) used medium with similar composition for isolation as well as maintenance of *Alcaligenes sp.* The isolate was allowed to grow in medium with pH ranging from acidic to basic and neutral pH was most suitable for its growth. A thermostable nitrilase produced by *Streptomyces* sp. MTCC 7546 was most active at 50°C and pH 7.4 (Khandelwal *et al*. 2007). However, Dennet and Blamey (2016) isolated a novel hyperthermophilic archaea *Pyrococcus sp*. M24D13 with nitrilase activity from the soils of Antarctica at 95°C in medium with pH 7.5. Carbon and nitrogen sources have a significant role in the enzyme production (Gong *et al.*, 2012). Nitrilases are inducible in nature requiring an external feeding of inducer for maximum enzyme activity. *G. subterraneus* MAC VI showed more affinity towards aliphatic or arylaliphatic nitriles. A reduction in cell growth was observed in presence of aromatic nitriles.

A. Optimization of culture conditions for production of nitrilase

Variation in culture conditions plays an important role in the enhancement of production of desirable enzymes. In order to increase the production of nitrilase optimization of culture conditions was performed by one factor at a time approach. An increase in nitrilase activity i.e. 0.009U/mgdcw to 0.064U/mgdcw was observed in M2 medium (Banerjee *et al*. 2003a) with pH 7.0 at 60°C with mandelonitrile as inducer. However, all other medium M3 (Dong *et al.* 2010), M4 (Almatawah *et al.* 1999), M5 (Nagamune *et al*. 1990), M6 (Kobayashi *et al.* 1989) showed a decrease not in growth of the cells as well as in enzyme activity. The isolate showed growth at all temperatures, however maximum nitrilase activity was observed at 60°C indicating that the isolate is thermophilic in nature. Though optimization by OVAT resulted in increase of enzyme activity but fails to assess the cumulative effect of various physicochemical parameters which lead to the production of enzyme by the microorganism. Doddapaneni *et al*. 2007 used factorial designs and regression analyses for generating empirical models which makes RSM a good statistical tool.

In order to further exploit the catalytic potential of *G. subterraneus* MAC VI response surface methodology (RSM) was performed as it offers a rapid procedure to select the most effective combination of variables. Four factors selected for RSM study were (A) ammonium acetate, (B) Yeast Extract, (C) Peptone and (D) K_2HPO_4. The range and the levels of these variables are given in Table 1. The results of RSM experiments (30 runs) for studying the effect of four variables along with the mean actual and predicted response (enzyme activity) are shown in Table 2.

Table 1: Coded values of independent variables at different levels used in Central Composite Design (CCD).

Independent Variables	**Symbols**	**-1**	**Levels**	**+1**
Ammonium Acetate	A	0.5	1.0	1.5
Yeast Extract	B	0.3	0.5	0.7
Peptone	C	0.3	0.5	0.7
K_2HPO_4	D	0.3	0.5	0.7

The corresponding analysis of variance (ANOVA) for Response surface quadratic model is presented in Table 3. The Model F-value of 38.4 implies that the model is significant. The lack-of-fit analysis gave non-significant P-values (>0.05) and F-values lower than the corresponding tabulated F-values, thus proving that the model obtained was highly significant and the coefficient determination R-squared (0.9727) indicated that the fitted models could explain at least 97 % of the total variation in the responses (Table 4) which indicate that the quadratic models were appropriate to fit and satisfactorily describe the experimental data pertaining to nitrilase activity. Only less than 2% of the total variations were not explained by the model. The predicted determination coefficient value (Pred-R-squared = 0.8483) was within reasonable agreement with the adjusted R-squared = 0.9727 which also indicated the significance of the model.

The highest nitirlase activity (0.076 U/mgdcw) was obtained in (g/100ml) ammonium acetate-1.0, yeast extract-0.5, peptone-0.5 and K_2HPO_4-0.5. The nitrilase activity before optimization was 0.069U/mgdcw and after optimization it increased to 0.076U/mgdcw indicating that use of RSM has increased the enzyme activity. Values of "Prob > F" less than 0.0500 indicate model terms are significant. Values greater than 0.1000 indicate the model terms are not significant. The "Lack of Fit F-value" of 1.65 implies the Lack of Fit is not significant relative to the pure error. Non-significant lack of fit is good. The 3D response surface plots described by the regression model were drawn to illustrate the effects of the independent variables on the response variables (Fig. 1). The interaction between each independent variable's pair along with the optimal values of the independent variables can be easily understood from 3D plots.

Table 2: Actual and predicted values of nitrilase recorded in experimental setup of RSM.

Std.	Run	Ammonium Acetate	Yeast extract	Peptone	K_2HPO_4	Actual Specific activity	Predicted specific activity
25	1	1.0	0.5	0.5	0.5	0.066	0.066
23	2	1.0	0.5	0.5	0.3	0.062	0.065
12	3	1.5	0.7	0.3	0.7	0.040	0.041
10	4	1.5	0.3	0.3	0.7	0.037	0.037
4	5	1.5	0.7	0.3	0.3	0.035	0.037
8	6	1.5	0.7	0.7	0.3	0.039	0.037
7	7	0.5	0.7	0.7	0.3	0.025	0.023
6	8	1.5	0.3	0.7	0.3	0.033	0.035
16	9	1.5	0.7	0.7	0.7	0.042	0.038
17	10	0.5	0.5	0.5	0.5	0.032	0.038
14	11	1.5	0.3	0.7	0.7	0.031	0.034
18	12	1.5	0.5	0.5	0.5	0.052	0.051
30	13	1.0	0.5	0.5	0.5	0.069	0.066
15	14	0.5	0.7	0.7	0.7	0.021	0.025
29	15	1.0	0.5	0.5	0.5	0.071	0.066
13	16	0.5	0.3	0.7	0.7	0.027	0.023
20	17	1.0	0.7	0.5	0.5	0.056	0.061
27	18	1.0	0.5	0.5	0.5	0.068	0.066
19	19	1.0	0.3	0.5	0.5	0.059	0.060
22	20	1.0	0.5	0.7	0.5	0.058	0.059
1	21	0.5	0.3	0.3	0.3	0.022	0.024
26	22	1.0	0.5	0.5	0.5	0.076	0.066
21	23	1.0	0.5	0.3	0.5	0.056	0.060
28	24	1.0	0.5	0.5	0.5	0.070	0.066
24	25	1.0	0.5	0.5	0.7	0.067	0.066
5	26	0.5	0.3	0.7	0.3	0.028	0.025
9	27	0.5	0.3	0.3	0.7	0.025	0.025
3	28	0.5	0.7	0.3	0.3	0.027	0.022
2	29	1.5	0.3	0.3	0.3	0.042	0.036
11	30	0.5	0.7	0.3	0.7	0.030	0.026

Table 3: ANOVA for Response Surface Quadratic model.

Source	Sum of squares	df	Mean squares	F Value	p-Value Prob>F	
Model	8.904E-003	14	6.360E-004	38.24	< 0.0001	Significant
A-Ammo. Acetate	6.480E-004	1	6.480E-004	38.95	< 0.0001	
B-Yeast Extract	9.389E-006	1	9.389E-006	0.56	0.4641	
C-Peptone	8.000E-006	1	8.000E-006	0.48	0.4986	
D-K_2HPO_4	5.000E-007	1	5.000E-007	0.030	0.8647	
AB	9.000E-006	1	9.000E-006	0.54	0.4733	
AC	2.250E-006	1	2.250E-006	0.14	0.7182	
AD	0.000	1	0.000	0.000	1.0000	
BC	2.500E-007	1	2.500E-007	0.015	0.9041	
BD	9.000E-006	1	9.000E-006	0.54	0.4733	
CD	6.250E-006	1	6.250E-006	0.38	0.5491	
A^2	1.166E-003	1	1.166E-003	70.07	< 0.0001	
B^2	1.540E-004	1	1.540E-004	9.26	0.0082	
C^2	1.747E-004	1	1.747E-004	10.50	0.0055	
D^2	2.171E-007	1	2.171E-007	0.013	0.9106	
Residual	2.495E-004	15	1.663E-005			
Lack of Fit	1.915E-004	10	1.915E-005	1.65	0.3021	not significant
Pure Error	5.800E-005	5	1.160E-005			
Cor Total	9.154E-003	29				

Table 4: Model Fitting Values.

S.No.	Model Terms	Values	S.No.	Model Terms	Values
1	Std. Dev.	4.079E-003	5	R-Squared	0.9727
2	Mean	0.046	6	Adj R-Squared	0.9473
3	C.V. %	8.87	7	Pred R-Squared	0.8483
4	Press	1.389E-003	8	Adeq Percision	15.567

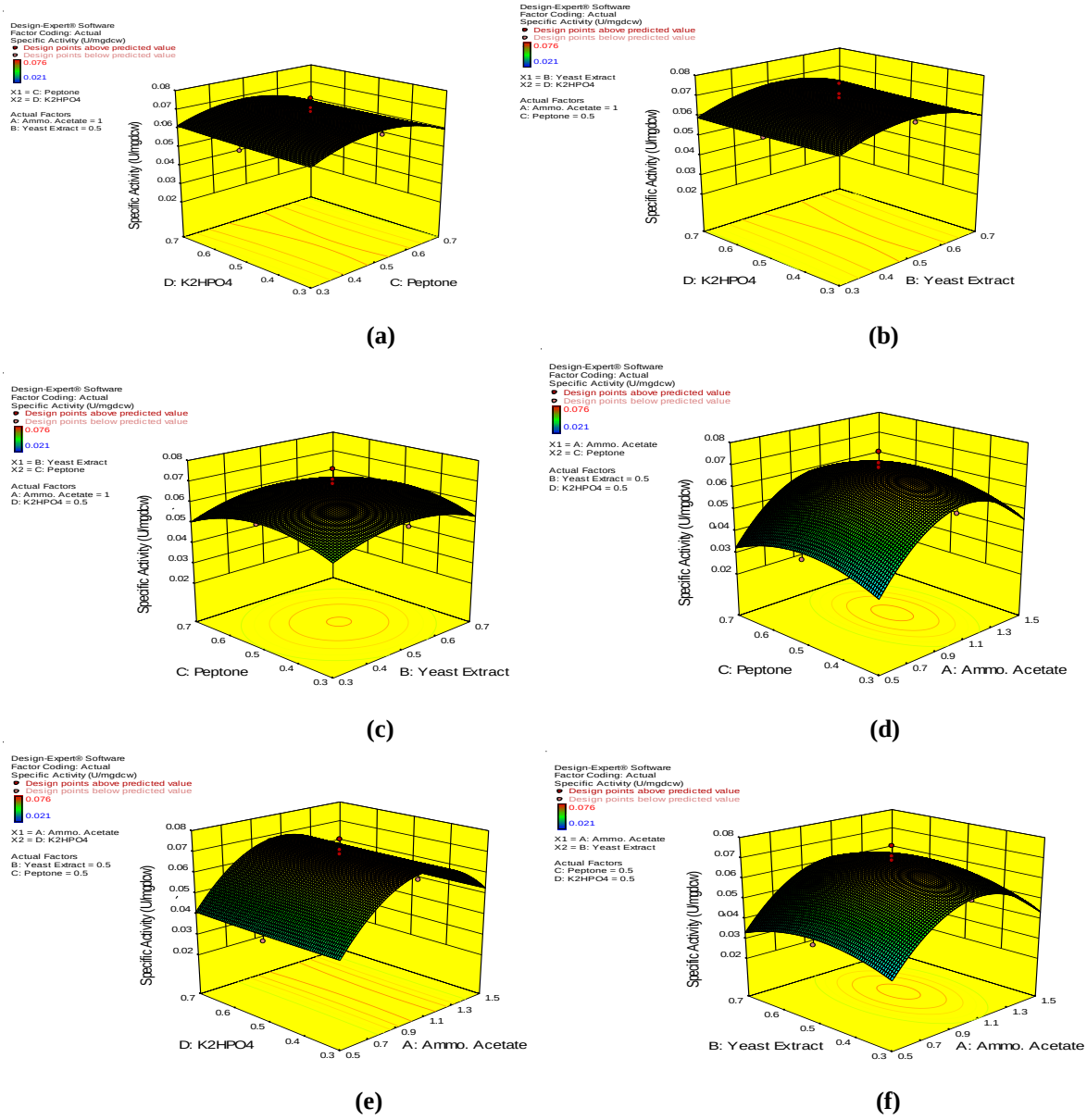


Fig. 1. 3D response plots of the RSM experiments for nitrilase of *G. subterraneus* MAC VI a) Interaction between (a) K_2 HPO_4 & Peptone (b) K_2HPO_4&Yeast Extract (c) Peptone &Yeast Extract (d) Peptone & Ammonium acetate (e) K_2 HPO_4&Ammonium acetate (f) Yeast Extract & Ammonium acetate.

B. Optimization of reaction conditions

Hyperinduced cells of *G. subterraneus* MAC VI were used for the optimization of reaction conditions by 'one variable at a time approach' followed by Response Surface Methodology. It was observed by OVAT maximum enzyme activity (0.095U/mgdcw) was observed in phosphate buffer (pH 7.0) at 80°C with 100µl cell volume and substrate concentration of 60mM. Thermo active nitrilase from *Bacillus pallidus* Dac521 has an optimum temperature of 65°C (Almatawah *et al.*, 1999). However, **t**he nitrilase from *G. subterraneus* MAC VI exhibited appreciable nitrilase activity even at 100°C. Optimization by OVAT approach indicated that temperature, pH, substrate concentration and cell volume were significant factors which enhanced nitrilase activity. Inorder to assess the combined effect of these factors further optimization was done by RSM.

The variables used for the factorial analysis were (A) reaction temperature, (B) pH of buffer, (C) cell volume and (D) substrate concentration (mandelonitrile). The range and the levels of these variables are given in Table 5. Maximum and minimum levels of variables chosen for trials (Run) in the Central Composite design along with predicted and actual nitrilase activity are shown in Table 6. The Model F-value of 3.39 in ANOVA implies that the model is significant (Table 7). There is 11.09% chance that a "Lack of Fit F-value". The lack-of-fit analysis gave non-significant P-values (>0.05) and F-values lower than the corresponding tabulated F-values, thus proving that the model obtained was significant.

The coefficient of determination (R-squared) for nitrilase activity of enzyme as a function of the independent variables was found to be 75%, which showed that the model correlated well with measured data and was statistically significant ($P \leq 0.05$). The corresponding analysis of variance (ANOVA) of the quadratic model along with the regression coefficient (R-squared), adjusted regression coefficient (adj.R-squared) and the predicted R-squared are given in Table 8. The F values below the tabulated F-values indicate that the model is significant. These results indicate that the quadratic model was appropriate to fit the experimental data satisfactorily for mandelic acid production.

Table 5: Coded values of independent variables at different levels used in Central Composite Design (CCD).

Independent Variables	**Symbols**	**Levels**		
		-1	**0**	**+1**
Temperature (ºC)	A	70	80	90
pH of buffer	B	6.5	7.0	7.5
Substrate conc. (mM)	C	50	100	150
Cell Volume (µl)	D	50	100	150

Table 6: Actual and predicted values of nitrilase recorded in experimental setup of Response Surface Methodology.

Std	**Run**	**Temp**	**pH**	**Substrate concentration**	**Cell volume**	**Nitrilase activity (U/mgdcw) (actual)**	**Nitrilase activity (U/mgdcw) (predicted)**
28	1	80	7.0	100	100	0.072	0.062
29	2	80	7.0	100	100	0.044	0.062
21	3	80	7.0	0	100	0	-0.007
15	4	70	7.5	150	150	0.061	0.06
23	5	80	7.0	100	0	0	0.025
7	6	70	7.5	150	50	0.024	0.036
26	7	80	7.0	100	100	0.078	0.062
22	8	80	7.0	200	100	0.064	0.080
27	9	80	7.0	100	100	0.041	0.062
2	10	90	6.5	50	50	0.062	0.050
16	11	90	7.5	150	150	0.088	0.094
13	12	70	6.5	150	150	0.038	0.039
14	13	90	6.5	150	150	0.084	0.075
8	14	90	7.5	150	50	0.149	0.111
17	15	60	7.0	100	100	0.084	0.052
4	16	90	7.5	50	50	0.012	0.013
24	17	80	7.0	100	200	0.034	0.018
5	18	70	6.5	150	50	0.048	0.049
3	19	70	7.5	50	50	0.008	0.004
30	20	80	7.0	100	100	0.07	0.062
6	21	90	6.5	150	50	0.144	0.125
12	22	90	7.5	50	150	0.03	0.015
19	23	80	6.0	100	100	0.076	0.080
9	24	70	6.5	50	150	0.026	0.50
25	25	80	7.0	100	100	0.068	0.062
20	26	80	8.0	100	100	0.065	0.067
18	27	100	7.0	100	100	0.055	0.096
11	28	70	7.5	50	150	0.025	0.047
1	29	90	6.5	50	50	0.044	0.040
10	30	90	6.5	50	150	0.029	0.019

Table 7: ANOVA for Response Surface Quadratic Model for nitrilase activity.

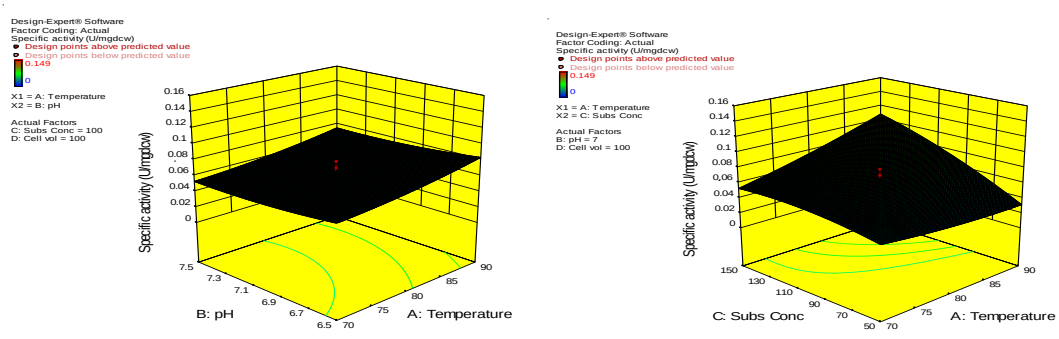
Source	Sum of squares	df	Mean square	F Value	P-Value Prob>F	
Model	0.028	14	1.995E-003	3.39	0.0125	Significant
A-Temp	2.948E-003	1	2.948E-003	5.01	0.0408	
B-pH	4.167E-004	1	4.167E-004	0.71	0.4132	
C-Subs conc	0.012	1	0.012	19.75	0.0005	
D-Cell vol	7.350E-005	1	7.350E-005	0.12	0.7287	
AB	2.500E-007	1	2.500E-007	4.250E-004	0.9838	
AC	4.356E-003	1	4.356E-003	7.40	0.0158	
AD	1.640E-003	1	1.640E-003	2.79	0.1157	
BC	5.523E-004	1	5.523E-004	0.94	0.3480	
BD	1.089E-003	1	1.089E-003	1.85	0.1937	
CD	3.802E-004	1	3.802E-004	0.65	0.4340	
A^2	2.538E-004	1	2.538E-004	0.43	0.5213	
B^2	2.972E-004	1	2.972E-004	0.51	0.4881	
C^2	1.100E-003	1	1.100E-003	1.87	0.1916	
D^2	2.789E-003	1	2.789E-003	4.74	0.0458	
Residual	8.824E-003	15	5.883E-004			
Lack of Fit	7.603E-003	10	7.603E-004	3.11	0.1109	Not significant
Pure Error	1.221E-003	5	2.442E-004			
Cor Total	0.037	29				

Table 8: Model fitting values.

S.No.	Model Terms	Values	S.No.	Model Terms	Values
1	Std. Dev.	0.024	**5**	R-squared	0.7599
2	Mean	0.054	**6**	Adj R-squared	0.5358
3	C.V. %	44.83	**7**	Pred R-squared	-0.2395
4	Press	0.046	**8**	Adeq Precision	7.711

A negative "Pred R-Squared" implies that the overall mean is a better predictor of our response than the current model. A ratio greater than 4 is desirable and ratio of 7.711 indicates an adequate signal so this model can be used to navigate the design space.

The 3D response surface plots described by the regression model were drawn to illustrate the effects of the independent variables on the response variables (Fig. 2). The shape of the corresponding contour indicate the significance of mutual interactions. Maximum nitrilase activity of 0.149 U/mgdcw was obtained when the reaction was performed at 90ºC with buffer pH 7.5, substrate concentration 50mM, and cell volume 50µl.



(a) **(b)**

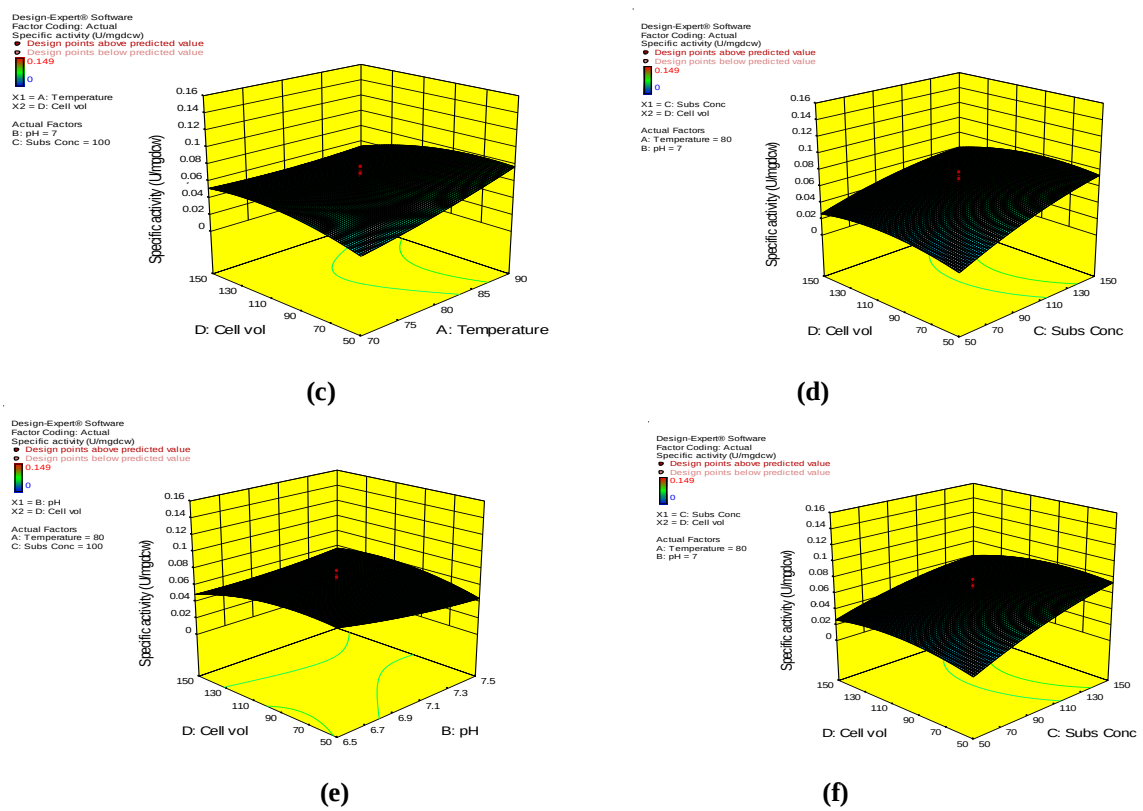


Fig. 2. 3D response plots of the RSM experiments for nitrilase of *G. subterraneus* MAC VI Interaction between (a) pH& Temperature (b) substrate conc. & Temperature (c) Cell volume &Temperature (d) substrate conc & pH (e) Cell volume & pH (f) cell volume & substrate conc.

C. Thermal and operational stability

Thermostability of the enzyme is an important characteristic which renders it to be exploited for biotransformation on industrial scale. *G. subterraneus* MAC VI was found to be extremely thermostable as it exhibited nitrilase activity even after 5 h at 100°C (Fig.3). However, Chen *et al.* 2015 while working on hyperthermophilic bacteria *Thermotoga maritima* MSB8 inferred that the enzyme exhibited good thermostability at 75°C while nitrilase from *Pyrococcus sp.* M24D13 was stable even after 8 h of incubation at 85°C (Dennet and Blamey, 2016).

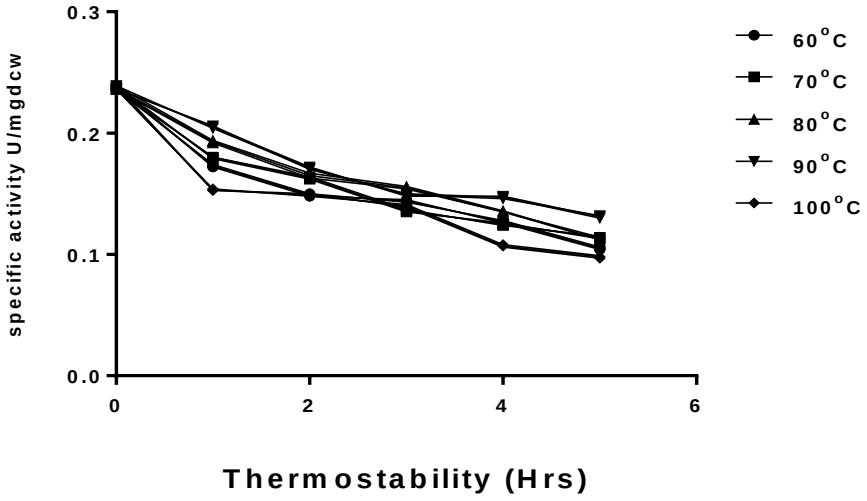


Fig. 3. Thermostability of nitrilase at different temperatures for 5h.

CONCLUSION

A new candidate for the production of thermostable nitrilase identified as *Geobacillus subterraneus* was isolated from hot water springs of Manikaran which was capable of degrading mandelonitrile. Optimization of culture conditions and process parameters enhanced nitrilase production. Thermo stability of the enzyme was an added advantage of this microbe as it was able to withstand 100°C for 5 h and still exhibited appreciable nitrilase activity.

ACKNOWLEDGEMENTS

This work was supported by the Department of Science and Technology, Government of India (grant number SB/YS/LS-19/2013). Authors are highly thankful to Honorable Vice-Chancellor, Prof. P.K. Khosla for providing necessary facilities for carrying out this research work.

REFERENCES

Almatawah, Q.A., Cramp, R. & Cowan, D.A. (1999). Characterization of an inducible nitrilase from thermophilic *Bacillus*. *Extremophiles*. **3**: 283-291.

Banerjee, A., Kaul, P., Sharma, R. & Banerjee, U.C. (2003a). A high-throughput amenable colorimetric assay for enantioselective screening of nitrilase producing microorganisms. *Journal Biomolecular Screening.* **8**: 559–565.

Banerjee, A., Sharma, R. & Banerjee, U.C. (2003b). A rapid and sensitive fluorometric assay method for the determination of nitrilase activity. *Biotechnology and Applied Biochemistry*. **37**: 289–293.

Chauhan, S., Seth, C.A. & Seth, A. (2015). Bioprospecting thermophilic microorganism from hot springs of Western Himalayas for xylanase production and its statistical optimization by RSM. *Journal of Pure Applied Microbiology.* **9**:1417-1428.

Chen, Z., Chen, H., Ni, Z., Rui, T., Zhang, T., Jia, J. & Yang, S. (2015). Expression and Characterization of a novel nitrilase from hyperthermophilic bacterium *Thermotoga maritima* MSB8. *Journal of Microbiology and Biotechnology*, **25(10)**: 1660–1669.

Cinti, D., Pizzino, L., Voltattorni, N., Quattrocchi, F. & Walia, V. (2009). Geochemistry of thermal waters along fault segments in the Beas and Parvati valleys (northwest Himalaya, Himachal Pradesh) and in the Sohna town (Haryana), India. *Geochemical Journal*, **43**: 65-76.

Dennet, G.V. & Blamey, J.M. (2016). A new thermophilic nitrilase from an Antarctic hyperthermophilic microorganism. *Frontiers in Bioengineering and Biotechnology.* **4** doi: 10.3389/fbioe.2016.00005.

Doddapaneni, K.K., Tatineni, R., Potumarthi, R. & Mangamoori, L.N. (2007). Optimization of media constituents through response surface methodology for improved production of alkaline proteases by *Serratia rubidaea*. *Journal of Chemical Technology and Biotechnology.* **82**: 721-729.

Doolittle, W.F. (1999). Phylogenetic classification and the universal tree. Science. **284**: 2124-2128.

Fawcett, J.K. & Scott, J.E. (1960). A rapid and precise method for the determination of urea. *Journal of Clinical Pathology*. **13**: 156-159.

Gong, J.S., Lu, Z.M., Li, H., Shi, J.S., Zhou, Z.M., Xu, Z.H. (2012). Nitrilases in nitrile biocatalysis: recent progress and forthcoming research. Microbial Cell Factories. **11**: 142-160.

Hamilton-Brehm, S.D., Schut, G.J. & Adams, M.W. (2005). Metabolic and evolutionary relationships among *Pyrococcus* species: genetic exchange within a hydrothermal vent environment. Journal of Bacteriology. **187(21)**: 7492-7299.

Kanokratana, P., Chanapan, S., Pootanakit, K. & Eurwilaichitr, L. (2004). Diversity and abundance of bacteria and archaea in the Bor Khlueng Hot Spring in Thailand. Journal of Basic Microbiology. **44(6)**: 430-44.

Khandelwal, A.K., Nigam, V.K., Choudhury, B., Mohan, M.K. & Ghosh, P. (2007). Optimization of nitrilase production from a new thermophilic isolate. Journal of Chemical Technology and Biotechnology. **82**: 646–651.

Kimura, H., Sugihara, K.K. & Hanada, S. (2006). Selective phylogenetic analysis targeted at 16s rRNA genes of thermophiles and hyperthermophiles in deep-subsurface geothermal environments. *Applied and Environmental Microbiology*.**72**: 21-27.

Kobayashi, M., Nagasawa, T., Yanaka, N. & Yamada, H. (1989). Nitrilase catalysed production of p-aminobenzoic acid from p- aminobenzonitrile with *Rhodococcus rhodochrous* J1. *Biotechnology Letters*. **11**: 27-30.

Li, H. & Zhang, X. (2005). Characterization of thermostable lipase thermophilic Geobacillus sp. TW1. *Protein Expression and Purification.* **42**:153–159.

Martinkova, L., Uhnakova, B., Patek, M., Nesvera, J. & Kren, V. (2009). Biodegradation potential of the genus *Rhodococcus*. *Environment International*. **35**: 162–177.

Nagamune, T., Kurata, H., Hirata, M., Honda, J., Koike, H., Ikeuchi, M., Inoue, Y. *et al*. (1990). Purification of inactivated photoresponsive nitrile hydratase. *Biochemical and Biophysical Communications.* **168 (2)**: 437-442.

Nagasawa, T., Shimizu, S. & Yamada, H. (1993). The superiority of the third generation catalyst *Rhodococcus rhodochrous* J1 nitrile hydratase for industrial production of acrylamide. *Applied Microbiology and Biotechnology*. **40**: 189–195.

Nichols, D.S., Sanderson, K., Buia, A., Van de Kamp, J., Holloway, P., Bowman, J.P., *et al*. (2002). Bioprospecting and biotechnology in Antarctica. The Antarctic: past, present and future, *Antarctic CRC Research Report*. **28**: 85-103.

Reigstad, L.J., Jorgensen, S.L. & Schleper, C. (2010). Diversity and abundance of Korarchaeota in terrestrial hot springs of Iceland and Kamchatka. *International Society for Microbial Ecology*. **4**: 346-356.

Santoshkumar, M., Nayak, A.S., Anjaneya, O., Timmanagouda, B. & Karegoudar, T.B. (2010). A plate method for screening of bacteria capable of degrading aliphatic nitriles. *Journal of Industrial Microbiology and Biotechnology.* **37**:111–115.

Sodhi, H.K., Sharma, K., Gupta, J.K. & Soni, K.S. (2005). Production of a thermostable α-amylase from *Bacillus* sp. PS-7 by solid state fermentation and its synergistic use in the hydrolysis of malt starch for alcohol production. *Process Biochemistry.* **40**: 525–534.

Verma, P., Yadav, A.N., Shukla, L., Saxena, A.K. & Suman, A. (2015). Hydrolytic enzymes production by thermotolerant *Bacillus altitudinis* IARI-MB-9 and *Gulbenkiania mobilis* IARI-MB-18 isolated from Manikaran hot springs. *International Journal of Advanced Research* **3(9)**: 1241-1250.

Yazbeck, D.R., Durao, P.J., Xie, Z. & Tao, J. (2006). A metal ion-based method for screening of nitrilases. *Journal of Molecular Catalysis B: Enzymatic.* **39**: 156-159.