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Enhanced Production of Amidotransferase From *Bacillus* Sp. ABP-6 By optimization of Nutritional Parameters Using Statistical Experimental Design

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Abstract: *The consortial effects of various nutritional parameters of medium on enhanced production of amidotransferase by Bacillus sp. APB-6 were studied using stepwise statistical experimental methodology. In addition to the effect of pH, temperature and inoculum size on enzyme production, a full factorial central composite design was chosen to explain nutritional constituents of medium viz. acetamide, glucose, yeast extract and sodium chloride for the analysis of the results. The Placket–Burman screening experiments suggested that sodium chloride, temperature, pH and n-methyl acetamide were the most influential media components. These four media components were optimized using a face centered design of response surface methodology (RSM) and the optimal combinations of the media constituents for enhanced amidohydrolase productions were determined as acetamide (3.0% (w/v), glucose 0.2% (w/v), yeast extract 1.5% (w/v), NaCl 0.35% (w/v), non-substrate inducer n-methyl acetamide 70 mM with inoculum 8% (v/v) at pH 7.0 and temperature 32.5^{o}C. Thus in the present studies, 4.10 fold enhanced enzyme production (198.2 units/ mg dry cells) was achieved using response surface methodology.*

Keyswords: *Amidohydrolase, RSM, Optimization, Bacillus sp.*

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I. INTRODUCTION

Amidase or amidohydrolase (E.C. 3.5.1.4) is an interesting member of nitrilase superfamily which catalyzes the hydrolysis of amides to carboxylic acid and ammonia and is used by prokaryotes in carbon and nitrogen fixation [1].These are amide bond-cleaving enzymes thus plays important role in the hydrolysis of various endogenous as well as foreign aliphatic and aromatic amides [2]. A number of amidases from microbial systems have been reported and explored for the acyl transfer activity [3]. In industries amidases are employed in combination with nitrile hydratases for the production of commercially important organic acids (acrylic acid, p-aminobenzoic acid, pyrazinoic acid, nicotinic acid etc.) through biotransformation of nitriles [4]. Different wide spectrum amidases exhibit acyl transfer activity (transfer of R-CO group) in the presence of hydroxylamine [5] which acts as an acyl acceptor and the amide acts as an acyl donor.The use of acyltransferase or bacteria with microbial enzymes with acyltransferase activity may be used to convert amides to Hydroxamic acids [6].The substrate specificity of most studied amidases is confined to the amides that contain the free amino group, i.e., to substituted amides [7]. Amidotransferase activity of amidase has been used for the biosynthesis of a range of hydroxamic acids, which have a high chelating potential. Several hydroxamic acids are used as drugs and have been reported as tumor inhibitors, anti-HIV and anticancerous [5, 8]. Some hydroxamic acids can conjugate with metal ions and thus find their use to eliminate metal ions in wastewater treatment and nuclear technology [9]. Some other hydroxamic acids (⟨-aminohydroxamic acid, acetohydroxamic acid, butyrohydroxamic acid etc.) have also been investigated as anti-human immunodeficiency virus agents, antimalarial agents and have also been recommended for treatment of ureaplasma infections and anaemia [10,11]. Some fatty hydroxamic acids have been studied as inhibitors of cyclooxygenase and 5-lipooxygenase with a potent anti-inflammatory activity [12].

The conventional method of medium optimization involves changing one parameter at a time and keeping the others at fixed levels. Furthermore, being linear in nature, the conventional method is incapable of determining the interactive effects of variables and is therefore unable to predict the 'true' optimum [13, 14]. RSM is a collection of statistical techniques that uses design of experiments (DoE) for building models, evaluating the effects of factors and searching for optimum conditions. It is a statistically designed experimental protocol in which several factors are simultaneously varied.In biotechnology, this technique has been used for a broad range of primary as well as secondary microbial metabolites, viz. enzymes, acids, terpenoids,

etc. Amidotransferase activity of amidase has been used for the biosynthesis of a range of hydroxamic acids, which have a high chelating potential. Several hydroxamic acids are used as drugs and have been reported as tumor inhibitors, anti-HIV and anticancerous.

In present work, production of amidotransferase from *Bacillus* sp. APB-6 using different carbon and nitrogen sources, temperature, pH and inducer was studied and was selected for further experiments. A systematic and sequential optimisation strategy was adopted to enhance the production of amidotransferase. An optimal medium composition for production of amidase was achieved in the following three steps: (1) screening of optimum carbon source,nitrogen source and inducer; (2) use of Placket–Burman experimental design to select the most influential media components; and (3) use of surface-centre central composite design(FCCCD)of RSM for optimisation of critical media constituents.

Microorganism

In the present study a nitrile metabolizing bacterium isolated in the Department of Biotechnology, Himachal Pradesh University, Shimla from the soil samples of Shimla (Himachal Pradesh, INDIA) has been explored for its amidotransferase (amidase) activity. This bacterium has been identified and deposited as *Bacillus* sp. APB-6 at Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh (INDIA) with accession number MTCC-7540. Since the amide transfer activity has been studied so the protein is being addressed as amidotransferase, though it is an amidase.

Chemicals

All the chemicals were of analytical grade. The nitriles and amides were from Alfa Aesar, A Johnson Matthey Company (earlier Lancaster Synthesis). Media components were from HiMedia (Mumbai) and the inorganic salts were of analytical grades.

Amidotransferase Assay

Reference: Brammar and Clarke (1964) [15].
Reagents: $FeCl_3$ reagent [6.0 % (w/v) in 2.0 % HCl (v/v)]

The standard curve was prepared using 80 ⌈g – 340 ⌈g of butyrohydroxamic acid. One unit (U) of amidotransferase activity was defined as that amount of enzyme which catalyzed the release of one micromole of butyro hydroxamic acid per min under assay conditions.

II. OPTIMIZATION OF PRODUCTION CONDITIONS FOR AMIDOTRANSFERASE OF *BACILLUS* SP. APB-6

Production Medium For Amidotransferase

A loopful of bacterial cells (*Bacillus* sp.APB-6) from the slant were seeded in 50 ml of modified nutrient broth containing 6 g peptone, 3 g beef extract, 1 g yeast extract, 10g glucose per liter of distilled water [16] pH 7.5 and incubated at 30°C for 24 h in an incubator shaker (160 rpm). Four ml of this seed culture (OD_{600}H14, 3.31 mg dcw ml^{-1}) was added to 50 ml of various media as listed in Table 1 in 250 ml Erlenmeyer flasks. These flasks were incubated at 30°C for 24 h at 150 rpm in an incubator shaker.

Optimization Of Production Conditions For Amidotransferase Of *Bacillus* Sp. APB-6

The *Bacillus* sp. APB-6 was grown in seventeen different media at 30°C (pH 9.5), out of which thirteen were already reported in literature and four self-formulated media were also tried, the media used were as follows **(**compositions are for g/l) table :

The cells were harvested by centrifuging the culture broth at 10,000 g for 15 min and were suspended in 0.1 M Glycine-NaOH buffer (pH 7.0) and after two washings with the same buffer, the cell suspension was referred to as 'whole resting cells' and assayed for enzyme activity.

Selection Of Suitable Carbon Source And Nitrogen Source

Various carbon sources viz. glucose, fructose, galactose, sorbitol, mannitol, xylose, arabinose, sucrose, maltose, lactose and starch were added to the production medium in the concentration of 0.2%. The cultured cells were assayed for amidotransferase activity.To study the effect of nitrogen sources on amidotransferase production from Bacillus sp. APB-6 nitrogen sources (3%) viz. casein enzyme, beef extract, ammonium chloride, di-ammonium hydrogen phosphate, tryptone, ammonium nitrate, casein acid, peptone, acetamide, soyatone and calcium nitrate were added to the production medium.

Effect Of Medium Ph And Size Of Inoculum

The bacterium was grown in pH range of 5.0-10.5 (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0 and 10.5) to investigate the effect of pH on the cell growth and production of amidotransferase.Twenty four hour old inoculum (OD_{600}H14, 3.40 mg dcw ml^{-1}) was added in the production medium (pH 8.0) at different concentrations (2%, 4%, 6%, 8%, 10%, 12%, 14% and 16% v/v) and incubated at 30°C for 24 h to study its effect on production of amidotransferase.

Optimization Of Production Temperature And Inducer

Bacillus sp.APB-6 was grown at different temperatures ranging from 25-60°C. Four ml of 24 h grown preculture was inoculated to 50 ml of selected production medium and flasks were incubated at 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C and 60°C for 24 h.The effect of various nitriles and amides for hyper induction of amidotransferase was studied. Different inducers viz. benzonitrile, isobutyronitrile, valeronitrile, acrylonitrile, acetamide, acrylamide, acetamide, butyramide, N-methylacetamide, methacrylamide, acrylamide, urea and acetonitrile (70 mM) were added in the production medium.

III. FACTORIAL DESIGN FOR THE ENHANCEMENT OF PRODUCTION OF AMIDOTRANSFERASE ACTIVITY OF *BACILLUS* SP. APB-6.

Screening of critical media components using a Plackett–Burman design

A total of eight process variables comprising components (yeast extract, glucose, sodium chloride, acetamide, pH, temperature, inoculums size and inducer concentration) variable were studied in Plackett–Burman screening experiments. Experiments were performed at various combinations of 'high' (H) and 'low' (L) values using design expert 9.0.3.1.

Experimental design and data analysis

The concentrations of the media components were optimized by using FCCD of RSM using design expert 9.0.3.1.Amidase activity was determined by using the assay method given by Brammar and Clarke [15].

Time course of amidotransferase induction in presence of N-methylacetamide

The growth curve and acyltransferase activity profile of *Bacillus* sp. APB-6 was studied in 50 ml production medium supplemented with 70 mM N-methylacetamide (as it emerged as the best inducer). Two ml of samples were withdrawn after an interval of 3 h up to 48 h and the amidotransferase activity was assayed.

IV. HYPER INDUCTION OF ACYLTRANSFERASE IN *BACILLUS* SP. APB-6 BY APPLICATION OF INDUCER IN DIFFERENT FEEDINGS

Once the time of incubation for maximum enzyme production was achieved, a study was carried out to find if the application of inducer in different feedings could enhance the enzyme activity and production of enzyme. To investigate the effect of step feeding of inducer different combinations were designed, as shown in the Table 2.

Table 2: Different combinations for feeding of inducer

Combination No.	N-methylacetamide (70 mM)
1	Only once at 0h for 27hrs
2	0h+6h
3	0h+12h
4	0h+18h
5	0h+24h
6	0h+27h
7	0h+6h+12h
8	0h+6h+12h+18h
9	0h+6h+12h+18h+24h
10	0h+6h+12h+18h+24h+27h

V. RESULTS AND DISCUSSION

Selection of medium for amidotransferase production

Among the different media used (Table 1), M12 [17] containing tryptone, NaCl, glucose and yeast extract proved to be the best medium for the production of amidotransferase. Maximum amidotransferase activity (48.31 U/mg dcw) and growth was recorded (21.35 mg dcw/ml) in this medium, hence it was selected for further studies (Fig. 1).

Selection of suitable carbon source and nitrogen source

Although the bacterium utilized all the sugars supplemented in the production medium for its growth, but maximum enzyme production (48.21 U/mg dcw) was achieved with glucose (Fig. 2).Acetamide proved to be the most effective nitrogen supplement as shown in Fig. 3.The maximum enzyme production was recorded 112.9 U/mg dcw. Similarly the *Alcaligenes* sp. MTCC 10674 showed highest acyl transfer activity in mineral salt medium when supplemented with glucose [3]. Yeast extract and meat peptone were selected as nitrogen source and sorbitol as carbon source for amidase production form *R. erythropolis* MTCC 1526 [18]. The presence of amides in the fermentation medium is known to enhance amidase production.

Effect of medium pH and inoculum size on amidotransferase activity of *Bacillus* sp. APB-6

Bacillus sp.APB-6 was stable at broad range of pH (6.0-10.0) both in terms of activity and growth. The maximum enzyme production was recorded at pH 8.0 (129.29 U/mg dcw, Fig 4). *Alcaligenes* sp. MTCC 10674 organism efficiently produced biomass and acyl transfer activity around neutral pH (7.5). Most of amidase producing organism which exhibit acyl transfer activity grow and produce enzyme around neutral pH [3]. Optimum growth (20.06 mg dcw/ml) (Fig. 5) as well as amidotransferase activity (130.03 U/mg dcw) was observed at 8% inoculum level.

Optimization of production temperature and inducer

This organism produced appreciably higher amount of enzyme at 30°C (129.23 U/mg dcw) as compared to other temperatures (Fig. 6). Similar results were obtained for *Alcaligenes* sp. MTCC 10674 which produced maximum acyl transfer activity at 30°C as reported for most of other amidase producing mesophilic organisms [3]. Comparable growth of the organism was also observed at 35 °C, whereas lesser and negligible growth was recorded at above 45°C and 50°C respectively.

Optimization of inducer (Fig.7) showed that 70 mM N-methylacetamide was optimum for induction of amidotransferase from *Bacillus* sp. APB-6 with enzyme activity 130.51 U/mg dcw. Similar inducer acetamide was optimized for amidase activities of *R. erythropolis* MTCC 1526 [18]. Isobutyronitrile (0.4% v/v) proved to be the best inducer for the acyl transfer activity of *Alcaligenes* sp. MTCC 10674 [3].

VI. FACTORIAL DESIGN FOR THE ENHANCEMENT OF PRODUCTION OF AMIDOTRANSFERASE ACTIVITY OF *BACILLUS* SP. APB-6.

Screening Of Significant Media Components Using A Plackett–Burman Design

The Plackett–Burman design served the purpose of ascertaining the critical influential process variables. High F-score value indicates greater significance of the input variable. Thus, depending on the F-score, sodium chloride, pH, temperature and inducer concentration, were found to be the most influential media components (Fig. 8).

Media optimization by RSM

The second-order polynomial equation was used to correlate the independent process variables with amidase production. The second order polynomial coefficient for each term of the equation was determined through multiple regression analysis using the Design Expert 9.0.3.1.After regression analysis; the second order response model was obtained.

Final Equation in Terms of Coded Factors:

R1= +187.77-2.47* A-7.57 * B-11.40* C+6.31* D+1.47* AB+9.28* AC+1.11* AD+3.71BC+17.83* BD-3.11* CD-35.2 * A^2-35.17* B^2-34.68* C^2-5.88* D^2

A low value of coefficient of variation (26.72 %) indicates the very high degree of precision and good reliability of the experimental values.

Table 3: Model fitting values

Std. Dev.	26.44		R-Squared	0.8978
Mean	98.95		Adj R-Squared	0.8024
C.V. %	26.72		Pred R-Squared	0.5258
PRESS	48635.59		Adeq Precision	8.640

The fit of the model can also be expressed by the coefficient of determination, R-square, which was found to be 0.8024, indicating that 80.24% of the variability in the response could be explained by the model. The closer the R-square value is to 1, the better is the model fit to experimental data and the less the distance between predicted and observed values. Adeq Precision measures the signal to noise ratio. A ratio more than 4 is desirable. Here, a ratio of 8.640 indicates an adequate signal (Table 3).

Table 4: ANOVA for Response Surface Quadratic model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob> F	
Model	92087.49	14	6577.68	9.41	< 0.0001	significant
A-sodium chloride	146.72	1	146.72	0.21	0.6534	
B-pH	1376.83	1	1376.83	1.97	0.1808	
C-Temperature	3120.41	1	3120.41	4.46	0.0518	
D-inducer	956.85	1	956.85	1.37	0.2602	
AB	34.40	1	34.40	0.049	0.8274	
AC	1379.01	1	1379.01	1.97	0.1805	
AD	19.67	1	19.67	0.028	0.8690	
BC	220.08	1	220.08	0.31	0.5830	
BD	5088.68	1	5088.68	7.28	0.0165	
CD	154.63	1	154.63	0.22	0.6449	
A^2	34164.35	1	34164.35	48.88	< 0.0001	
B^2	33927.59	1	33927.59	48.54	< 0.0001	
C^2	32988.81	1	32988.81	47.20	< 0.0001	
D^2	948.39	1	948.39	1.36	0.2623	
Residual	10484.24	15	698.95			
Lack of Fit	7763.49	10	776.35	1.43	0.3646	not significant
Pure Error	2720.75	5	544.15			
Cor Total	1.026E+005	29				

The Model F-value of 9.41 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.Values of "Prob> F" less than 0.0500 indicate model terms are significant.In this case BD, A^2, B^2, C^2 are significant model terms.Values greater than 0.1000 indicate the model terms are not significant.The "Lack of Fit F-value" of 1.43 implies the Lack of Fit is not significant relative to the pure error. There is a 36.46% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit (Table 4). A perturbation plot was obtained to observe the relative effect of media components on amidase activity fig (Fig 9). The perturbation plot indicates that (A) sodium chloride is most influential medium component; wheras (D) has least influence on amidotransferase activity.The three dimensional plots of the statistically significant interactions are shown in (Fig.10). These plots were obtained from the pair-wise combination of two independent variables, while keeping the other two variables at their centre point levels. The contour response plots highlight the roles of played by process variables and their interactive effects.

Validation of model

The maximum enzyme activity obtained by performing experiment 198.31 U/mg dcw which is closely related to 198.98 U /mg dcw predicted value calculated by ANNOVA analysis. A perturbation plot (Fig. 8) was obtained to observe the relative Effect of media components on amidase activity and showed optimum value for variable; 0.35% sodium chloride, 70 mM inducer, 7.0 pH and 32.5°C temperature. The perturbation plot indicates that sodium chloride (D) is the most influential medium component on amidotransferase activity. The model was validated by performing the experiment under optimum condition, which resulted 198.5 U/mg dcw thus proved the validity for the model. Amidase activities before and after optimisation were 48.31 units/ mg dry cells and 198.5 units/ mg dry cells, respectively. Thus, use of RSM increased production of amidase by 4.11 - fold.

Time course of amidotransferase induction in presence of N-methylacetamide

The bacterium was fast growing and attained stationary phase in 27 h (33.27 mg dcw/ml). Final pH of the culture increased with the growth of the bacterium (Fig. 11). Maximum enzyme activity, 214.34 U/mg dcw (after 24 h) and 33.39 U/mg dcw (after 30 h) was recorded (Fig.10).During the course of fermentation, maximum amidase activity was found at 36 h from *R. erythropolis* MTCC 1526 [18].

Hyper induction of acyltransferase in *Bacillus* sp. APB-6 by application of inducer in different feedings

Out of the ten different combinations designed for the application of inducer, the step-feeding of inducer at 0, 6 and 12 h of incubation showed maximum enzyme activity (215.36 U/ mg dcw).Although there was not much variation in growth of the bacterium with a single feed and multiple feedings of inducer but multiple feedings of inducer resulted in enhanced induction of enzyme (Fig. 11). After 24 h of incubation, hyper induction of acyl transfer activity was observed with the addition of 0.4% isobutyronitrile in the medium in *Alcaligenes* sp. MTCC 10674 [3].

VII.CONCLUSIONS

The production of amidotransferase was found to depend greatly on four media component (pH, temperature, sodium chloride and inducer). Using RSM it was possible to model individual and interactive effects of media components on production of amidase. Medium optimization by RSM effectively enhanced amidotransferase production by 4.11 fold.

VIII. ACKNOWLEDGEMENTS

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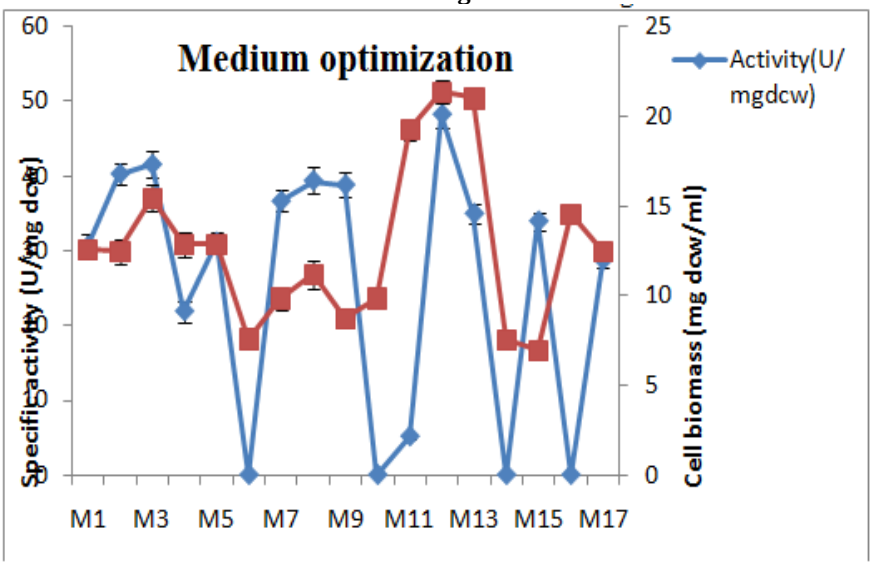

Fig. 1 Medium optimization

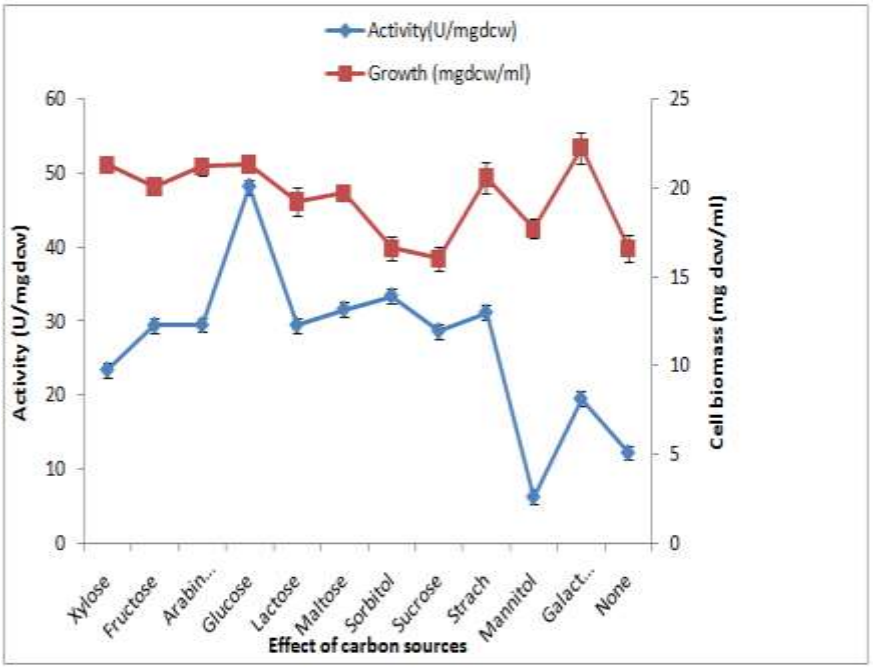

Fig. 2 Effect of various carbon sources

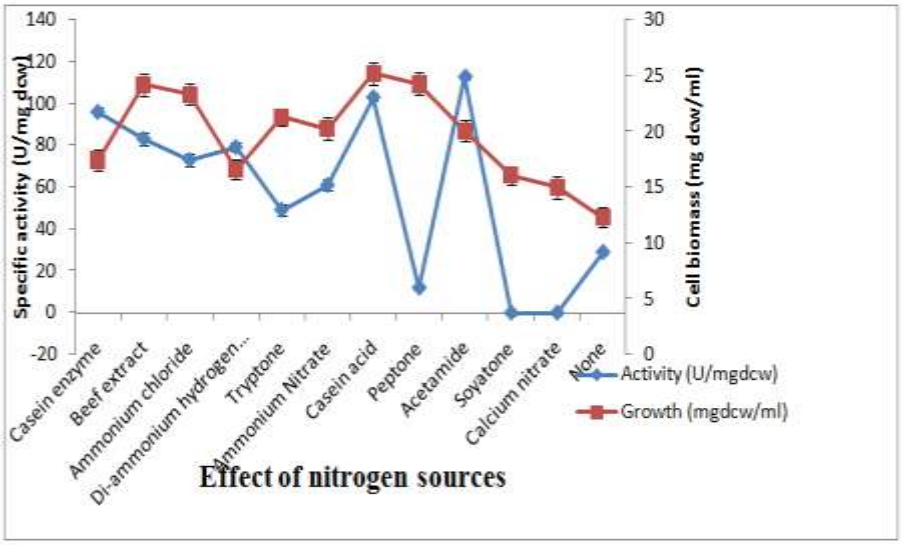

Fig. 3 Effect of various various nitrogen sources

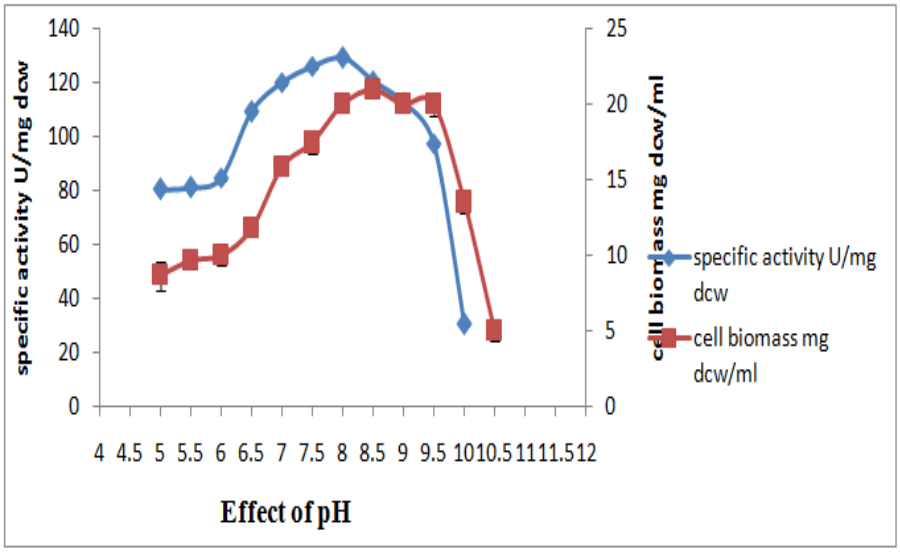

Fig. 4 Effect of pH

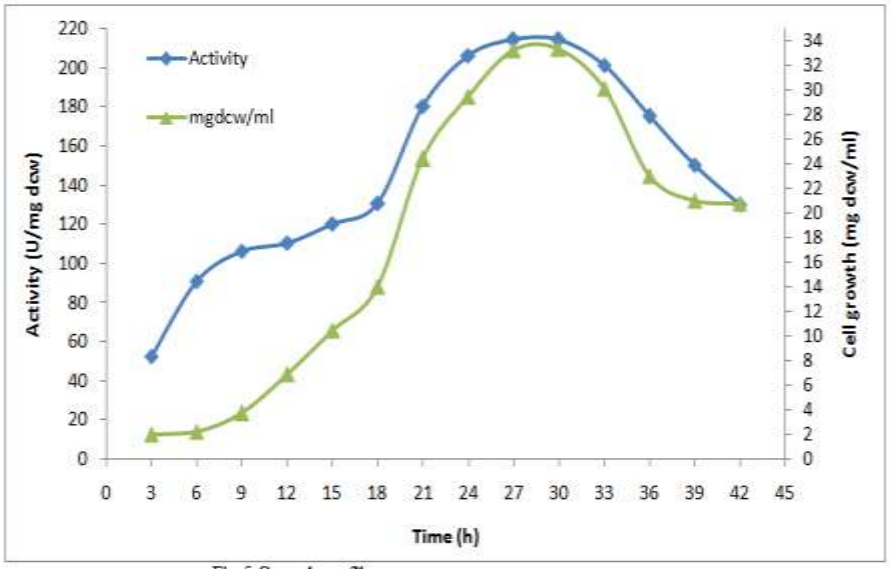

Fig.5 Growth profile.

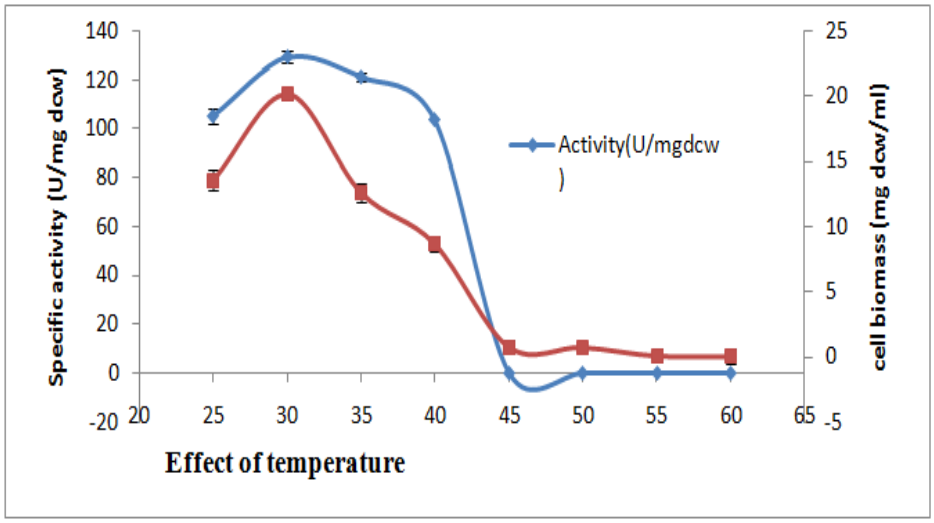

Fig.6 Effect of temperature

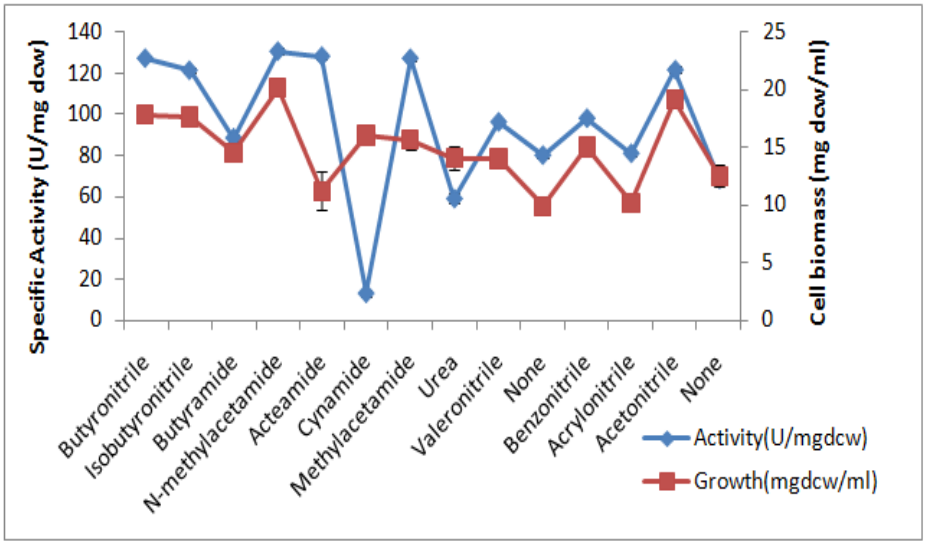

Fig. 7 Effect of various nitriles and amides on induction of amidotransferase.

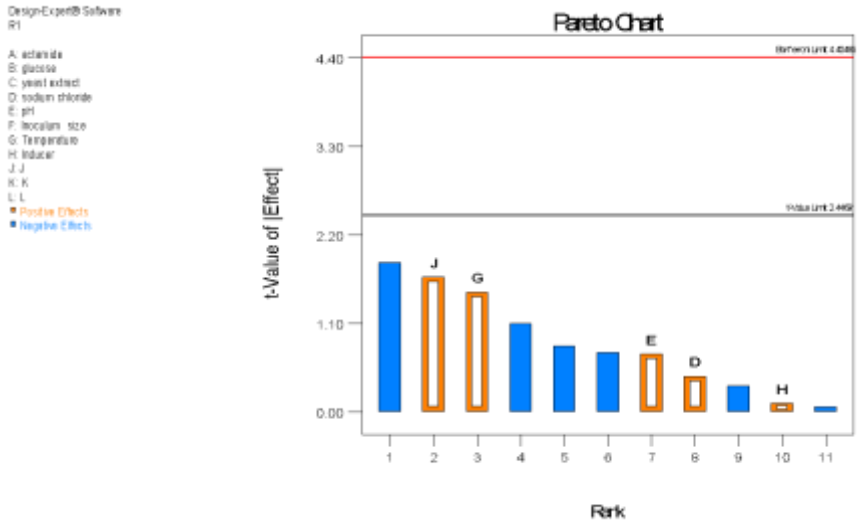

Fig.8 Pareto chart.

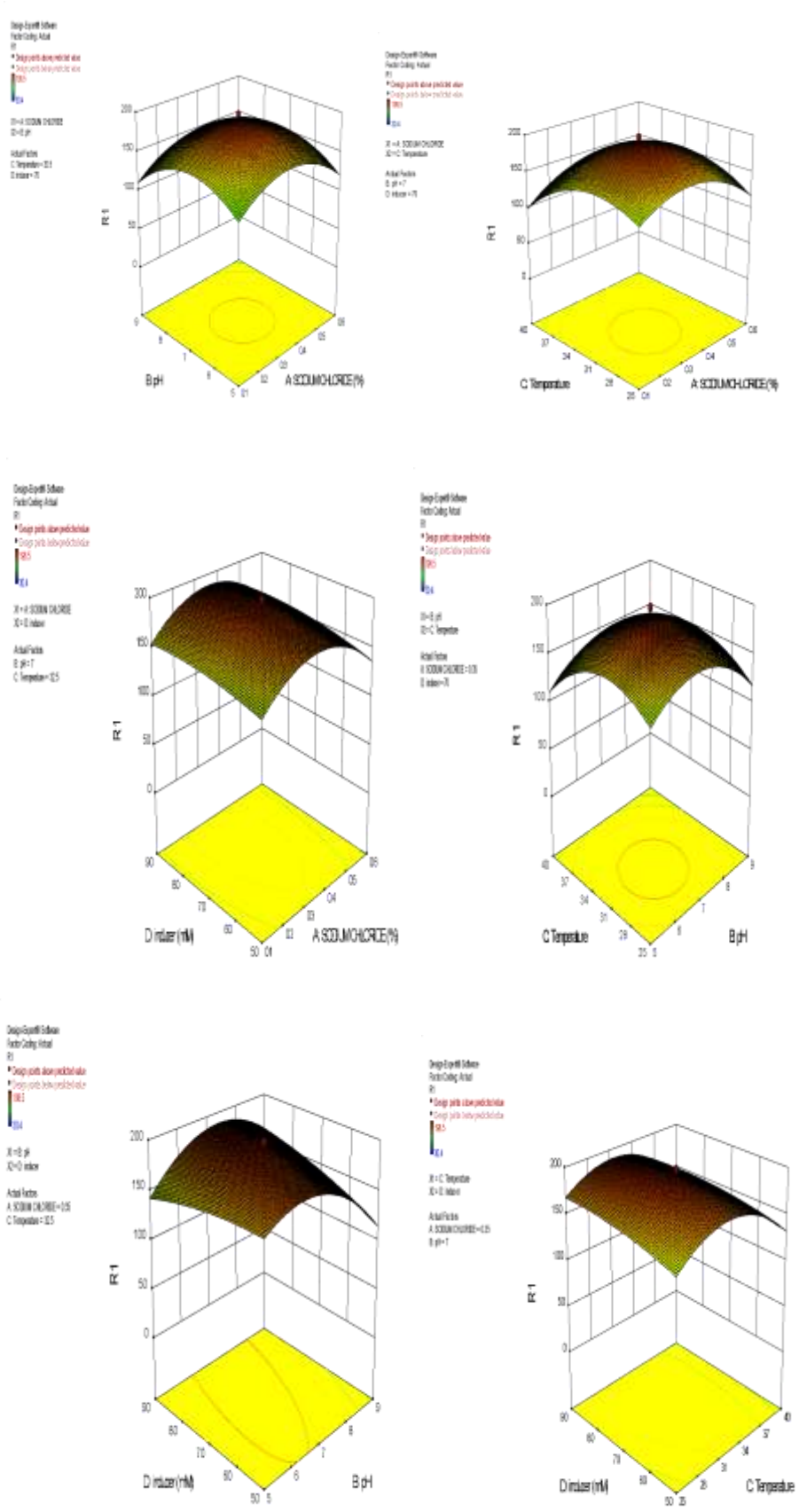

Fig. 9 3D graphs showing interaction between two variables.

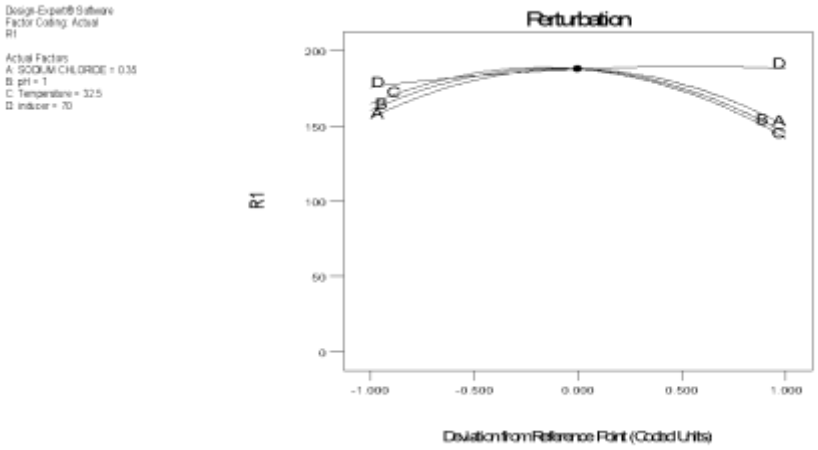

Fig.10 **Perturbation Plot.**

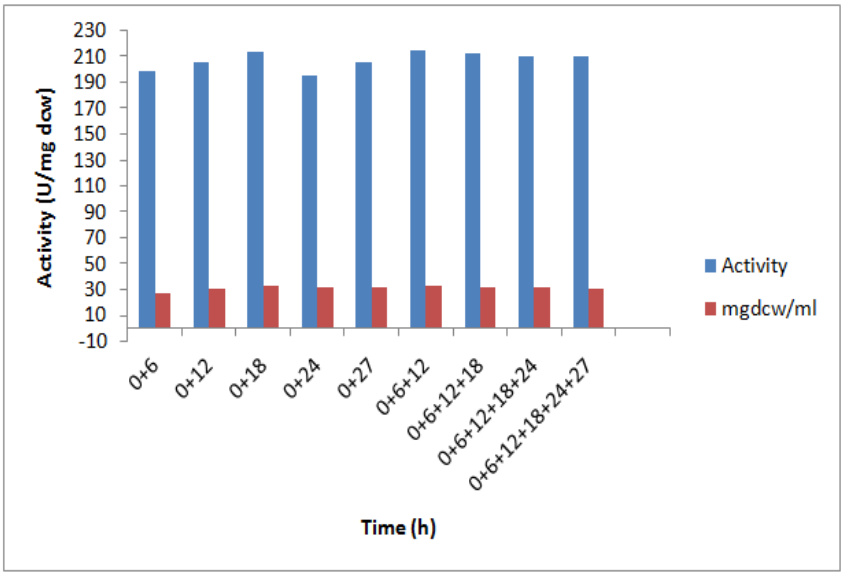

Fig.11 Effect of inducer feeding at different time interval on enzyme activity.

Table 1 : Composition Of Different Media Used

Media	Reference	Composition (g/l)
M1	GY Medium (Kobayashi et al. 1993).	Glycerol (10), KH_2PO_4(0.5), K_2HPO_4 (0.5), $MgSO_4.7H_2O$ (0.1),Yeast extract (1.0), Peptone (5).
M2	Modified Nutrient Broth[a] (Robaset al. 1993).	Bactopeptone (20), Sodium chloride (5), Glucose (2).
M3	Modified Nutrient Broth[a] (Piotraschkeet al. 1994).	Tryptone (30), Yeast extract (15), Sodium chloride (5).
M4	Modified Nutrient Broth (Black et al. 1996).	Peptone (12.5), Yeast extract (3), Beef extract(5), Sodium chloride (5).
M5	Nutrient Broth	Peptone (5), Beef extract (3).
M6	Enriched Nutrient Broth (Bhallaet al. 1997).	Peptone (5), Beef extract (3), Yeast extract (1) Glucose (10).
M7	MY Medium (Watanabe et al. 1987).	Glucose (15), Peptone (5), Yeast extract (3), Malt extract (3).
M8	Modified MY Medium (Kobayashi et al. 1989).	Glycerol (10), Peptone (5), Yeast extract(3), Malt extract (3).
M9	Mineral Salt Medium (Bhallaet al. 1992).	$Na_2HPO_4.2H_2O$ (2.5), KH_2PO_4 (2), $MgSO_4.7H_2O$ (0.5), $FeSO_4.7H_2O$ (0.03), $CaCl_2$. $2H_2O$ (0.06), Yeast extract (0.1).
M10	APY Mineral Salt Medium (Bhallaet al. 1992).	$(NH_4)_2HPO_4$ (5), Peptone (5.0), Yeast extract (3), K_2HPO_4 (5), $MgSO_4.7H_2O$ (0.2), $FeSO_4.7H_2O$ (0.02).
M11	Modified Nutrient Broth[b] (Robaset al. 1993).	Peptone (20), Sodium chloride (5), Glucose (2), Yeast extract (3), Beef extract(3).

M12	Modified Nutrient Broth[b] (Piotraschkeet al. 1994).	Tryptone (30), Yeast extract (15), Sodium chloride (5), Glucose (2).
M13	Modified Nutrient Broth[c] (Pandyet al. 2012).	Peptone (26), Sodium chloride (3), Arabinose (5), $(NH_4)_2HPO_4$(6.6), Beef extract (7).
M14	Self- formulated media MM 1.	KH_2PO_4 (5), Sodium chloride (5), $MgSO_4.7H_2O$ (2), $FeSO_4.7H_2O$ (0.2).
M15	Self- formulated media MM 2.	$(NH_4)_2HPO_4$ (5), $MgSO_4.7H_2O$ (2), $FeSO_4.7H_2O$ (0.2).
M16	Self- formulated media MM 3.	Glucose (10), Sodium chloride (5), KNO_3 (5.0)
M17	Self- formulated media MM 4.	Glucose (10).

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