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RESEARCH ARTICLE

Application of Response Surface Methodology in Process Parameter Optimization of Media for Production of Amylase

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ABSTRACT:

Agricultural wastes are being discarded and those wastes get accumulated in the environment. On the other hand, amylase production has become significant for its use in various industries like food, fermentation, brewing, detergent and paper industries. For this elevating demand, amylase production has to be increased. By using the agricultural waste as substrate source amylase enzyme can be produced in higher quantities. Using *Bacillus subtilis* as microbial source, agricultural waste can be utilised for the production of amylase in submerged fermentation as well as in solid state fermentation. Usage of synthetic media is very expensive and hence normal nutrient broth can be used. By using OVAT procedure, parameters such as temperature, pH, and substrate concentration can be varied and the optimum level at which enzyme production is higher can be determined. Response surface methodology is used for efficient production of enzyme. The enzyme produced in this process could be useful in reducing the demand of amylase in commercial use.

KEYWORDS: Amylase, OVAT, Submerged Fermentation, Response Surface Methodology.

INTRODUCTION:

Enzymes are macromolecular biocatalyst. These are responsible for many metabolic activities that take place inside the body. Also enzymes are highly useful in industrial purposes. For these industrial applications, enzymes must be produced in larger quantities. For production of enzymes in industrial scale[1], Agricultural waste usually contains a huge amount of starch in it. Starch is a polysaccharide which can be used to produce amylase by microorganisms through the process of fermentation[2].

Agricultural wastes such as sugarcane bagasse, wheat bran, rice bran, corn cob and wheat straw are cheapest and abundantly available natural carbon sources[3]. Attenuating the fluctuations in postprandial glycaemia and insulinaemia is important in the prevention and treatment of life-style associated diseases, notably diabetes mellitus and cardiovascular disease, and also has implications for obesity management[4]. With increase in its application spectrum, the demand is for the enzyme with specificity. Research is focused on developing thermo tolerant and pH tolerant a-amylase from microbes, modifying them genetically or applying site-directed mutagenesis to acquire desired properties in the enzyme[5].

The substrates are utilized rapidly and hence need to be constantly supplemented with nutrients[6]. Other variables

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that affect submerged fermentation include the pH level of the liquid, temperature and ionic strength. SMF is primarily used in the extraction of secondary metabolites that need to be used in liquid form[7].

Solid state fermentation (SSF) is a biomolecule manufacturing process used in the food, pharmaceutical, cosmetic, fuel and textile industries. This technology for the culture of microorganisms is an alternative to liquid or submerged fermentation, used predominantly for industrial purposes[8].

Microorganisms are capable of utilising the organic matter in wastes both as a source of energy and as carbon source for the synthesis of cell biomass. These wastes could serve as inexpensive fermentation source[9].

Response surface methodology (RSM) is a collection of mathematical and statistical techniques for empirical model building. The objective is to optimize a response (output variable) which is influenced by several independent variables (input variables). Central Composite[10].

MATERIALS AND METHODS:

Optimization of Amylase Production:

Bacterial Strain and Culture Media:

The medium for the production of *Lactobacillus acidophilus* consist of1% peptone, 0.5% sodium acetate tetra hydrate, 0.1% tween 80, 0.2% dipotassium hydrogen phosphate, 0.2% triammonium citrate, 0.02% magnesium sulfate tetra hydrate, 0.005% manganese sulfate tetra hydrate. The above chemicals were mixed and sterilized properly with the purified strain. The production medium was incubated for 24 hours at room temperature with RPM range of 150.

Media Optimization for Production of Amylase Enzyme in Submerged Fermentation:

Optimization of production medium has been carried out in OVAT (One Variable at A Time) procedure. The results of OVAT will then be utilized to run the design experiment in Response Surface Methodology (RSM). RSM is the statistical method to conduct the experiment. The procedures were carried out by above described shaken flask method.

One Variable at A Time analysis for amylase production:

The physical conditions of the media for the production of amylase enzyme were optimized by OVAT. The factors that were analyzed for the optimization of basal production medium includes temperature (°C), substrate concentration (%),and incubation time (hours). These parameters must be analyzed for efficient production of amylase within the given nutrient conditions. And those are as follows.

Effect of temperature on amylase production:

Temperature for production of amylase has been set with the reference of appropriate value at which the amylase production will be higher and also at which the production of amylase enzyme will be stable. The temperature variations can be set as 30°C, 35°C, 40°C, 45°C. This is because amylase will be stable at 37°C.

Effect of substrate concentration on amylase production:

By varying the substrate concentration, the required amount ofsubstrate for the efficient production of amylase can be identified.As the enzyme enquired here is amylase, any glucose source can be given. Here starch has been used as a substrate for the production of amylase. The substrate concentration variation has been set as 0.5%, 1%, 1.5%.

Effect of incubation period on amylase production:

Incubation period is one of the important criteria for efficient production of any enzyme. It determines the activity and also the amount of enzyme produced. With all the referred articles it has been concluded that activity of amylase ill reach its peak value at 72hrs. So the incubation period variation has been set as 24hrs, 48hrs, 72hrs, and 96hrs.

Statistical optimization by Response Surface Methodology (RSM):

RSM using Central Composite Design has been used to find out the analysis of optimum concentration of medium constituents. The experiments have been carried out with the help of Design Expert software 7.1.6 software package. RSM consist of a collection of statistical and mathematical techniques useful for developing, improving and optimizing the enzyme production process.

RSM defines the effect of the independent variables, alone or in combination, on the process. In addition to analyzing the effects of the independent variables, this experimental methodology generates a mathematical model that accurately describes the overall process.It is a general linear model in which attention is focused on where the optimum response value occurs.A computer generated design in which a contour plot is obtained to determine the optimal parameter setting. It has been successfully applied to optimizing conditions in food, chemical and biological processes.

Experimental design of RSM for optimizing of media components:

In statistics, the response surface methodology (RSM) explores the relationships between several explanatory variables and one or more response variables. The main idea of RSM is to use a sequence of designed experiments to obtain an optimal response. This model is only approximation, but this model is easy to estimate

and apply, even when little is known about the process. RSM is an empirical statistical modelling technique employed for multiple regression analysis using quantitative data obtained from properly designed experiments to solve multivariate equations simultaneously. RSM is used to determine the optimum nutrient concentrations, for the production of scleroglucan. A

Central composite rotatable experimental design (CCRD) for four independent variables was used. The relationship of the independent variables and the response was calculated by the second-order polynomial Eq.

The three variables under consideration has been optimized by the response surface approach by using the set of experimental design (central composite design with five coded levels) was performed. For the three factors, this design was made up of a full 2^3 factorial design with its eight points augmented with the replications of the center points (all factors at level 0) and the six star points, that is, points having for one factor an axial distance to the center of ±α, whereas the other two factors are at the level of 0. The axial distance α was chosen to be 1.68 to make the design orthogonal. A set of 20 experiments were carried out for the three variables.

In developing the regression equation, the test factors were coded according to the following equation:

$$Xi=\frac{(xi-xo)}{\Delta xi}$$

Where xi is the code value of the*i*th independent variable, Xi is the natural value of the *i*th independent variable, X0 the natural value of the *i*th independent variable at the centerpoint, and ΔXi is the step change value of variables. For a three-factor system, the model equation is:

(Y=b0+b1x1+b2x2+b3x3+b11x1^2+b22x2^2+b33x3^2 +b12x1x2+b13x1x3+b23x2x3)

Where: Y-predicted response; b0- intercept; b1, b2 and b3 – linear coefficients; b11, b22 and b33 – squared coefficients and b12, b13 and b23 – interaction coefficients.

Table 3.1 Design summary for amylase enzyme production in submerged fermentation

Variables	-1	0	+1
Temperature(°C)	35	40	45
Substrate concentration (%)	0.5	1	1.5
Incubation period(hours)	48	72	96

Table 3.2 Experimental design and 2^3 factorial design for submerged fermentation

RUN	VARIABLES		
	A:TEMPERA-TURE	B:SUBSTRATE CONCEN-TRATION	C:INCUBA-TION PERIOD
1	40	1	72
2	35	1.5	72
3	40	1	72
4	40	0.5	48
5	45	0.5	72
6	45	1	96
7	40	1	72
8	40	1.5	48
9	40	1	72
10	35	1	96
11	40	1.5	96
12	40	0.5	96
13	45	1	48
14	45	0.5	72
15	35	1.5	72
16	35	1	48
17	40	1	72

Enzyme Assay: Dinitro Salicylic Acid Assay:

Amylase activity has been measured with the help of DNS assay in which starch is used as a substrate. 1 ml of enzyme source is added with 1% starch dissolved in 0.1M phosphate buffer at 55°C for 15 min. With this 1 ml of 3, 5dinitro salicylic acid is added and boiled for 10 min. the final volume was made upto 12ml with D.H2O. Finally the OD was measured at 540 nm.

$$\text{Amylase activity (U/ml)} = \frac{\text{(mg of enzyme produced)}}{\text{(volume of enzyme solution)}} \times \text{incubation time}$$

Preparation of enzyme solution:

The enzyme solution has been prepared by centrifuging the crude enzyme solution in the incubated media at 5000 rpm for 10 min. The supernatant is taken as the enzyme source and the pellet is discarded. This supernatant is used as the enzyme solution for performing the assay.

RESULTS AND DISCUSSION:

Media Optimization for the Production of Amylase in Submerged Fermentation:

The results for the optimization studies of submerged fermentation process for the production of amylase has been obtained in the form of graphs and tables. Optimum values in the OVAT procedure has been given as an input for performing the RSM in design expert software. The designed experiment which acts as result in RSM is given in the form of table. The ANOVA results are also given in the corresponding tables as subscript alphabets. There will be no significant variation with the values.

Extracellular amylase production is strongly influenced by media components there are provided to the growth of microorganism. Those media components included variation in C/N ratio, presence of some easily metabolisable sugars, such as aeration, inoculum density, pH, temperature and period of incubation also affect the amount of enzyme produced. In the present study, culture conditions have been found to influence the quantity of amylase produced by the selected strain.

To optimize the media conditions, One Variable at A Time method and Response Surface Methodology have been used. One Variable at A Time method is carried out by varying the media conditions such as temperature, substrate concentration, and incubation time. After the significant values have been obtained in the OVAT procedure, Response Surface Methodology has been performed, more significant values have been obtained while performing OVAT.

By using the final equation in terms of coded values generated in Response Surface Methodology, Genetic Algorithm has been performed. Current best fitness and best individual values of amylase enzyme production in submerged fermentation will be generated.

Single Variable Analysis for Amylase Enzyme Production

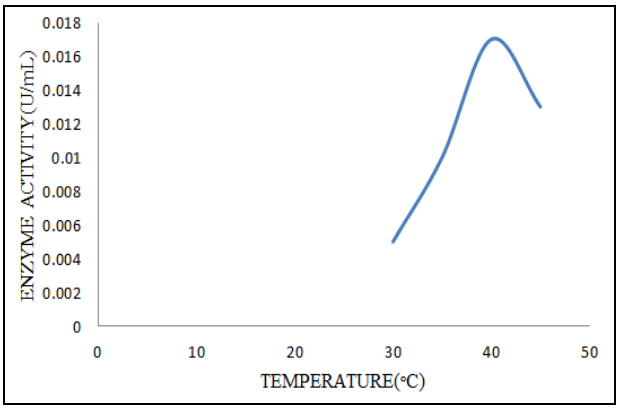

Figure 4.1 (a) Effect of temperature on amylase enzyme production

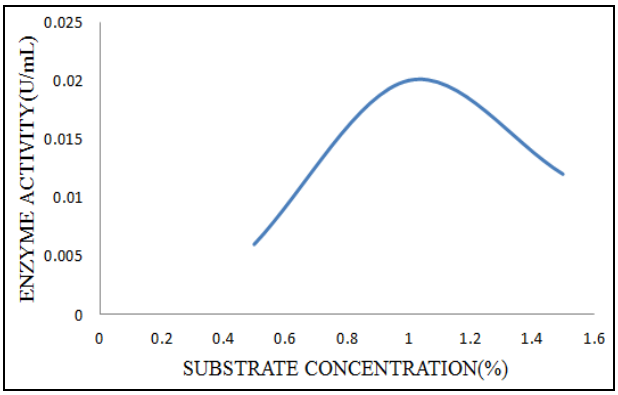

Figure 4.1 (b) Effect of substrate concentration on amylase enzyme production

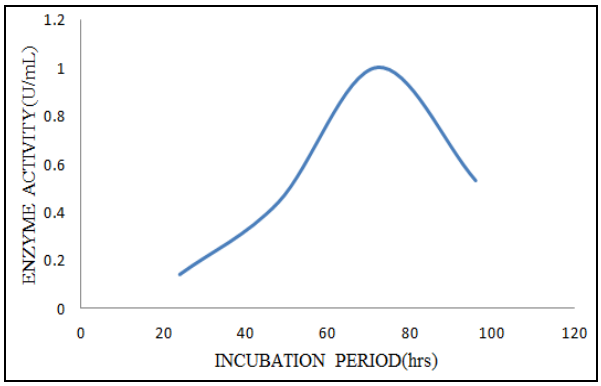

Figure 4.1 (c) Effect of incubation period on amylase production

DISCUSSION:

Studies have revealed that to produce amylase with the use of Bacillus subtilis, the temperature was found to be optimum at 38°C, the substrate concentration was found to be 1% and the incubation time was 24 hrs[8]. As this study incorporates Lactobacillus acidophilus culture, the temperature was found to optimum at 45^0C, the substrate concentration as 1% and the incubation time was found to be optimum at 72 hrs. This clearly shows that the substrate concentration remains same for both the cultures whereas incubation time and temperature varies depending upon the type of culture used.

Statistical optimization using Response Surface Methodology:

The optimum conditions for enzyme production were determined by means of the CCD under RSM. The results are displayed in Table 4.1

Table 4.1: optimization of temperature, substrate concentration and incubation period for the enzyme production by CCD under RSM

RUN	VARIABLES			
	A: TEMPERA -TURE	B:SUBSTRATE CONCEN- TRATION	C:INCUBA -TION PERIOD	ENZYME ACTIVITY (U/mL)
1	40	1	72	0.073
2	35	1.5	72	0.067
3	40	1	72	0.081
4	40	0.5	48	0.042
5	45	0.5	72	0.051
6	45	1	96	0.009
7	40	1	72	0.075
8	40	1.5	48	0.036
9	40	1	72	0.071
10	35	1	96	0.03
11	40	1.5	96	0.026
12	40	0.5	96	0.029
13	45	1	48	0.021
14	45	0.5	72	0.052
15	35	1.5	72	0.063
16	35	1	48	0.031
17	40	1	72	0.076

Statistical Analysis of amylase activity:

Table 4.2 sequential model sum of squares for amylase activity in submerged fermentation

Source	Sum of squares	Df	Mean square	F	p-value	
Mean vs Total	**0.041**	**1**	**0.041**	**0.37**	**0.7729**	
Linear vs Mean	**6.682E-004**	**3**	**2.227E-004**	**0.015**	**0.9975**	
2FI vs Linear	**3.350E-005**	**3**	**1.117E-005**	**< 0.0001**	**Suggested**	
Quadratic vs 2FI7.620E-003	**3**	**2.540E-003**	**223.51**	**0.53**	**0.6833**	**Aliased**
Cubic vs Quadratic	**2.275E-005**	**3**	**7.583E-006**			
Residual5.680E-005	**4**	**1.420E-005**				
Total 0.050	**17**	**2.924E-003**				

"Sequential Model Sum of Squares [Type I]": Select the highest order polynomial where the additional terms are significant and the model is not aliased.

Table 4.3 Lack of fit tests for amylase activity in submerged fermentation

Source	Sum of squares	Df	Mean square	F	p-value	
Linear	7.676E-003	9	8.529E-004	60.07	0.0007	
2FI	7.643E-003	6	1.274E-003	89.71	0.0003	
Quadratic	2.275E-005	3	7.583E-006	0.53	0.6833	Suggested
Cubic	0.000	0				Aliased
Pure error	5.680E-005	4	1.420E-005			

"Lack of Fit Tests": Want the selected model to have insignificant lack-of-fit.

Table 4.4: Model summary statistics for amylase activity in submerged fermentation

Source	Std. dev.	R-square	Adjusted R-square	Predicted R-square	PRESS	
Linear	0.024	0.0795	-0.1329	-0.5351	0.013	
2FI	0.028	0.0835	-0.4664	-2.0804	0.026	
Quadratic	3.371E-003	0.9905	0.9784	0.9461	4.528E-004	Suggested
Cubic	3.768E-003	0.9932	0.9730		+	Aliased

"Model Summary Statistics": Focus on the model maximizing the "Adjusted R-Squared" and the "Predicted R-Squared".

Table 4.5: ANOVA table for amylase activity in submerged fermentation
Analysis of Variance Table

SOURCE	SUM OF SQUARES	Df	MEAN SQUARE	F VALUE	p-VALUE Prob>F	SIGNIFICANCE
Model	8.322E-003	9	9.247E-004	81.37	<0.0001	Significant
A-Temperature	4.961E-004	1	4.961E-004	43.66	0.0003	
B-substrate concentration	1.013E-005	1	1.013E-005	0.89	0.3767	
C-incubation period	1.620E-004	1	1.620E-004	14.26	0.0069	
A1.000E-006	1	1.000E-006	0.088	0.7754		
AC3.025E005	1	3.025E-005	2.66	0.1468		
BC2.250E006	1	2.250E-006	0.20	0.6698		
$A^2$7.22E-004	1	7.226E-004	63.58	<0.0001		
$B^2$2.846E-005	1	2.846E-005	2.50	0.1575		
$C^2$6.52E-003	1	6.520E-003	573.70	<0.0001		
Residual	7.955E-005	7	1.136E-005			
Lack of Fit	2.275E-005	3	7.583E-006	0.53	0.6833	Not significant
Pure Error	5.680E-005	4	1.420E-005			
Cor Total	8.402E-003	16				

The Model F-value of 81.37 implies the model is significant. There is only a 0.01% chance that a "Model 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 A, C, A^2, $C^2$are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy),model reduction may improve your model.

The "Lack of Fit F-value" of 0.53 implies the Lack of Fit is not significant relative to the pure error. There is a 68.33% 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.

Std. Dev.	3.371E-003	R-Squared	0.9905
Mean	0.049	Adj R-Squared	0.9784
C.V. %	6.84	Pred R-Squared	0.9461
PRESS	4.528E-004	Adeq Precision	26.136

The "Pred R-Squared" of 0.9461 is in reasonable agreement with the "Adj R-Squared" of 0.9784.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 26.136 indicates an adequate signal. This model can be used to navigate the design space.

Table 4.6: Correlation coefficient for amylase activity in submerged fermentation

Factor	Coefficient estimated	Df	Std. error	95% confidence interval-low	95% confidence interval-high	Variation inflation factor
Intercept	0.075	1	1.508E-003	0.072	0.079	1.00
A-Temperature	-7.875E-003	1	1.192E-003	-0.011	-5.057E-003	1.00
B-Substrate concentration	-1.125E-003	1	1.192E-003	-3.943E-003	1.693E-003	1.00
C-Incubation time	-4.500E-003	1	1.192E-003	-7.318E-003	-1.682E-003	
AB5.000E-004	**1**	1.686E-003	-3.486E-003	4.486E-003	1.00	
AC-2.750E-003	1	1.686E-003	-6.736E-003	1.236E-003	1.00	
BC7.500E-004	1	1.686E-003	-3.236E-003	4.736E-003	1.00	
A^2-0.013	1	1.643E-003	-0.017	-9.215E-003	1.01	
B^2-2.600E-003	1	1.643E-003	-6.485E-003	1.285E-003	1.01	
C^2-0.039	1	1.643E-003	-0.043	-0.035	1.01	

Final equation in terms of coded factors:

Amylase activity =
++0.075-7.875E-003* A-1.125E-003* B-4.500E-003*C
+5.000E-004* A * B-2.750E-003 * A * C+
7.500E-004 * B * C-0.013 * A^2
-2.600E-003 * B^2-0.039* C^2

Final equation in terms of actual factors:

Amylase activity =
1.10250+0.041795*Temperature+6.05000E-003*Substrate concentration+0.010504 * Incubation time+2.00000E-004* Temperature * Substrate concentration-2.29167E-005* Temperature * Incubation time+6.25000E-005 * Substrate concentration * Incubation time-5.24000E-004* $Temperature^2$-0.010400 * Substrate $concentration^2$-6.83160E-005 * Incubation $time^2$

The p-values lower than 0.05 indicated that the model and model terms were statistically significant. All the factors and their square interactions (P<0.05) except for interaction of temperature-temperature and substrate concentration- substrate concentration were significant at the 95% confident level (R^2=0.9744), which suggested that there is an excellent relationship between the experimental and suggested procedure.

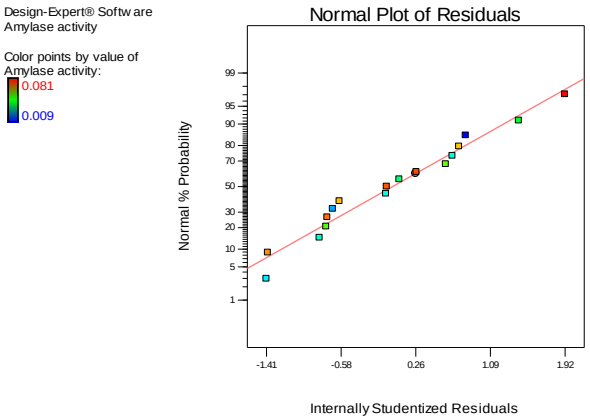

Fig 4.2(a): The above linear plot shows the normal plot of residues.

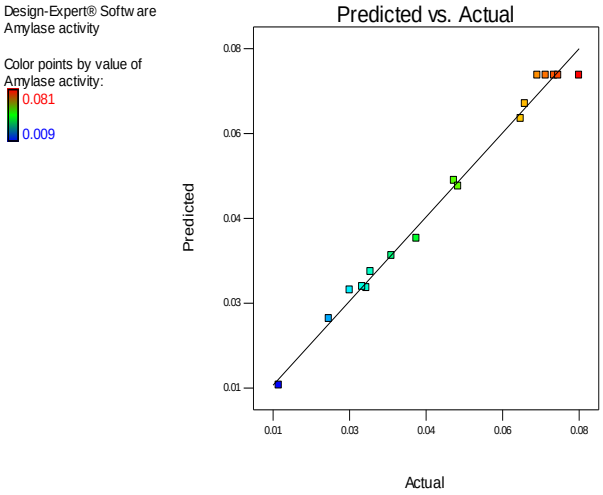

Fig 4.2(b): This plot shows the correlation between the predicted and actual results obtained.

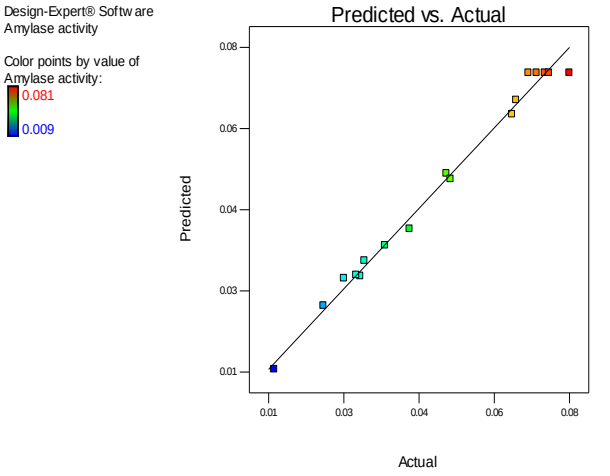

Fig 4.2(c): Box-cox plot for power transforms shows the any transforms between the variance

Figure 4.2: Diagnostic plots for amylase activity in submerged fermentation

Response Surface Methodology:

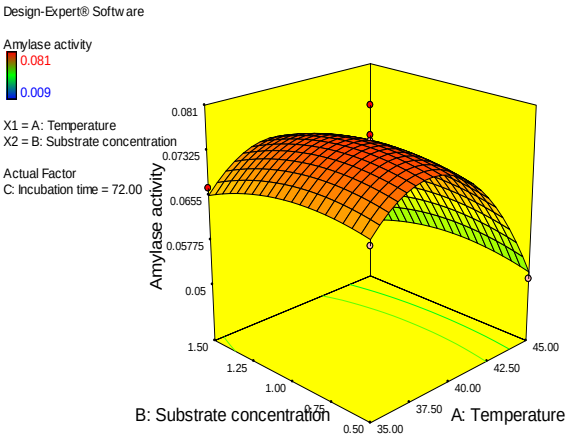

Fig 4.2.1(a): the above plot shows the 3D plot for substrate concentration vs temperature

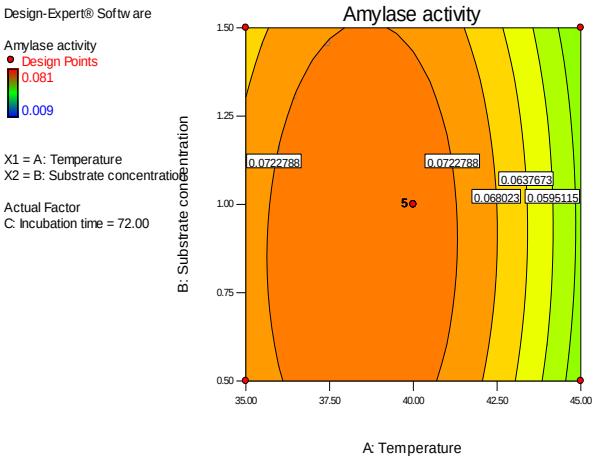

Fig 4.2.1(b): The above plot shows the contour plot forsubstrate concentration vs temperature

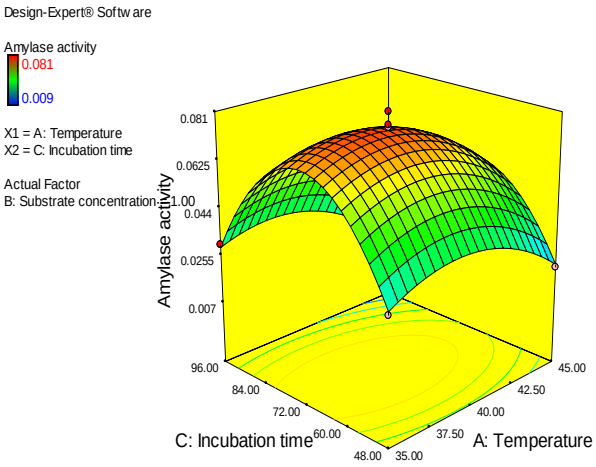

Fig 4.2.1 (c): The above plot implies the 3D plot for incubation time vs temperature

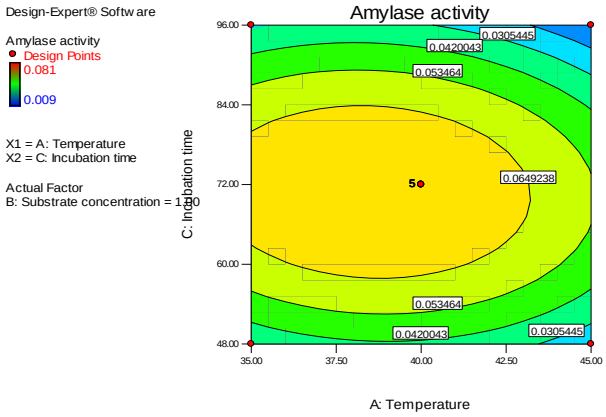

Fig 4.2.1(d): The above plot shows the contour plot for incubation time vs temperature

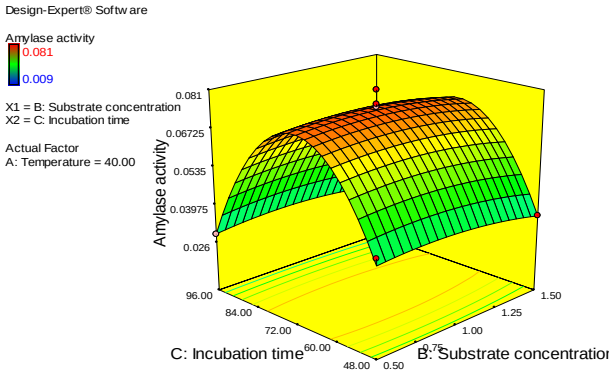

Fig 4.2.1(e): The above graph shows the 3D plot for incubation time vs substrate concentration

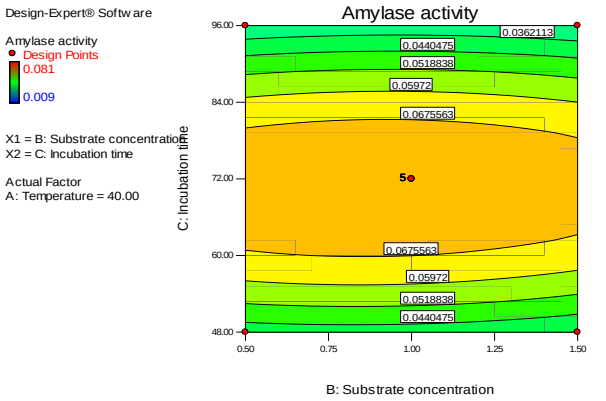

Fig 4.2.1(f): The above graph shows the contour plot for incubation time vs substrate concentration

DISCUSSION:

The studies revealed that the contour plot for the variables used must have limited level and should have acceptable values of interaction between the variables used[10]. The correct umbrella shaped graphs shows that the parameters have high effect on the enzyme production. In this study, incubation time and temperature have high effect on amylase production.

Table 4.7: Result for RSM for submerged fermentation (Maximum values)

Factor	Name	Level	Low Level	High Level	Std. Dev.	Coding
A	Temperature	38.50	35.00	45.00	0.00	Actual
B	Substrate concentration	0.87	0.50	1.50	0.00	Actual
C	Incubation time	70.82	48.00	96.00	0.00	Actual

Response	Prediction	SE Mean95%	CI low95%	CI high	SE Pred	95%PI low	95% PI
Amylase activity	0.0766345	1.479E-003	0.073	0.080	3.681E-003	0.068	0.085

Genetic Algorithm for enzyme production in submerged fermentation:

Genetic algorithm can be performed by using the final equation in coded factors generated in RSM. Then parameter values will be generated in genetic algorithm. The coded values can then be converted to actual values using the specified formula. The Parameter values(coded values) generated in genetic algorithm will then be converted into actual values using the formula

$$Xi = \frac{xi - xo}{\Delta xi}$$

The above equation is used to convert coded values to actual values.

Where Xi = coded values, xi = natural value at centre point, Δxi = step change, xo = natural value.

The following are the coded values generated in genetic algorithm.

- Temperature – 0.026
- Substrate concentration – 0.09
- Incubation period – 0.001

After performing genetic algorithm, the following are the results obtained for varied media conditions.

Temperature – 40.13°C

Substrate concentration – 1.045%

Incubation period – 72.024 hours

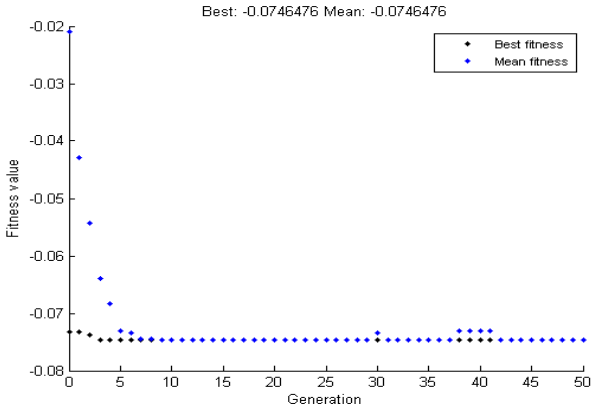

Fig 4.2.2 (a): The above graph shows the best fitness between the predicted and actual results

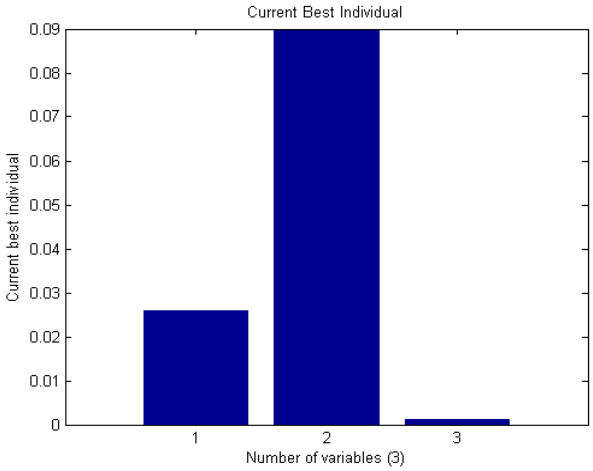

Fig 4.2.2 (b):

The above graph shows the current best individual that affect the enzyme activity more.

The variables implied in graph are 1.Temperature, 2. Substrate concentration, 3. Incubation time. Inference of the graph shows that substrate concentration is highly responsible for the enzyme activity.

DISCUSSION:

In the recent studies, research in genetic algorithm for the production of enzyme has revealed that GA will be used to identify current best fitness of the values obtained[7]. In this study, current best fitness of accurate values for the parameters set has been obtained as substrate concentration showed high fitness when compared with other parameters. This clearly shows that substrate concentration highly affects the enzyme production

CONCLUSION:

In the present investigation, amylase producing *Lactobacillus acidophilus* has been grown in media that was optimized by One Variable at A Time analysis, Central Composite Design and Genetic Algorithm. The One Variable at A Time method was carried out by varying the media conditions such as temperature, substrate concentration and incubation period. Currently, the industrial and environmental applications of the commercialization of amylase have an important role. Although, its application remains very less when compared with the hydrolytic enzymes. This enzyme

can be used in different formulation for innovative processing. This leads to substantial increase in utilization of the glucose source. Amylase production by *Lactobacillus acidophilus* significantly enhance through optimization of submerged fermentation. Production was correlated with the concentration of temperature, substrate concentration and incubation period. The overall optimized fermentation conditions obtained thereby, when verified experimentally, have brought about a significant improvement in the amylase production.

The performance of both the optimization approach in terms of computational time and convergence rate has been computed. The amylase producing bacteria grew well utilizing the glucose source such as starch and produced good amount of amylase activity in submerged fermentation and rate of formation of amylase is high at submerged fermentation (SMF) is the best suited method for fermentation techniques involving fungi and microorganisms that require less moisture content.

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