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Optimizing of Cultural Conditions for the Production of Pectinolytic Enzyme from Marine Actinomycetes by Submerged Fermentation Along With Statistical Approach

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Abstract: Actinomycetes species possess excellent enzymatic potential and are active degraders of pectic substances. Pectinases catalyze the hydrolysis of pectin (polygalacturonic acid) to galacturonic acid residues. The pectinase enzyme produced from Actinomycetes are highly stable and can withstand broader range of pH and moreover thermo-stable property makes an advantage over pectinase produced by other species. So, Actinomycetes are choosen to produce pectinase as it has numerous benefits and optimistic alternative than other species in future. This pectinase can be industrially used in extraction and clarification processes. In the present study, 52 Actinomycetes isolates from four locations along Visakhapatnam coast sample at a region (1 feet) of Bay of Bengal were recovered. A maximum of 9 isolates from the sample of Rushikonda Beach, 3 from Kursura, 27 from the sample of Thenneti and 13 from the R.K. Beach samples were obtained. All isolates were maintained on Starch casein Agar medium. Purified Actinomycetes isolates were subjected to pectinolytic activity by growing them on pectin agar plates and the pectinolytic activity was detected by visualizing a clear zone around the colony using iodine flooding method. Out of the 52 isolates, Thenneti sample isolates were found effective for hydrolysis of pectin. The isolate E5 has shown good zone of inhibition and was subjected to secondary screening under submerged fermentation. Physico-chemical parameters such as fermentation time, temperature, pH, inoculum age, inoculum volume were varied to analyze the optimum enzyme production. The highest yield of the pectinase was obtained at an initial medium pH of 6, when incubated at 35^0C for 6 days with a 5 day old culture and 5ml of the volume i.e., 159.65U/ml. The effect of carbon and nitrogen supplements revealed that Ammonium sulfate (nitrogen source) enhanced the pectinase activity to 187.79U/ml. A 25 factorial Central Composite Design (CCD) using Response Surface Methodology (RSM) was employed to optimize 5 variables (fermentation time, temperature, pH, inoculum age and inoculum volume). According to the results of Response Surface Methodology, a maximum yield of 202.7955 U/ml of pectinase was produced.

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Introduction

Actinomycetes are biotechnologically valuable bacteria which are well exploited for secondary metabolites. Screening, isolation and characterization of promising strains of actinomycetes producing potential secondary metabolites has been a major area of research by many groups worldwide for many years. Actinomycetes especially Streptomyces species are widely recognized as industrially important microorganisms as they are a rich source of several useful bioactive natural products with potential applications and are prolific producers of secondary metabolites, many of which have commercial importance as antibiotics, anti-parasitics and antifungal agents, herbicides, pesticides, anticancer or immunosuppressive agents as well as industrially important enzymes.

Pectic substances are complex high molecular mass glycosidic macromolecules found in higher plants. They are present in the primary cell wall and are the major components of the middle lamellae, a thin extracellular adhesive layer formed between the walls of adjacent young cells. In short, they are largely responsible for the structural integrity and cohesion of plant tissues. Three major pecticpolyssacharide groups are recognized, all containing D-galacturonic acid to a greater or a lesser extent.

1) Homogalacturonan (HG) HG is a linear polymer formed by D-galacturonic acid which can be acetylated and/or methyl esterified. It is the smooth regions of pectin. The molecule is classified according to its esterification level: pectin has at least 75 % of the carboxyl groups methylated; pectinic acid has less than 75 % of the carboxyl groups methylated; pectic acid or polygalacturonic acid has no methyl esterified carboxyl groups. Frequently the word pectin is used as a generic name for pectic substances (Jayani *et al.*, 2005).

2. Rhamnogalacturonan I (RGI) RGI is composed of the repeating disaccharide rhamnose-galacturonic acid. The galacturonic residues can be acetylated and both residues can carry side chains of neutral sugars as galactose, arabinose and xylose (Willats *et al.*, 2006).

3. Rhamnogalacturonan II (RGII) Despite its name, RGII is a homogalacturonan chain with complex side chains attached to the galacturonic residues. Vincken and coworkers (Vincken *et al.*, 2003), have proposed a pectin molecule structure model in which HG and RGII are long side chains of RGI backbone. Both RG chains are also called hairy regions of pectin molecule. In unripe citrus fruit, pectin is found as a water insoluble pectic substance, the protopectin, bounded to cellulose micro fibrils conferring rigidity on cell walls. During ripening the fruit enzymes alter the pectin structure by breaking the pectin backbone or side chains, resulting in a more soluble molecule (Kashyap *et al.*, 2001).

Pectic substances tend to form a gel structure when portions of HG are cross-linked forming a three dimensional crystalline network in which water and solutes are trapped. Various factors determine gelling properties including temperature, pectin type, esterification degree, acetylation degree, pH, sugar and other solutes, and mainly the interaction between calcium ions and pectin unesterified carboxyl groups. In high-ester pectins, the junction zones are formed by the cross-linking of HG by hydrogen bridges and hydrophobic forces between methoxyl groups, both promoted by high sugar concentration and low pH. Pecticpolysaccharides have been used as bioactive food ingredients and as detoxifying agents. It is an adequate infant food supplement (Gummadi and Panda, 2003). Pectinolytic Enzymes (Pectinase): Pectic enzymes are a group of complex enzymes, which occur in plants and microorganisms, where pectin serves as the substrate. Pectin, the main component of middle lamella is dissolved by the pectic enzymes.

Protopectinases, the enzyme that catalyses the solubilisation of protopectin was originally named by Brinton *et al.,* (1927). These enzymes release pectin from protopectin. Microbial protopectinases are produced at pH 5 and temperature 30 ^{0}C. One unit of protopectinase activity is defined as liberation of pectic substances equivalent to 1µ mol D-galacturonic acid/ml of reaction mixture at 30^{0}C in 30 minutes (Stutzenberger, 1992).

Industrially Pectinases are used in Fruit juice extraction, Textile processing and bio scouring of cotton fibers, Degumming of plant bast fibers, Retting of plant fibers, Waste water treatment, Coffee and tea fermentation, Paper and pulp industry, Animal feed and Purification of plant viruses, etc.

Protease Activities studies

Screening of isolates for Pectinase Activity: Purified actinomycetes were spotted on to pectin enriched agar plates which contained (g/l) pectin–5, yeast extract–5, agar–16 (pH 7.0). Plates were incubated at 28^{0}C for 3 days. After 3 days, clear zones were visualized using iodine solution (Beg *et al.*, 2000).

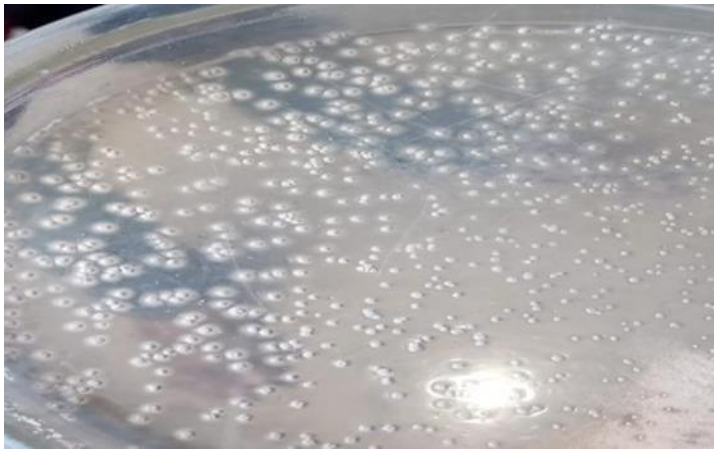

Figure 1. Colonial observation of Actinomycetes in petri dish for Thenneti sample

Assay of Pectinase: Pectinase activity was assayed by measuring the reducing sugars released from the action of pectinase on pectin using 3,5-dinitrosalicylic acid reagent. The reaction mixture consists of 0.8ml of pectin (1% w/v) and 0.2ml of enzyme in 2ml of sodium acetate buffer (0.1 M, pH 5). The reaction mixture was incubated at 40ºC for 10 min followed by the addition of 1 ml of NaOH and 1ml of DNS to the tubes to stop the reaction and the tubes were boiled in water bath until color change is observed. Then the solution is made up to 10ml using distilled water and the absorbance was read at 540 nm using visible spectrophotometer and its concentration was determined using galacturonic acid standard curve (Miller, 1959). The reducing sugars released by enzymatic hydrolysis were determined. A separate blank was set up to correct the non-enzymatic release of sugars. One unit of enzyme activity is defined as the amount of enzymes required to liberate 1µ mole of reducing sugars per minute with galacturonic acid as standard under the assay conditions.

Optimization Studies: In order to improve the yield of pectinase production various parameters were studied and optimized. They include effect of fermentation time, effect of fermentation temperature, effect of pH, effect of inoculum age. The impact of carbon and nitrogen (both organic and inorganic) sources were also studied. The experiments were carried out in such a way that the parameter optimized in one experiment was maintained in the subsequent investigation.

Effect of fermentation time: The effect of fermentation time on pectinase production for E5 isolate was studied at different time intervals of 1, 2, 3, 4, 5, 6, 7 and 8 days. Gradual increase in the pectinase activity was observed up to 6th day which showed maximum activity and the depletion of activity was observed since then due to decrease in the fermentation medium. The maximum enzyme activity observed is 116.94 U/ml.

Table 1. Effect of fermentation time for pectinase production for E5 isolate

Fermentation time (days)	Pectinase Activity (Units/ml)
1	42.35
2	59.82
3	77.94
4	95.35
5	112.18
6	**116.94**
7	99.47
8	79.06

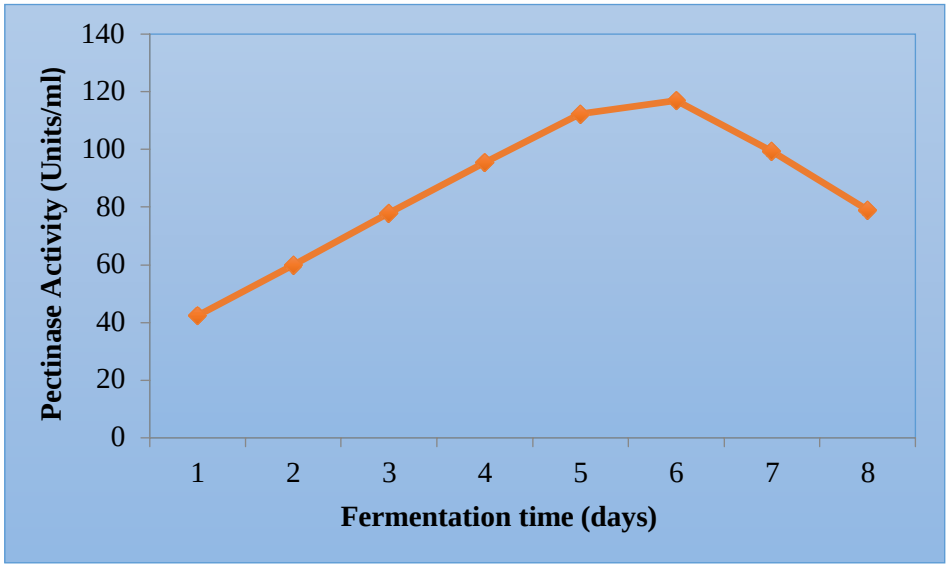

Figure 2. Effect of fermentation time for pectinase production for E5 isolate

Effect of fermentation temperature: The effect of fermentation time on pectinase production for E5 isolate was studied at different temperatures 20^{0}C, 25^{0}C, 30^{0}C, 35^{0}C, 40^{0}C and 45^{0}C. The optimum incubation temperature for the production of pectinase (124.65 U/ml) was found at 35^{0}C. As the temperature kept increasing decrease in the activity was observed as high temperatures could denature the extra cellular enzymes. Hence 35^{0}C temperature is considered as the optimum temperature for pectinase activity.

Table 2. Effect of fermentation temperature for pectinase production for E5 isolate

Fermentation temperature (^{0}C)	Pectinase Activity (Units/ml)
20	81.71
25	92.47
30	110.89
35	124.65
40	106.53
45	82.47

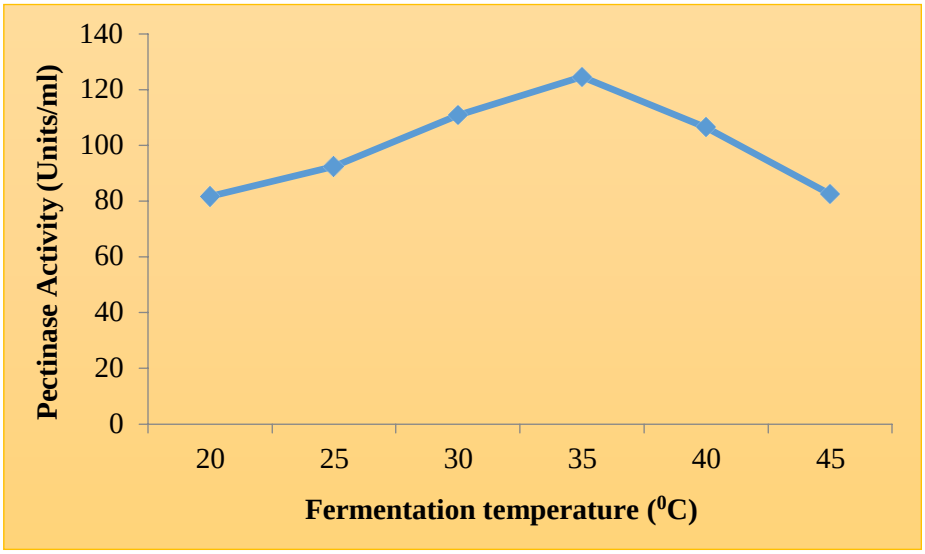

Figure 3. Effect of fermentation temperature for pectinase production for E5 isolate

Effect of inoculum age: The effect of inoculum age on pectinase production was studied by conducting the fermentation with different inoculum ages. The production media was inoculated with 1day old culture to 8 days old culture in different flasks. The flasks were incubated at 35^0C for 6 days. The fifth day old culture gave maximum production of pectinase (145.65 U/ml).This is due to the well growth of the actinomycetes on the 5^{th} day.

Table 3. Effect of inoculum age for pectinase production for E5 isolate

Inoculum age (days)	Pectinase Activity (Units/ml)
1	95.32
2	102.71
3	115.47
4	132.89
5	**145.65**
6	125.26
7	103.47
8	89.71

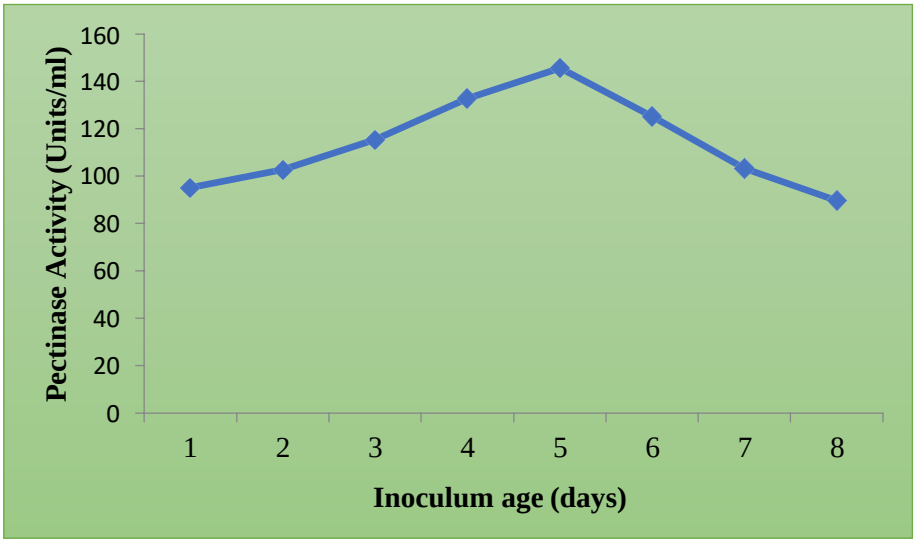

Figure 4. Effect of inoculum age for pectinase production for E5 isolate

Effect of pH: The optimum pH for pectinase production for E5 isoalte was determined by conducting the fermentation at different pH of the medium namely 2, 3, 4, 5, 6, 7 and 8 pH. The maximum enzyme production of 132.26 (U/ml) was obtained at pH 6. The pH regulates the growth and the synthesis of extracellular enzymes and also influences the transport of various components across the cell membranes, which supports the cell growth and product formation.

Table 4. Effect of pH for pectinase production for E5 isolate

pH	Pectinase Activity (Units/ml)
2	91.71
3	102.47
4	114.89
5	127.65
6	**132.26**
7	99.47
8	71.71

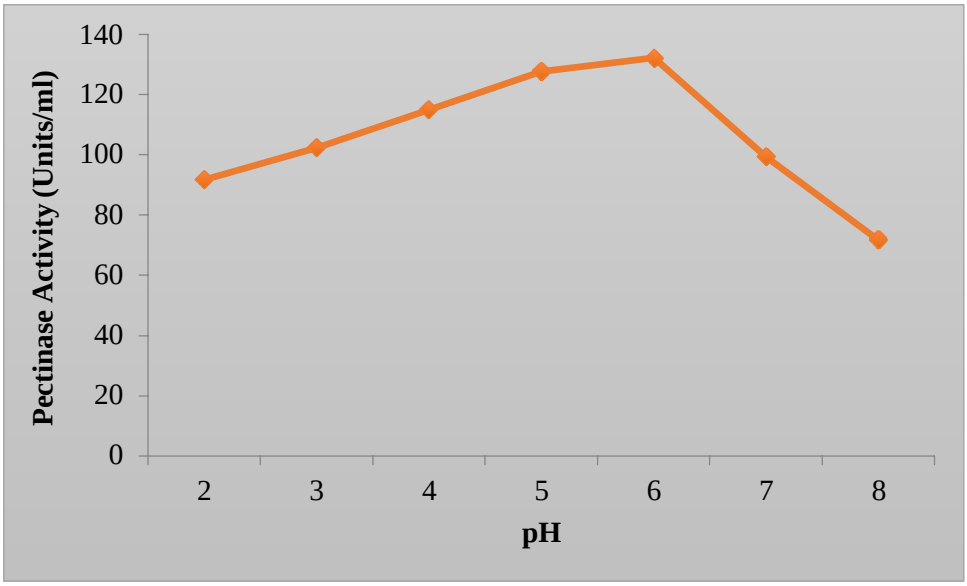

Figure 5. Effect of pH for pectinase production for E5 isolate

Effect of different carbon sources: Influence of various carbon supplements on pectinase production for E5 isolate was studied by adding various sugars namely glucose, lactose, maltose, sucrose, galactose and fructose at 0.5% (w/w) to the fermentation media. Among these different carbon sources used as enrichment, sucrose as the carbon source gave maximum pectinase activity (167.79 U/ml).

Table 5. Effect of carbon source for pectinase production for E5 isolate

Carbon source	Pectinase Activity (Units/ml)
Glucose	136.31
Lactose	110.67
Maltose	91.48
Sucrose	**167.79**
Galactose	135.15
Fructose	126.26

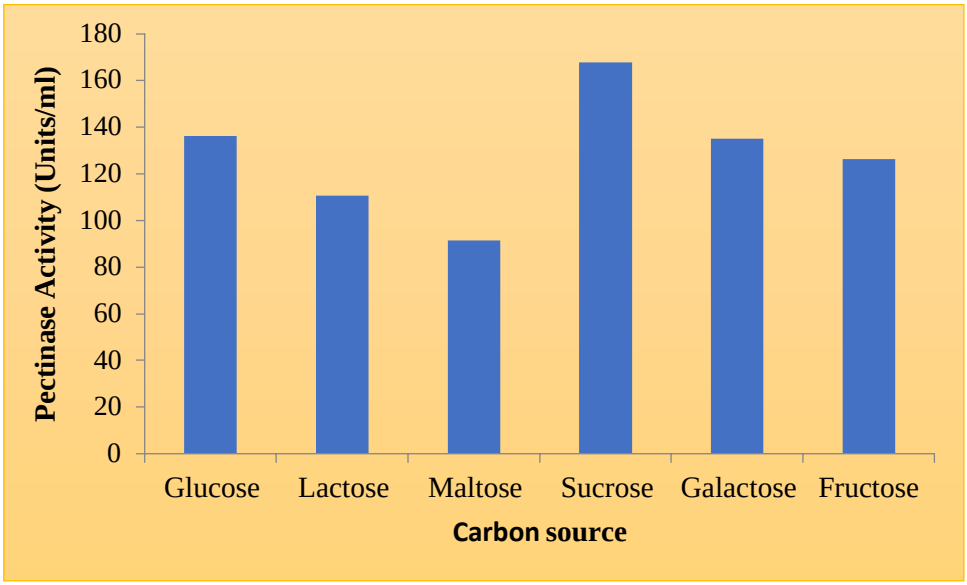

Figure 6. Effect of carbon source for pectinase production for E5 isolate

Effect of different nitrogen sources: Nitrogen sources which include both organic and inorganic sources are important enhancing or limiting factor for production. The different nitrogen sources (casein, Yeast extract, peptone Ammonium sulfate, beef extract and sodium nitrate of 0.5% (w/w) are tested. Among these Ammonium sulfate reported as a source of maximum production of pectinase enzyme (187.79U/ml).

Table 6. Effect of nitrogen source for pectinase production for E5 isolate

Nitrogen source	Pectinase Activity (Units/ml)
Casein	146.31
Yeast extract	120.67
Peptone	99.48
Ammonium sulfate	**187.79**
Beef extract	145.15
Sodium nitrate	136.26

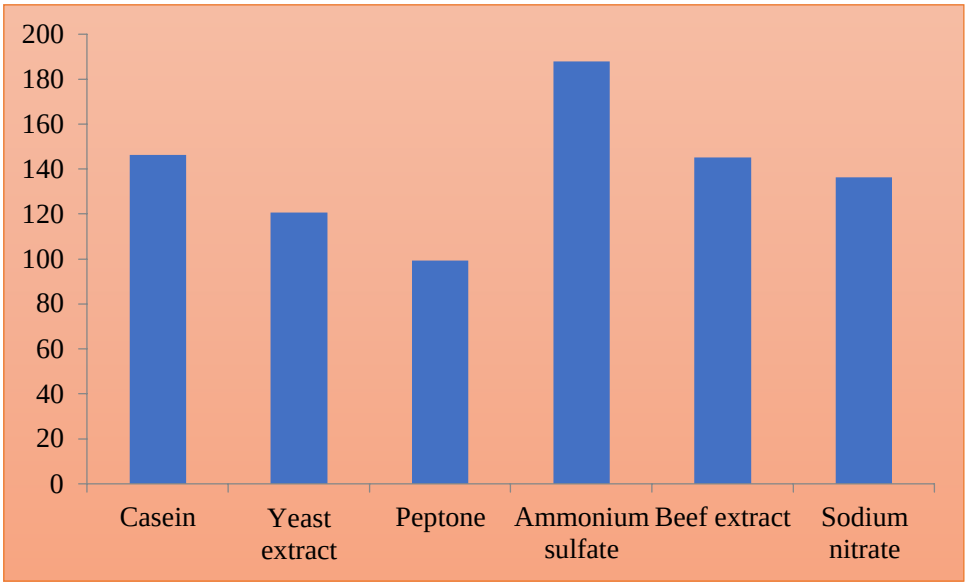

Figure 7. Effect of nitrogen source for pectinase production for E5 isolate

Central Composite Design (CCD): This is the most popular class of designs used for fitting second order models. Generally, the Central Composite Design (CCD) consists of a 2k factorial (or fractional factorial of resolution V) nf runs, 2k axial or star points, and nc centre runs. The two points that must be specified in the design are: The distance (α) of the axial runs from the design centre and the number of centre points is nc.

Response Surface Methodology (RSM): With the individual optimum values obtained from the previous experiments, which are obtained on one parameter at a time basis, a 25 full factorial, central composite design and RSM were used to get the accurate optimized values which are interdependent and which yield higher production of pectinase enzyme. The central values (zero level) chosen for experimental design were: fermentation time 6 days, fermentation temperature 35^0C, pH 6, inoculums age 5 day old, inoculums volume 5 ml. Table 7 shows the range and coded levels of these variables.

Table 7. Experimental range and levels of independent variables

Variable	Name	Range and levels				
		-2	-1	0	1	2
X_1	Fermentation time, days	4	5	**6**	7	8
X_2	Fermentation temperature, ^{0}C	25	30	**35**	40	45
X_3	pH	4	5	**6**	7	8
X_4	Inoculums age, days	3	4	**5**	6	7
X_5	Inoculums volume, ml	3	4	**5**	6	7

With these variables, a set of 30 number of experiments were planned with 16 factorial points, 8 star points and 6 central points and the pectinase enzyme produced was measured in each case and tabulated in table 8.

Table 8. Central composite design consisting of 30 experiments for the study of four experimental factors in coded and real values and comparison of experimental and predicted values of pectinase production

Run no.	Ferm. Time, days	Ferm. Temp., ^{0}C	Ferm. pH	I-age, days	I-Vol., ml	Pectinase produced (U/ml)		Residue
						Experimental	Predicted	
1	5(-1)	30(-1)	5(-1)	4(-1)	6(1)	136.00	143.19	-7.19
2	5(-1)	30(-1)	5(-1)	6(1)	4(-1)	136.70	145.27	-8.57
3	5(-1)	30(-1)	7(1)	4(-1)	4(-1)	134.20	139.80	-5.59
4	5(-1)	30(-1)	7(1)	6(1)	6(1)	134.80	140.18	-5.38
5	5(-1)	40(1)	5(-1)	4(-1)	4(-1)	142.40	147.16	-4.76
6	5(-1)	40(1)	5(-1)	6(1)	6(1)	142.90	147.44	-4.54
7	5(-1)	40(1)	7(1)	4(-1)	6(1)	153.60	155.17	-1.56
8	5(-1)	40(1)	7(1)	6(1)	4(-1)	153.90	156.85	-2.95
9	7(1)	30(-1)	5(-1)	4(-1)	4(-1)	139.70	149.10	-9.39
10	7(1)	30(-1)	5(-1)	6(1)	6(1)	140.60	149.78	-9.18
11	7(1)	30(-1)	7(1)	4(-1)	6(1)	137.30	143.51	-6.21
12	7(1)	30(-1)	7(1)	6(1)	4(-1)	137.90	145.49	-7.59
13	7(1)	40(1)	5(-1)	4(-1)	6(1)	141.50	146.86	-5.365
14	7(1)	40(1)	5(-1)	6(1)	4(-1)	142.30	149.05	-6.75
15	7(1)	40(1)	7(1)	4(-1)	4(-1)	162.70	166.47	-3.77
16	7(1)	40(1)	7(1)	6(1)	6(1)	163.10	166.66	-3.56
17	4(-2)	35(0)	6(0)	5(0)	5(0)	155.30	146.57	8.73
18	8(2)	35(0)	6(0)	5(0)	5(0)	171.40	157.04	14.36

19	6(0)	25(-2)	6(0)	5(0)	5(0)	168.90	150.89	18.01
20	6(0)	45(2)	6(0)	5(0)	5(0)	175.80	170.72	5.08
21	6(0)	35(0)	4(-2)	5(0)	5(0)	167.30	150.97	16.33
22	6(0)	35(0)	8(2)	5(0)	5(0)	166.80	160.04	6.76
23	6(0)	35(0)	6(0)	3(-2)	5(0)	178.90	168.52	10.38
24	6(0)	35(0)	6(0)	7(2)	5(0)	183.60	170.89	12.71
25	6(0)	35(0)	6(0)	5(0)	3(-2)	175.90	162.75	13.15
26	6(0)	35(0)	6(0)	5(0)	7(2)	171.10	161.15	9.95
27	6(0)	35(0)	6(0)	5(0)	5(0)	196.00	201.77	-5.77
28	6(0)	35(0)	6(0)	5(0)	5(0)	196.00	201.77	-5.77
29	6(0)	35(0)	6(0)	5(0)	5(0)	196.00	201.77	-5.77
30	6(0)	35(0)	6(0)	5(0)	5(0)	196.00	201.77	-5.77

By applying multiple regression analysis on the experimental data using STATISTICA 6.0, the following second order polynomial equation was found to represent the pectinase production adequately.

Y = -1407.49 + 143.76; X1 + 24.04; X2 +102.39; X3 + 74.64; X4 + 92.43; X5 -12.47; X1 2 - 0.41; X2 2 - 11.57; X3 2 - 8.02; X4 2 - 9.94; X5 2 + 0.04; X1X2 +1.15; X1X3 + 0.05; X1X4 -0.01; X1X5 + 0.91; X2X3 - 0.01 X2X4 - 0.06; X3X4 + 0.03; X3X5 + 1.32 X4X5

Where Y is the response which is pectinase production in U/ml and X1, X2, X3, X4, X5 are the real values of the test variables, viz: fermentation time (days), fermentation temperature (^{0}C), pH, inoculums age (days), respectively.

The coefficients of the regression model calculated are listed in table 9.

Table 9. Regression data of the model

	Coefficient	Regression	Std. Error	t-value	p-value
Constant	b_0	-1407.49	1.474038	-954.86	0.000000
X_1	b_1	143.76	0.166577	863.00	0.000000
X_1^2	b11	-12.47	0.008659	-1440.52	0.000000
X_2	b_2	24.04	0.034654	693.58	0.000000
X_2^2	b22	-0.41	0.000346	-1182.69	0.000000
X_3	b_3	102.39	0.166577	614.66	0.000000
X_3^2	b33	-11.57	0.008659	-1335.72	0.000000
X_4	b_4	74.64	0.160698	464.47	0.000000
X_4^2	b44	-8.02	0.008659	-925.72	0.000000
X_5	b_5	92.43	0.160698	575.16	0.000000
X_5^2	b55	-9.94	0.008659	-1147.75	0.000000
X_1 X_2	b12	0.04	0.002236	18.34	0.000000
X_1 X_3	b13	1.15	0.011178	102.88	0.000000
X_1 X_4	b14	0.05	0.011178	4.92	0.000824
X_1 X_5	b15	-0.01	0.011178	-1.12	0.292422
X_2 X_3	b23	0.91	0.002236	408.16	0.000000
X_2 X_4	b24	-0.01	0.002236	-6.04	0.000193
X_2 X_5	b25	-0.00	0.002236	-2.24	0.052142

X_3 X_4	b34	-0.06	0.011178	-5.59	0.000338
X_3 X_5	b35	0.03	0.011178	2.68	0.025051
X_4 X_5	b45	1.32	0.011178	118.53	0.000000
Significant at $p \leq 0.05$, Adjusted R2 = 0.9999					

The significance of each coefficient was determined by student's t-test and p-values were also listed in Table 9. The p-values were used as a tool to check the significance of the each of the coefficients which, in turn, are necessary to understand the pattern of the mutual interactions between the test variables. The larger the magnitude of the t-value and smaller the p-value, the more significant is the corresponding coefficient. The results of the second order response surface model fitting in the form of ANOVA are given in Table 10. It is required to test the significance and adequacy of the model. The Fisher variance ratio, the F-value (Sr 2 / Se 2), is a statistically valid measure of how well the factors describe the variation in the data about is mean. The greater the f-value is form unity, the more certain it is that the factors explain adequately the variation in the data about its mean and the estimated factor effects are real. The ANOVA of the regression model demonstrates that the model is highly significant. The goodness of the fitness of the model was checked by the determination coefficient R^2. The R^2 value is always between 0 and 1. The adjusted R^2 value is 0.9999 which is very high to advocate high significance of the model.

The parity plot (Figure 8) showed a satisfactory correlation between experimental and predicted values of pectinase produced wherein the points cluster around the diagonal line indicated the good fit of the model.

Table 10. ANOVA for the model

Source of variation	SS	df	Mean Square (MS)	F-value
Model	10374.91	20	518.7405	2,35,791.1363
Error	0.02	9	0.0022	
Total	10374.83			

Where df-Degree of freedom; S–Sum of squares; F–Factor F; P–Probability.

The Pareto Chart in figure 8. shows the graphic representation of variables studied.

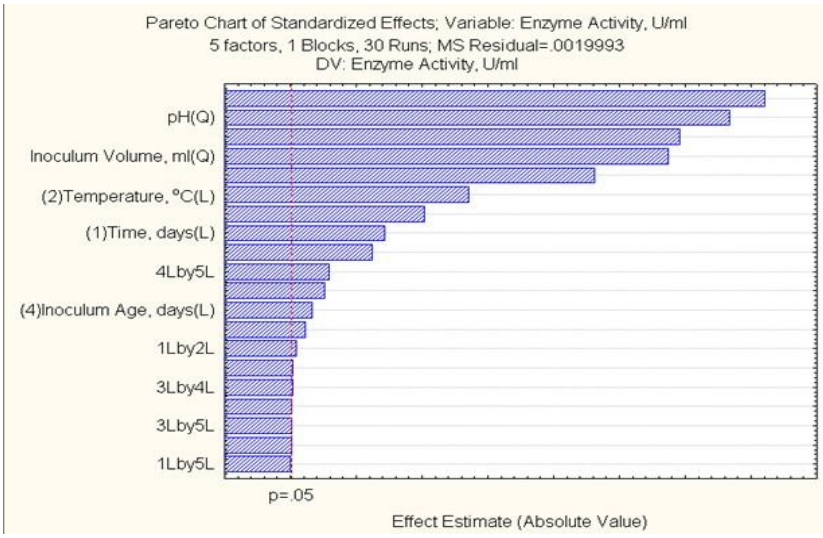

Figure 8. Pareto Chart showing variables studied

The parity plot (Figure 8) showed a satisfactory correlation between experimental and predicted values of pectinase produced wherein the points cluster around the diagonal line indicated the good fit of the model.

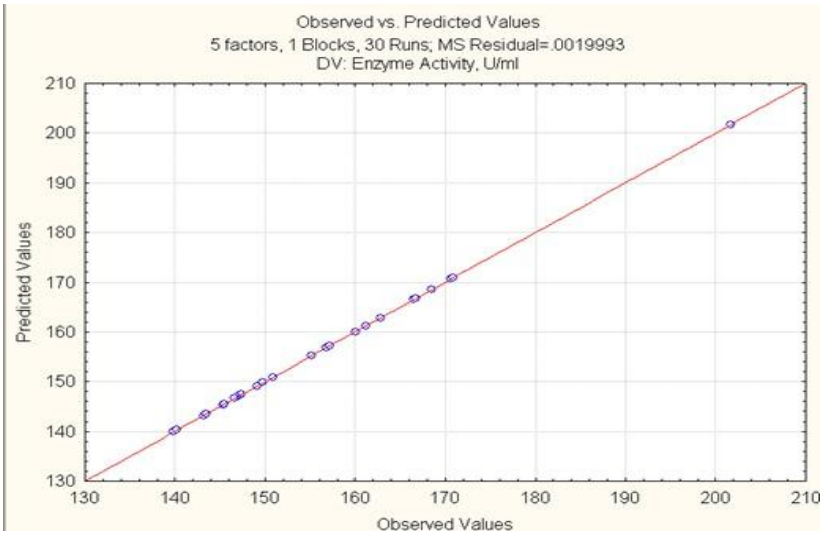

Figure 9. Parity plot showing the distribution of experimental vs. predicted values of pectinase production.

The experimental optimum values are compared with the CCD and RSM in table 11.

Table 11. Comparison of experimental values with CCD

Variable	Experimental	CCD
Fermentation time, days	6	6.11506
Fermentation temperature, ^{0}C	35	36.39123
Fermentation pH	6	6.15899
Inoculums age, days	5	5.03403
Inoculums volume, ml	5	4.98205

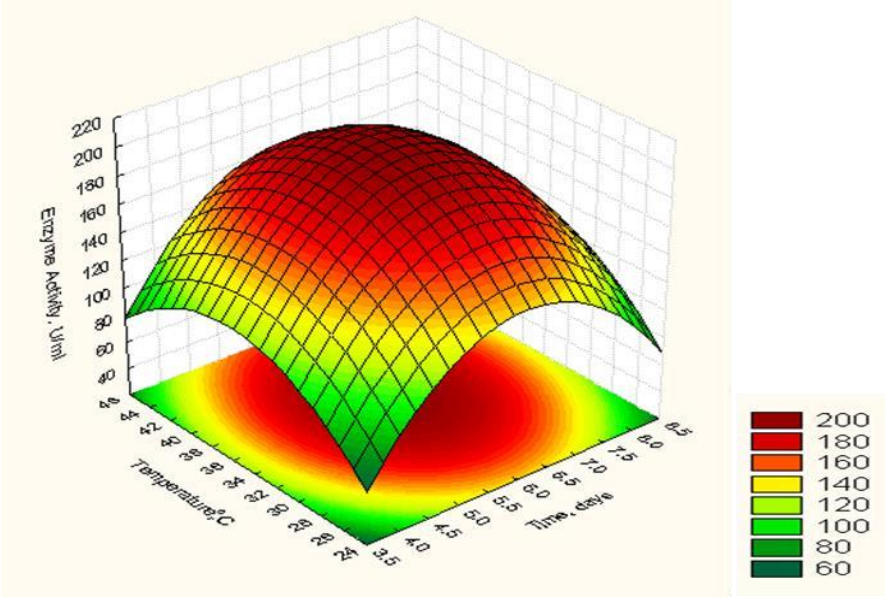

Figure 10. Surface contour plot showing the effect of time and temperature on pectinase production

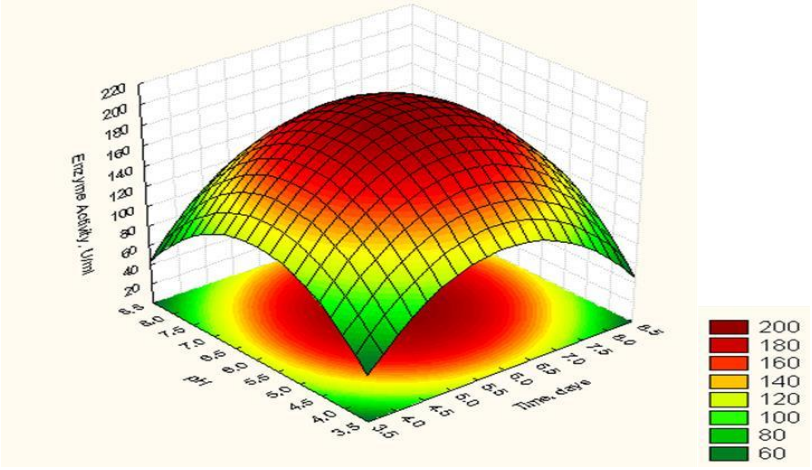

Figure 11. Surface contour plot showing the effect of time and pH on pectinase production

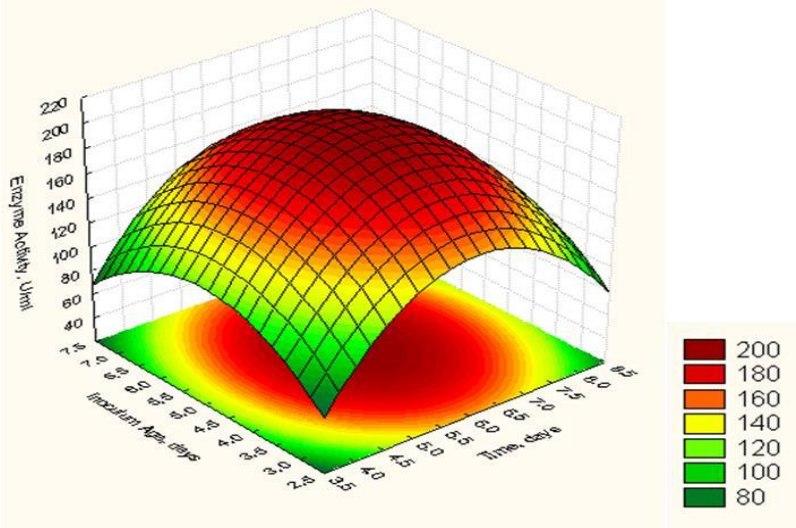

Figure 12. Surface contour plot showing the effect of time and inoculums age on enzyme activity

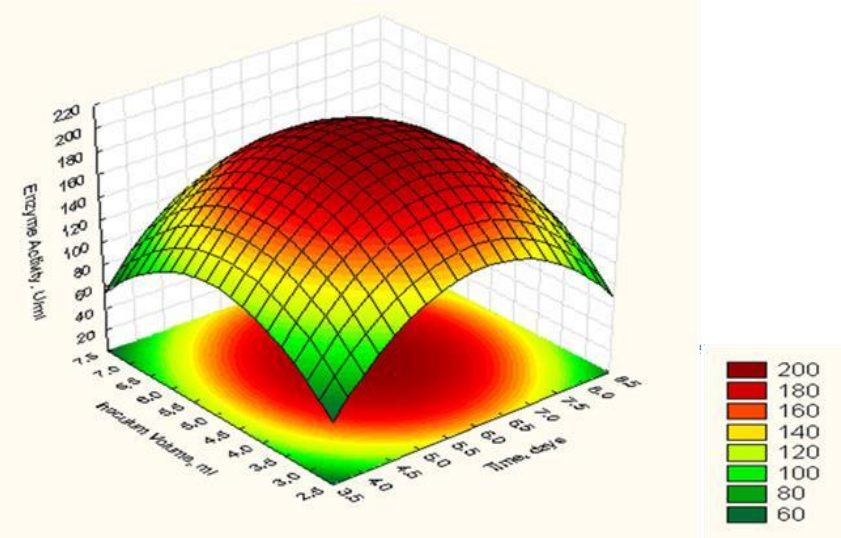

Figure 13. Surface contour plot for the effects of time and inoculums volume on pectinase production

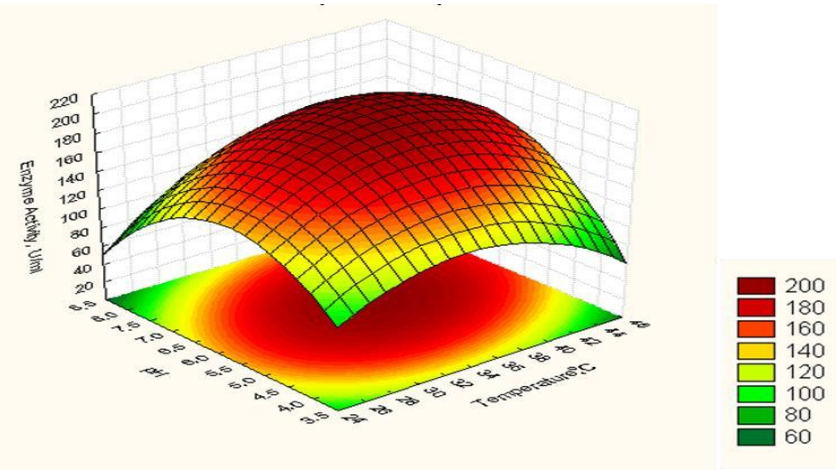

Figure 14. Surface contour plot showing the effect of temperature and pH on pectinase production

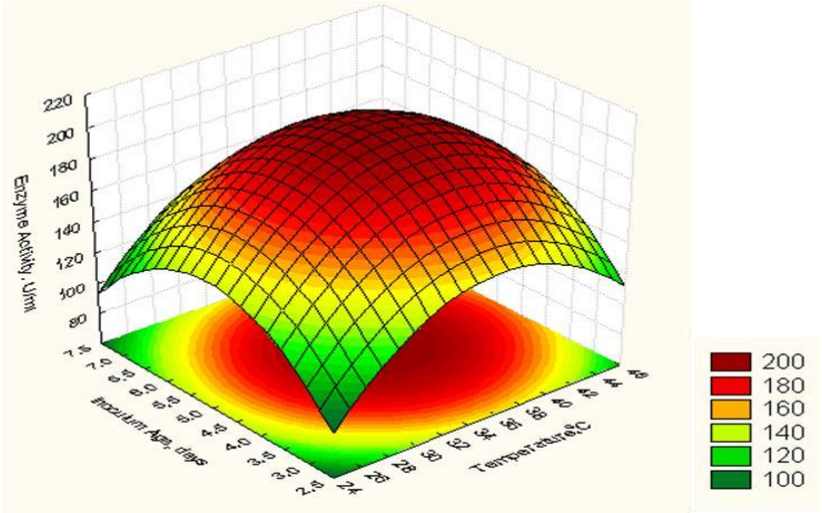

Figure 15. Surface contour plot showing the effect of temperature and inoculums age on pectinase production

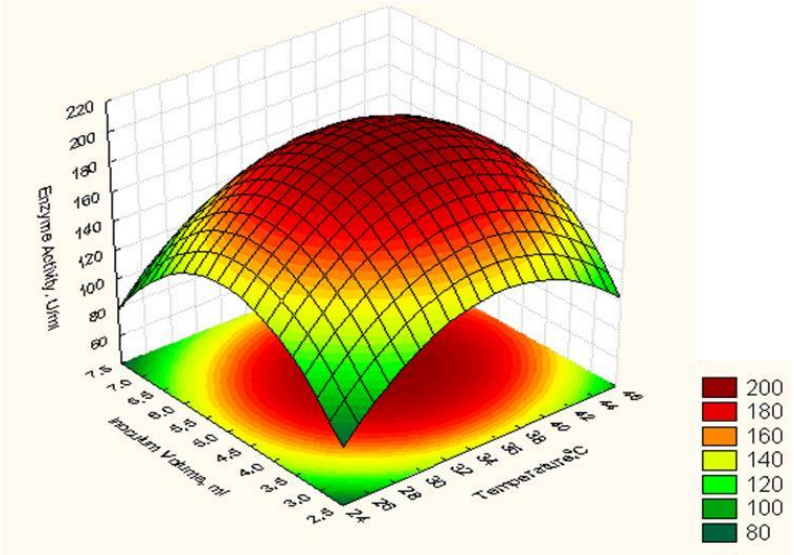

Figure 16. Surface contour plot showing the effect of temperature and inoculums volume on pectinase production

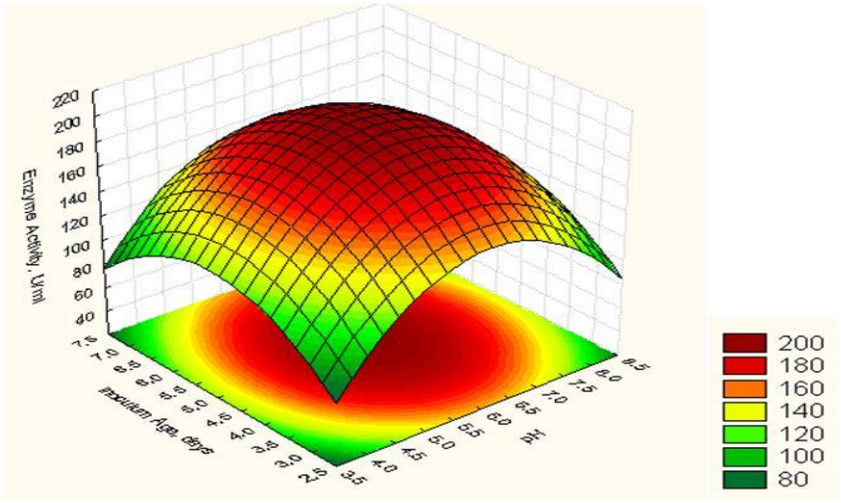

Figure 17. Surface contour plot showing the effect of inoculums age and pH on pectinase production

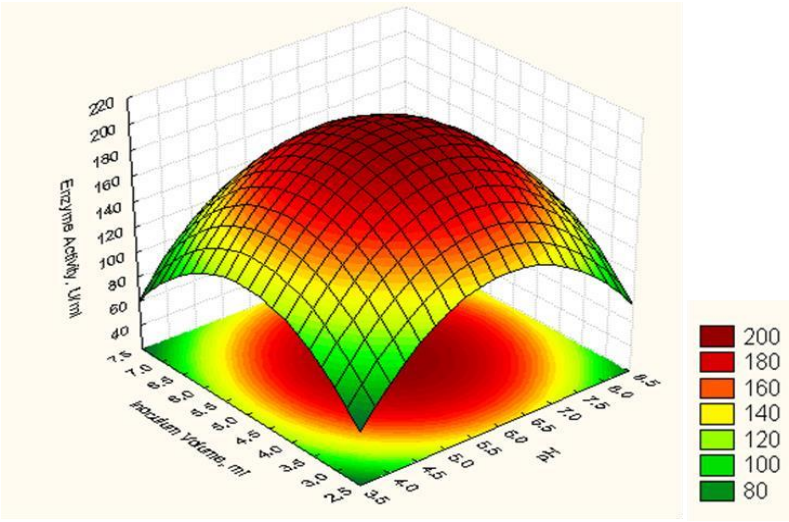

Figure 18. Surface contour plot showing the effect of inoculums volume and pH on pectinase production

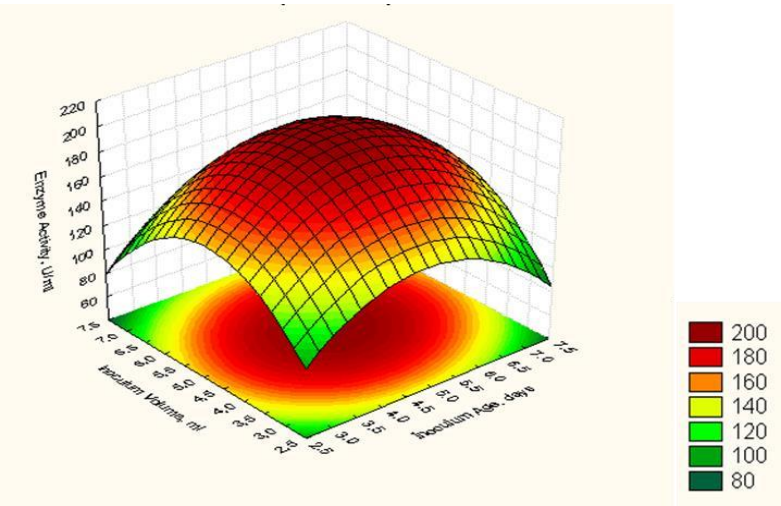

Figure 19. Surface contour plot showing the effect of inoculums age and inoculums volume on pectinase production

Conclusion

From the present research, it is clear that marine environment is a good source for isolation of actinomycetes. The pectinase produced from potential E5 isolate, obtained from Thenneti sample collected along the coast of Bay of Bengal at Visakhapatnam, India seems promising for efficient production of pectinase using pectin as the sole source of carbon. The pectinase enzyme has various applications in food and textile industries. Response Surface Methodology (RSM) was performed with Central Composite Design (CCD) which permitted studying and exploring fermentation conditions for the production of pectinase in 30 experimental runs with overall increase in enzyme production.

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