KINETIC STUDY ON PRODUCTION OF PROTEASE FROM SOIL SAMPLES

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Abstract
Many microorganisms produce wide variety of extra cellular enzymes including proteases which is important in rapidly growing industrial field. The major scope of the work is to optimize the production of protease enzyme by altering different variables like change in pH, temperature, initial substrate concentration and initial enzyme concentration. Microorganisms producing protease enzymes were isolated from 3 soil samples collected from local market from butcher shop by using serial dilution method. The samples were assayed and optical density was observed at 660nm at which protease activity was measured in terms of one unit of casein to produce color equivalent to 1.0 µmol of Tyrosine per min at pH 7.5 at 45oC. We could also demonstrate satisfactorily that the Michealis-Menten kinetics would adequately represent the production of protease.
Key Words: Protease, Michealis-Menten kinetics, Tyrosine, pH, temperature, laminar flow chamber, Sterilization.

I. INTRODUCTION

Proteases are enzymes that have a catalytic function of breaking down peptide bonds from a poly peptide chain in a particular protein. Proteases are involved in digesting long protein chains into shorter fragments by splitting the peptide bonds that link amino acid residues. Some detach the terminal amino acids from the protein chain exo-peptidases, such as amino peptidases, carboxy peptidase. This study majorly concentrates on protease activity from selected samples using different carbon sources (media). The samples were from (3 different water samples) from butcher shops. Different conditions were observed to attain maximum amount of protease.

II. RAW MATERIALS AND METHOD

2.1: Materials and Physical measurements: The chemicals and equipment used in this method are Autoclave Reactor, Incubator, Laminar air flow chamber, U.V Spectrophotometer. The chemicals used are Agar, Gelatin, Casein, Starch, $MgSO_4.7H_2O$ (Epson salt), Peptone, Beef extract, Yeast extract, $FeSO_4.\ 7H_2O$ (green vitrol).

2.2: Method:

2.2.1: Collection of sample for production of protease: It is essential to collect the potential microbial strain from various suitable sources. The main source of protease producing microbial strains includes stagnant water from sewages or from the drain near a place (soil) where butcher work has been done. Three such samples were collected.

2.2.2: Inoculum preparation: Samples are filtered and 10 ml of filtrate from each sample was taken. The 10 ml of filtrate were divided into two parts of 5 ml each and then they were transferred into Erlenmeyer flask (250 ml) containing 50 ml of sterile Inoculum media for the growth of protease producing microbial strain (sterilized). Inoculum should be placed on the gelatin agar plates. 1N NaOH, 1NHcl are used to maintain pH and gelatin pH maintained at 7. The prepared media was steam sterilized at required conditions and inoculated after cooling and then kept in a rotary shaker at 180 rpm at 55° C for about 48 hrs. Three mediums were prepared to check the growth by broth culturing (starch and glucose media). The prepared media was steam sterilized at a pressure of 15 psi, 121 ° C for 15 minutes. The sample was then inoculated after the cooling of media for 15-20 minutes and then was kept on a rotary shaker at 180 rpm at 55° C for about 48 hrs.

2.2.3: After 48hrs the growth of microbial strain is checked and the strain which was obtained was then cultured on the Agar media (in petri dish) having the following composition:

Constituents	Quantity (wt/vol)
Beef extract	0.1
Yeast	0.2
Peptone	0.5
NaCI	0.5
Agar	1.5
DH2O	100

Table 1: Beef extract Medium

The Petri dish containing the cultured strain was then put into the incubator for about 48 hrs. at 37° C. After 48 hrs growth was checked and strains so obtained were then further sub-cultured to get desired strain from the mixed colony of the different microbial strains for the production of protease enzyme.

Fig 1: Microbial Growth

2.2.4 Repeated Sub culturing:

Repeated sub culturing is performed to get the desired strain out of the mixed colonies of the microbes for the production of the protease enzyme. The sub culturing is performed carefully under aseptic condition in laminar flow to avoid any type of contamination.

2.2.5 Single colony isolation: The serial dilution method is performed for single colony isolation from mixed colonies of microbes to get the desired strain which is used in the maximum production of protease enzyme.

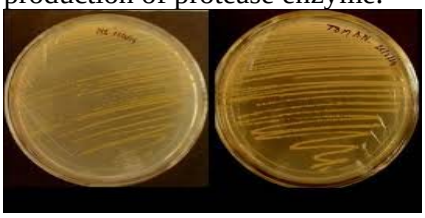


Fig 2: Single colony isolation by streaking method

These isolated colonies were checked for organism growth using a medium called CZAPEK-DOX AGAR medium. Fungal growth was not observed even after 72 hrs but single colonies of bacterial growth are observed. By streaking method the organism is preserved in test tubes.

Microorganism slant growth in test tubes

2.2.6 Standard solutions: for assay

1. A 50 mM Potassium Phosphate Buffer, pH 7.5 prepared using 11.4 mg/ml of potassium phosphate dibasic, tri hydrate in purified water and adjusting pH with 1M HCl. This solution is placed at 37°C prior to use.
2. A 0.65% weight/volume casein solution, prepared by mixing 6.5 mg/ml of casein in the 50 mM potassium phosphate buffer. Gradually increased the solution temperature with gentle stirring to 80-85 °C for about 10 minutes until a homogenous dispersion is achieved. It is very important not to boil the solution. The pH is then adjusted if necessary with NaOH and HCl.
3. A 110 mM Tri chloro acetic acid solution, prepared by diluting a 6.1N stock 1:55 with purified water.
4. 0.5 mM Folin & Ciolcaltea's, or Folin's Phenol Reagent, which is the solution that will react with tyrosine to generate a measurable color change that can be directly related to the activity of proteases.
5. A 500 mM Sodium Carbonate solution, prepared using 53 mg/ml of anhydrous sodium carbonate in purified water. An enzyme diluent solution, which consists of 10 mM Sodium Acetate Buffer with 5mM Calcium Acetate, pH 7.5, at 37°C.
6. 1.1 mM L-tyrosine Standard stock solution. Prepared using 0.2 mg/ml L-tyrosine in purified water and heated gently until the tyrosine dissolves.
7. Protease solution. Immediately before use, dissolve protease in enzyme diluent solution prepared in step 6.

3.1: Assay of enzyme Activity: Casein (1.0%) was dissolved in buffer (pH 8.0) by heating at 70° C. 2 ml of casein solution was mixed with 0.5 ml of

culture supernatant and incubated at 45° C in water bath for 50 min. The reaction was terminated by adding an equal volume of 10 % TCA followed by 10 min holding time. Suspension was filtered through Watmann's filter paper. To 1 ml of filtrate 5 ml of 0.5 Na_2CO_3 solutions and 0.5 ml of 3-fold diluted folin reagent were added and mixed thoroughly. The color development after 30 min of incubation at 30° C was measured as O.D at 660nm with blank as reference.

3.2 Determination of enzyme activity: Each sample will be assayed in 4 different samples, out of which one vial is taken as blank and other 3 vials with different dilutions of protease. To each set of four vials, add 5mls of our 0.65% casein solution. Let them equilibrate in a water bath at 37°C for about 5 minutes.

Add varying volumes of enzyme solution to the test sample vials, except the blank. Mix by swirling and incubate for 37°C for exactly ten minutes. The protease activity and consequential liberation of tyrosine during this incubation time will be measured and compared between test samples.

After this 10 minute incubation, add the 5 mls of the TCA reagent to each tube to stop the reaction. Then, add an appropriate volume of enzyme solution to each tube, even the blank, so that the final volume of enzyme solution in each tube is 1 ml. incubate the solutions at 37°C for 30 minutes. To the six vials, add the 1.1 mM tyrosine standard stock solutions with the following volumes in mls: 0.05, 0.10, 0.20, 0.40, and 0.50. Tyrosine is avoided in blank. Add required amount of water to make up to 2ml. After incubation time of 30 minutes all the samples are filtered to remove any particulates, then add 5 ml of sodium carbonate and 1 ml of Folin's reagent and pH is adjusted and incubated for some time. Gradation of color is observed correlating with amount of tyrosine added. Then Absorbance is noted using UV-Spectrophotometer.

3.3: Measuring Absorbance and Calculating Enzyme Activity

The absorbance of the samples is measured by a spectrophotometer using a wavelength of 660nm.

Volume of tyrosine(ml)	Micromoles of tyrosine released
0.01	0.011
0.02	0.022
0.04	0.044
0.05	0.055
0.08	0.088

Table 2: Micromoles of Tyrosine released

$$E\left(\frac{units}{ml}\right) = moles\ of\ tyrosine\ released * \frac{11}{1 * 10 * 20}$$

E= Enzyme activity, 11= total volume (ml) of assay, 10= time of assay (mins), 1= vol of enzyme (ml), 2= vol used in colorimetric determination (ml)

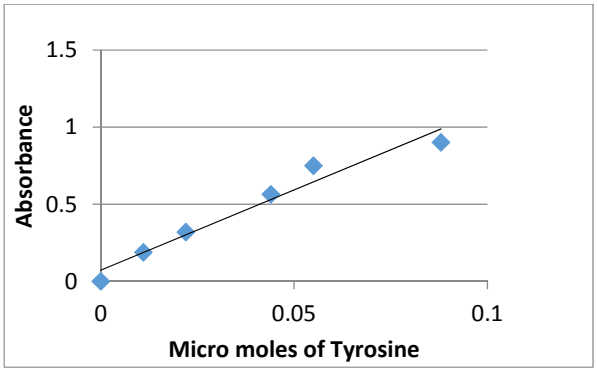
IV. RESULTS:

4.1 Standardization using Tyrosine:

Conc of Tyrosine	Absorbance at 660 nm	Absorbance at 750nm
0.01	0.116	0.216
0.02	0.326	0.432
0.03	0.542	0.691
0.04	0.763	0.860
0.05	0.806	0.912

Table 3: Standardization Values

Standardization graph:



Graph 1: standardization

4.1.1 Effect of Substrate concentration:

Name of sample	Trail	Gelatin	Albumin	Casein
D	I	0.632	0.586	0.985
III-F-B1	I	0.589	0.545	0.967

Table 4: Effect of Substrate concentration.

4.1.2 Effect of casein (substrate) concentration:

Selection of two best samples by varying casein (substrate) concentration

1. Casein with 0.45%
2. Casein with 0.65%
3. Casein with 0.75%

TEST-1(with casein 0.45%)

Test sample	Absorbance at 660nm	Absorbance at 750nm
I-F-B1	0.812	0.852
E1	0.102	0.291
I-C-B1	0.112	0.199
D1	0.961	0.996
A1	0.271	0.431
B1	0.901	0.931
C1	0.804	0.891
III-F-B1	0.789	0.861

Table 5: with Casein 0.45%

TEST-2(With Casein 0.65%)

Test sample	Absorbance at 660nm	Absorbance at 750nm
I-F-B1	0.706	0.758
E1	0.776	0.815
I-C-B1	0.010	0.008
D1	0.993	1.002
A1	0.750	0.793
B1	0.920	0.981
C1	0.812	0.838
III-F-B1	0.836	0.880

Table 6: With Casein 0.65%

TEST-3(With Casein 0.75%)

Test sample	Absorbance at 660nm	Absorbance at 750nm
I-F-B1	0.779	0.812
E1	0.269	0.392
I-C-B1	0.761	0.799
D1	0.809	0.891
A1	0.366	0.422
B1	0.832	0.899
C1	0.886	0.921

Table 7: with Casein 0.75%

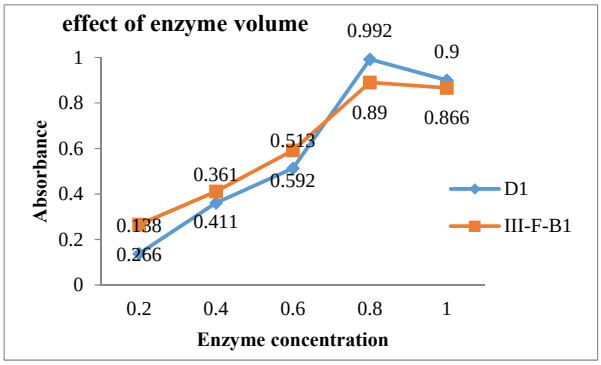
Observation: By above values we observed that samples (D1 & III-F-B1) gives optimum growth with 0.65% of casein for protease enzyme. Concentration is kept constant and further tests were conducted for the samples (D1 & III-F-B1)

4.1.3 Effect of enzyme volume:

Enzyme volume		0.2	0.4	0.6	0.8	1
D1	660	0.138	0.361	0.513	0.992	0.901
	750	0.916	0.431	0.622	0.99	1.006
III-F-B1	660	0.266	0.411	0.592	0.890	0.866
	750	0.311	0.523	0.613	0.910	0.899

Table 8: Effect of Enzyme volume

For sample D1& III-F-B1:



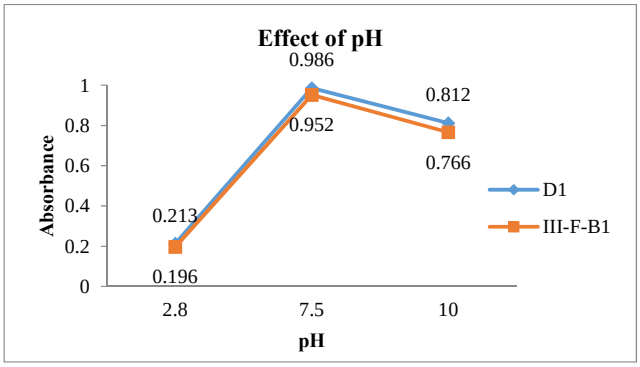
Graph 2: For sample D1& III-F-B1

Observation: From the above graph it is observed that the optimum enzyme volume for protease production is 0.8.

4.1.4 Effect of pH:

Sample	Phosphate buffer(10)	Acetate buffer(2.8)	Tris HCL Buffer(7.5)
D1	0.812	0.213	0.986
III-F-B1	0.766	0.196	0.952

Table 9: effect of pH



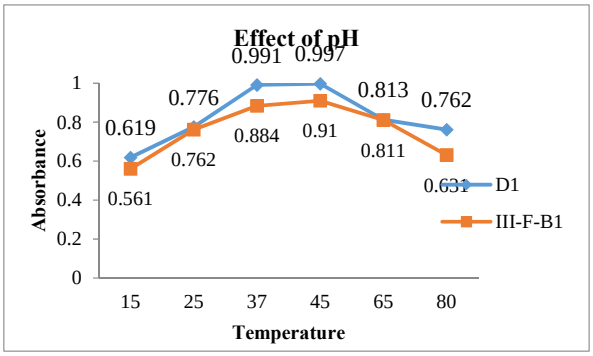
Graph 3: effect of pH

Observation: the above two graphs we observed that the optimum pH (HCL buffer) for protease production is 7.5.

4.1.5 Effect of temperature

Temp^{0}C	Sample	Absorbance (670nm)
15	D1	0.619
25		0.776
37		0.991
45		0.997
65		0.813
80		0.762
15	III-F-B1	0.561
25		0.762
37		0.884
45		0.910
65		0.811
80		0.631

Table 10: effect of Temperature



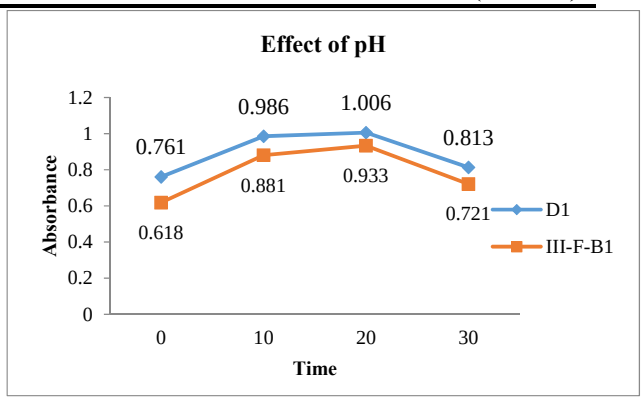
Graph 4: effect of Temperature

4.1.6 Effect of time:

Optimization by varying time [Casein conc. (0.65)/ Enzyme conc. (0.8) Fixed]

Time(min)	Sample	Absorbance at 660nm
0	D1	0.761
10		0.986
20		1.006
30		0.813
0	III-F-B1	0.618
10		0.881
20		0.933
30		0.721

Table 11: Effect of time



Graph 5: Effect of time

Observation: From the above two graphs us observed that the optimum time for protease production is 20mins.

4.2 CALCULATION OF KINETIC PARAMETERS BY USING

LINE-WEAVER BURK PLOT (michaelis-menten) EQUATION.

ARRHENIUS EQUATION:

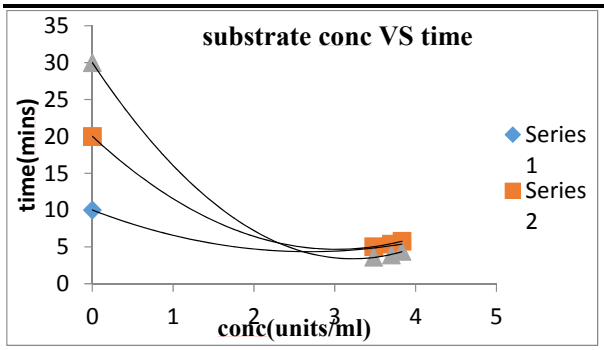
$$\frac{1}{V} = \frac{k_m + [S]}{V_{max}[S]} = \frac{k_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}$$

$$\ln(k) = \ln(A) - \frac{E_a}{R}\frac{1}{T}$$

4.2.1 Calculation of Kinetic parameters by Using Line-weaver Burk Plot:-

Time (mins)	Casein(4.5g m/lit) Units/ml of enzyme		Casein(6.5g m/lit) Units/ml of enzyme		Casein(7.5)g m/lit Units/ml of enzyme	
sample	D1	III-F-B1	D1	III-F-B1	D1	III-F-B1
0	4.54	3.48	4.72	3.83	4.62	3.7
10	5.96	4.9	6.16	5.46	6.07	5.04
20	6.01	5.04	6.22	5.79	6.12	5.4
30	4.11	3.6	5.04	4.4	4.82	3.9

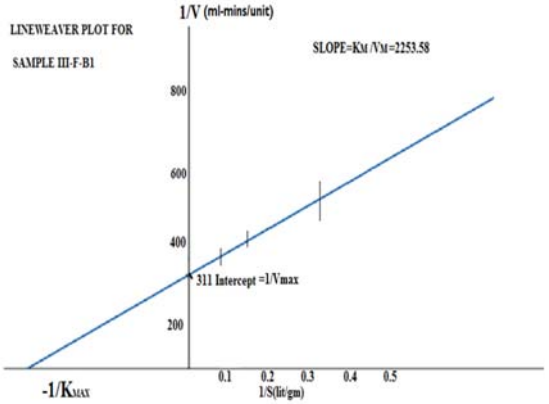
Table 12: for Sample D1 & III-F-B1



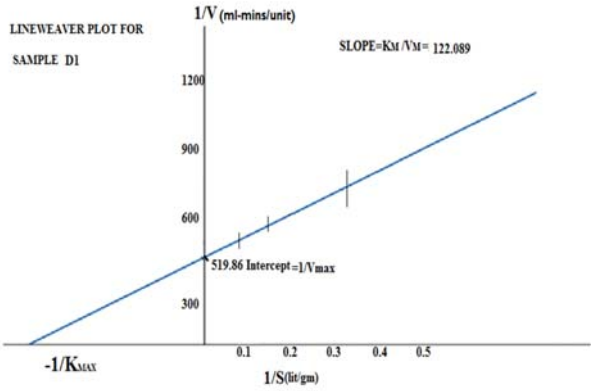
Graph 6: substrate Conc vs Time

Sample	D1			III-F-B1		
Conc.(gm/lit)	4.6	6.5	7.5	4.6	6.5	7.5
1/conc. (gm/lit)	0.22	0.153	0.133	0.22	0.153	0.133
r(units/ lit-min)	0.0116	0.0102	0.0062	0.005	0.00204	0.009
1/r(units/lit-min)	6.2	78.02	153.83	200	49.01	104.49

Table 13: for Sample D1 & III-F-B1



Graph 7: Line-weaver Burk Plot for Sample III-F-B1



Graph 8: Line-weaver Burk Plot for Sample D1

Result for Line-Weaver Burk plot:-

Samples	**r_{max}(units/ml-min)**	**Kmax(units/ml-min)**
D1	0.00192	0.2344
III-F-B1	0.0032	7.211

Table 14: Result for Line-weaver Burk plot

4.2.2 Calculation of Kinetic Parameters by using Arrhenius Equation:-

Sample III-F-B1:-

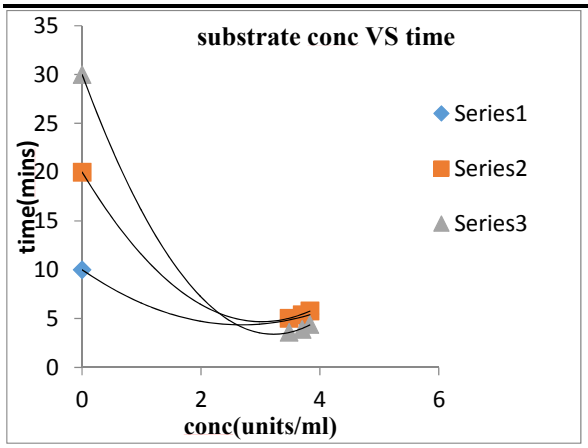
Time (min)	CONCENTRATION AT DIFFERENT TEMPERATURES (k units/ml)					
	288	298	310	318	338	353
0	1.16	1.31	1.61	1.72	2.01	2.12
10	3.02	3.12	4.12	4.21	4.31	4.46
20	3.59	4.36	6.13	6.13	4.91	4.86
30	3.26	4.12	4.53	4.53	5.36	5.49

Table 15:Sample III-F-B1

Sample D1:-

Time (min)	CONCENTRATION AT DIFFERENT TEMPERATURES (k units/ml)					
	288	298	310	318	338	353
0	1.08	1.19	1.56	1.58	1.96	2.06
10	2.96	2.99	3.56	4.86	3.42	3.48
20	3.42	4.10	5.92	5.97	4.86	4.12
30	3.12	4.06	4.61	4.63	3.56	3.33

Table 16: Sample D1



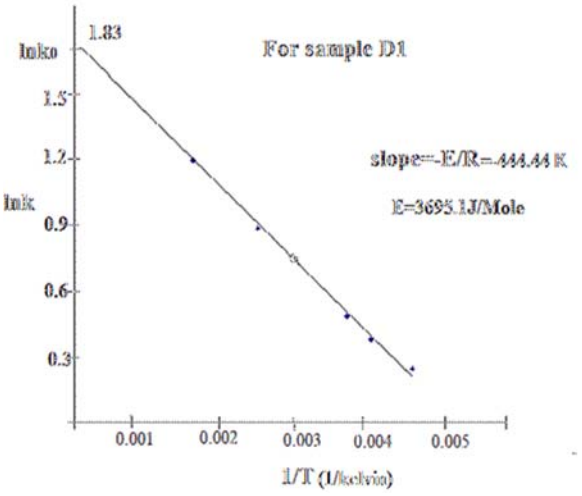
Graph 9: Substrate Conc. Vs Time

From above graph conc. Vs time:

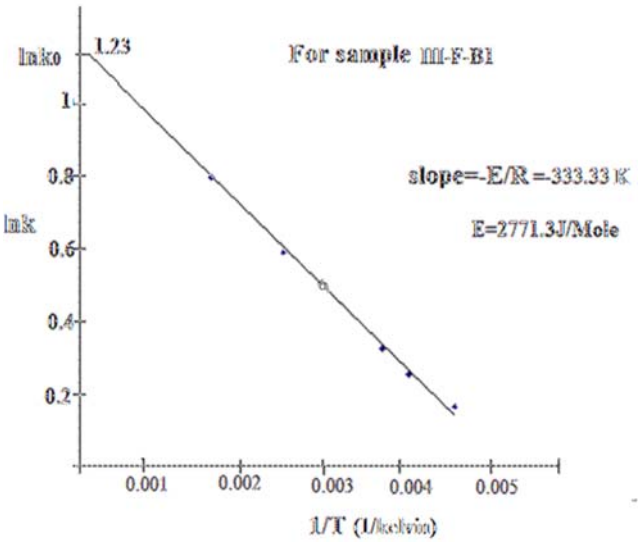
	Sample III-F-B1		Sample D1	
1/T(Kelvin Inverse)	K(units/ml-min)	lnk	K(units/ml-min)	lnk
0.00347	1.86	0.62	1.88	0.63
0.00356	1.81	0.59	1.8	0.59
0.00322	2.51	0.14	2	0.698
0.00314	2.41	0.91	3.28	1.19
0.00296	2.31	0.84	1.46	0.38

Table 17: Conc. Vs Time

*Plotted graph between **lnk vs 1/T** (for sample D1 & III-F-B1):*



Graph 10: Arrhenius Plot for D1



Graph 11: Arrhenius Plot for III-F-B1

Result for Arrhenius Plot:

Samples	**Frequency factor(K_0)**	**Activation energy(j/mol)**
D1	6.223	3695.1
III-F-B1	3.4212	2771.3

Table 18: Result for Arrhenius Plot

5. Discussion

Enzyme activity is optimum at:

- Substrate [casein (0.65%)wt/vol], Enzyme Concentration [0.8ml], pH [7.5], Residue time [20min], Temperature [45°C].
- The isolated new source of protease producing bacteria from the D1 & III-F-B1 samples and practically purified protease may be alternative source also for industrial applications.
- The maximum protease activity was found in glucose (media), suggesting that glucose is the best carbon source for the effective protease production.

6. Conclusion

Proteases are always very important in every field of life. Tremendous research work is going on in this field of science for enzyme production. The exploration of new cost effective process for enzyme production by exploiting industrial waste in the basic need of today in the current competitive enzyme market.

The optimum alkaline pH (7.5), reasonable activity at high temperature up to 45^0C and good

detergent compatibility characteristics make this alkaline protease a promising candidate to be used in the detergent industry.

The utilization of cheap substrate for protease production and high purification fold with easy protocols could make this enzyme economically feasible.

7. References

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