Kinetic Study of L-asparaginase From Alkaline *Bacillus sp.* Isolated From hypersaline environment

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ABSTRACT

Bacterial L-asparaginase is amidohydrolases that act on L-asparagine and produce L-aspartate and ammonia. These enzymes have been used in treatment of lymphoblastic leukaemia. In the present study, alkaline isolates were screened for potential producers of L-asparaginase using L-asparagine major source in M9 medium and phenol red as indicator. Further the isolates were been studied morphologically, biochemically to identify Bacillus sp, a novel strain. It produced largest zone of hydrolysis due to the bacteria utilised aspargine as nitrogen source. Bacillus sp. was explored for the production of extra-cellular L-asparaginase enzyme. The enzyme production was carried out by submerged fermentation.
The studied strains shows prominant enzyme activity as mesophillic Bacillus Sp. at pH 7.6 and temperature 70^0C with findings that metal ion Mg^{2+} as enhancer and EDTA as a inhibiter.

Keywords : L-asparaginase, mesophillic, *Bacillus sp*.

Introduction:

L-asparaginase (L-asparagine aminohydrolase, EC 3.5.1.1) the enzyme which converts L-asparagine to L-aspartic acid and ammonia, has been used as a chemotherapeutic agent **(Fisher and Wray, 2002).** L-Asparaginase has its application in food industry also. It helps in reducing the content of acrylamide in baked food products by hydrolysing the L-asparagine (**Mario Sanches *et al.* 2007** 3). More attention has been given to isolate L-asparaginase from microorganism (**Hill *et al.*, 1967**) It produced extracellular is advantageous and preferred over intracellular type because of higher accumulation of protein. The enzyme produces extracellular hence extraction and downstream processing were low economically.

The Lonar Lake situated in Central India included in National Geological Monuments of India (e-Journal Earth Science India, 2008) lies in an impact crater, within the zone of summer monsoon rainfall, and in the climatically sensitive region where the effects of tropical-multitude interactions are strongly seen. In India the Lonar Lake, popularly called as the Lonar soda lake is situated in the Buldhana district (19°58' N; 76°31' E) of the Maharashtra state Studies on microbial diversity of alkaline saline environments are important for two reasons. First, some of the earliest microbial life on earth might have been haloalkaliphiles, thus research on microbial community in soda lakes may gives clues into the evolution on life earth (Kunte *et al.*, 2002). Secondly, because of the presence of hyper saline conditions on Mars, terrestrial saline environments may act as good models for studies on life on Mars (Mancinelli *et al.*, 2004).

Isolation, identification and screening:

Water sample and Sediment sample were been collected from hypersaline condition i.e. Lonar lake in sterilize bottle and ziplock bags and were further processed for enrichment in alkaline nutrient media. The isolates were further subcultured for a well isolated colony in same medium.

Screening of L-asparaginase production by plate assay:

The pre-inoculated broth was spread on nutrient agar medium followed by morphological and gram's nature were detected. The isolated *Bacillus* cultures were spot on M9 medium containing 5% phenol red act as

an indicator for zone of hydrolysis of L-asparagine (as nitrogen source) as substrate. Phenol red at acidic pH is yellow and at alkaline pH turns pink, thus a pink zone is formed around microbial colonies producing L-asparaginase.

The largest zone was collected and inoculates on Horikoshi medium for future use. Plates were examined for change in color of medium from yellowish to pink due to change of pH indicating the positive asparaginase activity. Colonies with pink zones were considered as L-asparaginase-producing strains. Isolates exhibiting L-asparaginase activity were selected for further study. Control plates were prepared as un-inoculated medium and medium without dye.

Production of L-asparaginase by submerged fermentation:

Fresh inoculums of *Bacillus sp.* were prepared from the stock culture in fifty mL of M9 broth dispensed in 250 mL Erlenmeyer conical flasks. pH 8.5 was maintained and cultivated at shaker condition of 100 RPM for 48 hrs at 37^0C. After 48 hrs supernatant was obtained by centrifugation 5000 RPM for 20 minute of the 250ml Erlenmeyer flask.

Estimation of Total Protein (Folin- Lowery's method):

This method is about 10 time's sensitive than Biuret method. The reagent is called Folin Ciocalteneau reagent is quite complex and contains phosphomolybdate, phophotungstate, the aromatic amino acids. Tyrosine and tryptophan present in the proteins react with this and produce dark blue color.

The color formation is due to reduction of Phosphomolybdate by tyrosine and tryptophan present in protein and reaction of alkaline copper with protein.

Standard stock solution of protein i.e. Bovine albumin was prepared as 20 mg 40…., 200ml i.e. 200mg/ml. Then different dilutions were prepared from this stock solution. To each 1ml of sample, 5 ml of alkaline solution was added. Contents were mixed thoroughly on a Cyclomixer and allowed to stand for 15 min at room temperature. Then 0.5 ml of Folin Ciocalteneau i.e. reagent D was added with constant shaking the mixture was kept at room temperature for 30 min. Absorbance of coloured complex formed was read at 650 nm on spectrophotometer. A graph of concentration of protein against optical density was plotted and concentration of unknown sample protein was estimate.

L-asparaginase assay by Nesslarization method:

L-asparaginase activity was determined by measuring the amount of ammonia released by Nesslerization according to the method described by **Ionaduet *et al.* 1973.** The reaction mixture containing 1.5ml of 0.04M L-asparagine prepared in 0.5M Tris-HCl buffer of pH 8.0 and 1.5ml of an enzyme to make up the total volume to 3ml. The tubes were incubated at 37°C for 10minutes.

The reaction was stopped by adding 1.5ml of 1.5M trichloroacetic acid (TCA). The blank was prepared by adding enzyme after the addition of TCA. The precipitated protein was removed by centrifugation at 10,000 RPM for 5 min and the liberated ammonia in the supernatant was determined calorimetrically by direct Nesslerization by adding 0.5ml Nessler's reagent into tubes containing 0.5ml of clear supernatant and 3ml distilled water and incubated at 37^0C temperature for 10min.

A yellow coloration indicates the presence of ammonia: at higher concentrations, a brown precipitate may form. The yellow color was read using a UV-visible spectrophotometer (Optizen Pop –UV/Vis spectrophotometer) at 500nm. The amount of ammonia liberated was calculated using ammonium (ammonium chloride) standard curve. One unit (U) of L-asparaginase is defined as the amount of enzyme which catalyzed the formation of 1µmole of ammonia from L-asparagine per minute at 37°C and pH 8.0. The enzyme activity was expressed in terms of units per gram dry fermented substrate (U/gds).

Physiological study of extracted L-asparaginase:

Effect of enzyme concentration, effect of pH, effect of temperature, effect of substrate concentration, effect of inhibitor and effect of stimulator were studies and result was recorded.

Effect of pH on L-asparaginase activity:

L-asparaginase activity was studied as a function of pH in range between 7.2–9.0 L-asparaginase was active over broad pH ranges (4.5–10.5). The enzyme activity increase pH 7.6 with maximum activity. At higher pH enzyme activity was decreased. L-asparaginase is one of the amidases that are generally active and stable at neutral and alkaline pH, whereas, pH 7.0 to 9.0 was reported earlier to be optimum for amidase activity.

Effect of Temparature on L-asparaginase activity:

The temperature optimum of L-asparaginase from *Bacillus sp* is shown. It was active at wide range of temperature condition from 20–80°C. The maximum L-asparaginase activity obtained at 70°C. At higher temperature the L-asparaginase activity declined.

Effect of substrate concentration on the activity of L-asparaginase:

In this experiment, the influence of substrate concentration on L-asparaginase activity was examined by using different concentration of substrate ranging from 0.00015 to 0.0015 M to determine the optimum concentration of substrate required to give the highest L-asparaginase activity. The results showed a gradual increase in the enzyme activity with the increase in substrate concentration from 0.00105 to 0.00135 M. The optimum substrate concentration for L-asparaginase activity was observed at 0.0015 M.

Effect of Mg^{2+} concentration on the activity of L-asparaginase:

In this experiment, the influence of Mg^{2+}concentration on L-asparaginase activity was examined by using different concentration of Mg^{2+}ranging from 200 to 1000μm to determine the optimum concentration of Mg^{2+} required to give the highest L-asparaginase activity.

Effect of EDTA concentration on the activity of L-asparaginase:

In this experiment, the influence of EDTA concentration on L-asparaginase activity was examined by using different concentration of EDTA ranging from 200 to 1000 μm to determine the inhibitory concentration of EDTA which gives the lower the L-asparaginase activity.

Result & discussion:

The culture isolate from Lonar lake water and sediment samples were identified by streaking on nutrient agar plates and observation of colony (Table No.1)

Light micrograph showing the morphology and strain grown on M9 agar medium for 48 hrs at 37°C at magnification of 1500X (A) Plate assay showing zone of hydrolysis of asparagine *Bacillus sp*. Sample was brought from Lonar Lake which is situated in **Buldhana District, Maharashtra.**

Media of isolation of bacteria used was nutrient agar media gram's staining showed that the organism is aerobic, alkalophilic, mesophilic, Gram's positive rod. Gram's staining of freshly grown (incubated for 24 hrs at 37°C) native *Bacillus* isolates was carried out as per the standard staining procedure (Cappuccino and Sherman 2002) using the following three processes: staining with a water-soluble dye called crystal violet, decolourization and counterstaining with safranin. Substrate used for production of

L-asparaginase was the M9 media.The optimum temperature was found to be 70^0C. The maximum asparaginase activity shown at pH 7.6 as it was alkaline nature and incubation time we prepared was 48 hrs.

$$\text{Enzyme unit} = \frac{\text{Amount of product released X1000}}{\text{Reaction time X molecular weight of ammonia}}$$

$$= \frac{33X1000}{10 \text{ X } 17.031}$$

Hence,

Enzyme unit = 193.76 (U/gds).

Table No.1. Morphological characteristics of *Bacillus sp.*	
Observation	**Character**
2 mm in diameter	Size
rod	Shape
white	Color
entire	Margin
raise	Elevation
opaque	Opacity
Gram positive	Gram's nature

Table No. 2. Standard graph of ammonia					
Optical Dencity at 500 nm	**Nesslar's reagent (ml)**	**D/W (ml)**	**D/W (ml)**	**Stock sol (ml)**	**Conc of amm. Sulphate µm mol**
0.199	1	3	0.9	0.1	10
0.408	1	3	0.8	0.2	20
0.579	1	3	0.7	0.3	30
0.790	1	3	0.6	0.4	40
1.020	1	3	0.5	0.5	50
1.251	1	3	0.4	0.6	60
1.443	1	3	0.3	0.7	70
1.570	1	3	0.2	0.8	80
1.801	1	3	0.1	0.9	90
1.849	1	3	-	1	100
0.651	1	3	_	-	sample
Table No.3.Standard graph of total protein					
Optical Dencity at 650 nm	**Folin c. reagent (ml)**	**Alkaline Solution (ml)**	**D/W (ml)**	**Stock sol (ml)**	**Conc of protein µm mol**
0.053	0.5	5	0.9	0.1	20
0.097	0.5	5	0.8	0.2	40
0.149	0.5	5	0.7	0.3	60
0.211	0.5	5	0.6	0.4	80
0.281	0.5	5	0.5	0.5	100
0.298	0.5	5	0.4	0.6	120
0.356	0.5	5	0.3	0.7	140
0.420	0.5	5	0.2	0.8	160
0.452	0.5	5	0.1	0.9	180
0.505	0.5	5	-	1	200
0.291	0.5	5	-	-	sample

Table No.4.Effect of pH on L-asparaginase									
O.D.at 500 nm	**D/W (ml)**	**Nessler reagent (ml)**	**Super-natant (ml)**	**TCA (ml)**	**Incubation time (min)**	**Substrate Conc. (ml)**	**Buffer (ml)**	**Enzyme (ml)**	**pH**
0.635	3	0.5	0.5	1.5	10	1.5	1.5	1.5	7.2
0.744	3	0.5	0.5	1.5	10	1.5	1.5	1.5	7.6
0.700	3	0.5	0.5	1.5	10	1.5	1.5	1.5	8

0.645	3	0.5	0.5	1.5	10	1.5	1.5	1.5	8.6
0.650	3	0.5	0.5	1.5	10	1.5	1.5	1.5	9

Table No.5.Effect of Temperature on L-asparaginase

O.D.at 500 nm	D/W (ml)	Nessler reagent (ml)	Super-natant (ml)	TCA (ml)	Incubation time (min)	Substrate Conc. (ml)	Buffer (ml)	Enzyme (ml)	Temp. (^{0}C)
0.202	3	0.5	0.5	1.5	10	1.5	1.5	1.5	20
0.310	3	0.5	0.5	1.5	10	1.5	1.5	1.5	30
0.388	3	0.5	0.5	1.5	10	1.5	1.5	1.5	40
0.421	3	0.5	0.5	1.5	10	1.5	1.5	1.5	50
0.409	3	0.5	0.5	1.5	10	1.5	1.5	1.5	60
0.531	3	0.5	0.5	1.5	10	1.5	1.5	1.5	70
0.286	3	0.5	0.5	1.5	10	1.5	1.5	1.5	80

Table No.6 Effect of enzyme concentration on L-asparaginase

O.D.at 500 nm 70 C	O.D. at 500 nm 37 C	D/W (ml)	Nessler reagent (ml)	Super-natant (ml)	TCA (ml)	Incubation time (min)	[S] (ml)	Buffer (ml)	D/W (ml)	[E]
0.150	0.265	3	0.5	0.5	1.5	10	1.5	1.5	2.5	0.5
0.240	0.700	3	0.5	0.5	1.5	10	1.5	1.5	2	1
0.925	0.782	3	0.5	0.5	1.5	10	1.5	1.5	1.5	1.5
1.796	0.985	3	0.5	0.5	1.5	10	1.5	1.5	1	2
1.686	0.984	3	0.5	0.5	1.5	10	1.5	1.5	0.5	2.5

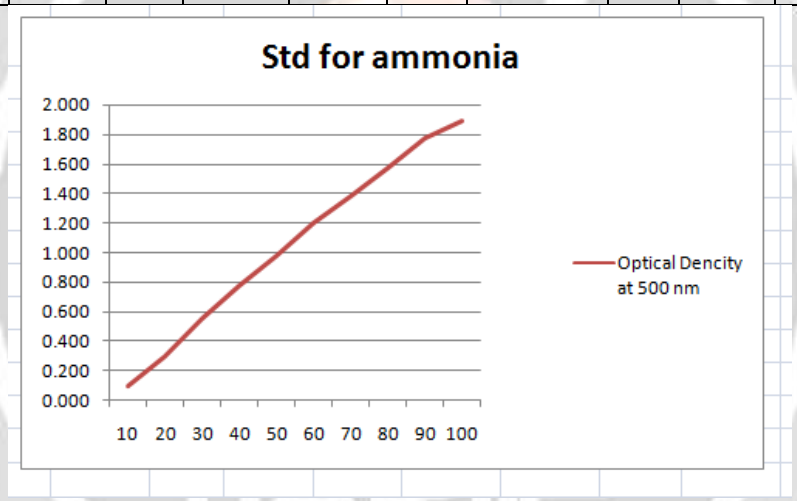
Table No.7 Effect of activter Mg2+ concentration on L-asparaginase

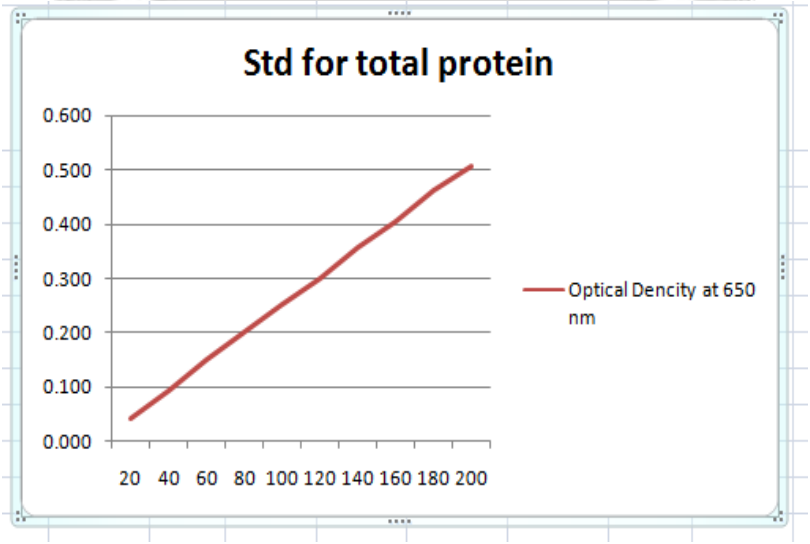
O.D.at 500 nm	D/W (ml)	Nessler reagent (ml)	Super-natant (ml)	TCA (ml)	Incubation time 70 C (min)	Substrate Conc. (ml)	Enzyme (ml)	Buffer (ml)	Stock+ D/W	Mg2+
0.160	3	0.5	0.5	1.5	10	1.5	1.5	1.5	0.2+0.8	200
0.204	3	0.5	0.5	1.5	10	1.5	1.5	1.5	0.4+0.6	400
0.227	3	0.5	0.5	1.5	10	1.5	1.5	1.5	0.6+0.4	600
0.282	3	0.5	0.5	1.5	10	1.5	1.5	1.5	0.8+0.2	800
0.318	3	0.5	0.5	1.5	10	1.5	1.5	1.5	1	1000

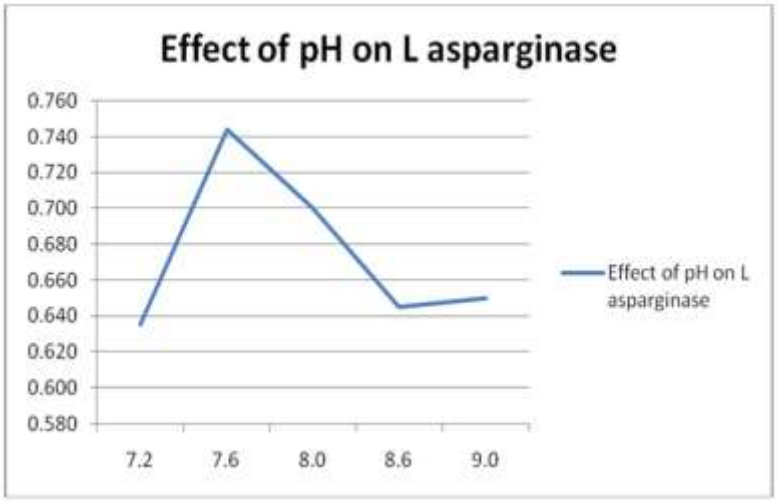
Table No.8 Effect of inhibiter EDTA concentration on L-asparaginase

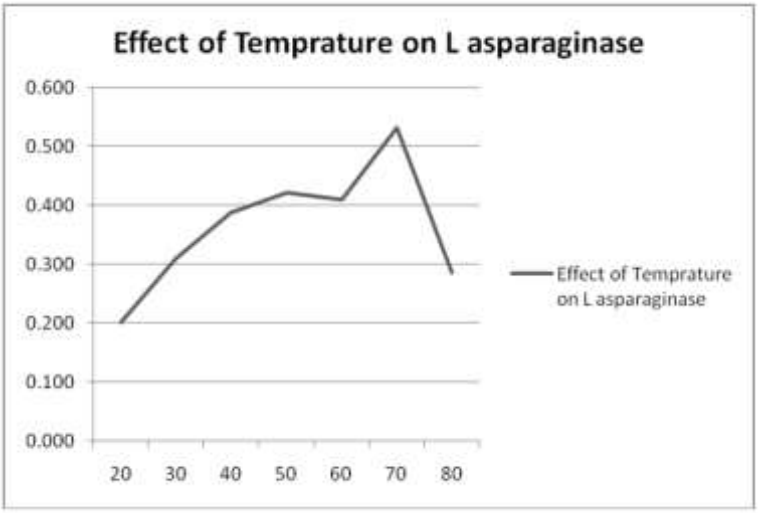
O.D.at 500 nm	D/W (ml)	Nessler reagent (ml)	Super-natant (ml)	TCA (ml)	Incubation time 70 C (min)	Substrate Conc. (ml)	Enzyme (ml)	Buffer (ml)	Stock+ D/W	EDTA
0.141	3	0.5	0.5	1.5	10	1.5	1.5	1.5	0.2+0.8	200
0.344	3	0.5	0.5	1.5	10	1.5	1.5	1.5	0.4+0.6	400
0.337	3	0.5	0.5	1.5	10	1.5	1.5	1.5	0.6+0.4	600
0.280	3	0.5	0.5	1.5	10	1.5	1.5	1.5	0.8+0.2	800
0.204	3	0.5	0.5	1.5	10	1.5	1.5	1.5	1	1000

Table No. 9 Effect of substrate concentration on L-asparaginase													
1/S	1/V	O.D. T (v)	O.D.at 500 nm	D/W (ml)	Nessler reagent (ml)	Super-natant (ml)	TCA (ml)	Incubation time (min)	E (ml)	Buffer pH 7.6 (ml)	D/W (ml)	Stock (ml)	Substrate Conc.
6666.67	154	0.0065	0.065	3	0.5	0.5	1.5	10	1.5	1.5	0.9	0.1	0.00015
3333.33	125	0.0080	0.080	3	0.5	0.5	1.5	10	1.5	1.5	0.8	0.2	0.00030
2222.22	50	0.0202	0.202	3	0.5	0.5	1.5	10	1.5	1.5	0.7	0.3	0.00045
1666.67	38	0.0266	0.266	3	0.5	0.5	1.5	10	1.5	1.5	0.6	0.4	0.00060
1333.33	40	0.0249	0.249	3	0.5	0.5	1.5	10	1.5	1.5	0.5	0.5	0.00075
1111.11	27	0.0376	0.376	3	0.5	0.5	1.5	10	1.5	1.5	0.4	0.6	0.00090
952.38	23	0.0441	0.441	3	0.5	0.5	1.5	10	1.5	1.5	0.3	0.7	0.00105
833.33	21	0.0469	0.469	3	0.5	0.5	1.5	10	1.5	1.5	0.2	0.8	0.00120
740.74	21	0.0474	0.474	3	0.5	0.5	1.5	10	1.5	1.5	0.1	0.9	0.00135
666.67	21	0.0483	0.483	3	0.5	0.5	1.5	10	1.5	1.5	-	1.0	0.00150






Effect of pH on L asparginase
0.760
0.740
0.720
0.700
0.680
0.660
0.640
0.620
0.600
0.580
7.2
7.6
8.0
8.6
9.0
Effect of pH on L asparginase


Effect of Temprature on L asparaginase
0.600
0.500
0.400
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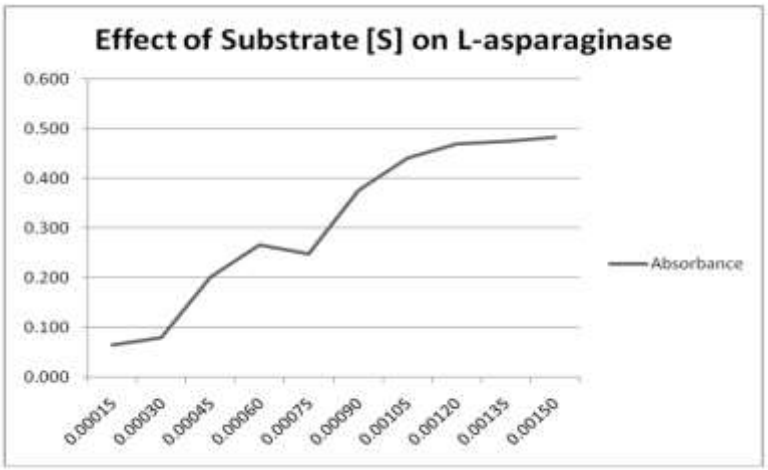
0.100
0.000
20
30
40
50
60
70
80
Effect of Temprature on L asparaginase


Effect of Substrate [S] on L-asparaginase
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0.500
0.400
0.300
0.200
0.100
0.000
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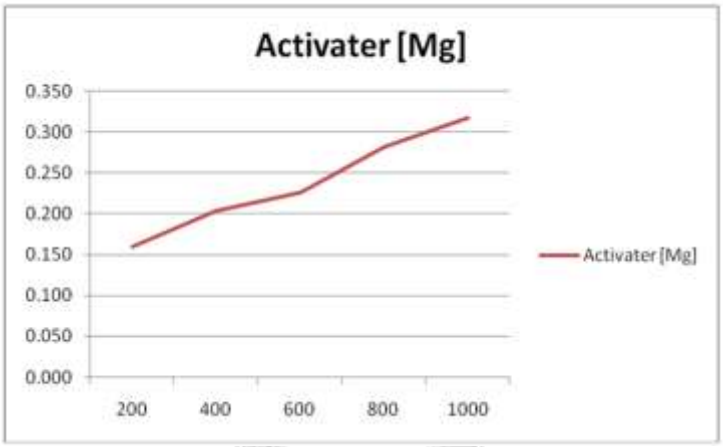
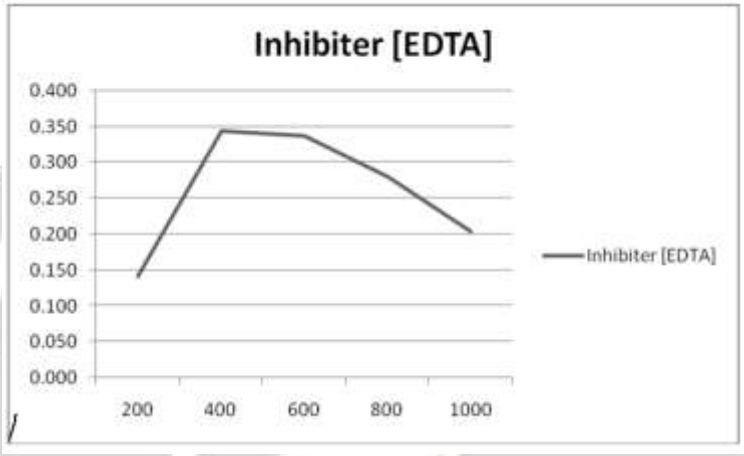
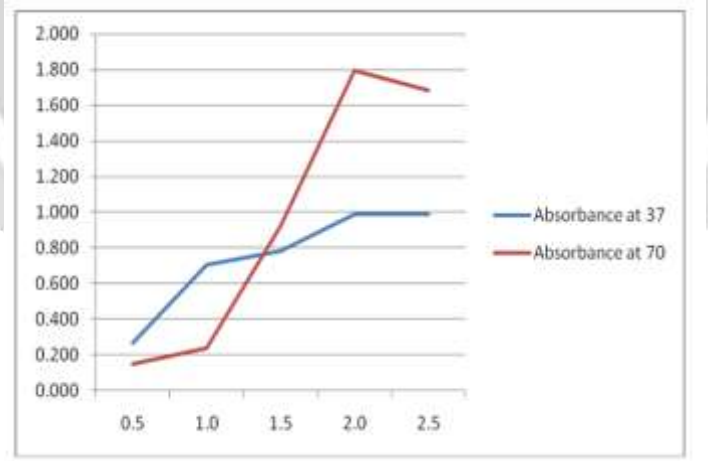
0.00060
0.00075
0.00090
0.00105
0.00120
0.00135
0.00150
Absorbance







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