International Journal of Trend in Research and Development, Volume 4(3), ISSN: 2394-9333
www.ijtrd.com

Study of Favorable Physico-Chemical Factors for Maximal Fungal Pectinase Production and Activity

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Abstract: The study was initiated by growing the mixed fungal culture on bread to produce the spores, followed by screening of effective strains on selective media containing pectin and studying them under varying ranges of temperature, pH and substrate concentrations to get the maximal growth conditions of the fungi. The ideal production conditions were found to be: pH-6.5, Temperature-40ºC, Substrate Concentration- 4%. The parameters for subsequent growth of the strains and for studying the maximum activity of the crude enzyme were maintained at the stated conditions. The value of V_{max} and K_m were then, determined using Lineweaver- Burke Reciprocal Plot.

Keywords: *Pectinase, Physico-Chemical, Fungal, Lineweaver Burke Plot.*

I. INTRODUCTION

Pectinase enzymes are extensively used in industries, waste water plants. In fact they also hold the key to effective agro-waste management, as they can utilize plant wastes as substrate and transform them into reclaimable pulp and produce various utilizable products. Microbial pectinases are important to phyto-pathological processes and decompose plant material, contributing to the natural carbon cycle. Pectinases are abundantly produced by saprophytic fungi.

The word "Pectinase" stands for a group of hydrolases, esterases and lyase enzymes capable of acting on pectic substances.

Pectic substances are complex high molecular mass glyosidic 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 [1,2].

Three major pectic polysaccharides groups are recognized, all containing D-galacturonic acid to a greater or a lesser extent.[3]

1) Homogalacturonan (HG)
2) Rhamno galacturonan I (RGI)
3) Rhamno galacturonan II (RGII)

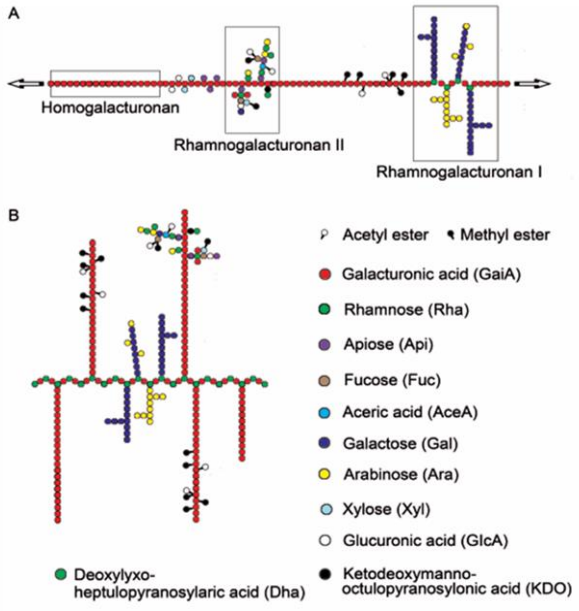
Pectinolytic enzymes are naturally produced by many organisms like bacteria, fungi, yeasts, insects, nematodes, protozoan and plants. Decaying plant tissue represents the most common substrate for pectinase-producing micro-organisms. Plant attack by pathogenic microorganisms usually starts by Pectinolytic enzymes attack since pectic substances are more accessible than other fibres in plant tissue [1].

They can be extracted from fungi such as *Aspergillus Niger*. Fungi produce these enzymes to break down the middle lamella in plants so that it can extract nutrients from the plant tissues and insert fungal hyphae. If pectinase is boiled it is denatured (unfolded) making it harder to connect with the pectin at the active site, and produce as much juice.

New enzymes for use in commercial applications with desirable biochemical and Physico-chemical characters and a low cost production have been the focus of much research [14]Enzyme breakdown of the biomolecules depends up on the type of enzyme, application, temperature, incubation time, agitation, concentration, pH and use of different enzyme preparations [15].

Several studies on microbial enzymes have shown the production of multiple pectinase forms which differ on molecular mass and kinetic properties [10, 11, 12]. Thus it has become the focal point of the said study and report. The production of multiple forms of enzymes improves the microorganism ability to adapt to environmental modifications [13]. Pectinase production has been observed in mixed strain fungi which possess varied conditions for optimum activity. Usually the enzyme works most efficiently within a range of 37 to 55' C and a pH of 3.0 to 6.5. These factors have been examined thoroughly for a lucid understanding of the activity.

Firstly, in the given report, one can find the evaluation of favourable Physico-chemical conditions of the maximal growth of the fungal micro-organism and then the partially purified dialysed enzyme activity so that the study delivers a moderate understanding of Pectinase Production.



II. MATERIALS AND METHODS

A. Isolation of fungal species

Bread was taken, it was sprinkled with adequate water and left in the open away from direct light where possibility of spore growth was high. After about a week, grey-black spores were observed on the bread surface. These spores were collected in Eppendorf tubes for further screening

B. Screening:

For screening of pectinase producing strains, we culture the spores, taken in suspension (spores in 10 ml water) of mixed strain in Modified Czapek-Dox media using pectin as the substrate (dark apple pectin, industrial grade) were used. The strain of interest breaks down the pectin supplied by producing pectinase enzyme. This helps them in their survival.

The Czapek-Dox medium was prepared by adding the compounds as per calculation : Modified Czapek-dox Media (per 150ml): Sucrose 1.5 g; $NaNO_3$ 0.6 g; $MgSO_4.7H_2O$ 0.1 g; KCl 0.1 g; KH_2PO_4 0.2 g;$FeSO_4.7H_2O$ 0.004 yeast extract 1g; pectin 3g;Agar 1.5%. The pH was adjusted to about 5.6(acidic environment is favorable). The agar was added and the volume made up to 150ml. The media was autoclaved at 121^0C for 15 mins. After the media cooled down to 50-55^0C the ampicillin was added and the conical flask shaken in swirling motion. The media was poured into four petri-dishes and left to solidify. The dishes were inoculated with spore suspension with a spreader. The petri-dishes were labeled and kept in incubator at 30^0C for seven days .After the stipulated time; the petri dishes were taken out for examination. Black coloured colonies were observed. (Fig.2)

C. Sub-culturing & Maintenance

Potato Dextrose Agar medium(Potato Dextrose Agar (PDA) media (Ingredients g/L): infusion from Potatoes 200 g, Dextrose 20 g, Agar 15 g, Final pH (at 30^0C) 5.6±0.2.) was used to sub culture the pectinase producing strains. The culture was done on agar slants. The media was prepared, autoclaved and ampicillin added after cooling. The media was poured in test tubes and solidified in slant position.

D. Study of most favorable culture conditions for enzyme production in fungi

Study of most favourable temperature for pectinase production: The temperature of the fermentation process can affect the growth as well as the product yield of the fungi and pseudo fungi in close relation (Torja and Mas 2003). Temperature can affect the sensitivity of the yeast to product concentration, growth rate, rate of fermentation, viability, length of lag phase and membrane function (Jack- son 2000). Four conical flasks (500 ml) were taken for pectinase production at four different temperatures-Room temperature,30°C,37°C & 50°C.Media (pH- 6.2, substrate concentration- 3%) was autoclaved. After cooling the media, at room temperature ampicillin and spore suspension was added. The flasks were kept for incubation at the different desired temperatures. Enzyme production was checked simultaneously from all the four flasks for five days.

Study of most favorable pH for pectinase production: Four conical flasks (500 ml) were taken, for pectinase production, at four different pH values, initially –3.5, 4.5, 5.5, 6.5.The pH of the media (substrate concentration 3%) for four flasks were maintained individually. The media filled flasks were autoclaved. After cooling the media, at room temperature ampicillin and spore suspension were added aseptically. Incubation was done at 37°C. Enzyme production was checked simultaneously from all the four flasks for five days.

Study of most favorable substrate concentration for pectinase production: According to Roumbouts and Pilnik (1980), culture media with an adequate balance of simple carbohydrates and pectin lead to the best production of pectinases in a submerged process. Due to their role as pectinase inducers, pectin or agricultural residues with high pectin content are essential components of the culture medium (Dhillon *et al*., 2004).Three conical flasks (500 ml) were taken for pectinase production at three different substrate concentrations-2%,3%.4%.The different substrate concentrations of the medias (pH- 6.5)were maintained. The media filled flasks were autoclaved. After cooling the media, at room temperature ampicillin and spore suspension were added aseptically. Incubation was done at 37°C.Enzyme production was checked simultaneously from all the flasks for five days.

E. Ideal conditions for maximum reaction of pectinase:

Method For Substrate Concentration Assay: Seven test tubes were labeled with concentrations 0, 0.5%, 1%, 1.5%, 2%, 2.5%, 3% .Stock solution, enzyme and buffer were added in each tube according to the labeled concentration and pH was maintained at 6.5.

These seven test tubes were kept for incubation at 37°C. For each concentration, set of five test tubes were taken each for collecting sample after 30 min. 200µl of sample was pipette out from each concentration after every 30 minute interval in their respective labeled test tubes. Then 1.5ml of DNSA in each test tube was added and incubation was done in boiling water bath for 10minutes. After cooling, the mixture was diluted to 50X (4.9ml of water with 100µl of the sample) and the OD at 540 nm was measured.

Method for Temperature Assay: Four test tubes were labelled, according to the desired temperatures 4°C, 25°C, 37°C, 50°C. Stock solution, enzyme and buffer were added in each test tube. Substrate concentration and pH was maintained at 3% and 6.5 respectively. For each temperature five test tubes were taken for collecting sample at interval of 30 min. One test tube was kept as control, containing only Stock (citric acid 11.8 g, tri sodium citrate 38.2 g and pectin .5 g) solution, buffer for each temperature. 200µl of sample was pipette out from each temperature point after every 30 minute interval in their respective labelled test tubes. Then 1.5ml of DNSA was added in each test tube and incubation in boiling water bath was done for 10minutes.After cooling, the mixture was diluted to 50X (4.9ml of water with 100µl of the sample) and the OD at 540 nm was measured.

Method for pH Assay: Four test tubes were labelled pH- 3.2, 4.2, 5.2, 6.2 Stock solutions, dialyzed enzyme and buffer were added to the test tubes making substrate concentration 3%, and their individual pH was maintained. Incubation was done at 37°C temperature. One control test tube was kept, containing only Stock solution, buffer for each temperature. For each pH five test tubes were taken for collecting samples at 30 min interval.200µl of sample from each pH test tube was pipette out after every 30 minute interval in their respective labelled test tubes. 1.5ml of DNSA was added in each test tube and was incubated in boiling water bath for 10minutes. After cooling, the mixture was diluted to 50X (4.8ml of water with 200µl of the sample) and the OD was measured at 540 nm.

F. Extraction and Purification of crude enzyme

In order to extract the crude enzyme the liquid culture broth was first filtered using Whatman No. 1 filter paper and then centrifuged at 7000rpm for 23 mins. After centrifugation the supernatant was collected in air tight vials and kept at 4^0C for further work.

Salt precipitation: 47.2 g of Ammonium sulfate was weight and used for 100 ml of suspension. Pinch-by-pinch Ammonium sulfate was added into the crude enzyme suspension for complete saturation maintaining ice-cold condition then the

solution was kept in 4^0C for overnight. The next day the solution was centrifuged at 9000 to 10000 rpm at 4^0C for 30 mins. Supernatant was discarded and the precipitation was suspended in citrate buffer.

***Dialysis*:** After salt precipitation 2 to 3 ml of enzyme extract was poured into dialysis bags and the sealed bags were kept into citrate buffer for 24 Hrs. After dialysis the concentrated enzyme was stored at 4^0C for future use.

G. Protein Estimation

For this assay we prepared BSA solutions of 5 various concentrations (10, 20,30,40,50 µg/ml) and one test sample (100µl enzyme extract and 900µl Bradford reagent).Added Bradford reagent into all the tubes and kept in dark for 15 mins and took their OD at 595 nm. From the graph we determined the total protein content of our enzyme extract.

H. Determination of V_{max} and K_m Value of the Enzyme

For this experiment enzyme is mixed with 10 to 20 (~11) different concentrated stock solutions in 1:5 volume ratio and incubated at 40^0C for 30 min. After incubation the DNSA test was conducted to determine the enzyme activity or product formation rate(dp/dt).

III. RESULTS &DISCUSSIONS

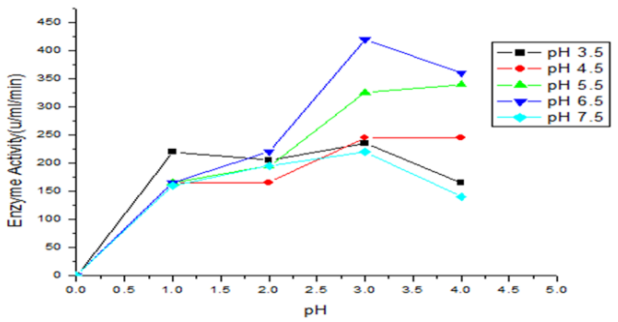
A. Study of most Favorable Conditions for Production of Pectinase

Effect of pH on Pectinase concentration: The Pectinase activity was found to increase with time at pH 3.5, 4.5, 5.5, 6.5 and 7.5 till the end of day 3.

Later on its activity decreased till the end of day 4. It reflects that the mixed fungal culture prefer pH 6.5 the most for its growth and metabolism. A maximum pectinase activity of 420 u/ml/min was obtained with mixed substrate for a fermentation period of 72h at temperature 35^0C and at pH value of 6.5. Hence the ideal pH value was chosen as 6.5.The results are evident from Table 1 and graph 1.

Table 1: Study of most favorable pH for maximum pectinase production

Day	Enzyme Activity(u/ml/min)				
	pH 3.5	pH 4.5	pH 5.5	pH 6.5	pH 7.5
1	220	165	165	165	160
2	205	165	195	220	195
3	235	245	325	420	220
4	165	245	340	360	140

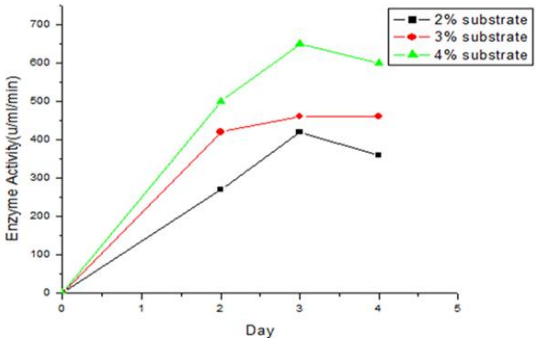


Graph 1: Representation of favourable temperature for maximum enzyme production

Effect of Substrate concentrations on production of total Pectinase: The Pectinase enzyme activity was found to increase exponentially reached at maximum of 650 u/ml/min at the end of day 3(48h) was obtained with 4% substrate (Pectin) concentration. Later on it was decreased till the end of day 4. The medium containing 4%pectin gave a maximum total pectinase activity of 650 u/ml/min and was chosen as the best substrate concentration for maximum pectinase enzyme production. The results have been given in Table 2 and Graph 2.

Table 2: Study of favourable substrate conc. for maximum production.

Day	Enzyme Activity(u/ml/min)		
	2% Substrate	3% Substrate	4% Substrate
2	270	420	500
3	420	460	650
4	360	460	600

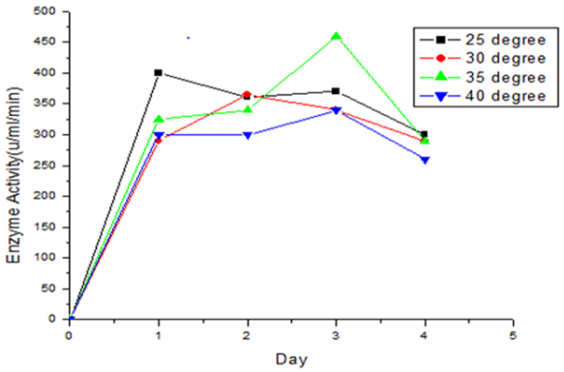


Graph 2: Representation of favourable the substrate conc. for max. pectinase production.

Effect of temperature on production of total pectinase: The optimum temperature of the pectinase enzyme production was studied using the media containing 4% pectin as substrate and pH 6.5. The experiments were conducted at different temperatures namely 25^0C, 30^0C, 35^0C and 40^0C by keeping all other conditions constant for the production period of 96h. Samples were drawn at regular interval of 24 h and analysed for pectinase activity. As temperature increases from 25^0C to 35^0C, the pectinase enzyme activity was found to increase and maximum pectinase activity of 460u/ml/min was found at 35^0C. Further increase in temperature, decreased the pectinase activity till the end of 96h. Hence, optimum temperature was chosen as 35^0C and was used for further studies. The results in table 3 and graph 3 are as follows:

Table 3: Study of favorable Temperature for maximum production.

Day	Enzyme Activity(u/ml/min)			
	25°C	30°C	35°C	40°C
1	400	290	325	300
2	360	365	340	300
3	370	340	460	340
4	300	290	290	260

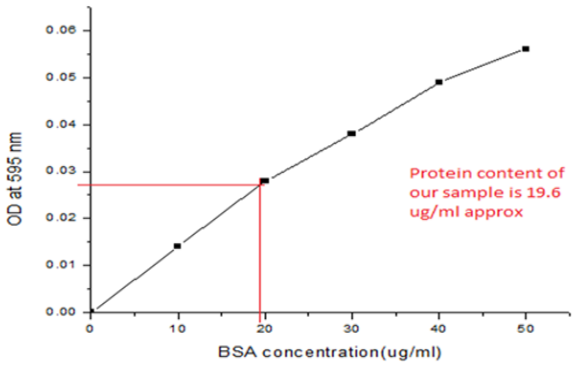


Graph 3: Representing the favorable Temperature for maximum production of pectinase

Total Protein estimation of Enzyme extract by Bradford assay: This assay was conducted using the dialyzed enzyme extract. The O.D. of test sample at 595 nm was 0.028 and amount of protein was determined from the graph.4.The concentration of protein equivalent to this OD is approximately 19.6 µg/ml.The result is given below:

Table 4: Values for generation of Standard Curve for Bradford assay.

BSA concentration(µg/ml)	OD at 595 nm
10	0.014
20	0.028
30	0.038
40	0.049
50	0.056



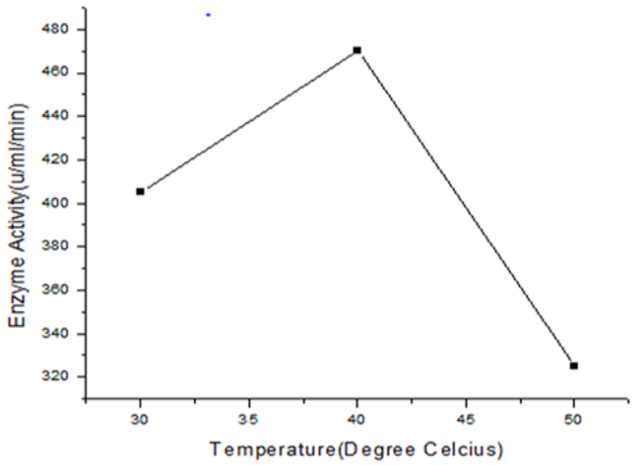
Graph 4: Representing the amount of protein in sample

B. Ideal Conditions for Reaction of Pectinase:

Effect of Temperature on the reactivity of Pectinase: For this experiment concentrated enzyme was mixed with 4% substrate stock in 1:5 volume ratios and incubated at 3 different temperatures for 2h 30mins. Samples were drawn at regular interval of 30mins and analysed for pectinase activity. The average activity of the enzyme at each time interval is given in Table 5 and From the Graph 5, it is evident that the enzyme (Pectinase) shows maximum activity at 40^0C.The decrease in the activity at higher temperature was may be due to the enzyme denaturation. At 40^0C the activity is 470u/ml/min. Hence it can be concluded that the ideal temperature for the enzyme to act upon its substrate is 40^0C. Results have been given in the table and graph below:

Table 5: Values for determining favourable temperature for enzymatic activity

Temperature(^{0}C)	Enzyme Activity(u/ml/min)
30	405
40	470
50	325

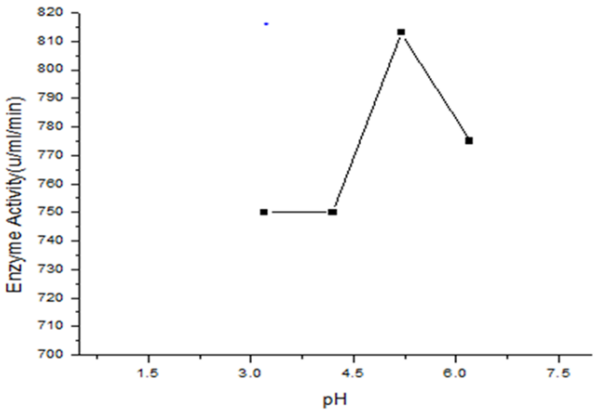


Graph 5: Determination of temperature for high enzyme activity

Effect of pH on the reactivity of Pectinase: For this experiment concentrated enzyme was mixed with 4% substrate stock in 1:5 volume ratios and incubated at 4 different pH at 40^0 for 2h 30mins. Samples were drawn at regular interval of 30mins and analyzed for pectinase activity. The average activity of the enzyme at each time interval is given in Table 6. From the Graph 6 it is evident that the enzyme (Pectinase) shows maximum activity at pH 5.2. At pH5.2 the activity is 813 u/ml/min. Hence it can be concluded that the ideal pH for the enzyme to act upon its substrate is pH 5.2. Table 6 and Graph 6 below explains the results.

Table 6: Values for determining favourable pH for maximum enzyme activity

pH	Enzyme Activity(u/ml/min)
3.2	750
4.2	750
5.2	813
6.2	775



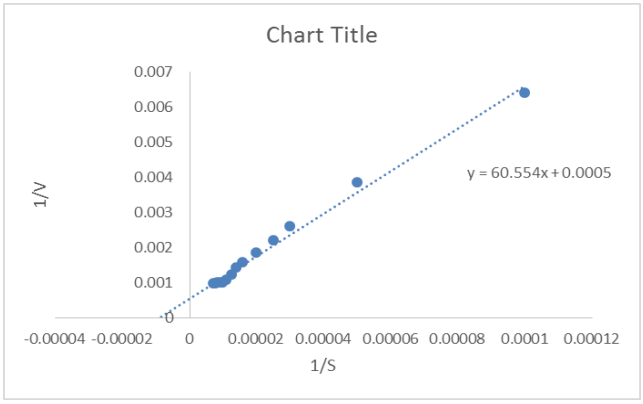
Graph 6. Determination of favourable pH for Maximum enzyme activity

***Determination of V_{max} and K_m Value of the Enzyme*:**

For this experiment enzyme is mixed with 10 to 20 (~11) different concentrated stock solutions in 1:5 volume ratio and incubated at 40^0C for 30 min. After incubation the DNSA test was conducted to determine the enzyme activity or product formation rate (dp/dt).The results are given in table 7 and Graph 7.

Table 7: Values for determining the V_{max} & K_m from Lineweaver –Burke reciprocal plot

Substrate Concentration(S) (g/100ml)	dp/dt (V) (µg/ml/min)	Substrate concentration (ug/ml)	1/s	1/v
1	156.13	10000	0.0001	0.006405
2	260.8	20000	0.00005	0.003834
3	385.4	30000	0.00003	0.002595
4	457.25	40000	0.000025	0.002187
5	543.75	50000	0.00002	0.001839
6	638	60000	0.000016	0.001567
7	708.5	70000	0.000014	0.001411
8	821.25	80000	0.0000125	0.001218
9	929	90000	0.000011	0.001076
10	1003.5	100000	0.00001	0.000997
11	1006	110000	0.000009	0.000994
12	998	120000	0.0000083	0.001002
13	1025	130000	0.0000076	0.000976
14	1020	140000	0.0000071	0.00098



Graph 7: Representing the reciprocal plot

The relation so generated from the Lineweaver-burke reciprocal is: y=60.554x + .0005. The standard reciprocal relation is given as:

$$\frac{1}{V} = \frac{Km}{V\max} \cdot \frac{1}{[S]} + \frac{1}{V\max}$$

Comparing both the relations, The K_m thus obtained is 121.108 mg/ml and the V_{max} value is 2000 µg/ml/min~2 mg/ml/min

CONCLUSION

Study of media and process parameters namely temperature and pH were carried out using fungal mixed culture in liquid culture. The maximum total pectinase activity of 420 u/ml/min was achieved using the media with 4 % pectin(by wt.). The optimum temperature was found to be 40^0C and Ideal pH was found to be 6.5 for maximum production of pectinase. The kinetics of pectinase production was studied using the determined media under optimum condition of temperature and pH. The kinetic parameters were determined using Lineweaver Burke plot and are given below km =121.108mg/ml and Vmax = 2 mg/ml/min

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