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OPTIMIZATION OF DISC DIFFUSION ASSAY TO STUDY PECTINASE ACTIVITY IN AGAR GEL

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ABSTRACT:

The activity pattern of Pectinase in gel matrix was studied. The behaviour of hydrolytic enzyme in gels as compared to that of its behaviour in solution is quite different. The study of this behaviour is required to better understand the enzyme action in nature. Each enzyme is quite specific in acting on a particular substrate to produce particular product. This specificity can be exploited for the application of enzymes. Present study was designed to understand the diffusion of enzymes in gels, enzyme substrate reaction in gel, enzyme parameter optimization and to determine the effect of substrate on enzyme activity in Agar gel.
The optimum pH and temperature were found to be 3 and 600C respectively. This study has established that enzyme action can be carried out and well studied in gels.

Keywords: Pectinase,Enzyme Activity, Agar-gel, disc diffusion

[I] INTRODUCTION

There are two fundamental conditions for life. First the living entity must be able to self replicate and second it must be able to catalyze biochemical reactions efficiently and selectively. Enzymes are biological catalyst, required for second condition. Each enzyme is quite specific in acting on a particular substrate or substrates to produce a particular product or products [1]. Pectinase is an enzyme found in many microorganisms and plants [2,3,4]. In microorganisms many times it acts as a virulence factor. Pectic enzymes also play a significant role in fruit development and ripening. Normally pectinase assays are performed in liquid media. However change in physical state change the enzyme activity [1]. Therefore the present study was done to design and optimized the '*disc diffusion assay*' to study Pectinase activity in agar gel.

[II] MATERIALS AND METHODS

2.1. Enzyme and Chemicals

Pectinase was produced from the laboratory isolate of *Aspergillus niger* [5] Pectin as substrate and Agar-agar (type1) was procured from Hi-media Laboratories' Pvt. Limited, Mumbai , India. Other routine chemicals and reagents used of analytical grade.

2.2. Disc Diffusion Assay

Assays were performed in agar gel matrix. The conditions were optimized in laboratories. Initially disc diffusion was performed to check whether the activity can be demonstrated or not. Once it was conformed, further assay were carried out.

1 %(w/v) pectin was prepared in 1% (w/v) agar in buffer of different pH . The thickness of agar was kept at 2 mm only. The assays were performed in 45 mm diameter plates. A 5mm diameter cellulose paper (Whatman No. 1) disc was punched and impregnated with 10 µl of 0.5% pectinase solution. The disc was placed on pectin agar plate and incubated for diffusion and reaction. The plates were developed by adding 1% (w/v) Cetyltrimetyl Ammonium Bromide (C-TAB) and the zone of clearance was measured.

The studies on determining the effect of pH, temperature disc diffusion optimization were carried out and studies on substrate concentration were also carried out.

The effect of pH was determined by preparing the substrate and agar gel in the corresponding buffer. The assessment of temperature effect was also performed in gels at different pH values.

As the experiments were carried out in gel, diffusion phenomenon plays an important role in overall enzyme activity. An enzyme concentration therefore was varied and corresponding results were also recorded and interpreted. .

To assess the *effect of substrate concentration*, varying concentration of substrate, from 0.5% to 8% (w/v) were used in the assay procedure.

[III] RESULTS

3.1. Effect of pH and temperature on enzyme activity

The effect of pH and temperature on Pectinase was simultaneously studied. The pH range was from 3 to 6 and temperature range was 10-70^{0}c. The optimum activity was shown at pH=3 temperature 60^{0}c. (Figure 1). This enzyme was active at very acidic pH and show very little activity at pH=6. (Table 1)

3.2. Effect of Pectinase concentration on enzyme activity

It is expected that increase in enzyme concentration should increase the activity. However it was found that except for 0.5% to 1% transition, there is no significant increase in activity for a particular incubation period. (Table 2)

3.3. Effect of incubation time on enzyme activity

The impact of incubation time on the substrate consumption and subsequently on enzyme activity was assessed. the time period was exponentially increased starting from 10 min till 1280 It was noted that increase in incubation period increases the enzyme activity (Table 3 and Figure 3). However this increase is very rapid initially that later on further incubation reduced. This study lead to establishing assay incubation for overnight duration

3.4. Effect of substrate concentration-Diffusion Limitation

To observe the effect of substrate concentration on Pectinase, pectin hydrolyzing activity was assayed by disc diffusion method [6]. It was found that enzyme activity show a decrease in zone diameter with increase in substrate concentration (Table 4) leading to a prima facia conclusion that, increase in concentration decreases activity. However careful consideration on the amount of substrate

consumed yielded a different picture. It was found that with the increase in substrate concentration there is corresponding increase in the amount of substrate consumed. The pattern of substrate effect is comparable with conventional pattern of effect of substrate concentration enzyme activity.

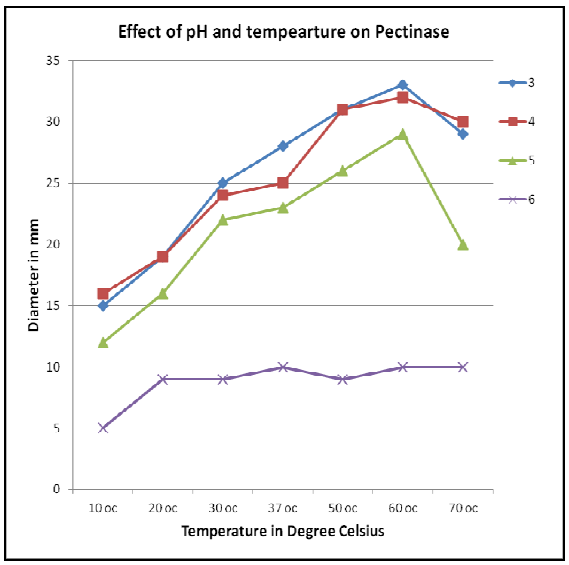

Fig: 1.. Effect of temperature and pH on Pectinase activity as determined by Disc diffusion assay

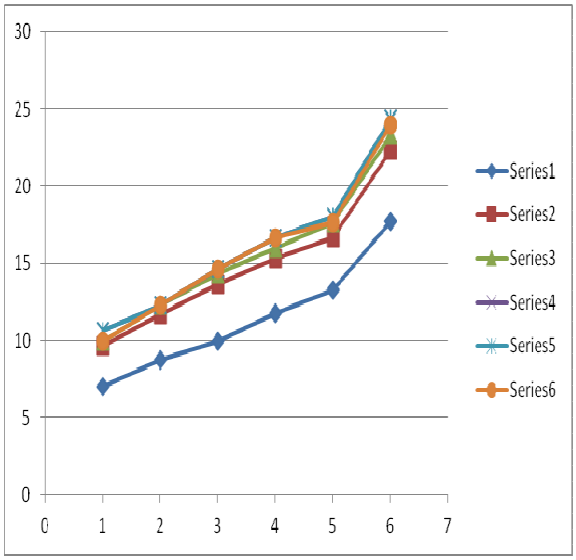

Fig:2. Effect of pectinase concentration on enzyme activity at different incubation times

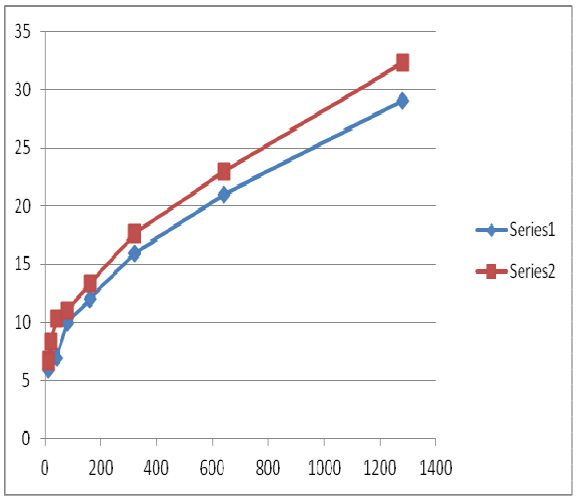

Fig:3. Effect of incubation time on enzyme activity

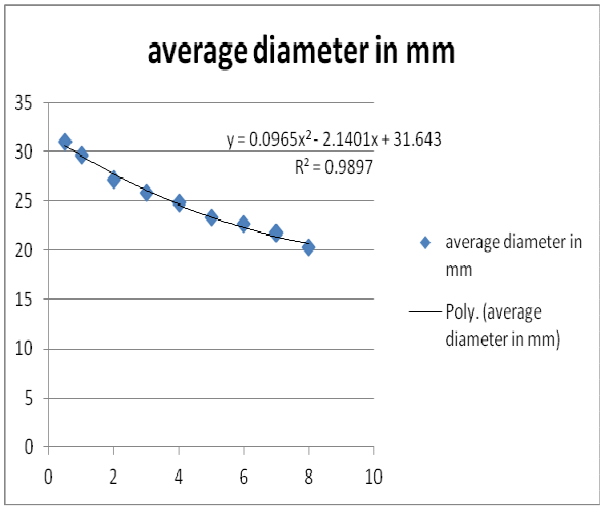

Fig: 4. The behaviour of enzyme activity with respect to change in substrate concentration

Sr. No	Temperature	pH			
		3	4	5	6
1	10 °c	15	16	12	5
2	20 °c	19	19	16	9
3	30 °c	25	24	22	9
4	37 °c	28	25	23	10
5	50 °c	31	31	26	9
6	60 °c	33	32	29	10
7	70 °c	29	30	20	10

Table 1 : Effect of pH and temperature on Pectinase in terms of zone diameter in mm

Time (hour)	Enzyme Concentration					
	0.50%	1%	2%	4%	8%	16%
1	7	9.67	10	10.67	10.67	10
2	8.75	11.67	12.33	12.33	12.33	12.33
3	10	13.67	14.33	14.67	14.67	14.67
4	11.75	15.33	16	16.67	16.67	16.67
5	13.25	16.67	17.67	18	18	17.67
6	17.75	22.33	23.33	24.33	24.33	24

Table 2: Effect of Pectinase concentration on enzyme activity in term of zone diameter in mm (values are averages of three observations)

Time in Minute	10	20	40	80	160	320	640	1280
0.5% Pectinase	6.0	7.0	7.0	10.0	12.0	16.0	21.0	29.0
1.0% Pectinase	6.66	8.33	10.33	11	13.33	17.66	23.0	32.33

Table 3: Effect of incubation time on enzyme activity as measured in terms of diameter of zone in mm

modulating enzyme activity [7]. hence change in one parameter may be compensated by other and a stable activity profile is obtained.

The increase in incubation time however has a significant effect. This is due to sufficient interaction taking place between enzyme and substrate. (Figure 2 and 3)

This behaviour of enzymes may be partly attributed to the presence of agar gel that probably act as imposing a quasi immobilization conditions.

The diffusion behaviour of enzyme in gel is very striking. Initially it follows a linear activity pattern with respect to time at a fixed substrate concentration. However, with the increase in time at low enzyme concentration the relationship between enzyme activities [diameter of zone] looses linear relationship. (figure 2;table 2) On careful analyses it was found hat for lower

ubstrate concentration in %	Diameter of Zone (mm)						Average diameter mm	volume of zone (ml)	substrate consumed
	1	2	3	4	5	6			
0.5	31	31	31	31	31	31	31.00	1.509	7.544
1	30	30	30	30	30	30	29.62	1.378	13.779
2	27	28	27	27	28	27	27.12	1.155	23.103
3	26	26	26	26	26	26	25.87	1.051	31.534
4	25	25	25	25	25	25	24.75	0.962	38.469
5	24	24	24	24	24	24	23.37	0.858	42.892
6	24	23	23	24	23	23	22.62	0.804	48.220
7	23	22	22	23	22	22	21.75	0.743	51.990
8	21	20	21	21	20	21	20.25	0.644	51.504

Table 4: Effect of substrate concentration on Pectinase (0.5%) activity and the substrate consumed

[IV] DISCUSSION

From the forgoing results it is established that enzyme show a typical relationship ratio. It is very clear from the results of the simultaneous experiment carried out at different pH and temperature combination (table 1) that at an alternate temperature a desired level activity can be obtained by changing a corresponding pH. This seems quite likely because each of them is

enzyme concentration up to 8 % enzyme follows a binomial relationship in the form of,

$$Y = ax^2 + bx + c$$

While,

a = diffusivity coefficient depends on the nature of matrix, concentration of Substrate, volume concentration.

b = coefficient that depend on the kinetic property of enzyme,

c = proportion to the diameter of disc used for imperilment of enzyme.

As enzyme concentration increase the value of 'a' decreased significantly, and for the used substrate concentration at 1 %, rise in enzyme concentration to beyond 16 % converts binomial relationship in to linear relationship and the behaviour can be model as Y =mx + c.

Where, Y= enzyme activity

X= enzyme concentration

The observation that with the increases in substrate concentration at a fixed enzyme amount, diameter of zone of hydrolyses decrease may lead to erroneous conclusion that there is diffusion limitation on enzyme diffusion. This is probably true, but the reason is all together different as with increasing substrate concentration for additional substrate is available near the enzyme source. Therefore, the enzyme is engaged in reaction with the additional substrate. This can be established from the fact that the amount of substrate converted into product increases with the increasing substrate concentration. This behavior of enzyme in gels is highly consistent and may be exploited to determine the kinetic parameters V_{max} and K_M.

[V] CONCLUSION

Disc diffusion assay was found to be useful method for study of pectinase enzyme activity.

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