Chapter 5

RESULTS & DISCUSSION

5.1 ANALYTICAL METHODS

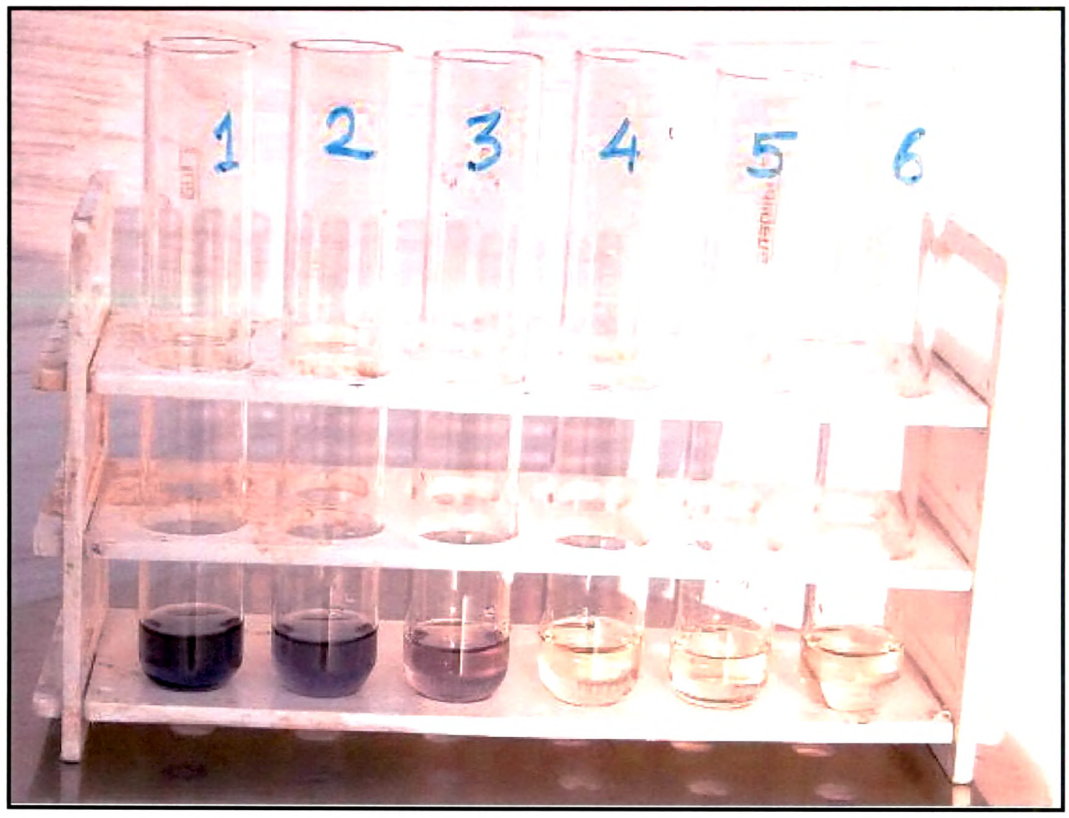
5.1.1 Estimation of fungal alpha amylase activity

Estimation of raw material fungal alpha amylase was done by Indian Pharmacopoeial method [1]. Fungal amylase is an amylolytic enzyme, which can degrade α - 1, 4 glucosidic bonds of starch to liberate smaller dextrins and maltose. The Indian Pharmacopoeial method estimates the substrate, starch remaining after the assay time interval for enzyme hydrolysis under defined conditions to evaluate the enzymatic activity.

The starch reacts with iodine solution to give blue colour complex. The smaller dextrins give yellow colour with iodine. Disappearance of the blue colour in the test medium indicates to the completion of starch hydrolysis as shown in Figure 5.1 where test tube number 4 indicates complete disappearance of blue colour.

In this method, to get accurate results, different sets of experiments are required to be repeated. Initially assay range is determined which is broad range about 10 % assay as per Indian Pharmacopoeia. Thereafter, this range is narrowed down by performing closer assay range experiments. Therefore this method is time consuming and also many excipients such as colour, antioxidants from formulations interfere with colour development, so this method is not suitable for formulations.

Figure 5.1 Indian Pharmacopoeial methods for estimation of fungal alpha amylase showing disappearance of the blue colour in the test medium indicates the completion of starch hydrolysis



5.1.2 Estimation of fungal alpha amylase activity from formulations

Estimation of fungal alpha amylase from formulation was done by radial diffusion method [2]. The substrate starch is dissolved in agar gel and fungal alpha amylase enzyme containing formulations are placed in the agar well. The enzyme diffuses out through the agar, turning the substrate into product as it goes. If it can actively digest starch it will create a starchless area around the well. Iodine stain is used to cause starch to turn a dark purple. Clear zones that are not purple are areas that the enzyme has digested the starch to dextrins.

5.1.3 Performance characteristics of radial diffusion agar plate method

Typical radial diffusion agar plate is shown in Figure 5.2, where clear zones that are not purple are areas that the enzyme has digested the starch to dextrins. The diameter of the zone is proportional to the enzyme concentration. The performance characteristics of radial diffusion agar plate method were carried out as per United States Pharmacopoeia [3] and ICH guidelines [4].

5.1.3a Accuracy

Accuracy was assessed using nine determinations over a three concentration levels. Known quantities of the analyte were added to the placebo for oral liquid formulation to make final concentrations 25 mcg /ml, 50 mcg/ml and 75 mcg/ml, in triplicate and analyzed by radial diffusion method. The assay results obtained are given in Table 5.1. The percentage recovery values obtained lie within the standard limit of 98 to 102%, which confirms that the proposed method is accurate.

5.1.3b Precision

Precision was assessed using six determinations of sample having concentration of 50 mcg/ml, analyzed by radial diffusion method. The assay results obtained are given in Table 5.2. The relative standard deviation (RSD) values mentioned in Table 5.2 are less than 2 % confirming that the analytical method is precise.

5.1.3c Specificity

To check the specificity of the method, acetate buffer pH 5 and placebo of oral liquid formulation was subjected to radial diffusion method. This study showed that the placebo as well as acetate buffer pH 5 does not show any zone of starch digestion. This confirms that the excipients used in manufacturing of oral liquid do not interfere with starch digestion.

5.1.3d Linearity and range

The linearity of response was determined by preparing solution containing 25, 37.5, 50, 62.5, 75 mcg/ml of fungal alpha amylase and placed each in triplicate on starch-agar plate. The mean potency obtained is given in Table 5.3 and graph of linearity is shown in Figure 5.3. The slope, intercept and correlation coefficient (r) were calculated from the results. By applying the proposed method, linearity was obeyed in the concentration range of 25 mcg/ml – 75 mcg/ml and correlation coefficient 0.999998 indicates good linearity between concentration and peak area. The slope value 2.00576 indicates the sensitivity of method.

5.1.3e Ruggedness

The reproducibility of analytical method was performed on oral liquid sample and made final concentration containing a 50 mcg/ml fungal alpha amylase by different analysts, on different days, different elapsed assay times. The assay results obtained are given in Table 5.4. The RSD value of analysis performed is less than 2 %, which demonstrates that the method developed is rugged.

5.1.3f Robustness

The robustness of analytical method was performed by change in temperature of incubation and change in buffer pH. The assay results obtained are given in Table 5.5. The robustness of the method showed that there were no marked changes in the starch digestion, which demonstrates that the method developed is robust.

5.1.3g Stability of analytical solution

Sample solutions having final concentration 50 mcg/ml of fungal alpha amylase were kept for 30, 60, 90, 120 and 150 minutes in stopper flask at ambient temperature. These samples solution were analyzed against standard solutions, which were freshly prepared. Changes in assay against initial values were calculated and given in Table 5.6. The stability study of analytical solution showed that the analytical solution is stable up to 90 minutes on storage at ambient temperature.

Table 5.1 Results of accuracy study of radial diffusion method for fungal alpha amylase

Assay No.	Concentration (mcg/ml)	Amount recovered (mcg/ml)	Amount recovered (%) (98.0 %-102 %)	RSD (%) NMT 2.0 %
1	25.0	25.056	100.22	0.4874
		24.813	99.25	
		24.920	99.68	
2	50.0	49.940	99.88	0.4967
		50.040	100.08	
		49.570	99.14	
3	75.0	74.990	99.99	0.4971
		74.865	99.82	
		74.300	99.06	
		Mean (assay %)	99.680	
		SD	0.4284	
		RSD (%)	0.430	

Table 5.2 Results of precision study of radial diffusion method for fungal alpha amylase

Assay No.	Amount recovered (%)
1	99.89
2	100.28
3	99.94
4	99.23
5	100.31
6	99.64
Mean (assay %)	99.89
SD	0.4071
RSD (%)	0.4076

Table 5.3 Results of linearity study of radial diffusion method for fungal alpha amylase

Level	Concentration mcg/ml	Nominal potency (%)	Potency recovered (%)
1	25.0	50.0	49.95
2	37.5	75.0	74.99
3	50.0	100.0	100.12
4	62.5	125.0	125.15
5	75.0	150.0	150.48
Slope			2.00576
Intercept			- 0.125
Correlation coefficient (r)			0.999998

Table 5.4 Results of ruggedness study of radial diffusion method for fungal alpha amylase

Parameter		Assay (%)	RSD of Assay Values (NMT 2.0%)
Interday	Day-1	100.12	0.1878
		99.97	
	Day-2	99.68	
		100.01	
Intraday	Morning	100.65	0.5810
		99.88	
	Evening	99.95	
		99.23	
Analyst	Analyst-I	100.12	0.5073
		99.85	
	Analyst-II	98.97	
		99.89	

Table 5.5 Results of robustness study of radial diffusion method for fungal alpha amylase

Parameter		Assay (%)	RSD of Assay Values (%) (NMT 2.0%)
Temperature of Incubation	35 °C	99.89	0.4620
		98.99	
	37°C	100.10	
		99.97	
	39°C	99.88	
		99.19	
Buffer pH	4.8	99.63	0.4126
		99.10	
	5.0	99.95	
		100.07	
	5.2	99.98	
		99.24	

Table 5.6 Results of analytical solution stability of radial diffusion method for fungal alpha amylase

Time of Exposure (minutes)	Assay (%)	Change in Assay against initial values (%)
0	100.02	0
30	99.98	0.04
60	99.15	0.87
90	98.45	1.57
120	97.76	2.26
150	97.13	2.89

Figure 5.2 Typical radial diffusion assay starch-agar plate showing clear zones of starch digestion produced by fungal alpha amylase and blue colour indicates the presence of undigested starch

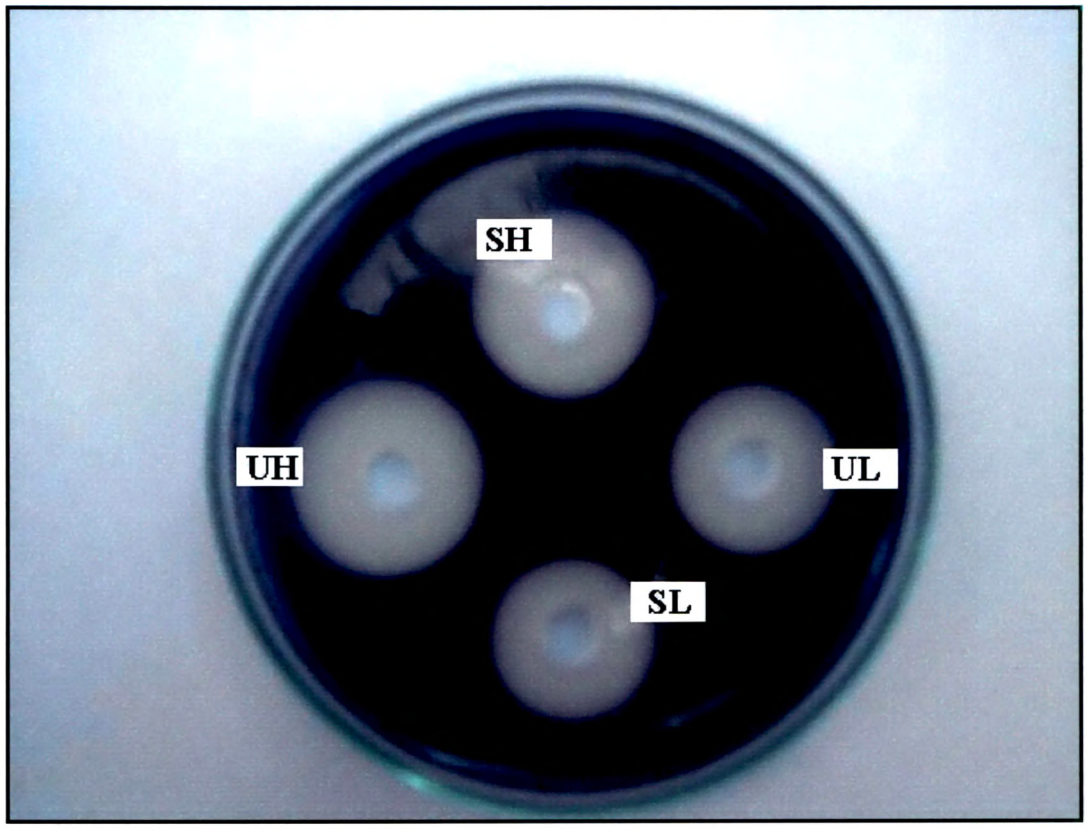
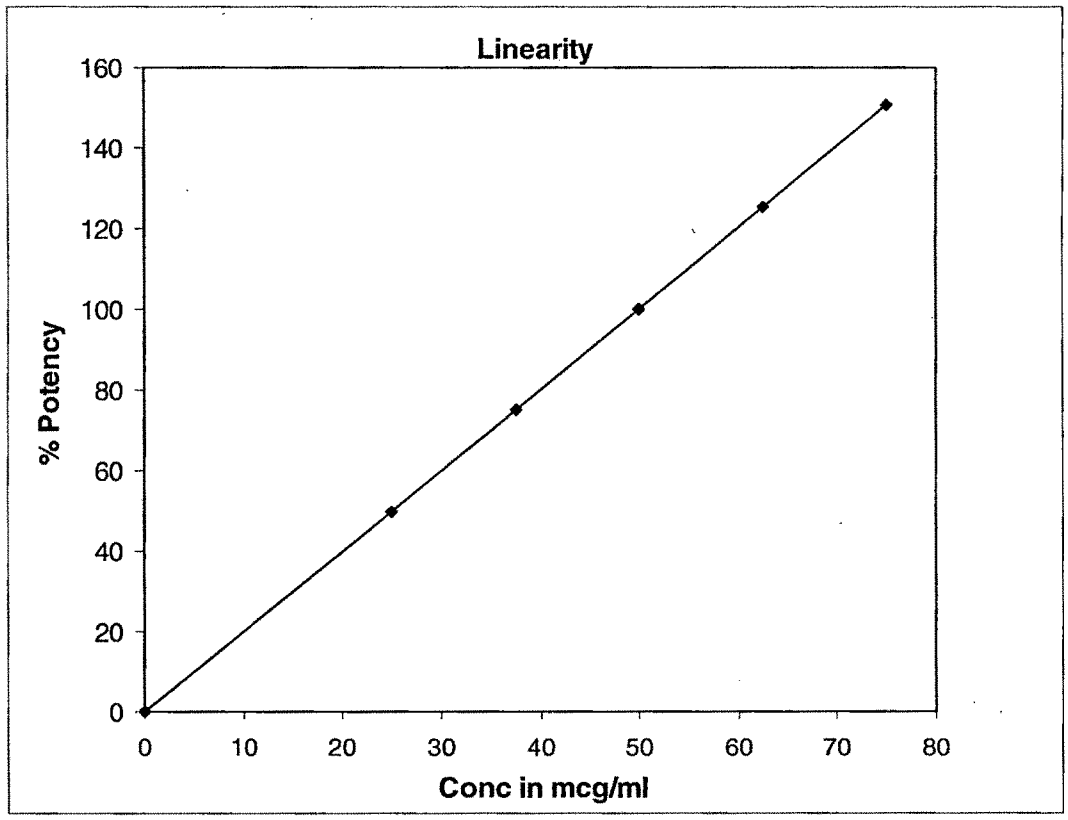


Figure 5.3 Linearity plot of the radial diffusion method



5.2 EFFICACY OF FUNGAL ALPHA AMYLASE IN FORMULATION CONDITION

5.2.1 Market sample study

Market survey revealed that there is only oral liquid formulation is available in market containing fungal alpha amylase (1: 1200) 50 mg and pepsin (1:3000) 10 mg per 5 ml.

To know the stability of fungal alpha amylase in market samples, eight leading market brands of oral liquid containing fungal alpha amylase and pepsin were analysed for pH and content of enzymes and results are given in Table 5.7 and Table 5.8. For initial analysis, market brands manufactured within two months were selected and for near expiry analysis, market brands whose expiry remained for two months were selected.

The analytical data clearly indicates that none of the product is stable up to expiry date. Pepsin is found to be stable up to expiry with overages of about 50 %. Fungal alpha amylase is not stable even with about 50 % overages. Also it is found that all market samples were at pH 4 to 5, which is not the stability pH range for fungal alpha amylase [5].

Therefore main aim of this study was to stabilize the fungal alpha amylase in formulation.

5.2.2 Effect of pH on fungal alpha amylase activity

Percentage activities of fungal alpha amylase recovered at different pH from 1 to 9 are given in Table 5.9 and Figure 5.4. The results indicate that fungal alpha amylase is stable in the range of pH 6 to 9 and the results are in the agreement of reported stability pH 6 to 9 [5, 6, 7].

Table 5.7 Initial analysis of market brands of fungal alpha amylase

Product	A	B	C	D	E	F	G	H
Expiry (months)	12	18	15	18	18	18	12	12
pH	4.730	4.168	4.870	4.877	4.443	4.342	4.610	5.053
% Assay of FAA	129.20	155.91	130.09	178.11	148.87	155.45	125.65	130.51
% Assay of PEP	120.15	142.23	130.67	148.0	146.38	148.55	125.02	132.46

FAA- Fungal alpha amylase, PEP- Pepsin

Table 5.8 Near expiry analysis of market brands of fungal alpha amylase

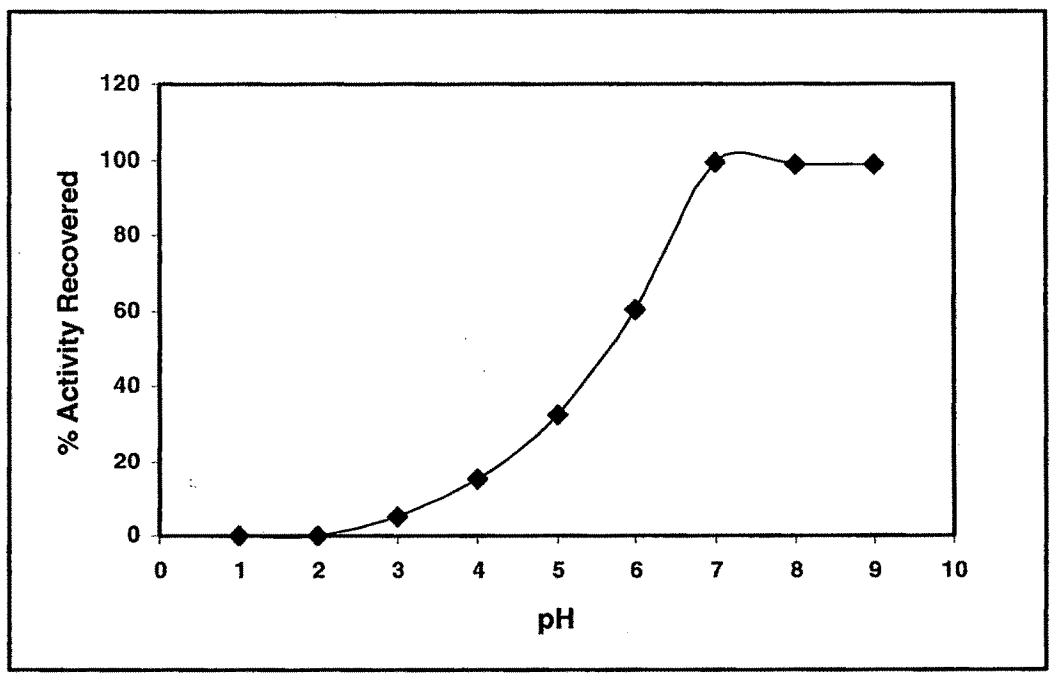
	A	B	C	D	E	F	G	H
pH	4.502	4.036	4.771	4.958	4.725	4.552	4.809	5.128
% Assay of FAA	58.14	38.29	43.56	43.52	49.33	55.12	45.25	50.11
% Assay of PEP	99.23	105.14	112.5	110.75	106.33	112.91	102.54	108.36

FAA- Fungal alpha amylase, PEP- Pepsin

Table 5.9 Effect of pH on fungal alpha amylase activity

pH	% Activity Recovered
1	0
2	0
3	5.12
4	15.23
5	32.27
6	60.44
7	98.99
8	98.95
9	98.88

Figure 5.4 The effect of pH on activity of fungal alpha amylase



5.2.3 Effect of temperature on fungal alpha amylase activity

5.2.3a Solid state

Results of stability study of fungal alpha amylase powder at refrigerated condition (2°-8°C), 30 °C, 45 °C and 40°C with 75 % RH for three month is given in Table 5.10. The stability results show that fungal alpha amylase is stable at 2° – 8°C and 30°C. If we extrapolate the data of 2°-8°C and 30°C as shown in Figure 5.5, then expected shelf life will be about 27 months if stored at 2° – 8°C and about 13 months if stored at 30°C.

5.2.3b Liquid state

Stability study results of fungal alpha amylase in mixed phosphate buffer pH 7 at 2.16 mg/ml concentration at refrigerated condition (2°-8°C), 30 °C, and 45 °C for three month is shown in Table 5.11. The stability result indicates that fungal alpha amylase is stable at 2°–8°C and 30°C. The data of 2-8°C and 30°C if extrapolated, then expected shelf life will be about 6 months if stored at 2°–8°C and about 3 months if stored at 30°C as represented in Figure 5.6.

Table 5.10 Stability data cum characterization of fungal alpha amylase powder

Parameter	Period	Condition/Results			
		RC (2-8°C)	30°C	45°C	40°C+ 75% RH
1.Moisture Content (%)	Initial	1.75	1.75	1.75	1.75
	1st Month	1.73	1.69	1.55	4.33
	2nd Month	1.70	1.57	1.05	3.85
	3rd Month	1.65	1.35	0.77	3.84
2. % Assay	Initial	101.23	101.23	101.23	101.23
	1st Month	100.78	100.28	94.42	97.86
	2nd Month	100.36	99.33	87.60	94.42
	3rd Month	99.94	98.40	80.89	90.89

RC- Refrigerated Condition

Figure 5.5 Shelf life estimation for fungal alpha amylase powder

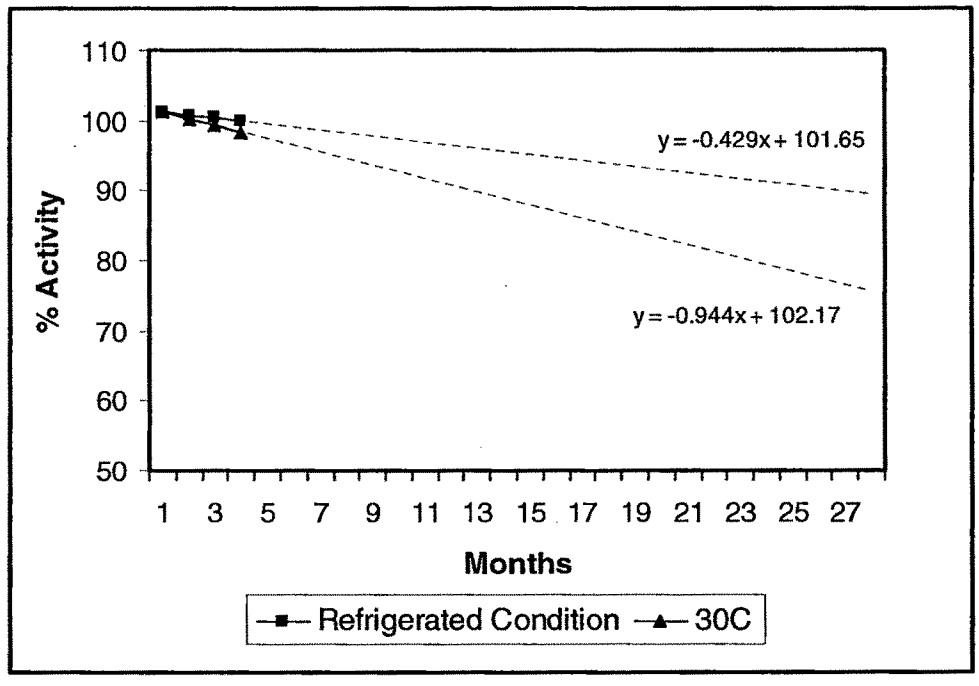
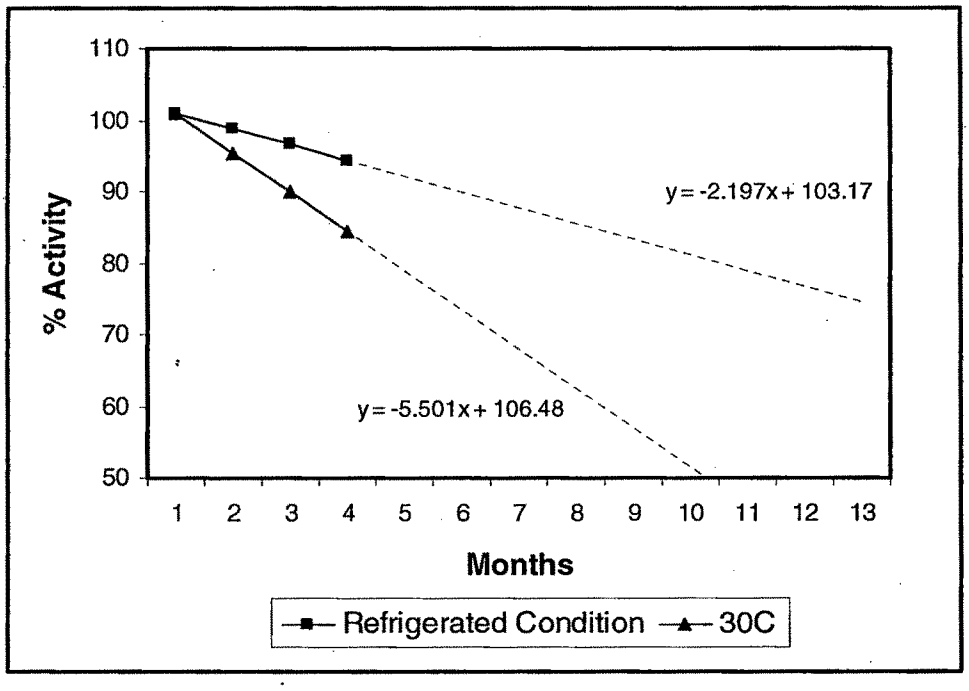


Table 5.11 Stability data of fungal alpha amylase in mixed phosphate buffer pH 7.0

Parameter	Period	Condition/Results		
		RC (2-8°C)	30°C	45°C
% Assay	Initial	100.98	100.98	100.98
	1st Month	98.76	95.46	57.10
	2nd Month	96.56	89.98	13.10
	3rd Month	94.39	84.47	Nil

RC- Refrigerated Condition

Figure 5.6 Shelf life estimation for fungal alpha amylase in mixed phosphate buffer pH 7.0



5.2.4 Effect of salts on fungal alpha amylase activity

The effects of calcium chloride, magnesium chloride and sodium chloride on fungal alpha amylase in mixed phosphate buffer pH 7 are given in Table 5.12.

Table 5.12 Effects of salts on fungal alpha amylase activity

Salts	% Assay		
	Initial	1st month	
		30°C	45°C
Without salt	100.98	95.46	57.10
With sodium chloride	100.08	97.49	78.12
With calcium chloride	100.45	99.09	86.51
With magnesium chloride	99.98	97.30	76.33

From the results, it is observed that calcium chloride remarkably increases the stability of fungal alpha amylase than magnesium chloride and sodium chloride.

Among the several enzymes used for thermostabilization, the calcium ion is known to be very effective owing to its structural stabilization. Calcium has been shown to regulate the stability and reactivity of a wide variety of biological proteins. In particular, its binding to alpha amylase is essential in activating and stabilizing the enzyme proteins [8-26].

5.2.5 Enzyme-cofactor interaction

5.2.5a Characterization

Solution containing fungal alpha amylase (2.16 mg/ml) and cofactor calcium chloride (0.4 mg/ml) in mixed phosphate buffer pH 7 at room temperature was subjected to ultraviolet spectroscopy, fluorescence spectroscopy and infrared spectroscopy and compared with fungal alpha amylase (2.16 mg/ml) in mixed phosphate buffer pH 7 and results are given below.

5.2.5a.1 UV spectroscopy studies

Protein or enzymes in a solution absorbs ultraviolet light at a wavelength of 280 nm, due to presence of aromatic amino acids, mainly tyrosine, tryptophan, in the protein structure [27, 28]. UV Absorbance spectrum of fungal alpha amylase (Figure 5.7) shows the λ max at 278 nm and UV absorbance spectrum of fungal alpha amylase with calcium chloride (Figure 5.8) also shows the λ max at 278 nm. This data shows that there are no alterations found in the aromatic residues of the fungal alpha amylase when interacted with cofactor.

5.2.5a.2 Fluorescence spectroscopy studies

A fluorescence spectrum (Figure 5.9) was recorded for fungal alpha amylase in mixed phosphate buffer pH 7 at 425 nm excitation wavelength and emission wavelength in the range 200- 500 nm. Similarly Figure 5.10 depicts the fluorescence spectra for fungal alpha amylase with calcium chloride solution. It is observed that both samples shown λ max at about 275 nm and shown identical fluorescence spectra. It is confirm that there is no change in the structure of fungal alpha amylase after interacting with cofactor.

5.2.5a.3 FTIR spectroscopy

The FTIR spectrum for fungal alpha amylase (Figure 5.11) shows the characteristic absorption bands of a protein. The absorption band at 3447.10 cm^{-1} is due to the N-H stretching, and the band at 1637.71 cm^{-1} is due to N-H bending. Similarly the FTIR spectrum for fungal alpha amylase

with calcium chloride solution (Figure 5.12) shows the absorption band at 3445.18 cm^{-1} is due to the N-H stretching and the band at 1633.85 cm^{-1} is due to N-H bending. The similar FTIR spectrum obtained confirms that there is no chemical interaction between fungal alpha amylase and cofactor.

Figure 5.7 UV absorbance scan of fungal alpha amylase

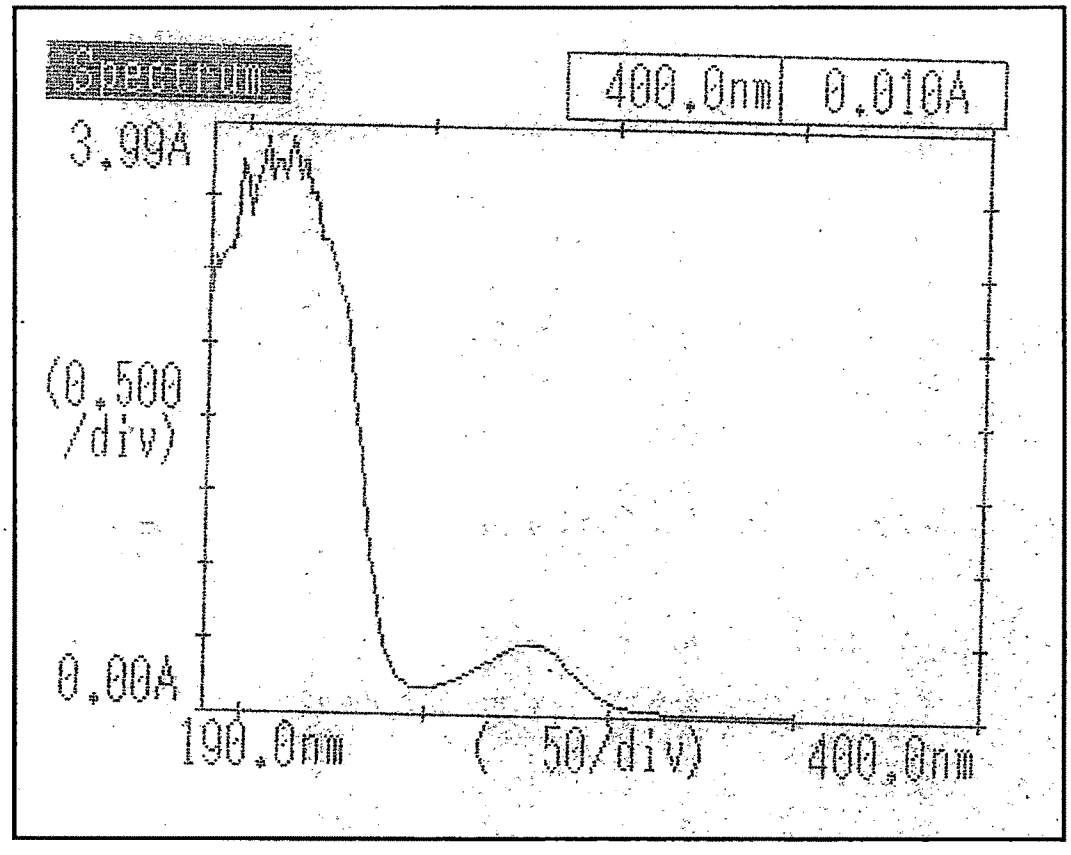


Figure 5.8 UV absorbance scan of fungal alpha amylase in presence of calcium chloride

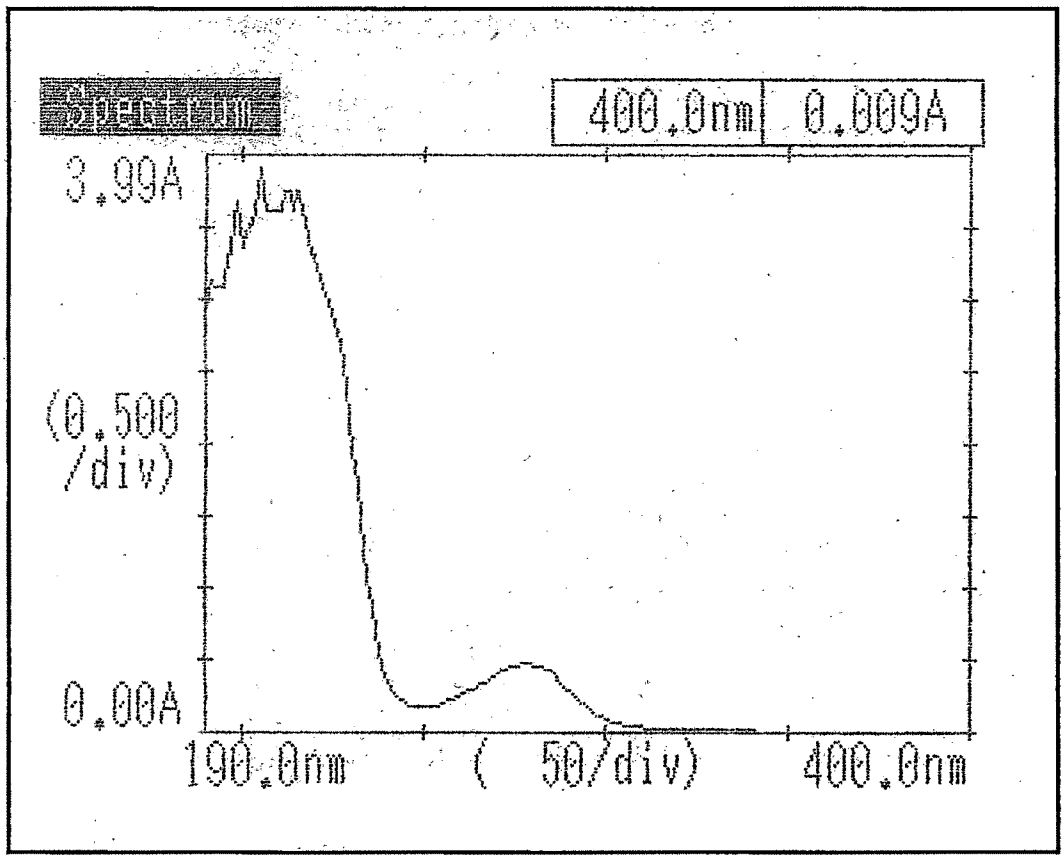


Figure 5.9 Fluorescence spectrum of fungal alpha amylase

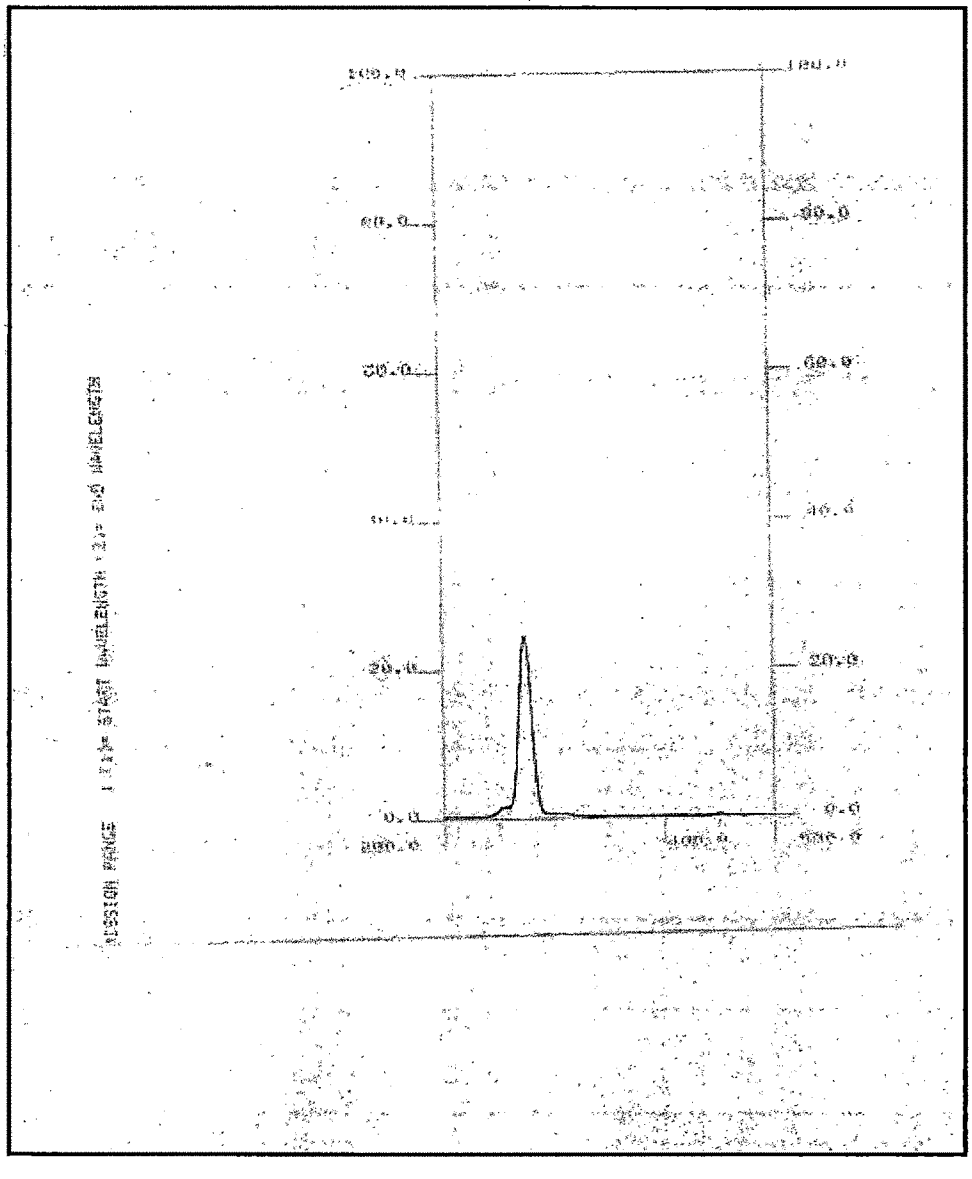


Figure 5.10 Fluorescence spectrum of fungal alpha amylase with calcium chloride

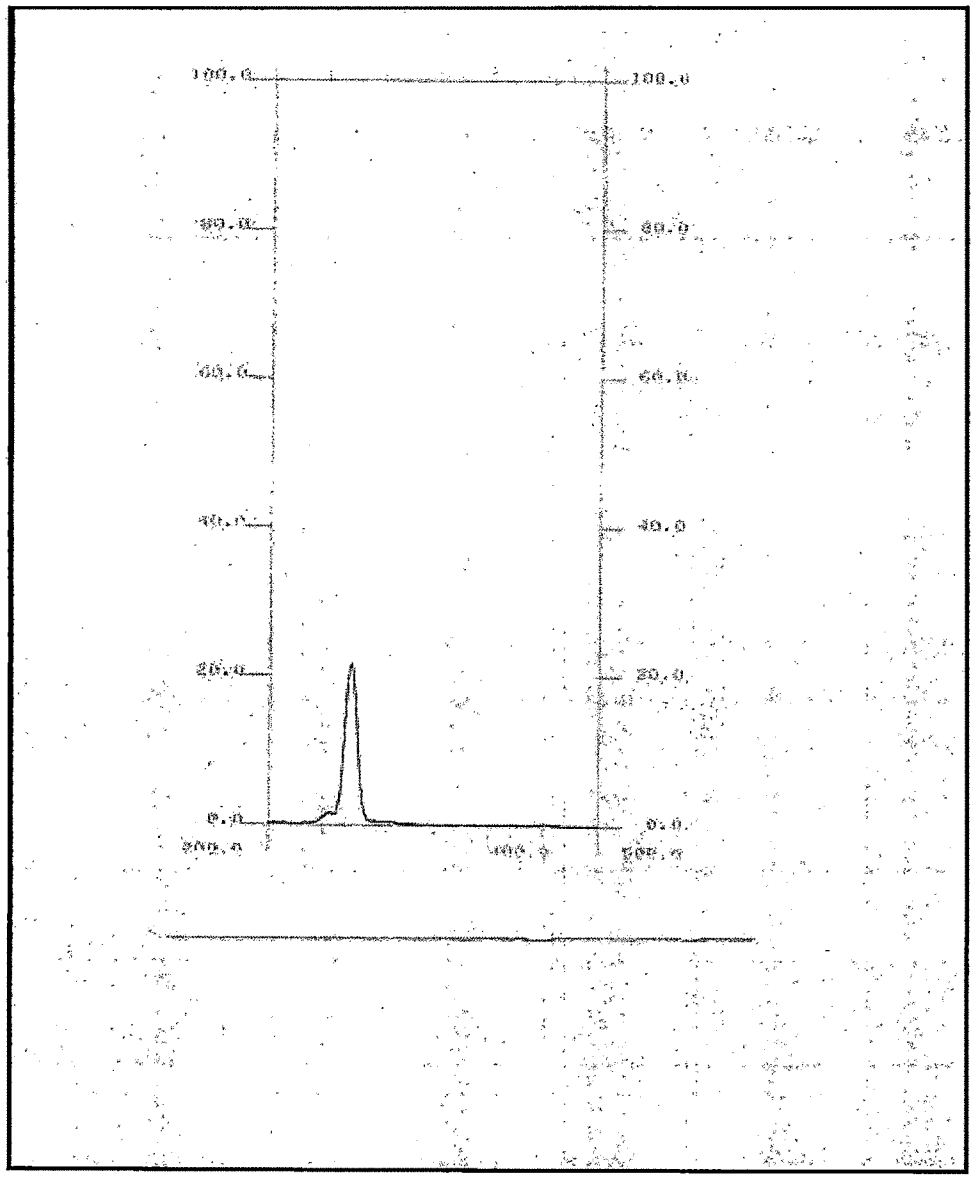


Figure 5.11 FTIR spectrum of fungal alpha amylase

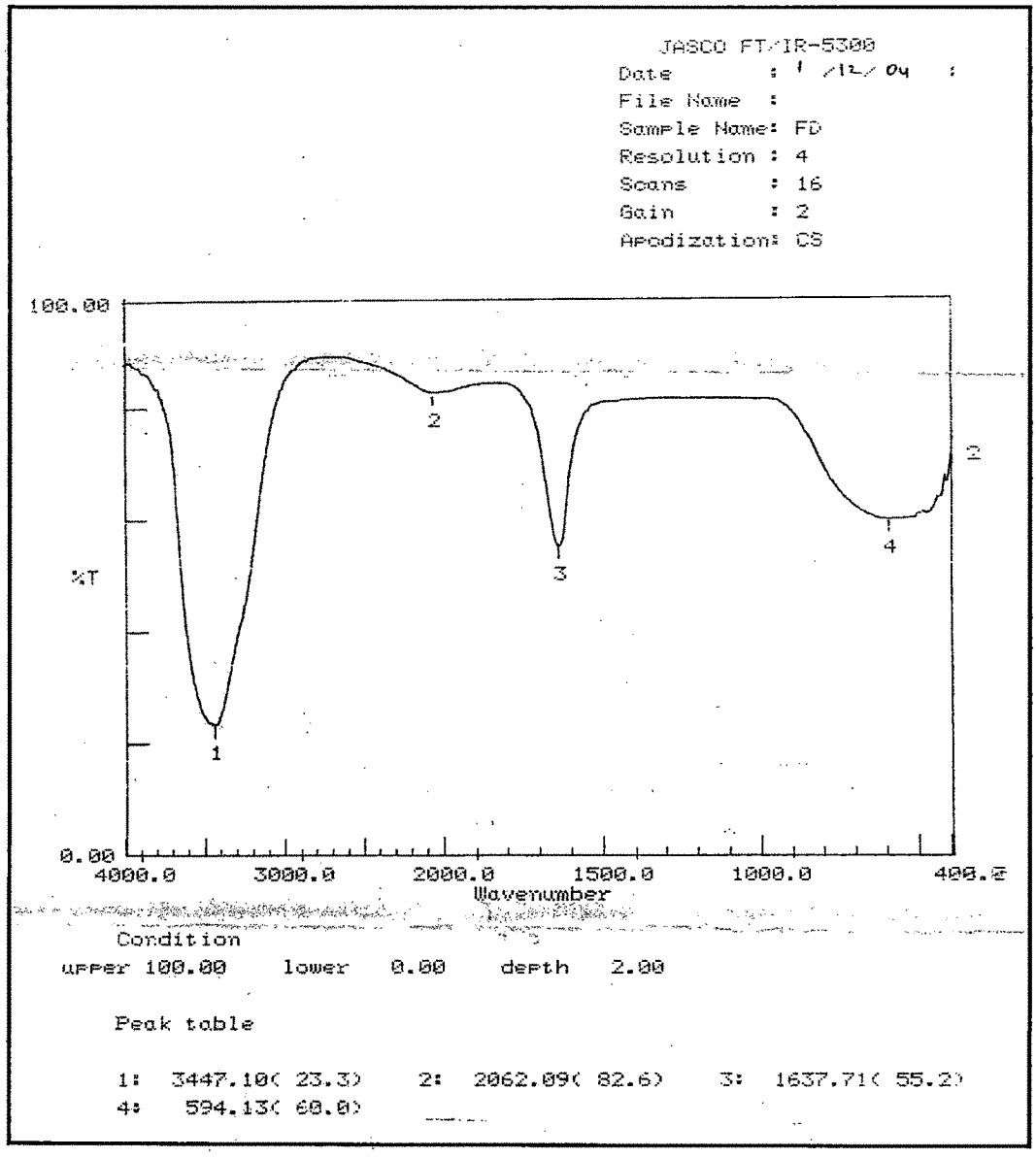
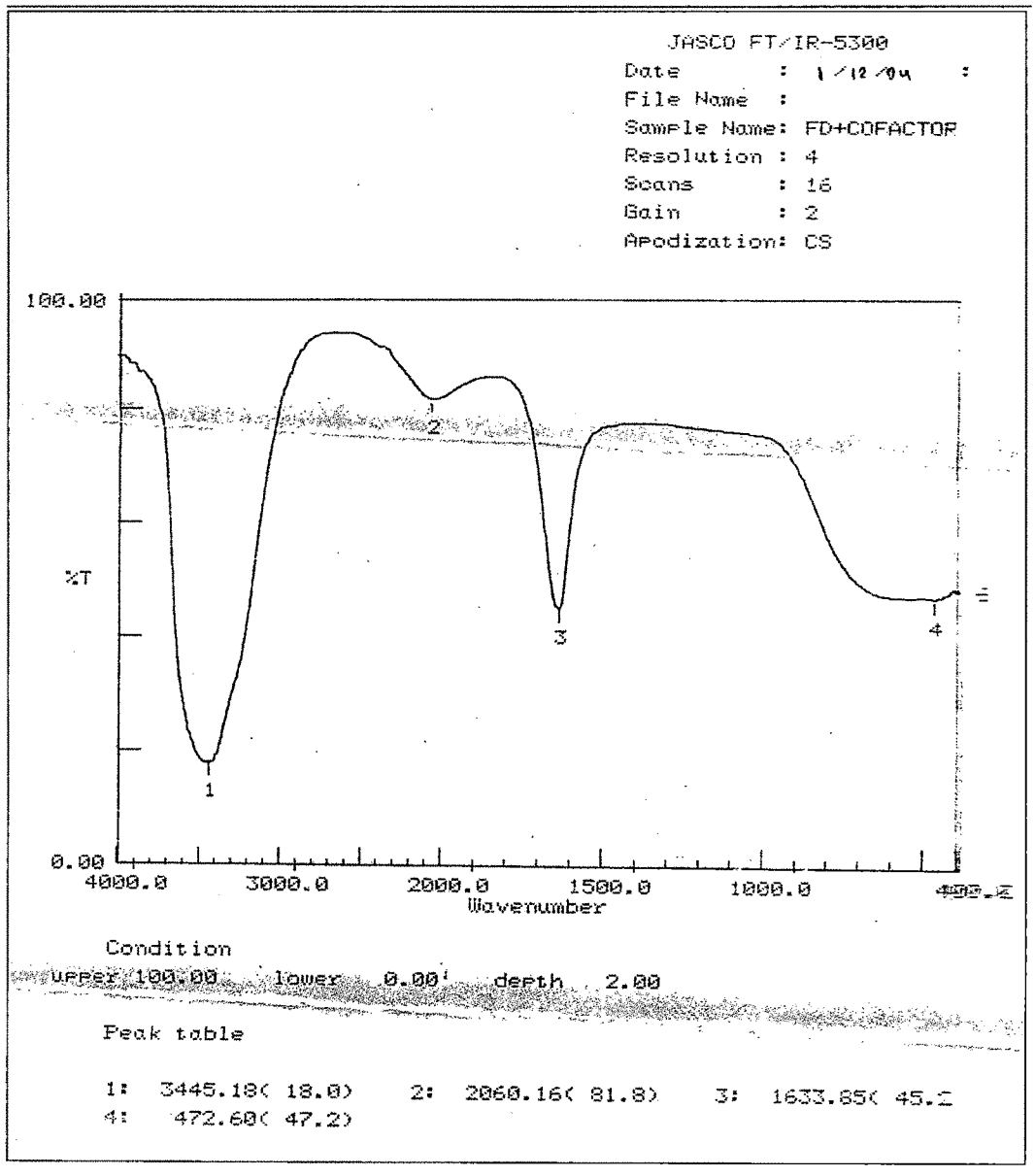


Figure 5.12 FTIR spectrum of fungal alpha amylase with calcium chloride



5.2.5b Effects of disodium EDTA

Metal binding characteristics of alpha amylase from various origins have been studied previously [10, 29, 30], but most of these studies focused on the catalytic activity of the enzyme in relationship to its calcium content. Loss of enzyme activity could be due to a partial removal of calcium from the enzyme by a chelating agent [19, 20, 30-32].

In presence of 10 mM disodium EDTA, fungal alpha amylase [1:5555] activity recovered after four hours is given in Table 5.13, wherein activity recovered was only 29.54 %. These results suggest that calcium is removed by chelating agent, disodium EDTA and after removal of calcium ions enzyme activity drastically reduces. This shows that calcium ion is essential for activity and stability of fungal alpha amylase.

Table 5.13 % Activity of fugal alpha amylase recovered in presence of disodium EDTA

	Initial % activity	% Activity after 4 hours.
Fungal alpha amylase in phosphate buffer	99.98	99.55
Fungal alpha amylase in phosphate buffer along with disodium EDTA	100.45	29.54

5.3 PREPARATION AND STABILIZATION OF FUNGAL ALPHA AMYLASE FORMULATION

5.3.1 Stabilization of fungal alpha amylase formulation by addition of cofactor & refolding aids

5.3.1a Oral liquid formulations

5.3.1a.1 Oral liquid formulations without addition of cofactor and refolding aid

Oral liquid formulation batches without addition of cofactor and refolding aids, characterization and stability data of Batch Co-L-1 and Batch Co-L-2 are given in Table 5.14. Both batches were not stable at 45°C up to three months. Both batches were having similar formulation except final pH. Batch Co-L-2 with pH 7 is found to be more stable than Batch Co-L-1 with pH 5. In previous section 5.2.2, fungal alpha amylase is found to be stable in the range of pH 6 to 9.

5.3.1a.2 Oral liquid formulation with refolding aid

Characterization and stability data of oral liquid formulation batches Co-L-3 to Co-L- 5 with refolding aids, are given in Table 5.15. These batches were not found to be stable at 45°C up to three months. The Batch Co-L-4 containing refolding aid polyethylene glycol 400 is more stable than the refolding aid glycerin and sorbitol 70% solution. In previous work, refolding aids such as polyethylene glycol, sorbitol, were used to refold the denatured enzyme [33, 34]. But in this present work, formulation is not found to stable even with the addition of refolding aids.

5.3.1a.3 Oral liquid formulation with cofactor

Characterization and stability data of oral liquid formulations batches Co-L-6 to Co-L-9 prepared with cofactor calcium chloride is given in Table 5.16 and 5.17; batches Co-L-10 to Co-L-13 prepared with calcium gluconate is given in Table 5.17 and 5.18; batches Co-L-14 to Co-L-17 prepared with calcium lactate is given in Table 5.18 and 5.19 and batches Co-L-18 to Co-L-21 prepared with calcium propionate is given in Table 5.20 and 5.21.

Batch Co-L-9 prepared with calcium chloride (0.04 %) and with final pH 7, was found to be very stable than other calcium salts. This may be due to its content of chloride ions from calcium chloride, which is also playing a role in stabilizing the enzymes [35].

Comparative stability data of 45°C is shown in figure 5.13. Among all these batches, Batch Co-L-9 is shown to be stable and expected shelf life was estimated to be up to 21.7 months by using general thumb rule [36], where if product is stable at 45°C for minimum of 3 months then it will be stable for 2 years.

It can be concluded that different approaches tried for stabilization of liquid oral formulations, addition of cofactor approach was found to give stable product with shelf life of about 21 months.

Table 5.14 Stability cum characterization of oral liquid formulation without addition of cofactor and refolding aid- Batch Co-L-1 and Co-L-2

Condition	Parameter	Initial	1st Month	2nd Month	3rd Month
Batch Co-L-1					
45°C	Description	A	A	A	A
	pH	5.012	4.969	4.912	4.893
	Sp.Gravity	1.223	1.223	1.224	1.225
	% Assay FAA	148.12	81.89	16.29	Nil
	% Assay PEP	149.23	135.33	118.14	103.62
30°C	Description	A	A	A	A
	pH	5.012	4.989	4.954	4.946
	Sp.Gravity	1.223	1.222	1.221	1.221
	% Assay FAA	148.12	139.80	131.51	123.21
	% Assay PEP	149.23	143.95	139.78	135.66
Refrigerated Condition [2°-8°C]	Description	A	A	A	A
	pH	5.012	5.010	5.009	4.995
	Sp.Gravity	1.223	1.223	1.222	1.222
	% Assay FAA	148.12	143.96	139.81	135.61
	% Assay PEP	149.23	148.35	147.19	145.67
Batch Co-L-2					
45°C	Description	A	A	A	A
	pH	6.997	6.954	6.915	6.893
	Sp.Gravity	1.225	1.224	1.221	1.220
	% Assay FAA	149.31	101.82	54.43	7.09
	% Assay PEP	149.08	134.09	118.57	103.64
30°C	Description	A	A	A	A
	pH	6.997	6.991	6.986	6.981
	Sp.Gravity	1.225	1.225	1.223	1.223
	% Assay FAA	149.31	143.36	137.41	131.52
	% Assay PEP	149.08	147.32	145.28	143.18
Refrigerated Condition [2°-8°C]	Description	A	A	A	A
	pH	6.997	6.995	6.990	6.899
	Sp.Gravity	1.225	1.225	1.225	1.224
	% Assay FAA	149.31	146.30	143.37	140.23
	% Assay PEP	149.08	148.23	147.19	145.99

A- Clear light yellow syrupy liquid with cardamom flavour, FAA- Fungal alpha amylase, PEP- Pepsin

Table 5.15 Stability cum characterization of oral liquid formulation with refolding aid -Batch Co-L- 3 to Batch Co-L- 5

Parameter	Batch Co-L - 3				Batch Co-L - 4				Batch Co-L - 5			
	Initial	1st Month	2nd Month	3rd Month	Initial	1st Month	2nd Month	3rd Month	Initial	1st Month	2nd Month	3rd Month
45°C												
Description	A	A	A	A	A	A	A	A	A	A	A	A
pH	7.010	6.978	6.926	6.897	7.009	6.974	6.931	6.893	7.008	6.959	6.910	6.883
Sp.Gravity	1.245	1.245	1.246	1.247	1.236	1.237	1.238	1.240	1.205	1.206	1.208	1.209
% Assay FAA	149.12	101.83	54.50	7.26	149.45	106.18	62.99	19.75	148.99	101.78	54.59	7.43
% Assay PEP	148.98	130.29	115.09	98.96	148.23	132.58	116.87	99.78	149.11	134.57	118.10	99.93
30°C												
Description	A	A	A	A	A	A	A	A	A	A	A	A
pH	7.010	7.003	6.998	6.991	7.009	7.001	6.995	6.992	7.008	6.997	6.992	6.989
Sp.Gravity	1.245	1.244	1.244	1.243	1.236	1.235	1.234	1.234	1.205	1.205	1.204	1.202
% Assay FAA	149.12	143.18	137.27	131.22	149.45	144.00	138.61	133.23	148.99	143.08	137.17	131.29
% Assay PEP	146.98	145.12	142.42	139.15	148.23	146.20	143.15	140.35	149.11	146.98	143.48	141.23
Refrigerated Condition [2° – 8°C]												
Description	A	A	A	A	A	A	A	A	A	A	A	A
pH	7.010	7.008	7.001	6.997	7.009	7.004	7.001	6.996	7.008	7.001	6.998	6.992
Sp.Gravity	1.245	1.245	1.244	1.243	1.236	1.235	1.234	1.231	1.205	1.205	1.204	1.204
% Assay FAA	149.12	146.17	144.13	140.24	149.45	146.47	144.04	141.23	148.99	146.00	143.11	140.13
% Assay PEP	146.98	146.52	144.06	141.18	148.23	148.01	145.67	142.82	149.11	148.99	146.24	144.11

A- Clear light yellow syrupy liquid with cardamom flavour, FAA- Fungal alpha amylase, PEP- Pepsin

Table 5.16 Stability cum characterization of oral liquid formulation with refolding aid -Batch Co-L- 6 to Batch Co-L- 8

Parameter	Batch Co-L - 6				Batch Co-L - 7				Batch Co-L - 8			
	Initial	1st Month	2nd Month	3rd Month	Initial	1st Month	2nd Month	3rd Month	Initial	1st Month	2nd Month	3rd Month
45°C												
Description	A	A	A	A	A	A	A	A	A	A	A	A
pH	5.016	4.974	4.924	4.897	5.009	4.960	4.915	4.899	5.007	4.958	4.916	4.901
Sp.Gravity	1.221	1.221	1.222	1.223	1.223	1.224	1.224	1.225	1.228	1.229	1.230	1.230
% Assay FAA	148.24	112.38	76.55	40.71	149.17	121.60	94.99	68.36	149.08	117.57	86.00	54.58
% Assay PEP	149.41	135.54	120.58	105.69	148.99	133.99	119.07	104.93	149.11	134.11	119.36	104.22
30°C												
Description	A	A	A	A	A	A	A	A	A	A	A	A
pH	5.016	5.010	5.008	5.000	5.009	5.002	4.999	4.995	5.007	5.001	4.997	4.993
Sp.Gravity	1.221	1.221	1.220	1.219	1.223	1.223	1.222	1.222	1.228	1.228	1.229	1.228
% Assay FAA	148.24	143.76	139.22	134.78	149.17	144.89	141.41	138.25	149.08	145.14	141.20	137.25
% Assay PEP	149.41	145.90	142.37	140.23	148.99	145.94	142.48	140.82	149.11	145.87	142.64	141.02
Refrigerated Condition [2° - 8°C]												
Description	A	A	A	A	A	A	A	A	A	A	A	A
pH	5.016	5.012	5.010	5.003	5.009	5.005	5.001	4.997	5.007	5.003	5.000	4.998
Sp.Gravity	1.221	1.221	1.220	1.220	1.223	1.222	1.222	1.221	1.228	1.227	1.226	1.227
% Assay FAA	148.24	145.99	143.74	141.52	149.17	146.55	144.89	142.21	149.08	147.11	145.12	143.16
% Assay PEP	149.41	147.55	144.29	142.78	148.99	147.82	144.37	142.77	149.11	147.41	144.83	143.79

A- Clear light yellow syrupy liquid with cardamom flavour, FAA- Fungal alpha amylase, PEP- Pepsin

Table 5.17 Stability cum characterization of oral liquid formulation with refolding aid -Batch Co-L- 9 to Batch Co-L- 11

Parameter	Batch Co-L - 9				Batch Co-L - 10				Batch Co-L - 11			
	Initial	1st Month	2nd Month	3rd Month	Initial	1st Month	2nd Month	3rd Month	Initial	1st Month	2nd Month	3rd Month
45°C												
Description	A	A	A	A	A	A	A	A	A	A	A	A
pH	7.008	6.975	6.945	9.910	5.008	4.969	4.931	4.888	5.010	4.960	4.925	4.890
Sp.Gravity	1.223	1.223	1.224	1.225	1.229	1.229	1.229	1.230	1.227	1.228	1.229	1.229
% Assay FAA	149.77	128.02	104.02	84.61	148.94	101.77	54.60	7.51	149.33	112.80	76.31	39.88
% Assay PEP	149.43	139.88	126.44	110.17	149.23	135.12	120.44	106.28	148.96	133.96	119.27	105.66
30°C												
Description	A	A	A	A	A	A	A	A	A	A	A	A
pH	7.008	7.005	7.002	6.999	5.008	5.005	5.002	5.000	5.010	5.007	5.003	5.001
Sp.Gravity	1.223	1.222	1.223	1.223	1.229	1.229	1.228	1.228	1.228	1.227	1.226	1.226
% Assay FAA	149.77	147.05	144.31	141.55	148.94	143.05	137.16	131.22	149.33	144.73	140.20	135.59
% Assay PEP	149.43	147.23	144.21	141.97	149.23	147.01	144.12	141.38	148.96	146.96	144.99	141.13
Refrigerated Condition [2° – 8°C]												
Description	A	A	A	A	A	A	A	A	A	A	A	A
pH	7.008	7.006	7.005	7.004	5.008	5.007	5.005	5.005	5.010	5.009	5.009	5.008
Sp.Gravity	1.223	1.223	1.222	1.222	1.229	1.228	1.228	1.228	1.227	1.227	1.227	1.226
% Assay FAA	149.77	148.27	146.75	145.20	148.94	145.99	143.00	140.07	149.33	147.05	144.76	142.48
% Assay PEP	149.43	148.52	147.06	144.99	149.23	148.22	147.10	145.09	148.96	147.69	146.28	144.97

A- Clear light yellow syrupy liquid with cardamom flavour, FAA- Fungal alpha amylase, PEP- Pepsin

Table 5.18 Stability cum characterization of oral liquid formulation with refolding aid- Batch Co-L- 12 to Batch Co-L- 14

Parameter	**Batch Co-L - 12**				**Batch Co-L - 13**				**Batch Co-L - 14**			
	Initial	1st Month	2nd Month	3rd Month	Initial	1st Month	2nd Month	3rd Month	Initial	1st Month	2nd Month	3rd Month
45°C												
Description	A	A	A	A	A	A	A	A	A	A	A	A
pH	5.001	4.968	4.929	4.889	7.005	6.978	6.952	6.933	5.006	4.970	4.932	4.898
Sp.Gravity	1.225	1.225	1.226	1.227	1.228	1.228	1.230	1.231	1.226	1.227	1.227	1.228
% Assay FAA	149.58	108.14	66.69	25.29	149.24	116.55	83.80	51.25	149.09	109.67	70.29	30.92
% Assay PEP	149.12	135.65	120.69	104.33	148.99	133.85	118.54	103.75	149.15	135.74	118.76	102.33
30°C												
Description	A	A	A	A	A	A	A	A	A	A	A	A
pH	5.001	4.997	4.995	4.990	7.005	7.002	6.998	6.996	5.006	5.002	4.999	4.997
Sp.Gravity	1.225	1.225	1.224	1.224	1.228	1.228	1.228	1.229	1.226	1.226	1.227	1.226
% Assay FAA	149.58	144.37	139.11	134.03	149.24	145.15	141.06	136.79	149.09	144.15	139.24	134.21
% Assay PEP	149.12	147.24	145.27	143.11	148.99	146.99	144.57	139.97	149.15	147.67	145.77	142.08
RC- Refrigerated Condition [2° – 8°C]												
Description	A	A	A	A	A	A	A	A	A	A	A	A
pH	5.001	5.000	4.998	4.996	7.005	7.004	7.001	6.999	5.006	5.004	5.002	5.001
Sp.Gravity	1.225	1.225	1.225	1.224	1.228	1.228	1.229	1.229	1.226	1.226	1.227	1.226
% Assay FAA	149.58	146.95	144.40	141.76	149.24	147.18	145.15	143.08	149.09	146.60	144.13	141.70
% Assay PEP	149.12	148.33	147.54	145.89	148.99	147.82	146.58	144.39	149.15	148.36	147.43	145.98

A- Clear light yellow syrupy liquid with cardamom flavour, FAA- Fungal alpha amylase, PEP- Pepsin

Table 5.19 Stability cum characterization of oral liquid formulation with refolding aid- Batch Co-L- 15 to Batch Co-L- 17

Parameter	**Batch Co-L - 15**				**Batch Co-L - 16**				**Batch Co-L - 17**			
	Initial	1st Month	2nd Month	3rd Month	Initial	1st Month	2nd Month	3rd Month	Initial	1st Month	2nd Month	3rd Month
45°C												
Description	A	A	A	A	A	A	A	A	A	A	A	A
pH	5.001	4.958	4.921	4.887	5.008	4.962	4.931	4.896	7.006	6.978	6.951	6.927
Sp.Gravity	1.225	1.226	1.227	1.229	1.228	1.228	1.229	1.231	1.225	1.226	1.227	1.227
% Assay FAA	149.12	119.54	90.05	60.45	149.25	115.32	81.55	47.67	149.39	121.44	93.50	65.46
% Assay PEP	149.55	135.18	120.08	102.82	148.47	133.27	118.22	102.33	148.99	133.99	118.58	103.87
30°C												
Description	A	A	A	A	A	A	A	A	A	A	A	A
pH	5.001	6.998	6.995	6.991	5.008	5.002	6.998	6.994	7.006	7.003	7.000	6.998
Sp.Gravity	1.225	1.225	1.226	1.226	1.228	1.228	1.229	1.230	1.225	1.225	1.225	1.226
% Assay FAA	149.12	145.42	141.70	137.98	149.25	145.01	140.77	136.45	149.39	145.88	142.40	138.79
% Assay PEP	149.55	147.28	145.54	143.33	148.47	146.41	144.28	142.09	148.99	146.89	144.77	142.59
Refrigerated Condition [2° - 8°C]												
Description	A	A	A	A	A	A	A	A	A	A	A	A
pH	5.001	5.000	5.001	4.999	5.008	5.007	5.007	5.004	7.006	7.006	7.005	7.002
Sp.Gravity	1.225	1.225	1.226	1.226	1.228	1.227	1.226	1.225	1.225	1.225	1.224	1.225
% Assay FAA	149.12	147.22	145.39	143.52	149.25	147.13	145.05	142.78	149.39	147.60	145.90	144.11
% Assay PEP	149.55	148.29	147.21	145.47	148.47	147.39	146.17	144.99	148.99	147.46	146.21	143.98

A- Clear light yellow syrupy liquid with cardamom flavour, FAA- Fungal alpha amylase, PEP- Pepsin

Table 5.20 Stability cum characterization of oral liquid formulation with refolding aid Batch Co-L- 18 to Batch Co-L- 20

Parameter	**Batch Co-L - 18**				**Batch Co-L - 19**				**Batch Co-L - 20**			
	Initial	1st Month	2nd Month	3rd Month	Initial	1st Month	2nd Month	3rd Month	Initial	1st Month	2nd Month	3rd Month
45°C												
Description	A	A	A	A	A	A	A	A	A	A	A	A
pH	5.008	4.967	4.928	4.894	4.999	4.956	4.926	4.872	5.003	4.962	4.925	4.883
Sp.Gravity	1.226	1.226	1.228	1.229	1.225	1.226	1.226	1.228	1.228	1.228	1.229	1.229
% Assay FAA	149.12	106.12	63.09	20.35	149.34	117.67	86.01	54.40	149.29	112.77	76.32	39.85
% Assay PEP	149.34	135.28	120.38	104.79	148.98	133.97	118.48	103.88	149.51	134.89	118.93	104.08
30°C												
Description	A	A	A	A	A	A	A	A	A	A	A	A
pH	5.008	5.002	4.999	4.997	4.999	4.995	4.992	4.988	5.003	5.001	4.998	4.995
Sp.Gravity	1.226	1.226	1.227	1.227	1.225	1.225	1.226	1.226	1.228	1.228	1.229	1.228
% Assay FAA	149.12	143.74	138.32	132.89	149.34	145.37	141.23	137.47	149.29	144.70	140.11	135.50
% Assay PEP	149.34	147.58	145.12	143.07	148.98	146.88	144.87	142.66	149.51	147.55	145.47	143.54
Refrigerated Condition [2° - 8°C]												
Description	A	A	A	A	A	A	A	A	A	A	A	A
pH	5.008	5.006	5.005	5.005	4.999	4.998	4.996	4.995	5.003	5.002	5.000	4.998
Sp.Gravity	1.226	1.226	1.225	1.225	1.225	1.225	1225	1.224	1.228	1.227	1.227	1.226
% Assay FAA	149.12	146.36	143.57	141.01	149.34	147.31	145.38	143.40	149.29	147.00	144.72	142.38
% Assay PEP	149.34	148.32	147.25	145.77	148.98	147.56	146.25	144.88	149.51	148.39	147.55	145.97

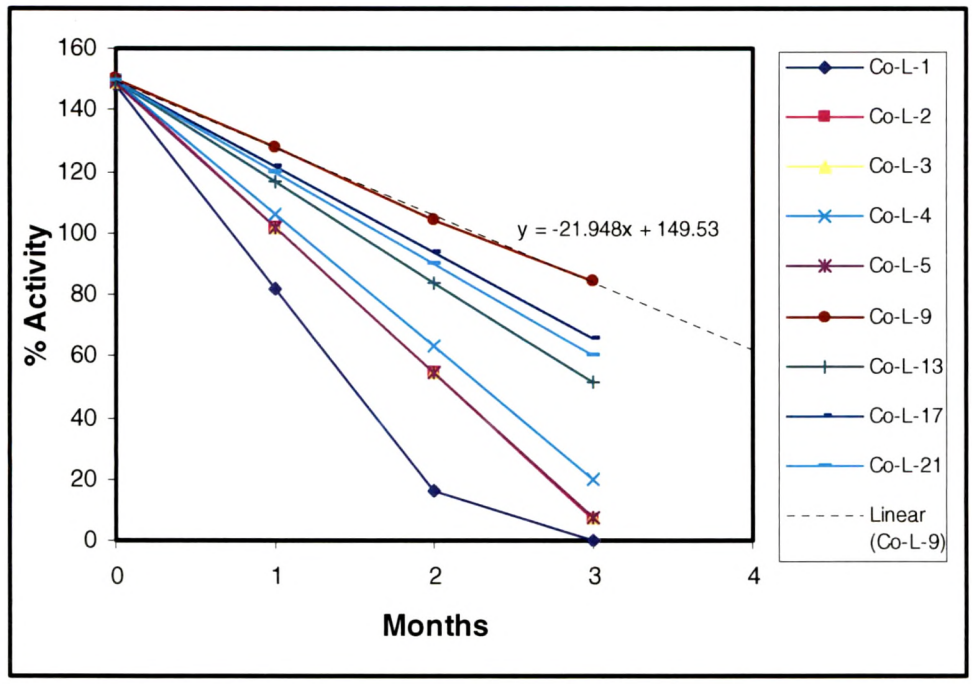
A- Clear light yellow syrupy liquid with cardamom flavour, FAA- Fungal alpha amylase, PEP- Pepsin

Table 5.21 Stability cum characterization of oral liquid formulation with refolding aid -Batch Co-L- 21

Condition	Parameter	Initial	1st Month	2nd Month	3rd Month
Batch Co-L-21					
45°C	Description	A	A	A	A
	pH	7.003	6.978	6.945	6.918
	Sp.Gravity	1.225	1.225	1.226	1.227
	% Assay FAA	149.28	119.62	90.05	60.43
	% Assay PEP	149.11	134.28	119.08	103.69
30°C	Description	A	A	A	A
	pH	7.003	7.000	6.997	6.995
	Sp.Gravity	1.225	1.225	1.226	1.226
	% Assay FAA	149.28	145.56	141.80	138.16
	% Assay PEP	149.11	147.28	145.38	143.88
Refrigerated Condition [2°-8°C]	Description	A	A	A	A
	pH	7.003	7.002	7.000	6.999
	Sp.Gravity	1.225	1.225	1.225	1.224
	% Assay FAA	149.28	147.38	145.55	143.70
	% Assay PEP	149.11	148.18	147.44	145.98

A- Clear light yellow syrupy liquid with cardamom flavour, FAA- Fungal alpha amylase, PEP- Pepsin

Figure 5.13 Comparison of oral liquid formulations stability data at 45°C



5.3.1b Dry syrup formulation

Characterization of dry syrup formulation batches Co-DS-1 to Co-DS-4 is given in Table 5.22. The data indicates that flow property of dry syrup prepared by wet granulation method (Batch Co-DS-1 & Co-DS-2) is better than dry syrup prepared by dry mixing method (Batch Co-DS-3 & Co-DS-4). Compressibility index values below 15 % usually give rise to good flow characteristics [37], all formulation's compressibility index was found to be within 15 %, which indicates the good flow property. Values for angle of repose ≤ 30° usually indicate a free flowing material and angles ≥ 40° suggest a poorly flowing material [38], here batch Co-DS-1 and Co-DS-2 shows angle of repose less than 30° but Batch Co-DS-3 and Co-DS-4 shows more than 30° but less than 40° which suggests that the flow of granules in not poor. Moisture content is quite well controlled in batches prepared by dry mixing method.

Reconstituted syrups of all batches were found to give almost similar characteristic and reconstituted syrups was found to be stable up to 15 days at room temperature (Table 5.23).

Stability study data is given in Table 5.24 and 5.25 and comparative stability data of temperature 45°C is depicted in Figure 5.14. From the figure it is clear that Batch Co-DS-4 is stable formulation and expected shelf life was estimated to be up to 26 months. Batch Co-DS-4 also showed better stability at 40°Cand 75 % Relative Humidity. It can be seen that calcium chloride present in Batch Co-DS-4 played a major role in stabilizing this formulation and dry mixing method gives better stability over wet granulation.

Table 5.22 Characterization of dry Syrup batches Co-DS-1 to Co-DS-4

Parameter	**Co-DS- 1**	**Co-DS- 2**	**Co-DS- 3**	**Co-DS- 4**
Description	A	A	B	B
Bulk density (gm/ml)	0.858	0.865	0.779	0.781
Tapped density (gm/ml)	0.913	0.920	0.881	0.892
Compressibility index (%)	5.5	5.98	11.58	12.44
Angle of repose (°)	24	25	33	35
Moisture content (%)	0.85	0.89	0.30	0.28
% Assay FAA	144.89	149.21	149.33	149.29
% Assay PEP	148.67	148.82	148.68	149.11

A –Yellow coloured, pineapple flavoured free flowing granules

B- Very light yellow coloured, pineapple flavoured free flowing granules

Table 5.23 Characterization cum stability of reconstituted dry syrup Batch Co-DS-1 to Co-DS-4 at 30°C

Parameter		Description	Sp. Gravity	pH	% Assay FAA	% Assay PEP
Co-DS- 1	Initial	A	1.123	5.018	144.76	148.85
	15 days	A	1.124	5.026	134.29	147.74
Co-DS- 2	Initial	A	1.125	5.012	149.23	148.58
	15 days	A	1.125	5.020	139.45	147.85
Co-DS- 3	Initial	A	1.222	4.885	149.13	148.52
	15 days	A	1.223	4.890	140.13	147.95
Co-DS- 4	Initial	A	1.228	4.882	149.17	149.22
	15 days	A	1.227	4.886	145.17	148.23

A- Slight hazy light yellow syrupy liquid with pineapple flavour,

FAA- Fungal alpha amylase, PEP- Pepsin

Table 5.24 Stability study data of dry syrup -Batch Co-DS-1 and Co-DS-2

Parameter	**Batch Co-DS-1**			**Batch Co-L-2**		
	1st Month	2nd Month	3rd Month	1st Month	2nd Month	3rd Month
45°C						
Description	A	A	A	A	A	A
MC (%)	0.80	0.76	0.75	0.83	0.80	0.78
% Assay FAA	108.26	71.73	35.29	122.88	96.60	70.30
% Assay PEP	136.68	124.99	111.58	136.58	123.99	111.87
40°C+75 % RH						
Description	A	A	A	A	A	A
MC (%)	0.86	0.89	0.91	0.91	0.95	0.99
% Assay FAA	126.51	108.28	90.15	145.90	142.63	139.29
% Assay PEP	142.23	136.46	130.51	142.81	136.80	129.67
30°C						
Description	A	A	A	A	A	A
MC (%)	0.84	0.82	0.80	0.88	0.87	0.86
% Assay FAA	140.19	135.60	131.07	147.49	145.76	144.02
% Assay PEP	147.28	145.60	143.89	146.98	145.48	143.28

A –Yellow coloured, pineapple flavoured free flowing granules

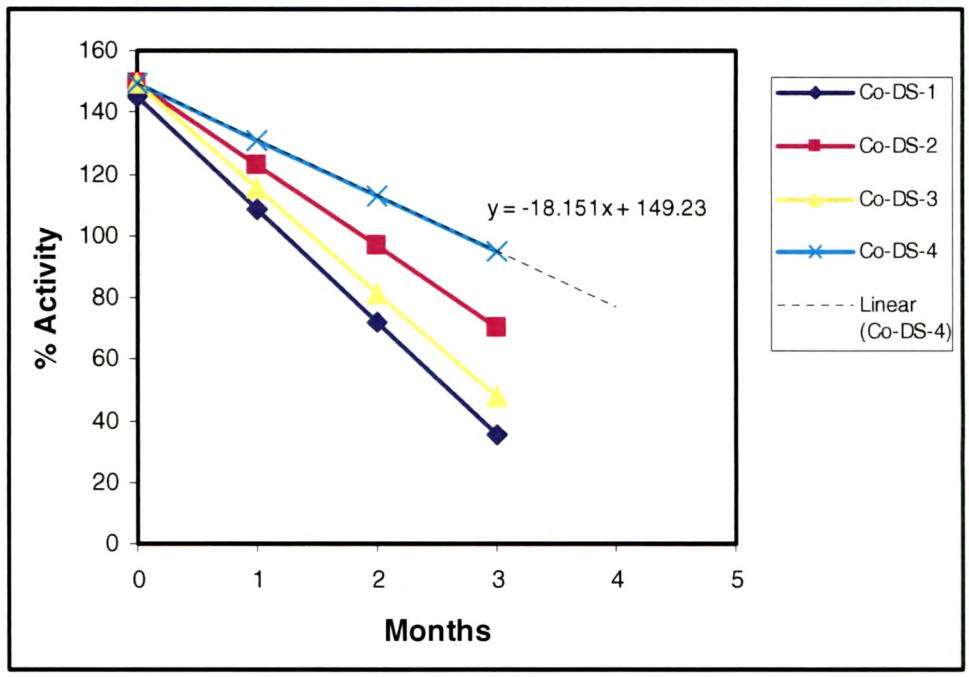
MC- Moisture content, FAA- Fungal alpha amylase, PEP- Pepsin

Table 5.25 Stability study data of dry syrup -Batch Co-DS- 3 and Co-DS- 4

Parameter	**Batch Co-DS- 3**			**Batch Co-L- 4**		
	1st Month	2nd Month	3rd Month	1st Month	2nd Month	3rd Month
45°C						
Description	A	A	A	A	A	A
MC (%)	0.29	0.28	0.26	0.27	0.26	0.23
% Assay FAA	115.40	81.54	47.65	131.06	112.78	94.88
% Assay PEP	136.58	124.39	112.05	137.01	124.72	112.34
40°C+75 % RH						
Description	A	A	A	A	A	A
MC (%)	0.31	0.31	0.32	0.28	0.30	0.31
% Assay FAA	132.35	115.41	98.50	147.96	146.21	144.73
% Assay PEP	142.50	136.33	130.11	142.92	136.28	130.13
30°C						
Description	A	A	A	A	A	A
MC (%)	0.30	0.29	0.28	0.27	0.26	0.24
% Assay FAA	145.07	140.85	136.61	147.00	144.70	142.23
% Assay PEP	147.13	145.58	144.07	147.52	146.09	144.24

A- Very light yellow coloured, pineapple flavoured free flowing granules, MC- Moisture content, FAA- Fungal alpha amylase, PEP- Pepsin

Figure 5.14 Comparison of dry syrup formulations stability data at 45°C



5.3.1c Oral drop formulations

Similar approach as that of oral liquid formulation was tried for stabilizing the oral drops. Here label claim is different than oral liquid formulation where instead of pepsin, papain is used and carminative oils are also used.

Oral drop formulations characterization and stability data of batches Co-D-1 and Co-D-2 is given in Table 5.26 and Batches Co-D-3 and Co-D-4 is given in Table 5.27.

Comparative stability data of 45°C is shown in figure 5.15. Among all these batches, Batch Co-D-3 is shown to be stable product and expected shelf life was estimated to be up to 20 months. It is revealed that 0.06 % calcium chloride is required for stability of oral drop formulations whereas calcium chloride concentration 0.04 % or 0.08 % is not enough to give stable product.

Table 5.26 Stability cum characterization of oral drop formulations- Batch Co-D-1 and Co-D-2

Condition	Parameter	Initial	1st Month	2nd Month	3rd Month
Batch Co-D-1					
45°C	Description	A	A	A	A
	pH	7.002	6.966	6.937	6.908
	Sp.Gravity	1.187	1.188	1.189	1.191
	% Assay FAA	149.28	112.81	76.32	40.01
	% Assay PAP	149.14	136.47	124.09	112.14
30°C	Description	A	A	A	A
	pH	7.002	6.999	6.997	6.996
	Sp.Gravity	1.187	1.188	1.188	1.889
	% Assay FAA	149.28	144.70	140.16	135.06
	% Assay PAP	149.14	147.35	145.60	144.11
Refrigerated Condition [2°-8°C]	Description	A	A	A	A
	pH	7.002	7.001	6.998	6.998
	Sp.Gravity	1.187	1.187	1.186	1.186
	% Assay FAA	149.28	147.02	144.72	142.40
	% Assay PAP	149.14	147.89	146.67	145.47
Batch Co-L-2					
45°C	Description	A	A	A	A
	pH	6.998	6.958	6.928	6.907
	Sp.Gravity	1.185	1.186	1.188	1.189
	% Assay FAA	149.52	117.76	86.11	54.30
	% Assay PAP	149.24	136.51	124.15	110.22
30°C	Description	A	A	A	A
	pH	6.998	6.996	6.992	6.988
	Sp.Gravity	1.185	1.185	1.186	1.186
	% Assay FAA	149.52	145.55	141.52	137.59
	% Assay PAP	149.24	147.08	145.84	144.44
Refrigerated Condition [2°-8°C]	Description	A	A	A	A
	pH	6.998	6.998	6.996	6.996
	Sp.Gravity	1.185	1.185	1.184	1.185
	% Assay FAA	149.52	147.46	145.31	143.35
	% Assay PAP	149.24	147.99	146.74	145.53

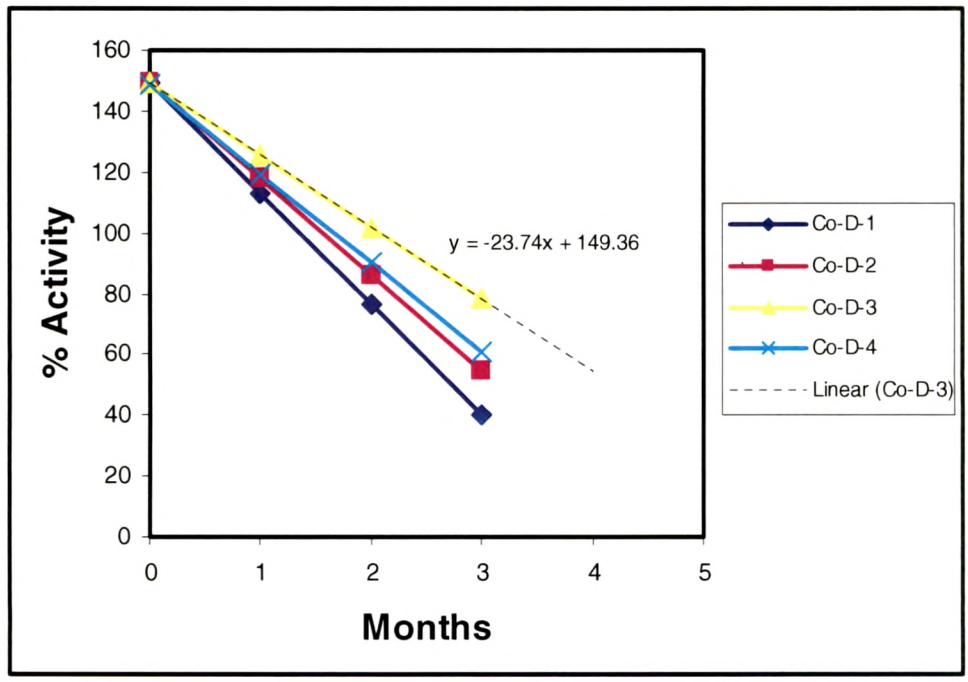
A- Clear, light orange, flavoured syrupy liquid, FAA- Fungal alpha amylase, PAP- Papain

Table 5.27 Stability cum characterization of oral drop formulations-Batch Co-D- 3 and Co-D- 4

Condition	Parameter	Initial	1st Month	2nd Month	3rd Month
Batch Co-D- 3					
45°C	Description	A	A	A	A
	pH	7.008	6.969	6.933	6.903
	Sp.Gravity	1.188	1.189	1.190	1.192
	% Assay FAA	149.36	125.62	101.85	78.15
	% Assay PAP	148.55	135.70	122.85	110.24
30°C	Description	A	A	A	A
	pH	7.008	7.004	7.000	6.998
	Sp.Gravity	1.188	1.188	1.189	1.190
	% Assay FAA	149.36	146.38	143.41	140.39
	% Assay PAP	148.55	146.58	145.29	143.67
Refrigerated Condition [2°-8°C]]	Description	A	A	A	A
	pH	7.008	7.006	7.005	7.005
	Sp.Gravity	1.188	1.188	1.188	1.187
	% Assay FAA	149.36	147.85	146.36	144.91
	% Assay PAP	148.55	147.33	146.31	145.06
Batch Co-L- 4					
45°C	Description	A	A	A	A
	pH	7.010	6.972	6.945	6.912
	Sp.Gravity	1.182	1.183	1.185	1.185
	% Assay FAA	148.93	119.45	90.06	60.55
	% Assay PAP	149.18	136.68	124.22	111.59
30°C	Description	A	A	A	A
	pH	7.010	7.005	7.001	6.998
	Sp.Gravity	1.182	1.182	1.183	1.183
	% Assay FAA	148.93	145.22	141.51	137.88
	% Assay PAP	149.18	147.28	145.68	144.33
Refrigerated Condition [2°-8°C]	Description	A	A	A	A
	pH	7.010	7.008	7.005	7.002
	Sp.Gravity	1.182	1.182	1.183	1.182
	% Assay FAA	148.93	147.08	145.22	143.40
	% Assay PAP	149.18	147.89	146.71	145.49

A- Clear, light orange, flavoured syrupy liquid, FAA- Fungal alpha amylase, PAP- Papain

Figure 5.15 Comparison of oral drop formulations stability data at 45°C



5.3.1d Hard gelatin capsule formulation

Characterization of hard gelatin capsule blend of Batch Co-C-1 and Co-C-2 is shown in Table 5.28.The data indicates good flow property of capsule blend where compressibility index values of both batches was about 16 % and angle of repose is 32° which suggests good flow characteristics [37, 38]. Moisture content in both batches was about 0.5 % which is quite well accepted for capsule formulation.

Characterization of hard gelatin capsule is given in Table 5.29, where both formulations shown almost similar characterization. These formulations were complying as per the standards given for general capsule in Indian Pharmacopoeia [39].

Stability data of Batch Co-C-1 and Co-C-2 is shown in Table 5.30 and comparative stability data of 45°C is shown in figure 5.16. Among these batches, Batch Co-C-2 is shown to be stable product and expected shelf life was estimated to be up to 24.4 months. Also batch Co-C-2 shown good stability over 40°C and 75 % Relative Humidity. It is confirmed that due to addition of calcium chloride in batch Co-C-2 formulation, stability is improved.

Table 5.28 Characterization of capsule blend-Batch Co-C-1 and Co-C-2

Parameter	**Co-C- 1**	**Co-C- 2**
Description	A	A
Bulk density (gm/ml)	0.427	0.428
Tapped density (gm/ml)	0.512	0.509
Compressibility index (%)	16.60	15.91
Angle of repose (°)	32	32
Moisture content (%)	0.50	0.53

A –Light yellow coloured, free flowing powder

Table 5.29 Characterization of hard gelatin capsule-Batch Co-C-1 and Batch Co-C-2

	Co-C- 1	**Co-C- 2**
Description	A	A
Average net content per capsule (mg)	131.02	130.78
Average weight of intact capsule (mg)	181.65	180.89
Uniformity of weight (% RSD) (n= 20)	± 2.041	± 1.928
Locking length (mm) ± SD (n= 20)	15.8 ± 0.076	15.8 ± 0.081
Moisture content (%)	0.51	0.55
Disintegration time	7' 20"	7' 18"
% Assay of FAA	149.12	148.97
% Assay of PEP	148.44	149.13

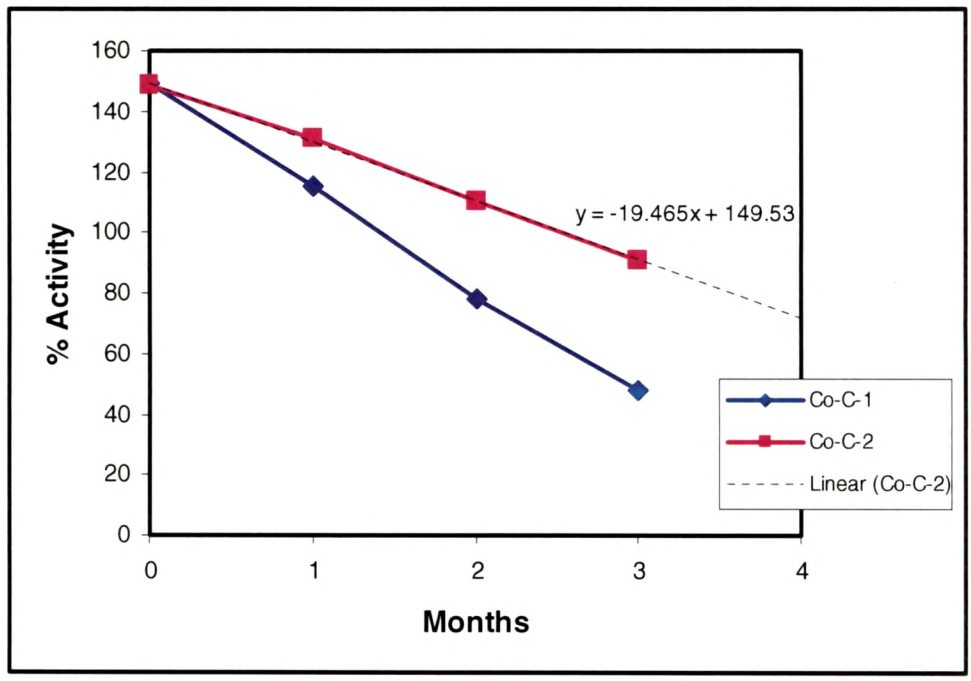
A- Ivory –Ivory size "3" hard gelatin capsule containing light yellow powder, FAA- Fungal alpha amylase, PEP- Pepsin

Table 5.30 Stability study of hard gelatin capsule- Batch Co-C- 1 and Co-C- 2

Parameter	**Batch Co-C-1**			**Batch Co-C-2**		
	1st Month	2nd Month	3rd Month	1st Month	2nd Month	3rd Month
45°C						
Description	A	A	A	A	A	A
DT	7' 26"	7' 50"	8' 02"	7' 33"	7' 56"	8' 11"
MC (%)	0.50	0.48	0.45	0.54	0.53	0.51
% Assay FAA	115.33	78.22	47.65	131.05	110.31	91.00
% Assay PEP	136.31	124.19	112.06	137.09	124.86	112.71
40°C+75 % RH						
Description	A	A	A	A	A	A
DT	7' 30"	7' 58"	8' 11"	7' 42"	8' 09"	8' 27"
MC (%)	0.51	0.52	0.54	0.56	0.57	0.59
% Assay FAA	132.23	115.20	98.38	139.30	129.61	119.98
% Assay PEP	142.31	136.12	129.92	143.02	136.84	130.75
30°C						
Description	A	A	A	A	A	A
DT	7' 23"	7' 35"	7' 41"	7' 28"	7' 33"	7' 51"
MC (%)	0.51	0.51	0.52	0.55	0.55	0.56
% Assay FAA	144.89	140.62	136.40	146.55	144.12	141.71
% Assay PEP	146.88	145.30	143.76	147.55	146.06	144.41

A- Ivory –Ivory size "3" hard gelatin capsule containing light yellow powder, MC- Moisture Content, DT – Disintegration Time, FAA- Fungal alpha amylase, PEP- Pepsin

Figure 5.16 Comparison of hard gelatin capsule formulations stability data at 45°C



5.3.2 Enterosoluble formulations preparation

Section 5.2.2 shows that fungal alpha amylase is not stable in acidic medium. Upon oral administration of this enzyme, there may be possibility of degradation of amylase enzyme. There are some references which quotes the degradation of fungal alpha amylase in acidic condition or in stomach [40, 41]. Therefore stability of fungal alpha amylase over different gut pH was studied. Stomach pH varies according to fasting and feeding condition. Empty stomach pH is about 1.2 and stomach pH after feeding is above pH 4.5 for 1-2 hours [42]. Therefore fungal alpha amylase was treated at different stomach pH condition and which is then subsequently treated at intestinal pH 6.8.

5.3.2.1 In-vitro fungal alpha amylase stability in gut

5.3.2.1a Stability at pH 1.2

Results of stability of fungal alpha amylase alone, with cofactor, with beta-cyclodextrin at pH 1.2 are given in Table 5.31 and all these samples were totally degraded in acidic pH 1.2 within one hour.

Table 5.31 In-vitro stability of fungal alpha amylase at pH 1.2

pH	Stirring Time (Hour)	% Activity Recovered
FAA		
1.2	1	Nil
1.2	2	Nil
1.2	3	Nil
FAA + calcium chloride		
1.2	1	Nil
1.2	2	Nil
1.2	3	Nil
FAA + beta-cyclodextrin complex		
1.2	1	Nil
1.2	2	Nil
1.2	3	Nil

FAA- Fungal alpha amylase

5.3.2.1b Stability at different pH 1.2 – 5.0 – 6.8

Results of stability of fungal alpha amylase alone, with cofactor, with beta-cyclodextrin at pH 1.2-5.0-6.8 are given in Table 5.32. These samples were totally degraded in acidic pH 1.2 and 5, and showed activity recovery at pH 6.8 which is about 3 to 3.5 % which is may be due to renaturation of fungal alpha amylase.

Table 5.32 In-vitro stability of fungal alpha amylase at different pH 1.2 – 5.0 – 6.8

pH	% Activity Recovered
FAA	
1.2	Nil
5.0	Nil
6.8	3.31
FAA + calcium chloride	
1.2	Nil
5.0	Nil
6.8	3.52
FAA + beta-cyclodextrin Complex	
1.2	Nil
5.0	Nil
6.8	3.55

FAA- Fungal alpha amylase

5.3.2.1c Stability at different pH 3.0 – 5.0 – 6.8

Stability of fungal alpha amylase alone, with cofactor, with beta-cyclodextrin at pH 3.0-5.0-6.8 is given in Table 5.33. These samples shown activity recovery at pH 3 about 3.5 %, and pH 5.0 very less activity recovered which is about 0.5 % and at pH 6.8 about 4.3 %.

Table 5.33 In-vitro stability of fungal alpha amylase at different pH 3.0 – 5.0 – 6.8

pH	% Activity Recovered
FAA	
3.0	3.35
5.0	0.56
6.8	4.29
FAA + calcium chloride	
3.0	3.41
5.0	0.52
6.8	4.34
FAA + beta-cyclodextrin complex	
3.0	3.78
5.0	0.66
6.8	4.32

FAA- Fungal alpha amylase

5.3.2.1d Stability at different pH 4.0 – 5.0 – 6.8

Stability of fungal alpha amylase alone, with cofactor, with beta-cyclodextrin at pH 4.0-5.0-6.8 is given in Table 5.34. These samples shown activity recovery at pH 4 about 20.0 %, and pH 5.0 less activity recovered which is about 8.6 % and at pH 6.8 about 25.0 %.

Table 5.34 In-vitro stability of fungal alpha amylase at different pH 4.0 – 5.0 – 6.8

pH	% Activity Recovered
FAA	
4.0	19.89
5.0	8.65
6.8	25.06
FAA + calcium chloride	
4.0	20.05
5.0	8.69
6.8	25.02
FAA + beta-cyclodextrin complex	
4.0	21.23
5.0	8.71
6.8	25.11

FAA- Fungal alpha amylase

5.3.2.1e Stability at different pH 5.0 – 6.8

Stability of fungal alpha amylase alone, with cofactor, with beta-cyclodextrin at pH 5.0-6.8 is given in Table 5.35. These samples shown activity recovery above 95.0 % at pH 5 and pH 6.8.

Table 5.35 In-vitro stability of fungal alpha amylase at different pH 5.0 – 6.8

pH	% Activity Recovered
FAA	
5.0	95.89
6.8	94.85
FAA + calcium chloride	
5.0	96.12
6.8	95.08
FAA + beta-cyclodextrin complex	
5.0	94.23
6.8	96.33

FAA- Fungal alpha amylase

5.3.2.2 Fungal alpha amylase market sample in-vitro stability in gut

The stability study of fungal alpha amylase at different pH suggests that enzyme will show full activity if stomach pH after feeding is 5. But this is not the case; most of the references have given the stomach pH 3 after feeding [41]. To check the in-vitro stability of market samples, pH 3.0 – 6.8 is chosen for study and results are given in Table 5.36. Out of eight market samples, four market samples shown total degradation of amylase enzyme and others showed about 45 % activity recovery. Batch Co-L-9 which is most stable oral liquid formulated also shown about 45 % activity recovery.

Table 5.36 Fungal alpha amylase market sample in-vitro stability in gut

Market Sample	% Activity Recovered	
	pH 3.0	pH 6.8
A	Nil	Nil
B	30.55	43.52
C	Nil	Nil
D	32.05	47.44
E	35.23	42.61
F	42.21	44.02
G	Nil	Nil
H	Nil	Nil
Co-L-9	41.80	45.91

5.3.2.3 Enterosoluble formulation preparation

Fungal alpha amylase in-vitro stability at gut pH and market sample containing fungal alpha amylase in-vitro stability confirms that fungal alpha amylase is not remaining active at stomach pH; it partially degrades and may be available only up to 45 % for starch digestion. It is reported that our natural salivary amylase can digest starch up to 30 - 40 % in the stomach and it fully degrades in the stomach [40]. This shows that there is a need to protect the fungal alpha amylase against the acidic environment of stomach by means of enteric coating to get full amylase activity which is required in the intestine in disease condition.

5.3.2.3a Enteric-sugar coated tablet formulation

Granules were prepared for enteric-sugar coated tablet by nonaqueous granulation method and important parameters of the tablet constituents that may affect the characteristics of the final tablet were assessed before manufacture. The evaluation of granules is given in Table 5.37, wherein the granules were showing 13.22 % compressibility index and 23° angle of repose which suggests that granules were having good flow property. Data of particle size distribution indicates that the granules were homogenously mixed. The proportion of fines was less than 40 % which indicates the good flow rate of granules. It is reported that when the proportion of fine particles exceeds approximately 40 %, there is dramatic fall in the flow rate [42]. Thus the assessment of granules property gives an indication of suitability of the pre-compression mix for use in the tablet press.

Evaluation parameters of uncoated tablets are shown in Table 5.38. The results of diameter, thickness, hardness are expressed as mean ± standard deviation and shows that tablets were uniform. The tablets were compressed at average weight of 65 mg and weight variation was under 2.5 %.Tablets friability was found to be 0.23 % and no capped tablets were observed which is necessary to withstand chipping, abrasion and breakage during the expected tablet life under the conditions of coating, storage and handling. The disintegration time was below 2 minutes which is within Pharmacopoeial limit. Moisture content was found to be 1.23 % which was not increased after granulation process. The uncoated tablets were complied all the general evaluation parameters given in Indian Pharmacopoeia.

Enteric coated tablets evaluation is depicted in Table 5.39. Enteric coated tablets weight gain was found to be about 8 % of uncoated tablet weight. The results of diameter, thickness are expressed as mean ± standard deviation and shows that tablets were uniform in diameter and thickness. The tablets weight variation was under 3.0 %. Moisture content was found to be 1.21 % which was not increased during coating process. Disintegration of enteric coated tablets was performed as per Indian Pharmacopoeia. In acid stage after 2 hours tablets did not show any signs of cracks or removal of coating, thereafter in mixed phosphate buffer tablets

were disintegrated at 14'16". Fungal alpha amylase release was carried out in Indian Pharmacopoeial paddle type dissolution apparatus, in two stages that is acid stage and buffer stage. After acid stage, tablets were remained intact and there were no signs of cracks or removal of coating layer. As fungal alpha amylase does not remain stable in acidic medium, its content could not be estimated at acid stage. At phosphate buffer stage, fungal alpha amylase release after 45 minutes found to be 145.39 % (96.93 % of the label claim). These results suggest that enteric coating polymer cellulose acetate phthalate has given complete resistance to acid medium. The enteric coated tablets were complied all the general evaluation parameters given in Indian Pharmacopoeia.

Evaluation parameters of enteric sugar coated tablets are given in Table 5.40. Weight gain about 50 % was increased during sugar coating over the enteric coated tablets. The results of diameter, thickness are expressed as mean ± standard deviation and shows that tablets were uniform in diameter and thickness. The tablets weight variation was under 3.5 %. Moisture content was found to be 2.04 % which was increased during coating process. Disintegration of enteric sugar tablets was performed as per the test given for enteric coated tablets in Indian Pharmacopoeia. In acid stage after 2 hours sugar coating was completely removed and tablets did not show any signs of cracks or removal of enteric coating (figure 5.17), thereafter in mixed phosphate buffer tablets were disintegrated at 15'29". Fungal alpha amylase release was carried out in Indian Pharmacopoeial paddle type dissolution apparatus, in two stages that is acid stage and buffer stage. After acid stage, sugar coat layer was removed and enteric coat were remained intact and there were no signs of cracks or removal of coating layer. Pepsin was estimated from acid medium and total 140.23 % (93.49 % of the label claim) release was estimated. At phosphate buffer stage, fungal alpha amylase release after 45 minutes found to be 143.09 % (95.39 % of the label claim). These results suggests that this is the targeted drug delivery system where pepsin will release in stomach where it is needed to digest the protein and fungal alpha amylase will release into the intestine in full amount where maximum starch digestion takes place [40,41].

Stability data of Batch ES-T-1 is shown in Table 5.41 and stability data of 45°C is shown in figure 5.18. This batch was shown to be stable and expected shelf life was estimated to be up to 26 months. Also this batch showed good stability over 40°C and 75 % Relative Humidity where moisture was not increased more than 2.12 %. Fungal alpha amylase release was decreased from initial month value 143.29 % to 89.80 % at 3rd month at 45°C; this is not due to release of drug from tablet but due to available fungal alpha amylase was less that is 94.53 %.

Table 5.37 Evaluation of granules prepared for enteric sugar coated tablet

Parameter		**Batch ES-T-1**
Description		Light yellow free flowing granules
Bulk density (gm/ml)		0.545
Tapped density (gm/ml)		0.628
Compressibility index (%)		13.22
Angle of repose (°)		23
Moisture content (%)		1.15
Particle size distribution (%) retained on mesh	16	2.3
	20	20.2
	40	30.7
	60	9.1
	80	12.0
	100	10.2
	200	5.3
	200 pass	10.2
Granules to fine ratio		62.43:37.57

Table 5.38 Evaluation of uncoated tablets prepared for enteric sugar coated tablet

Parameter	**Batch ES-T-1**
Description	Very light yellow, round, biconvex uncoated tablets
Average weight (mg)	65.08
Uniformity of weight (% RSD) (n=20)	± 2.312
Diameter (mm) ± SD (n= 10)	5.56 mm ± 0.043
Thickness (mm) ± SD (n= 10)	2.61 mm ± 1.431
Disintegration time	1' 35"
Friability (%)	0.23
Hardness (kg/cm^2) ± SD (n= 10)	3.2 ± 0.287
Moisture content (%)	1.23
% Assay of fungal alpha amylase	149.58

Table 5.39 Evaluation of enteric tablets prepared for enteric sugar coated tablet

Parameter		**Batch ES-T-1**
Description		white, round, biconvex enteric coated tablets
Average weight (mg)		70.25
Uniformity of weight (% RSD) (n= 20)		± 2.565
Diameter (mm) ±SD (n= 10)		5.61 mm ± 0.029
Thickness (mm) ± SD (n= 10)		2.68 mm ± 0.089
Disintegration time	Acid stage	Intact, shows no signs of any crack
	Buffer stage	14' 16"
% Drug release ± SD (n=6)	Buffer stage	145.39 ± 2.135 of fungal alpha amylase
Moisture content (%)		1.21
% Assay of fungal alpha amylase		149.61

Table 5.40 Evaluation of enteric sugar coated tablet

Parameter		**Batch ES-T-1**
Description		white, round, biconvex sugar coated tablets with glossy finish
Average weight (mg)		105.62
Uniformity of weight (% RSD) (n= 20)		± 3.442
Diameter (mm) ± SD (n= 10)		5.83 ± 0.032
Thickness (mm) ± SD (n=10)		3.29 ± 0.102
Disintegration time	Acid stage	Sugar coat completely disintegrated and core tablet is intact, shows no signs of any crack
	Buffer stage	15' 29"
% Drug release ± SD (n=6)	Acid stage	140.23 ± 2.464 of pepsin
	Buffer stage	143.09 ± 2.223 of fungal alpha amylase
Moisture Content (%)		2.04
% Assay of fungal alpha amylase		148.55
% Assay of pepsin		148.79

Figure 5.17 Enteric sugar coated tablet showing complete disintegration of sugar coat and remaining intact enteric coated tablet in acidic medium

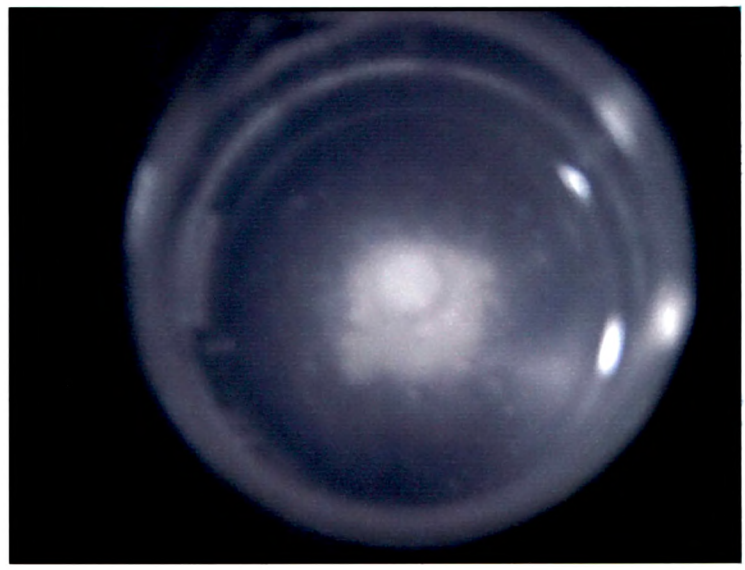
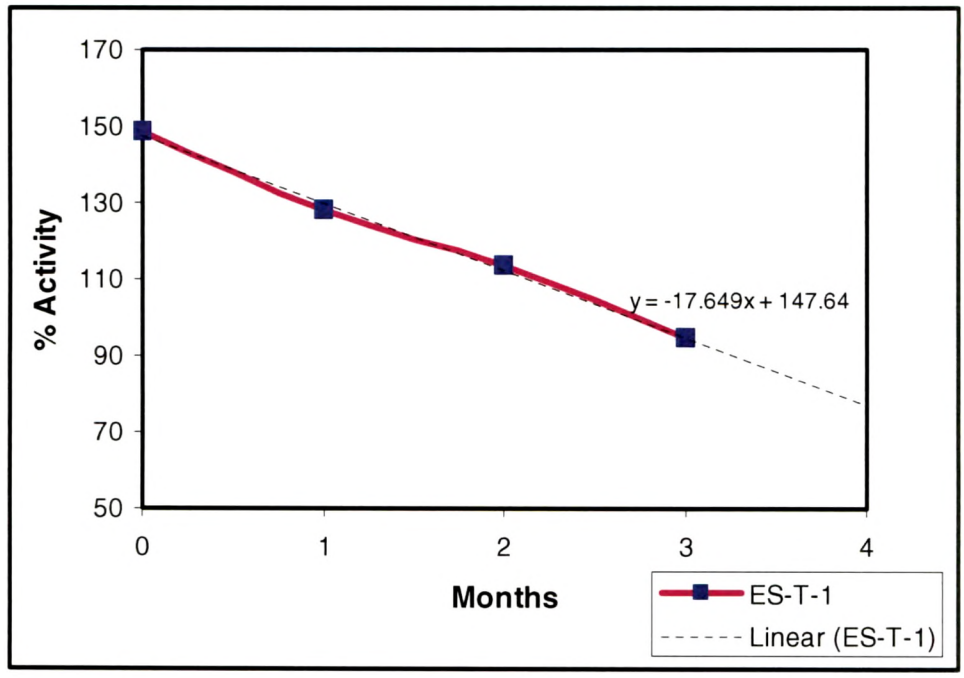


Table 5.41 Stability study data of enteric sugar coated -Batch ES-T-1

Parameter		**Batch ES-T-1**		
		1st Month	2nd Month	3rd Month
45°C				
Description		A	A	A
MC (%)		2.01	1.99	1.97
% Assay FAA		130.51	112.48	94.53
% Assay PEP		136.67	124.55	112.39
Disintegration time	Acid stage	B	B	B
	Buffer stage	16' 47"	17' 23"	18' 44"
% Drug release ± SD (n=6)	% PEP dissolved in acid stage	128.46 ± 2.028	114.53 ± 1.675	101.25 ± 2.102
	% FAA dissolved in mixed phosphate buffer pH 6.8	123.98 ± 2.368	106.86 ± 2.112	89.80 ± 2.892
40°C+75 % RH				
Description		A	A	A
MC (%)		2.06	2.08	2.12
% Assay FAA		139.28	130.54	121.50
% Assay PEP		142.61	136.56	130.19
Disintegration time	Acid stage	B	B	B
	Buffer stage	15' 15"	15' 00"	14' 51"
% Drug release ± SD (n=6)	% PEP dissolved in acid stage	134.06 ± 1.882	126.99 ± 2.235	122.37 ± 1.899
	% FAA dissolved in mixed phosphate buffer pH 6.8	133.12 ± 1.984	124.02 ± 2.132	116.85 ± 2.095
30°C				
Description		A	A	A
MC (%)		2.03	2.04	2.05
% Assay FAA		146.26	144.08	141.65
% Assay PEP		147.24	145.57	144.12
Disintegration time	Acid stage	B	B	B
	Buffer stage	15' 52"	16' 23"	16' 49"
% Drug release ± SD (n=6)	% PEP dissolved in acid stage	137.84 ± 1.747	135.23 ± 2.079	132.69 ± 2.107
	% FAA dissolved in mixed phosphate buffer pH 6.8	138.52 ± 1.898	135.86 ± 2.108	131.71 ± 2.284

A- White, round, biconvex sugar coated tablets with glossy finish
B- Sugar coat were completely disintegrated and core tablets were intact, shows no signs of any crack

Figure 5.18 Stability data of enteric sugar coated tablet Batch ES-T-1 at 45°C



5.3.2.3b Sugar coated matrix tablet formulation

Matrix tablets were prepared using polymer cellulose acetate phthalate [43] with aim to protect the drug from acid environment. Cellulose acetate phthalate was used in dry mixing as well as in binding. The evaluation of granules of Batch ES-MT-1 to ES-MT-4 is shown in Table 5.42 and Batch ES-MT-5 to ES-MT-7 is shown in Table 5.43. All batches except Batch ES-MT-1, shown compressibility index from 15.19 % to 11.56 %, angle of repose from 28° to 23° and proportion of fine is less than 40 %, which suggests the good flow property. Batch ES-MT-1 shows poor flow property may be due to lowest concentration of cellulose acetate phthalate (2 %) which ultimately results in less binding property. Data of particle size distribution indicates that the granules were homogenously mixed. Moisture content was found in the range from 1.52 % to 0.86 %.

As proportion of cellulose acetate phthalate increases, the flow parameters such as compressibility index, angle of repose, proportion of granules were improved. Batch ES-MT-7 where maximum cellulose acetate phthalate content is used (21%) has shown very good flow property.

The evaluations of matrix tablets were given in Table 5.44. The results of diameter, thickness, hardness are expressed as mean ± standard deviation and shows that tablets were uniform. The tablets were compressed at average weight of 100 mg and weight variation was under 2.5 %.Tablets friability was found to be less than 0.56 % and no capped tablets were observed. Moisture content was found to be below 1.6 % which was not increased after compression process. Drug release was estimated at acid stage and buffer stage. At acid stage, content of fungal alpha amylase could not be estimated due to its total degradation in acid medium. After acid stage, tablets were subjected to buffer stage and hourly drug release was estimated and drug release is depicted in Table 5.45 and Figure 5.19. From the release pattern, it is observed that Batch ES-MT-7 released the fungal alpha amylase up to 144.30 % over 3 hours and the results revealed that there is no loss of fungal alpha amylase at acid stage whereas Batch ES-MT-1, only 4.47 % amylase released in buffer stage and remainder amount released at acid stage.

From the Table 5.45, it can be concluded that as cellulose acetate phthalate amount increases, the more resistance to acid increases. AT acid stage Batch ES-MT-7 were remained as it is whereas at buffer stage, Batch ES-MT-7 tablets were swelled and formed spongy mass and up to 4 hours tablets remained as spongy mass.

Batch ES-MT-7 was selected and subjected for further sugar coating containing pepsin and evaluation parameters of matrix sugar coating tablets are given in Table 5.46.

Weight gain of about 55 % was increased during sugar coating over the matrix tablets. The results of diameter, thickness are expressed as mean ± standard deviation and shows that tablets were uniform in diameter and thickness. The tablets weight variation was under 3.6 %. Moisture content was found to be 2.25 % which was increased during coating process from 1.57 %.

Fungal alpha amylase release was carried out in Indian Pharmacopoeial paddle type dissolution apparatus, in two stages that is acid stage and buffer stage. During acid stage, sugar coat layer was removed and enteric coat were remained intact and there were no signs of cracks or removal of coating layer. Pepsin was estimated from acid medium and total 146.23 % (97.49 % of the label claim) release was estimated. At phosphate buffer stage, fungal alpha amylase release was found to be 40.52 % (27.01 % of the label claim) after 1 hour, 96.48 % (64.32 % of the label claim) after 2 hours, 143.21 % (95.47 % of the label claim) after 3 hours and 143.20 % (95.47 % of the label claim). Within 3 hours matrix sugar coated tablets delivered the maximum content of fungal alpha amylase.

This matrix sugar coated tablets will deliver pepsin as immediate release in stomach and fungal alpha amylase will be delivered in intestine in slow release over 3-4 hours with assumption that fungal alpha amylase will digest maximum amount of starch in the intestine. Stability data of Batch ES-MT-7 is shown in Table 5.47 and stability data of 45°C is shown in figure 5.20. This batch was shown to be stable and expected shelf life was

estimated to be up to 24 months. Also this batch showed good stability over 40°C and 75 % Relative Humidity where moisture was not increased more than 2.30 %. Fungal alpha amylase release was decreased from initial month value 143.21 % to 86.05 % at 3rd month at 45°C, low release was due to available content of fungal alpha amylase was less that is 91.25 %.

Table 5.42 Evaluation of granules prepared for sugar coated matrix tablets- Batch ES-MT-1 to ES-MT-4

Parameter		**Batch ES-MT-1**	**Batch ES-MT-2**	**Batch ES-MT-3**	**Batch ES-MT-4**
Description		A	A	A	A
Bulk density (gm/ml)		0.497	0.519	0.522	0.541
Tapped density (gm/ml)		0.608	0.612	0.615	0.623
Compressibility index (%)		18.25	15.19	15.12	13.16
Angle of repose (°)		34	28	27	24
Moisture content (%)		0.89	0.88	0.86	0.96
Particle size distribution (%) retained on mesh	16	2.21	2.10	2.01	1.85
	20	22.10	22.55	24.07	24.21
	40	24.89	27.53	26.88	27.40
	60	9.01	8.15	8.10	7.80
	80	10.08	7.93	8.65	11.66
	100	4.89	6.08	5.99	6.42
	200	12.9	12.22	11.08	8.11
	200 pass	13.92	13.44	13.22	12.55
Granules to fine ratio		58.21:41.79	60.33:39.67	61.06:38.94	61.26:38.74

Table 5.43 Evaluation of granules prepared for sugar coated matrix tablets- Batch ES-MT-5 to ES-MT-7

Parameter		**Batch ES-MT-5**	**Batch ES-MT-6**	**Batch ES-MT-7**
Description		A	A	A
Bulk density (gm/ml)		0.549	0.553	0.566
Tapped density (gm/ml)		0.628	0.631	0.640
Compressibility index (%)		12.57	12.36	11.56
Angle of repose (°)		24	23	23
Moisture content (%)		1.12	1.23	1.52
Particle size distribution (%) retained on mesh	16	1.77	1.55	1.19
	20	25.42	27.62	29.38
	40	29.11	28.8	30.47
	60	7.28	6.28	5.19
	80	13.12	11.24	12.25
	100	4.98	4.58	3.34
	200	6.07	11.45	10.33
	200 pass	12.25	8.48	7.85
Granules to fine ratio		63.58:36.42	64.25:35.75	66.23:33.77

Table 5.44 Evaluation of uncoated tablets prepared for sugar coated matrix tablets- Batch ES-MT-1 to ES-MT-7

Parameter	**Batch ES-MT-1**	**Batch ES-MT-2**	**Batch ES-MT-3**	**Batch ES-MT-4**	**Batch ES-MT-5**	**Batch ES-MT-6**	**Batch ES-MT-7**
Description	A	A	A	A	A	A	A
Average weight (mg)	100.21	100.25	99.98	101.10	100.39	100.58	100.07
Uniformity of weight(%) ± SD (n=20)	± 2.201	± 2.158	± 2.234	± 2.135	± 2.366	± 2.227	± 2.203
Diameter (mm) ± SD	6.44 ± 0.027	6.44 ± 0.032	6.44 ± 0.029	6.44 ± 0.027	6.44 ± 0.031	6.44 ± 0.029	6.44 ± 0.022
Thickness (mm) ± SD	2.53 ± 0.089	2.49 ± 0.091	2.49 ± 0.090	2.47 ± 0.098	2.43 ± 0.088	2.42 ± 0.096	2.39 ± 0.087
Friability (%)	0.56	0.41	0.40	0.35	0.33	0.26	0.24
Hardness (kg/cm^2) ± SD	2.5 ± 0.243	3.1 ± 0.238	3.2 ± 0.225	3.7 ± 0.237	3.6 ± 0.223	3.5 ± 0.246	3.6 ± 0.228
Moisture content (%)	0.90	0.91	0.90	1.02	1.15	1.26	1.57
% Assay of fungal alpha amylase	148.89	148.29	149.58	149.85	149.10	148.99	149.11

Table 5.45 Fungal alpha amylase release from uncoated matrix tablets Batch ES-MT-1 to ES-MT-7 at buffer stage

Batch	% Drug release ± SD			
	Buffer stage			
	1 hour	2 hour	3 hour	4 hour
ES-MT-1	4.47 ± 1.321	4.45 ± 1.563	4.48 ± 1.347	4.47 ± 1.653
ES-MT-2	22.73 ± 1.682	47.45 ± 1.445	47.41 ± 1.567	47.43 ± 1.625
ES-MT-3	31.63 ± 2.878	67.32 ± 1.877	67.30 ± 2.581	67.29 ± 1.739
ES-MT-4	38.96 ± 2.345	81.93 ± 1.865	81.90 ± 1.925	81.90 ± 2.097
ES-MT-5	33.88 ± 2.458	73.51 ± 2.338	107.35 ± 1.628	107.25 ± 1.873
ES-MT-6	41.65 ± 1.890	82.42 ± 2.398	126.64 ± 2.486	126.50 ± 2.547
ES-MT-7	47.11 ± 2.791	95.36 ± 2.673	144.33 ± 2.045	144.30 ± 2.45

Figure 5.19 Graph representing the drug release from uncoated matrix tablets at buffer stage

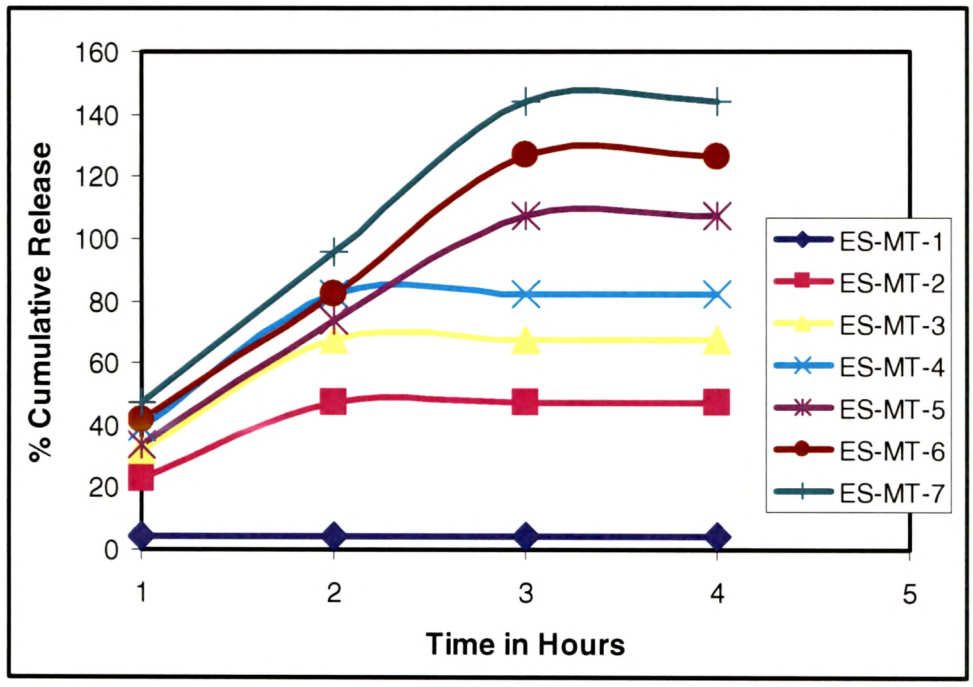


Table 5.46 Evaluation of sugar coated matrix tablets - ES-MT-7

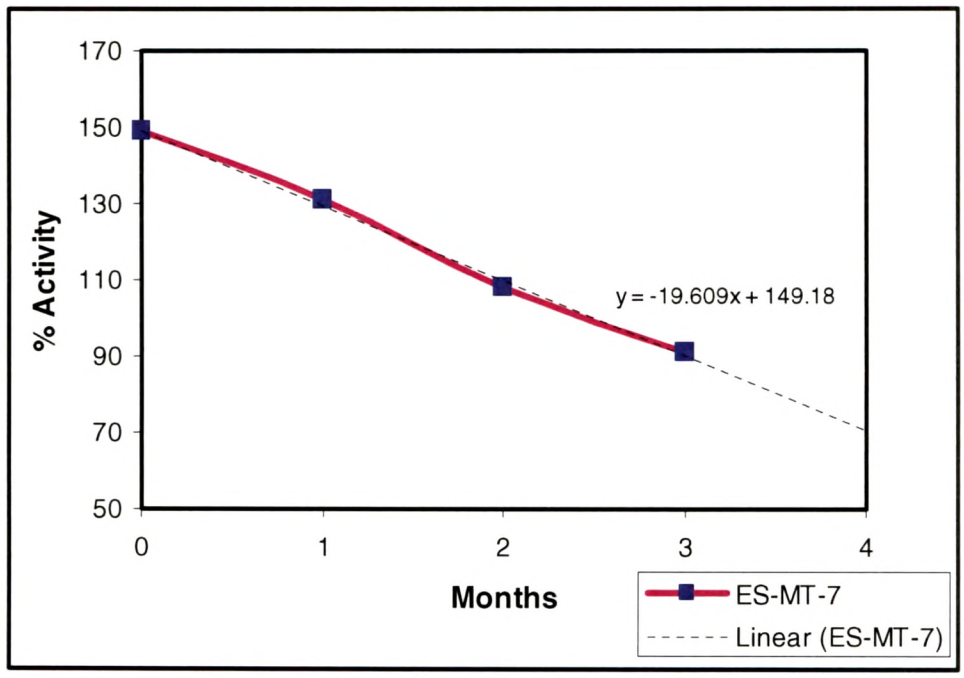
Parameter			**Batch ES-MT-7**
Description			white, round, biconvex sugar coated tablets with glossy finish
Average weight (mg)			155.12
Uniformity of weight (% RSD) (n=20)			± 3.5334
Diameter (mm) ± SD (n=10)			6.67 ± 0.041
Thickness (mm) ± SD (n=10)			2.99 ± 0.112
% Drug Release ± SD (n=6)	Acid Stage	2 hr	146.23 ± 1.893 of pepsin
	Buffer Stage	1 hr	40.52 ± 2.586 of fungal alpha amylase
		2 hr	96.48 ± 2.893 of fungal alpha amylase
		3 hr	143.21 ± 2.528 of fungal alpha amylase
		4 hr	143.20 ± 2.459 of fungal alpha amylase
Moisture Content (%)			2.25
% Assay of fungal alpha amylase			148.87
% Assay of pepsin			148.55

Table 5.47 Stability data of sugar coated matrix tablets

Parameter			**Batch ES-MT-7**		
			1st Month	2nd Month	3rd Month
45°C					
Description			A	A	A
MC (%)			2.22	2.18	2.14
% Assay FAA			131.08	107.85	91.25
% Assay PEP			136.28	123.86	111.53
% Drug release ± SD (n=6)	Acid stage PEP Release	2 hr	130.83 ± 1.857	115.59 ± 2.047	100.11 ± 2.231
	Buffer stage FAA release	1 hr	41.45 ± 2.231	35.16 ± 2.120	28.56 ± 2.783
		2 hr	81.86 ± 2.280	69.30 ± 2.456	59.12 ± 2.121
		3 hr	124.25 ± 2.124	105.45 ± 1.984	86.09 ± 2.136
		4 hr	124.20 ± 2.055	105.43 ± 2.127	86.05 ± 2.654
40°C+75 % RH					
Description			A	A	A
MC (%)			2.26	2.28	2.30
% Assay FAA			139.14	129.30	118.99
% Assay PEP			142.12	136.34	130.17
% Drug release ± SD (n=6)	Acid stage PEP release	2 hr	135.04 ± 1.683	122.38 ± 2.313	112.35 ± 2.108
	Buffer stage FAA release	1 hr	44.43 ± 1.847	41.32 ± 2.007	37.86 ± 2.241
		2 hr	87.68 ± 1.997	83.12 2.117	7.68 ± 1.858
		3 hr	134.54 ± 1.931	120.98 ± 2.033	115.28 ± 2.101
		4 hr	134.52 ± 2.311	120.97 ± 2.010	115.21 ± 2.112
30°C					
Description			A	A	A
MC (%)			2.25	2.26	2.27
% Assay FAA			146.43	143.89	141.57
% Assay PEP			147.03	145.49	143.25
% Drug release ± SD (n=6)	Acid stage PEP release	2 hr	139.15 ± 2.341	136.27 ±1.924	133.36 ± 2.019
	Buffer stage FAA release	1 hr	45.89 ± 2.102	43.58 ± 1.945	42.24 ± 2.413
		2 hr	90.89 ± 1.878	91.36 ± 1.541	89.28 ± 2.114
		3 hr	139.28 ± 2.152	135.57 ± 1.787	133.54 ± 1.889
		4 hr	139.30 ± 1.894	135.55 ± 2.238	133.55 ± 2.022

A- white, round, biconvex sugar coated tablets with glossy finish
PEP- Pepsin, FAA- Fungal alpha amylase

Figure 5.20 Stability data of sugar coated matrix tablet Batch ES-MT-7 at 45°C



5.3.2.3c Pellets formulation

Separate enteric coated pellets of fungal alpha amylase and uncoated pellets of pepsin were prepared and were filled in capsule. Here main aim was to protect the fungal alpha amylase from acid environment by way of enteric coating.

Evaluation of fungal alpha amylase enteric coated pellets is shown in Table 5.48.

Flow parameters such as compressibility index was determined and compressibility index was found to be 4.26 % which suggests very good flowability of pellets and low compressibility index is may be due to round shape of pellets which is ideal shape. Data of particle size distribution indicates that the pellets were homogenously mixed. Thus the assessment of pellets property gives an indication of suitability of filling into the hard gelatin capsule. Moisture content was found to be 1.58 % which was controlled during entire process. Disintegration of enteric pellets was performed as per Indian Pharmacopoeia. In acid stage after 2 hours pellets did not show any signs of cracks or removal of coating, thereafter in mixed phosphate buffer pellets were disintegrated at 12'35". Fungal alpha amylase release was carried out in Indian Pharmacopoeial paddle type dissolution apparatus, in two stages that is acid stage and buffer stage. After acid stage, pellets were remained intact and there were no signs of cracks or removal of coating layer. As fungal alpha amylase does not remain stable in acidic medium, its content could not be estimated at acid stage. At phosphate buffer stage, fungal alpha amylase release after 45 minutes found to be 145.89 % (97.26 % of the label claim). These results suggest that enteric coating polymer cellulose acetate phthalate has given complete resistance to acid medium. The content of fungal alpha amylase was found to be 16.93 mg per 100 mg pellets.

Evaluation of pepsin uncoated pellets is shown in Table 5.49 wherein compressibility index was determined and compressibility index was found to be 5.36 % which suggests very good flowability of pellets. Data of particle size distribution indicates that the pellets were homogenously mixed.

Moisture content was found to be 1.47 % which was controlled during manufacturing process. Disintegration time was found to be 4' 16" and content of pepsin was found to be 18.4 mg per 100 mg of pellets. The fungal alpha amylase enteric coated pellets are shown in Figure 5.21 where round shape can be clearly seen.

The fungal alpha amylase enteric coated pellets and pepsin pellets were mixed and filled in hard gelatin capsules and evaluation is depicted in Table 5.50. Average net weight per capsule was 525 mg which contains 50 mg of fungal alpha amylase with 50 % overages and 10 mg of pepsin with 50 % overages. Uniformity of weight and locking length data shows that the capsules are uniformly filled. Moisture content was found to be 1.63 % which was not increased during filling and packing process.

Drug release was carried out in Indian Pharmacopoeial paddle type dissolution apparatus, in two stages that is acid stage and buffer stage. During acid stage, those pellets contains pepsin was disintegrated and enteric coated fungal alpha amylase was remained intact. Pepsin release was estimated from acid medium and total 146.66 % (97.77 % of the label claim) release was estimated. At phosphate buffer stage, fungal alpha amylase release after 45 minutes found to be 145.96 % (97.31 % of the label claim). Figure 5.22 clearly demonstrates that in acid medium, enteric coated pellets of fungal alpha amylase are intact whereas pellets of pepsin are disintegrated.

Stability data of hard gelatin capsule batch ESP-C-1 is given in Table 5.51 and stability data of 45°C is shown in Figure 5.23. This batch is shown to be stable and expected shelf life was estimated to be up to 32 months. Fungal alpha amylase release was decreased from initial month value 145.96 % to 99.42 % at 3rd month at 45°C. At 40°C and 75 % Relative Humidity, this batch showed good stability where moisture was not increased more than 1.71 %.

Table 5.48 Evaluation of fungal alpha amylase enteric coated pellets

Parameter		**Batch ES-P-1**
Description		White, round free flowing pellets
Bulk density (gm/ml)		0.651
Tapped density (gm/ml)		0.680
Compressibility index (%)		4.26
Moisture content (%)		1.58
Particle size distribution (%) retained on mesh	12	3.38
	14	14.85
	16	8.59
	18	57.39
	20	6.81
	40	6.88
	40 pass	2.10
Disintegration time	Acid stage	Intact, shows no signs of any crack
	Buffer stage	12' 35"
% Drug release ± SD (n=6)	Buffer stage	145.89± 1.658 of fungal alpha amylase
Content of fungal alpha amylase		16.93 mg per 100 mg pellets

Table 5.49 Evaluation of pepsin uncoated pellets

Parameter		**Batch ES-T-1**
Description		White, round free flowing pellets
Bulk density (gm/ml)		0.636
Tapped density (gm/ml)		0.672
Compressibility index (%)		5.36
Moisture content (%)		1.47
Particle size distribution (%) retained on mesh	12	3.69
	14	13.24
	16	10.12
	18	57.96
	20	5.99
	40	7.09
	40 pass	1.91
Disintegration time		4' 16"
Content of pepsin		18.40 mg 100 mg pellets

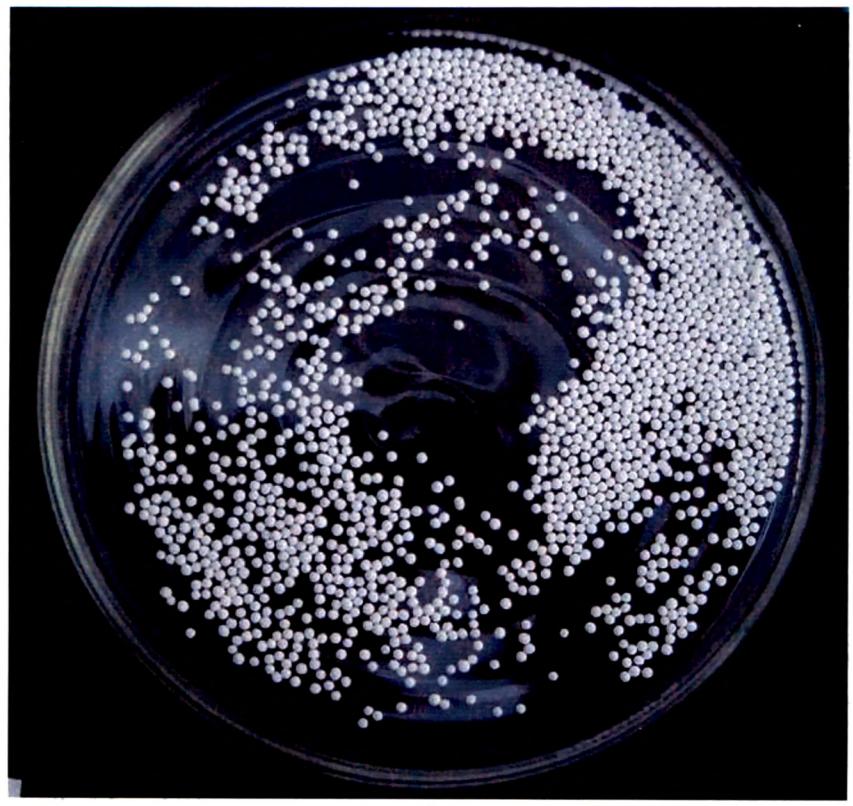
Table 5.50 Evaluation of hard gelatin capsule containing fungal alpha amylase enteric coated pellets and pepsin uncoated pellets

Parameter		**ESP-C-1**
Description		A
Average net content per capsule (mg)		525.13
Average weight of intact capsule (mg)		621.34
Uniformity of weight (% RSD) (n=20)		± 2.698
Locking length (mm) ± SD (n=20)		21.7 ± 0.078
Moisture content (%)		1.63
% Drug release ± SD (n=6)	Acid stage	146.66 ± 2.028 of PEP
	Buffer stage	145.96 ± 1.889 of FAA
% Assay of FAA		148.98
% Assay of PEP		149.08

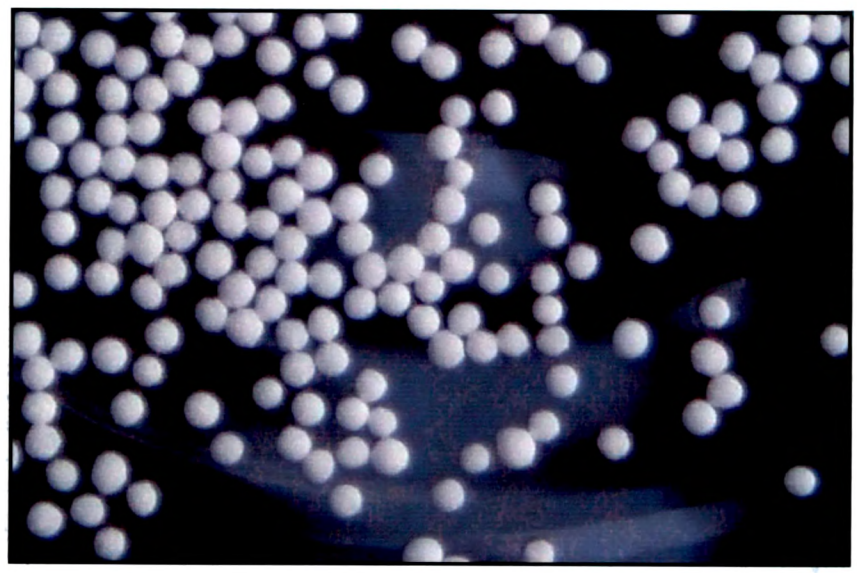
A- Clear Transparent-Clear Transparent Hard Gelatin Capsule containing white round pellets

FAA- Fungal alpha amylase, PEP- Pepsin

Figure 5.21 Figure A and B showing round shaped fungal alpha amylase enteric coated pellets



A



B

Figure 5.22 Figure showing disintegrated pepsin pellets and intact fungal alpha amylase enteric coated pellets

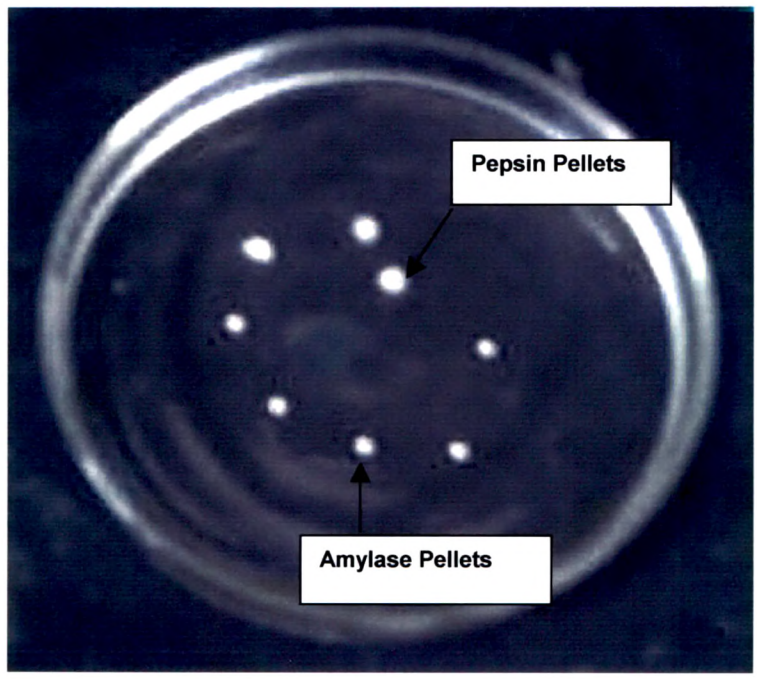
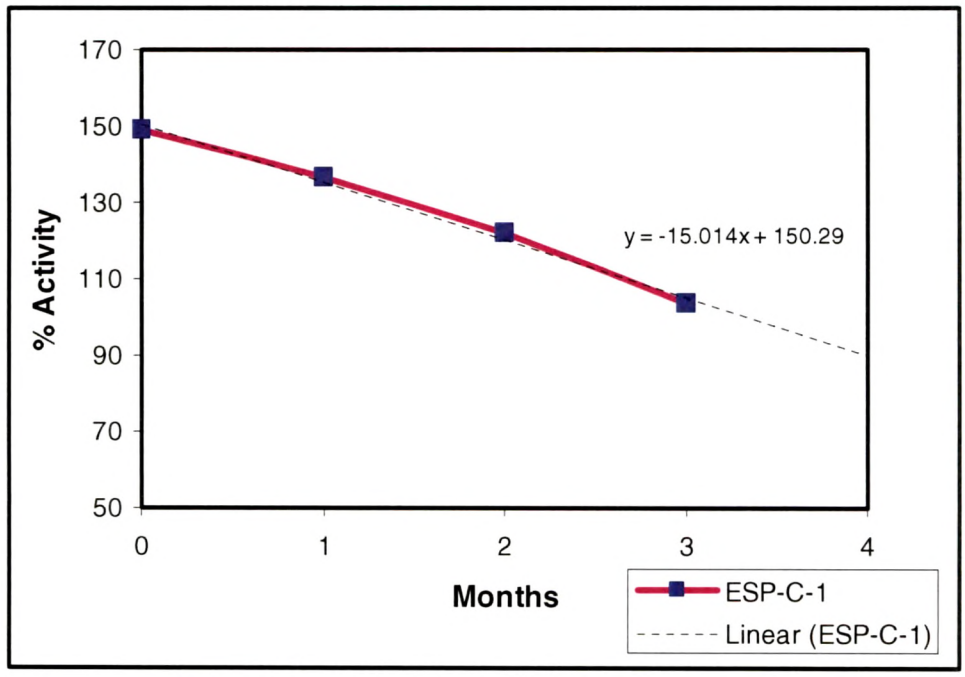


Table 5.51 Stability study of hard gelatin capsule containing fungal alpha amylase enteric coated pellets and pepsin uncoated pellets -Batch ESP-C-1

Parameter		**Batch ESP-C-1**		
		1st Month	2nd Month	3rd Month
45°C				
Description		A	A	A
MC (%)		1.61	1.58	1.53
% Assay FAA		134.19	119.59	104.61
% Assay PEP		136.62	124.22	110.07
% Drug release ± SD (n=6)	% PEP dissolved in acid stage	131.11 ± 2.047	118.00 ± 1.664	103.15 ± 1.981
	% FAA dissolved in mixed phosphate buffer pH 6.8	126.13 ± 1.853	111.21 ± 2.011	99.42 ± 2.411
40°C+75 % RH				
Description		A	A	A
MC (%)		1.65	1.69	1.71
% Assay FAA		141.60	133.23	126.82
% Assay PEP		142.90	136.74	131.06
% Drug release ± SD (n=6)	% PEP dissolved in acid stage	134.76 ± 2.255	129.09 ± 1.663	121.11 ± 2.104
	% FAA dissolved in mixed phosphate buffer pH 6.8	133.10 ± 2.781	125.13 ± 1.870	118.93 ± 2.114
30°C				
Description		A	A	A
MC (%)		1.63	1.65	1.66
% Assay FAA		147.13	145.09	143.41
% Assay PEP		147.53	146.05	144.52
% Drug release ± SD (n=6)	% PEP dissolved in acid stage	140.13 ± 2.121	136.25 ± 1.925	133.58 ± 1.863
	% FAA dissolved in mixed phosphate buffer pH 6.8	136.88 ± 2.647	134.99 ± 2.217	134.81 ± 2.337

A- Clear transparent-clear transparent hard gelatin capsule containing white round pellets

Figure 5.23 Stability data of hard gelatin capsule containing fungal alpha amylase enteric coated pellets and pepsin uncoated pellets Batch ESP- C-1 at 45°C



5.3.2.3d Enteric coated Powder preparation

Enteric coating of fungal alpha amylase powder was carried out by spray drying method using enteric polymer cellulose acetate phthalate and drug to polymer ratio 1:0.5, 1:1 were tried. Yield obtained was 85.1% for Batch ECP-1 and 88.3 % for Batch ECP-2.

The evaluation of spray dried powder is given in Table 5.52. Batch ECP-1 and ECP-2 were shown almost similar flow properties. Compressibility index were found to be 7.61 % and 7.048 % and angle of repose were found to be 23° and 21° respectively for Batch ECP-1 and ECP-2, which suggests that both batches were having good flow property. Moisture content was found to be less than 1 %. Total fungal alpha amylase content was found to be 30.13 % and 26.56 % for Batch ECP-1 and Batch ECP-2 respectively.

5.3.2.3d.1 Particle size distribution

Figure 5.24 shows the particle size distribution of the spray dried powders of Batch ECP-1 and ECP-2 respectively investigated by laser diffraction. Both batch powders showed a uniform size distribution. The SEM pictures for spray-dried enteric coated powder of fungal alpha amylase , showing only fine particles with diameters less than 20 μm, would however suggest that the larger particles observed during particle sizing for these powders solely reflect aggregation behaviour under the measurement conditions. The parameters observed during particle size analysis are summarised in Table 5.53.

5.3.2.3d.2 Scanning electron microscopy (SEM)

The Scanning electron micrographs of the Batch ECP-1 and ECP-2 are shown in Figures 5.25 to 5.29. For both drug-polymer ratio 1:0.5 (Batch ECP-1) and ratio 1:1 (Batch ECP-2), the spray-dried microparticles exhibited shriveled surfaces, apparently derived from originally spherical particles distorted by loss of internal volume as a result of solvent evaporation [44].

SEM of Batch ECP-2 shown more number of spherical particles than Batch ECP-1 this may be due to more content of enteric polymer. All SEM figures

clearly shows that the particle size obtained for both batches are less than 20 μm.

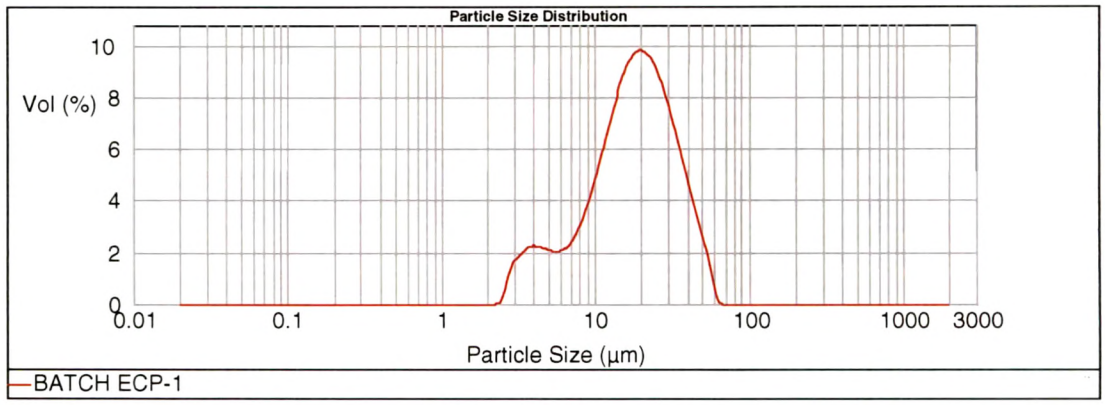
Fungal alpha amylase release was estimated after 45 minutes at buffer stage, found to be 77.13 % and 96.29 % respectively for Batch ECP-1 and ECP-2. This data reveals that fungal alpha amylase from Batch ECP-1 was released about 22.87 % at acid stage which was not desired profile whereas Batch ECP-2 shown only about 3.7 % release at acid stage. Thus Batch ECP-2 was selected for preparation of different dosage form.

Table 5.52 Evaluation of enteric coated fungal alpha amylase powder Batch ECP-1 and ECP-2

Parameter		**Batch ECP-1**	**Batch ECP-2**
Description		White free flowing powder	White free flowing powder
Bulk density (gm/ml)		0.473	0.488
Tapped density (gm/ml)		0.512	0.525
Compressibility index (%)		7.61	7.048
Angle of repose (°)		23	21
Moisture content (%)		0.91	0.97
FAA content (% w/w)		30.13	26.56
% Drug release ± SD (n=3)	Buffer stage	77.13 ± 2.443	96.29 ± 2.021

Figure 5.24 Particle size distribution of enteric coated powder of fungal alpha amylase Batch ECP-1 and ECP-2

Batch ECP-1



Batch ECP-2

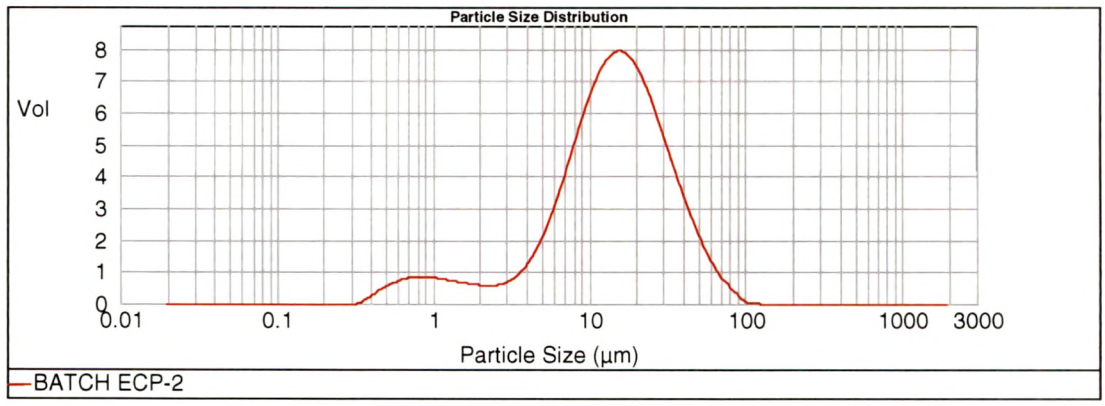


Table 5.53 Particle size analysis of enteric coated powder of fungal alpha amylase Batch ECP-1 and ECP-2

	D (v,10) (µm)	**D (v,50) (µm)**	**D (v,90) (µm)**	**VMD**	**Span**
ECP-1	5.756	18.111	37.377	20.209	1.746
ECP-2	3.692	14.627	37.577	18.431	2.317

D [v, x], particle diameter at x % of the volume distribution.

Span, width of the volume distribution relative to the median diameter (D [v, 90] - D [v, 10]/D [v, 50]).

VMD- Volume Median Diameter

Figure 5.25 Scanning electron micrograph A) Magnification X 1000 and B) Magnification X 1500 of enteric coated fungal alpha amylase Batch ECP-1

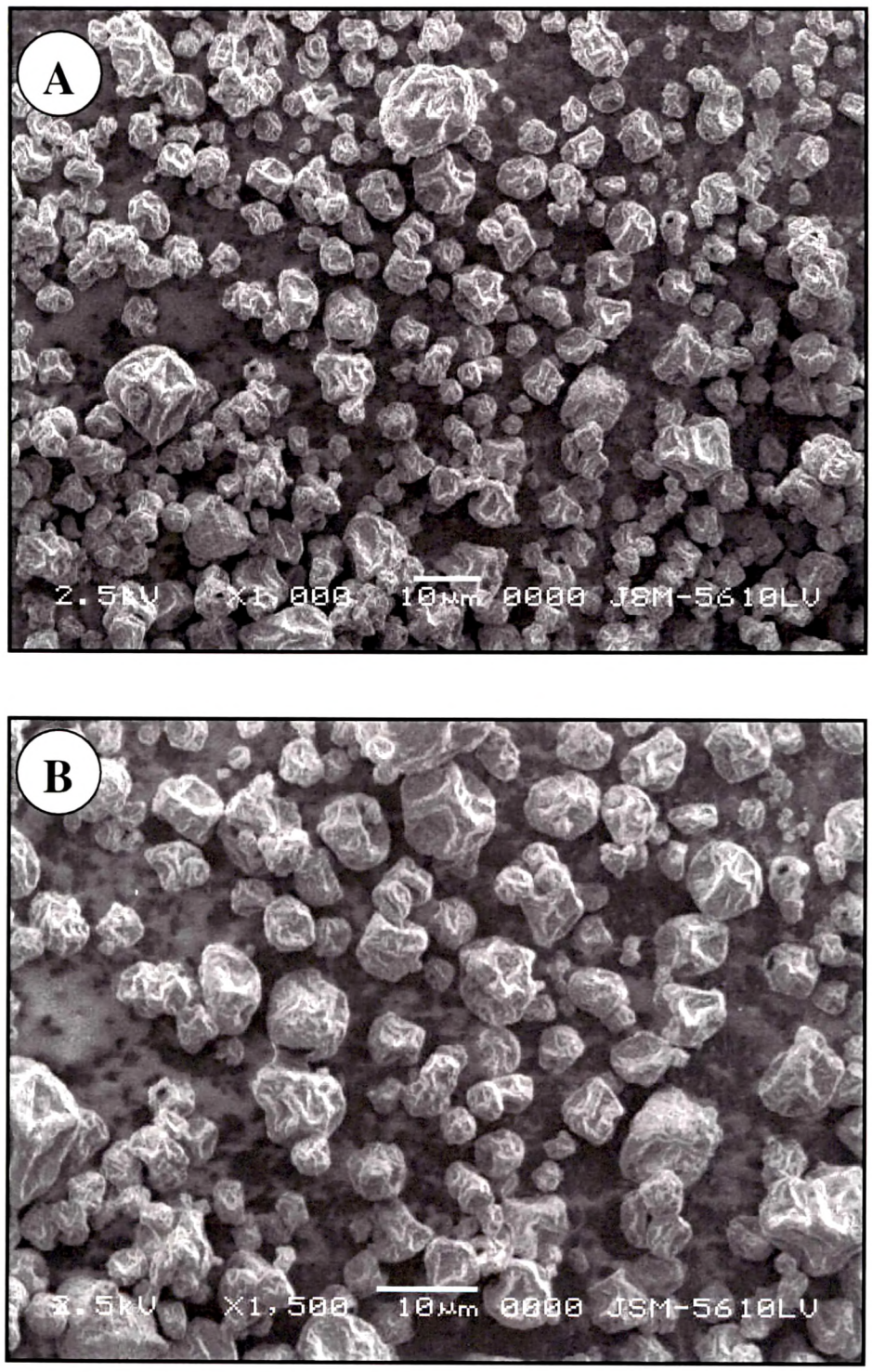
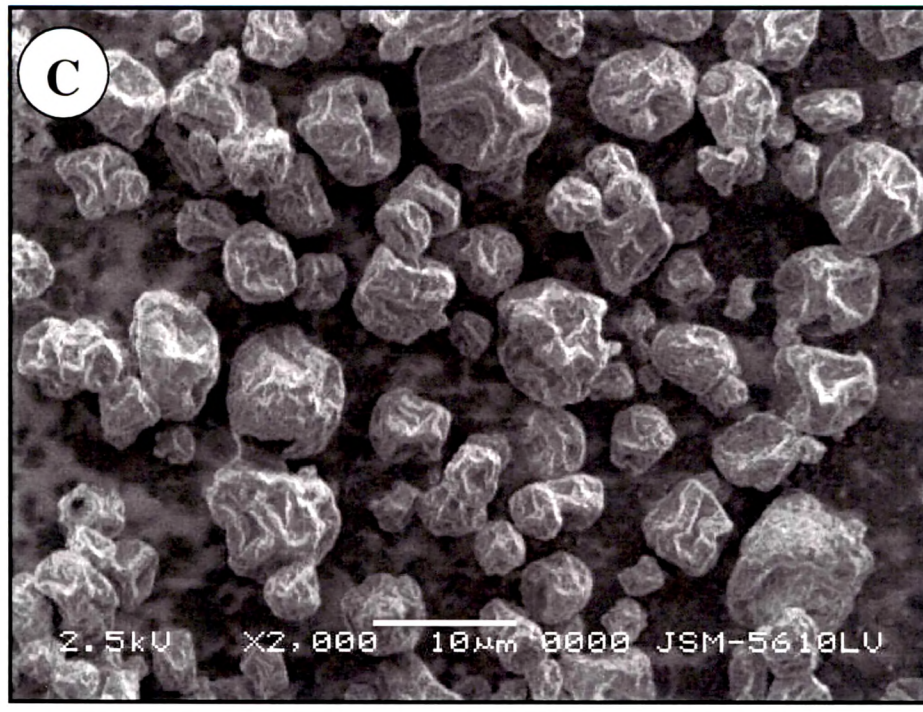


Figure 5.26 Scanning electron micrograph C) Magnification X 2000 and D) Magnification X 4000 of enteric coated fungal alpha amylase Batch ECP-1



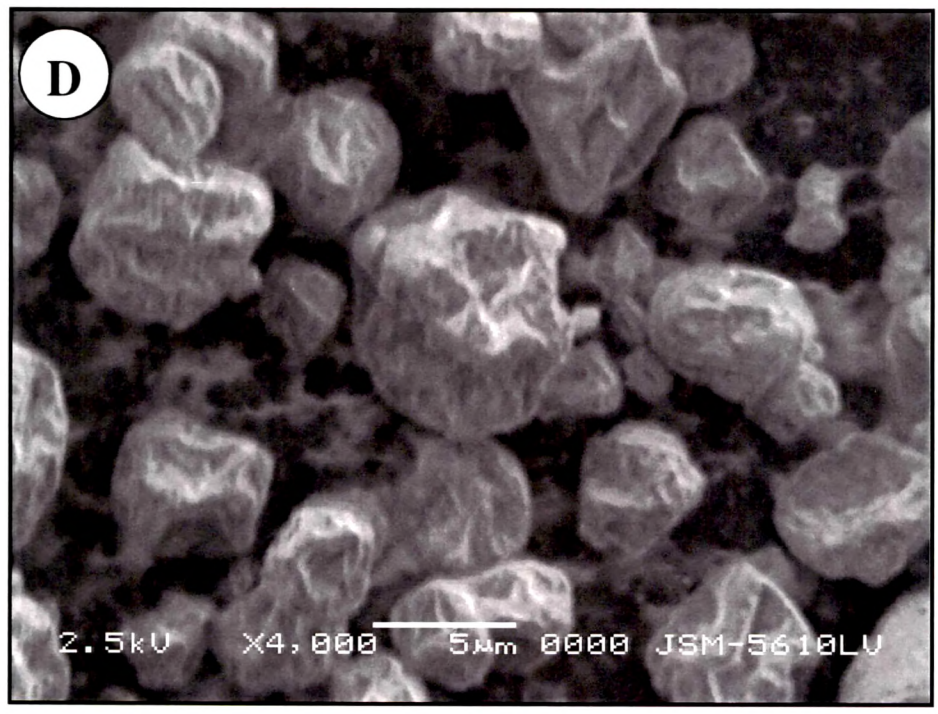


Figure 5.27 Scanning electron micrograph A) Magnification X 1000 and B) Magnification X 1500 of enteric coated fungal alpha amylase Batch ECP-2

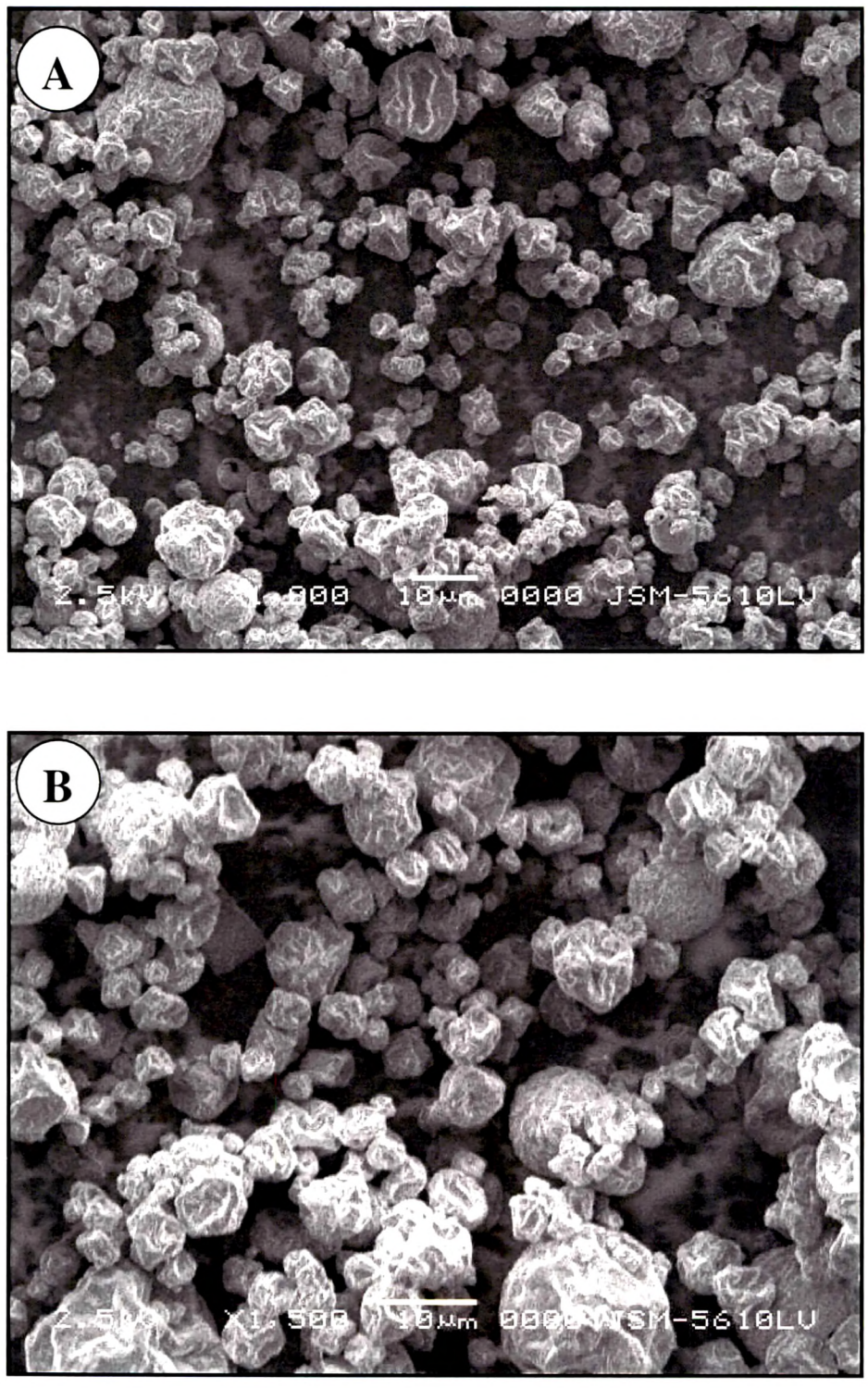


Figure 5.28 Scanning electron micrograph C) Magnification X 2000 and D) Magnification X 3500 of enteric coated fungal alpha amylase Batch ECP-2

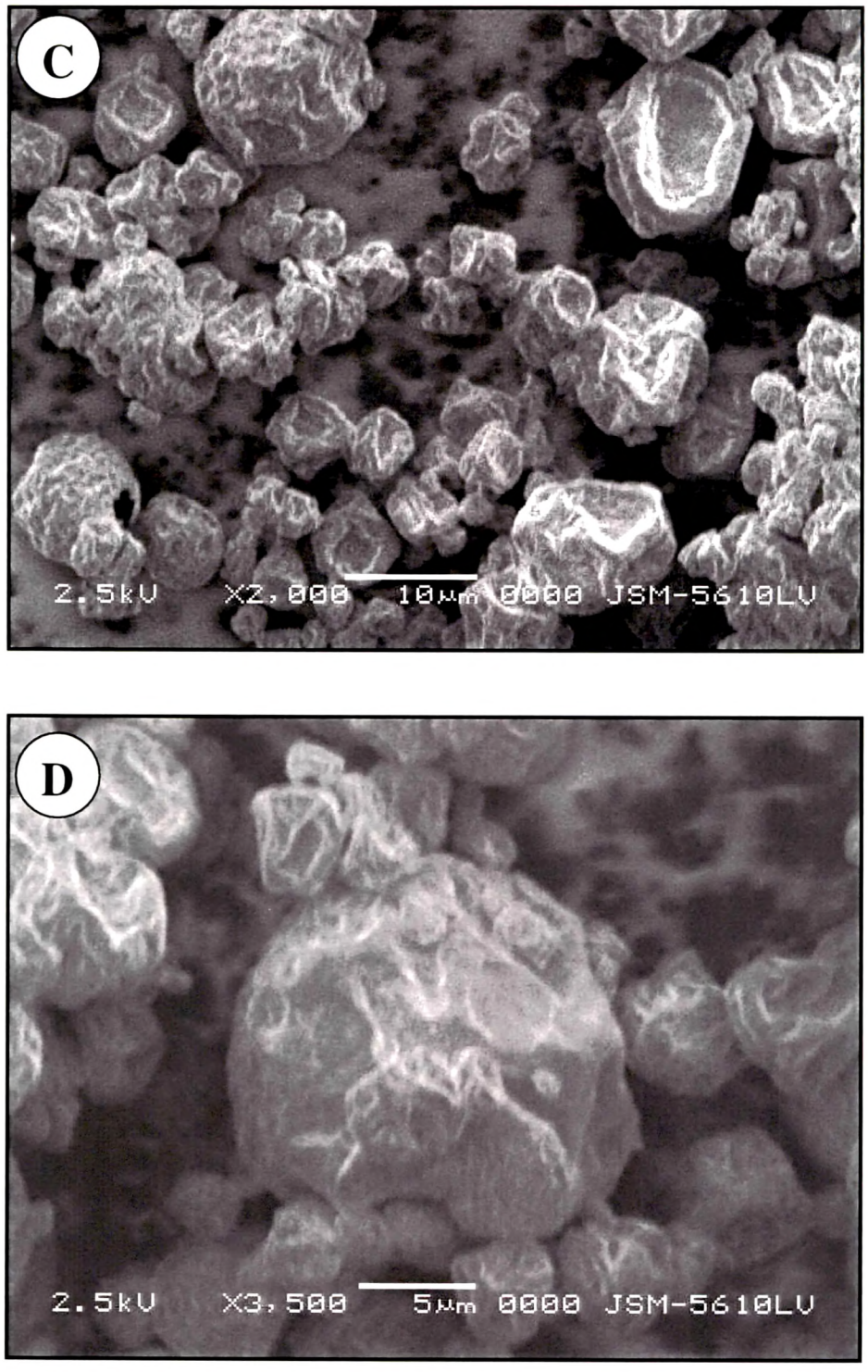


Figure 5.29 Scanning electron micrograph E) Magnification X 6500 of enteric coated fungal alpha amylase Batch ECP-2

5.3.2.3d.3 Hard gelatin capsule formulation

Characterization of hard gelatin capsule blend of Batch ECP-C-1 is shown in Table 5.54. The compressibility index 17.11 % and angle of repose 26° indicated good flow property of capsule blend. Moisture content was found to be 1.26 % which is well controlled.

Characterization of hard gelatin capsule is given in Table 5.55, where average weight, weight variation and locking length shows that the capsules are uniform.

Disintegration of hard gelatin capsule was 9' 12" which is within the limit described in Indian Pharmacopoeia. Drug release was carried out in Indian Pharmacopoeial paddle type dissolution apparatus, in two stages that is acid stage and buffer stage. During acid stage, all capsules were disintegrated and pepsin content was found to be 141.28 % (94.18 %). At phosphate buffer stage, fungal alpha amylase release after 45 minutes found to be 139.48 % (92.99 %). These results suggest that enteric coated fungal alpha amylase was dissolved at buffer stage

Stability data of Batch ECP-C-1 is shown in Table 5.56 and stability data of 45°C is shown in Figure 5.30 and results suggest that Batch ECP-C-1 is shown to be stable and expected shelf life was estimated to be up to 28.4 months. Stability over 40°C and 75 % Relative Humidity also shown stable product. It is confirmed that due to addition of calcium chloride and enteric coating, stability is improved.

Table 5.54 Characterization of hard gelatin capsule blend containing enteric coated fungal alpha amylase and pepsin powder-Batch ECP-C-1

Parameter	**ECP-C- 1**
Description	A
Bulk density (gm/ml)	0.431
Tapped density (gm/ml)	0.520
Compressibility index (%)	17.11
Angle of repose (°)	26
Moisture content (%)	1.26

A – White coloured, free flowing powder

Table 5.55 Characterization of hard gelatin capsule containing enteric coated fungal alpha amylase and pepsin powder-Batch ECP-C-1

		ECP-C- 1
Description		A
Average net content per capsule (mg)		140.09
Average weight of intact capsule (mg)		190.21
Uniformity of weight (%RSD) (n=20)		± 2.559
Locking length (mm) ± SD (n=20)		15.9 ± 0.065
Moisture content (%)		1.33
Disintegration time		9' 12"
% Assay of FAA		148.38
% Assay of PEP		149.17
% Drug release ± SD (n=6)	Acid stage	141.28 ± 2.155 of PEP
	Buffer stage	139.48 ± 1.881 of FAA

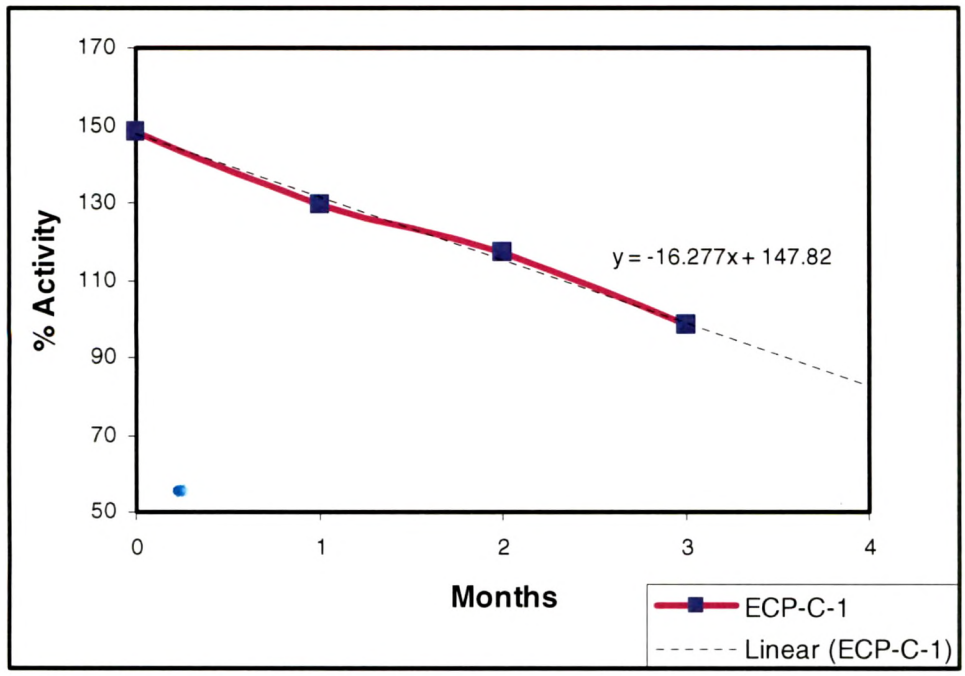
A- Ivory –Ivory size "3" Hard Gelatin capsule containing white powder, FAA- Fungal alpha amylase, PEP- Pepsin

Table 5.56 Stability study of hard gelatin capsule containing enteric coated fungal alpha amylase and pepsin powder-Batch ECP-C-1

Parameter		**Batch ECP-C-1**		
		1st Month	2nd Month	3rd Month
45°C				
Description		A	A	A
MC (%)		1.31	1.29	1.25
% Assay FAA		131.69	115.02	98.26
% Assay PEP		135.98	124.28	110.28
Disintegration time		9' 55"	10' 11"	10' 55"
% Drug release ± SD (n=6)	% PEP dissolved in acid stage	129.14 ± 1.335	117.06 ± 1.980	103.66 ± 2.102
	% FAA dissolved in mixed phosphate buffer pH 6.8	112.47 ± 2.120	107.49 ± 1.865	92.33 ± 2.358
40°C+75 % RH				
Description		A	A	A
MC (%)		1.35	1.37	1.40
% Assay FAA		139.95	131.28	122.39
% Assay PEP		142.0	135.88	130.69
Disintegration time		9' 10"	9' 09"	9' 00"
% Drug release ± SD (n=6)	% PEP dissolved in acid stage	133.92 ± 2.364	127.72 ± 1.778	122.84 ± 2.411
	% FAA dissolved in mixed phosphate buffer pH 6.8	131.55 ± 2.147	123.40 ± 1.724	113.81 ± 2.015
30°C				
Description		A	A	A
MC (%)		1.33	1.34	1.36
% Assay FAA		146.29	144.08	141.99
% Assay PEP		147.52	145.23	143.70
Disintegration time		9' 14"	9' 36"	9' 55"
% Drug release ± SD (n=6)	% PEP dissolved in acid stage	140.14 ± 1.984	137.96 ± 2.117	136.44 ± 2.052
	% FAA dissolved in mixed phosphate buffer pH 6.8	137.51 ± 2.028	134.89 ± 1.657	132.58 ± 2.034

A- Ivory –Ivory size "3" Hard Gelatin capsule containing white powder, FAA- Fungal alpha amylase, PEP- Pepsin

Figure 5.30 Stability data of hard gelatin Capsule containing enteric coated fungal alpha amylase and pepsin powder-Batch ECP-C-1 at 45°C



5.3.2.3d.4 Tablet formulation

Granules were prepared for tablet by nonaqueous granulation method and the evaluation of granules is given in Table 5.57, wherein the granules were showing 12.30 % compressibility index and 24° angle of repose which suggests that granules were having good flow property. Data of particle size distribution indicates that the granules were homogenously mixed. The proportion of fines was less than 40 % which indicates the good flow rate of granules. Thus the assessment of granules property gives an indication of suitability of the pre-compression mix for use in the tablet press.

Evaluation parameters of uncoated tablets are shown in Table 5.58. The results of diameter, thickness, hardness are expressed as mean ± standard deviation and shows that tablets were uniform. The tablets were compressed at average weight of 100 mg and weight variation was under 2.5 %.Tablets friability was found to be 0.38 % and no capped tablets were observed. The disintegration time was below 7 minutes which is within Pharmacopoeial limit. Moisture content was found to be 1.21 % which was not increased after granulation process. The uncoated tablets were complied all the general evaluation parameters given in Indian Pharmacopoeia.

Drug release was carried out in Indian Pharmacopoeial paddle type dissolution apparatus, in two stages that is acid stage and buffer stage. During acid stage, all tablets were disintegrated and pepsin was estimated to be 140.83 % (93.88 % of the label claim). At phosphate buffer stage, fungal alpha amylase release after 45 minutes found to be 138.54 % (92.36 % of the label claim).

Stability data of Batch ES-T-1 is shown in Table 5.59 and stability data of 45°C is shown in figure 5.31. This batch is shown to be stable and expected shelf life was estimated to be up to 29.6 months. Fungal alpha amylase release was decreased from initial month value 138.54 % to 94.48 % at 3rd month at 45°C. Also this batch showed good stability over 40°C and 75 % Relative Humidity where moisture was not increased more than 1.27 %.

Table 5.57 Evaluation of granules prepared for tablets containing enteric coated fungal alpha amylase and pepsin powder-Batch ECP-T-1

Parameter		**Batch ECP-T-1**
Description		A
Bulk density (gm/ml)		0.542
Tapped density (gm/ml)		0.618
Compressibility index (%)		12.30
Angle of repose (°)		24
Moisture content (%)		1.16
Particle size distribution (%) retained on mesh	16	1.94
	20	34.28
	40	17.87
	60	8.29
	80	18.12
	100	6.98
	200	5.07
	200 pass	7.45
Granules to fine ratio		62.38:37.62

A- White free flowing granules

Table 5.58 Evaluation of tablets containing enteric coated fungal alpha amylase and pepsin powder-Batch ECP-T-1

Parameter		**Batch ECP-T-1**
Description		A
Average weight (mg)		100.09
Uniformity of weight (% RSD) (n=20)		± 2.112 %
Disintegration time		6' 24"
Diameter (mm) ± SD		6.42 ± 0.028
Thickness (mm) ± SD		2.63 ± 0.072
Friability (%)		0.38
Hardness (kg/cm^2)		3.1
Moisture content (%)		1.21
% Assay of FAA		148.96
% Assay of PEP		149.13
% Drug release ± SD (n=6)	% PEP dissolved in acid stage	140.83 ± 2.021
	% FAA dissolved in mixed phosphate buffer pH 6.8	138.54 ± 1.792

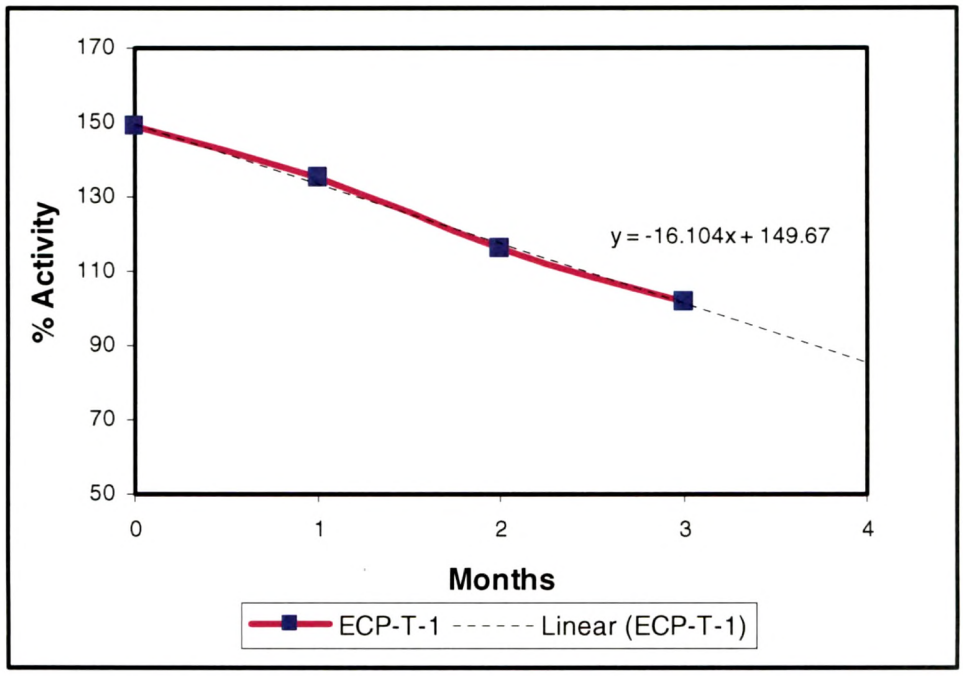
A- White, round, flat bevel edged uncoated tablet with break line on one side, FAA- Fungal alpha amylase, PEP- Pepsin

Table 5.59 Stability study of tablets containing enteric coated fungal alpha amylase and pepsin powder-Batch ECP-T-1

Parameter		**Batch ECP-T-1**		
		1st Month	2nd Month	3rd Month
45°C				
Description		A	A	A
MC (%)		1.20	1.18	1.15
% Assay FAA		135.21	116.28	101.59
% Assay PEP		135.98	124.85	112.66
Disintegration time		6' 58"	7' 30"	8' 09"
% Drug release ± SD (n = 6)	% PEP dissolved in acid stage	127.56 ± 1.992	117.20 ± 2.117	104.90 ± 1.874
	% FAA dissolved in mixed phosphate buffer pH 6.8	123.80 ± 1.668	107.56 ± 2.187	94.48 ± 2.576
40°C+75 % RH				
Description		A	A	A
MC (%)		1.22	1.24	1.27
% Assay FAA		141.09	132.69	125.17
% Assay PEP		143.02	136.78	130.72
Disintegration time		6' 20"	6' 02"	5' 59"
% Drug release ± SD (n = 6)	% PEP dissolved in acid stage	134.14 ± 2.044	126.89 ± 1.921	122.88 ± 1.637
	% FAA dissolved in mixed phosphate buffer pH 6.8	129.80 ± 2.125	122.34 ± 1.993	115.78 2.331
30°C				
Description		A	A	A
MC (%)		1.21	1.22	1.24
% Assay FAA		146.98	144.85	143.12
% Assay PEP		147.25	146.05	143.97
Disintegration time		6' 29"	6' 32"	6' 38"
% Drug release ± SD (n = 6)	% PEP dissolved in acid stage	138.42 ± 2.115	136.28 ± 1.784	133.89 ± 1.903
	% FAA dissolved in mixed phosphate buffer pH 6.8	136.56 ± 2.174	134.41 ± 2.228	131.64 ± 1.773

A- White, round, flat bevel edged uncoated tablet with break line on one side, FAA- Fungal alpha amylase, PEP- Pepsin

Figure 5.31 Stability data of tablets containing enteric coated fungal alpha amylase and pepsin powder-Batch ECP-T-1 at 45°C



5.3.2.3d.5 Dry syrup formulation

Characterization of dry syrup formulation batches ECP-DS-1 is given in Table 5.60. The compressibility index was found to be 12.13 % and angle of repose was found to be 31° which indicates the good flowability of dry syrup blend. Moisture content was found to be 0.57 % which is very low.

Reconstituted syrups of batch ECP-DS-1 was found to be stable up to 15 days at room temperature (Table 5.61).

Stability study data is given in Table 5.62 and stability data of temperature 45°C is depicted in Figure 5.32. From the figure it is clear that Batch ECP-DS-1 is stable formulation and expected shelf life was estimated to be up to 30 months. Batch ECP-DS-1 also showed better stability at 40°C and 75 % Relative Humidity.

Table 5.60 Evaluation of dry syrup containing enteric coated fungal alpha amylase and pepsin powder Batch ECP-DS-1

Parameter		**ECP-DS- 1**
Description		A
Bulk density (gm/ml)		0.768
Tapped density (gm/ml)		0.874
Compressibility index (%)		12.13
Angle of repose (°)		31
Moisture content (%)		0.57
% Assay FAA		148.88
% Assay PEP		149.33
% Drug release ± SD (n = 6)	% PEP dissolved in acid stage	147.68 ± 1.863
	% FAA dissolved in mixed phosphate buffer pH 6.8	141.43 ± 2.238

A- Very light yellow coloured, pineapple flavoured free flowing granules

Table 5.61 Evaluation of reconstituted dry syrup containing enteric coated fungal alpha amylase and pepsin powder Batch ECP-DS-1 and stability at 30°C

Parameter	**ECP-DS- 1**	
	Initial	15 days
Description	A	A
Sp.Gravity	1.218	1.218
pH	4.574	4.571
% Assay FAA	148.76	145.48
% Assay PEP	149.30	148.10

A- Light yellow syrupy suspension with pineapple flavour,

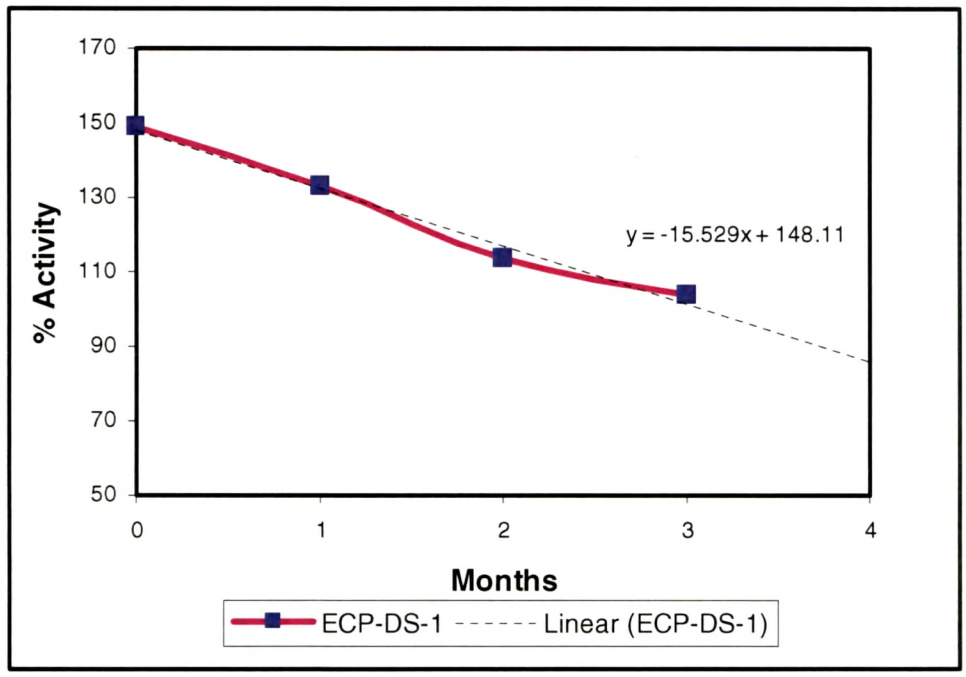
FAA- Fungal alpha amylase, PEP- Pepsin

Table 5.62 Stability study of dry syrup containing enteric coated fungal alpha amylase and pepsin powder Batch ECP-DS-1

Parameter		**Batch ECP-DS-1**		
		1st Month	2nd Month	3rd Month
45°C				
Description		A	A	A
Moisture content (%)		0.52	0.50	0.47
% Assay FAA		133.17	113.54	101.66
% Assay PEP		137.20	125.05	112.57
% Drug release ± SD (n = 6)	% PEP dissolved in acid stage	134.44 ± 1.936	123.02 ± 2.011	108.19 ± 1.612
	% FAA dissolved in mixed phosphate buffer pH 6.8	122.50 ± 2.003	107.66 ± 2.131	93.58 ± 1.969
40°C+75 % RH				
Description		A	A	A
Moisture content (%)		0.58	0.61	0.65
% Assay FAA		141.02	132.27	125.13
% Assay PEP		143.12	137.06	130.88
% Drug release ± SD (n = 6)	% PEP dissolved in acid stage	140.25 ± 1.882	133.31 ± 2.056	126.95 ± 1.861
	% FAA dissolved in mixed phosphate buffer pH 6.8	131.85 ± 2.017	124.06 ± 2.237	116.62 ± 2.045
30°C				
Description		A	A	A
Moisture content (%)		0.57	0.59	0.61
% Assay FAA		146.91	144.46	142.68
% Assay PEP		147.72	146.22	144.63
% Drug Release ± SD (n = 6)	% PEP dissolved in acid stage	144.76 ± 2.113	143.27 ± 2.030	140.39 ± 1.944
	% FAA dissolved in mixed phosphate buffer pH 6.8	138.09 ± 2.589	136.16 ± 2.210	134.11 ± 1.954

A- Very light yellow coloured, pineapple flavoured free flowing granules

Figure 5.32 Stability data of dry syrup containing enteric coated fungal alpha amylase and pepsin powder-Batch ECP-DS-1 at 45°C



5.3.3 INCLUSION COMPLEX

5.3.3.1 EXPERIMENTAL SECTION

Inclusion complex of fungal alpha amylase and beta-cyclodextrin were carried out and following studies were performed.

5.3.3.1a Formation of inclusion complex

5.3.3.1a.1 Aggregation inhibition study

Fungal alpha amylase was denatured by urea as denaturing agent [45].This denatured solution was rapidly diluted with Tris buffer at pH 8.5 and at every minute, aggregation was monitored by light scattering at 400 nm and results are given in Table 5.63.

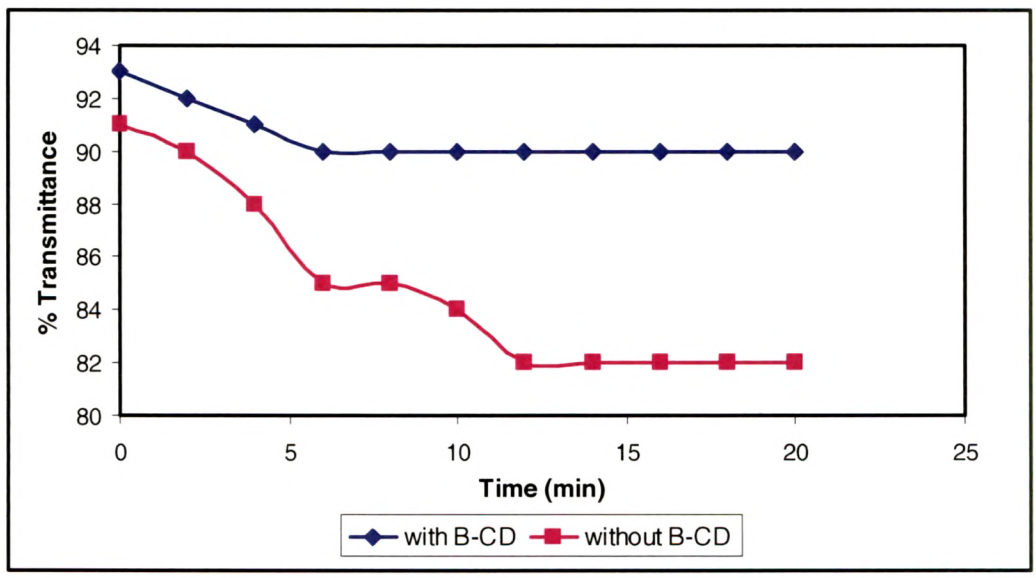
Similarly, denatured fungal alpha amylase solution was rapidly diluted with Tris buffer solution having pH 8.5 containing beta-cyclodextrin. At every minute, aggregation was monitored by light scattering at 400 nm and results are given Table 5.63.

From the results it is observed that aggregation was increased with time and then stabilized after approximately 5 minutes. When the denatured protein was renatured in the presence of beta-cyclodextrin under the same conditions, light scattering due to aggregation was significantly reduced. (Figure 5.33). Inhibition of alpha amylase aggregation was enhanced with beta-cyclodextrin in the renaturation buffer.

Table 5.63 Aggregation inhibition of fungal alpha amylase by beta-cyclodextrin

Time	% T with beta-cyclodextrin	% T without beta-cyclodextrin
0	93	91
2	92	90
4	91	88
6	90	85
8	90	85
10	90	84
12	90	82
14	90	82
16	90	82
18	90	82
20	90	82

Figure 5.33 Aggregation inhibition of fungal alpha amylase by beta-cyclodextrin



5.3.3.1a.2 Recovery of activity in excess of initial activity

Inclusion complex of fungal alpha amylase and beta-cyclodextrin were carried out at 1:1 to 10:1 Host: Guest [beta-cyclodextrin: fungal alpha amylase] molecular ratio by adding fungal alpha amylase solution in 50 mM Tris buffer having pH 8.5 and to this solution beta-cyclodextrin was added and stirred slowly at 37°C for 2 hours. After 2 hours of stirring, fungal alpha amylase activity was estimated and results are depicted in Table 5.64 and Figure 5.34 where Molecular ratios 1:1, 2:1 and 3:1 shown more fungal alpha amylase activity and out of this ratios, 3:1 ratio has shown maximum activity recovery, 185 %.

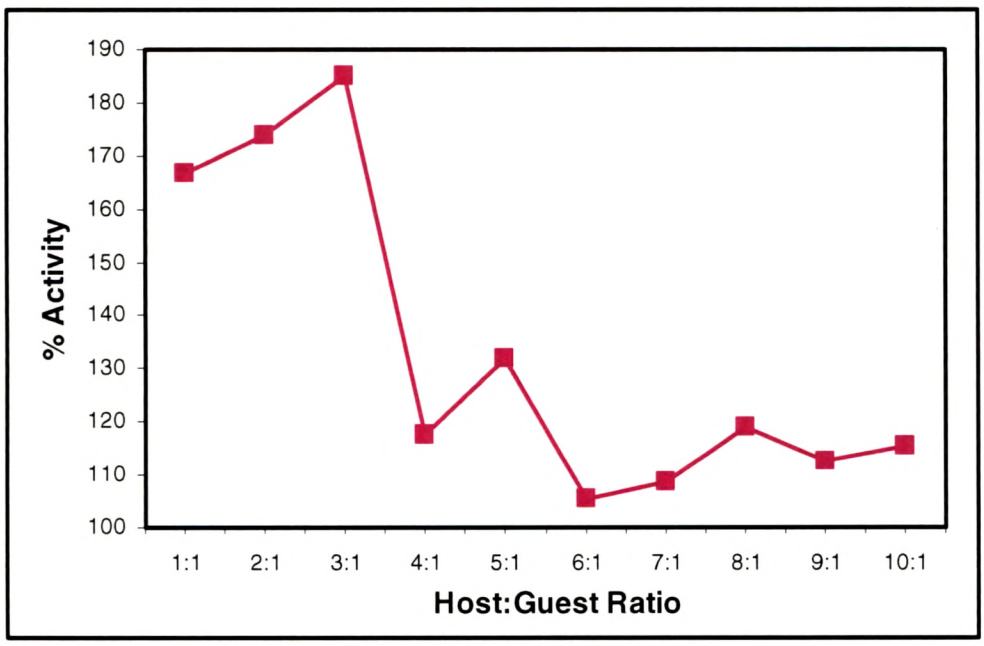
Given enzyme or protein sample may contain both active and inactive protein. The inactive protein may be present due to denaturation during protein purification and/or storage. If such a sample were subjected to refolding by the present method, then recovery of the activity of the inactive protein, in addition to the activity of the initially active protein, can yield greater than 100% recovery of activity [46, 47].

The results indicate that fungal alpha amylase enzyme present in the commercially available sample was a mixture of active (correctly folded) and inactive (denatured or incorrectly folded) forms. Thus, the present refolding method is useful to recover the inactive form present in a sample of fungal alpha amylase.

Table 5.64 Recovery of fungal alpha amylase activity in excess of initial activity

Host: Guest Ratio	Initial % activity	% Activity recovered
1:1	100.12	166.68
2:1	100.39	173.90
3:1	100.02	185.00
4:1	100.85	117.70
5:1	100.27	131.95
6:1	100.37	105.27
7:1	99.98	108.52
8:1	100.11	118.88
9:1	100.68	112.60
10:1	99.97	115.29

Figure 5.34 Recovery of fungal alpha amylase activity in excess of initial activity



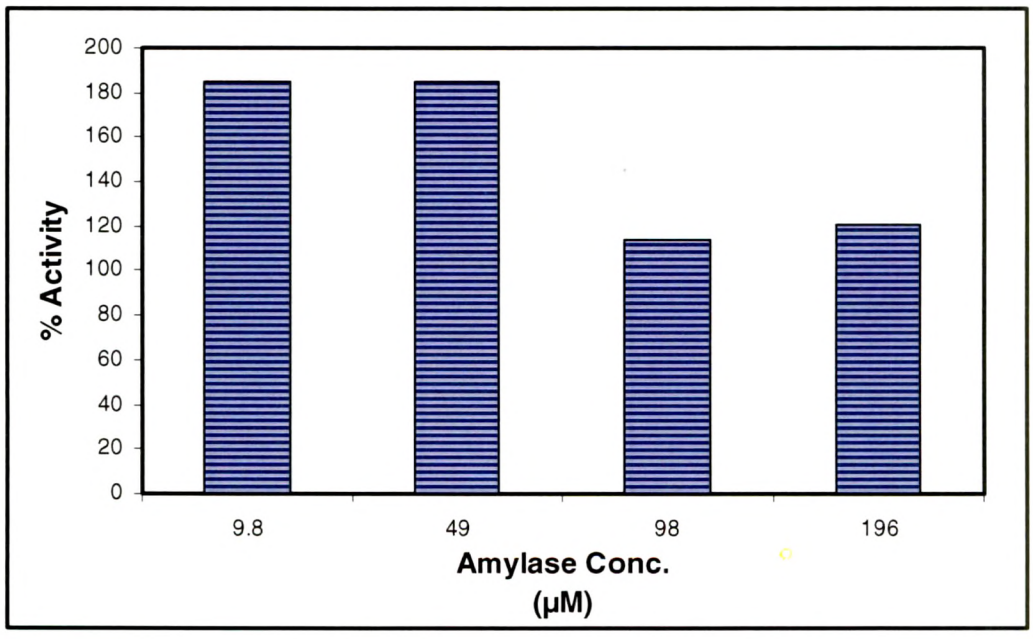
5.3.3.1a.3 Effect of enzyme concentration on inclusion complex

Inclusion complex of fungal alpha amylase in different concentration 9.8, 49, 98 and 196 μM were carried out with beta-cyclodextrin at molecular ratio 3:1 and results of recovery of fungal alpha amylase was given in Table 5.65 and figure 5.35. Recovery of active amylase decreased at higher protein concentrations due to increased protein aggregation [51].

Table 5.65 Effect of enzyme concentration on inclusion complex

Sr.No.	fungal alpha amylase concentration (M)	% Activity recovered
1	9.8	185.23
2	49	184.56
3	98	114.18
4	196	120.32

Figure 5.35 Effect of enzyme concentration on inclusion complex



5.3.3.1a.4 Effect of stirring time

Inclusion complex of fungal alpha amylase at 9.8 μM concentration was carried out with beta-cyclodextrin at molecular ratio 3:1 and stirred for 2 hours, 4 hours, 6 hours, 8 hours and 10 hours and results of recovery of fungal alpha amylase is shown in Table 5.66 and it is observed that two hours of stirring is enough to form the inclusion complex.

Table 5.66 Effect of stirring time on inclusion complex

Stirring Time (hours)	% Activity Recovered
2	185.12
4	185.11
6	185.12
8	185.10
10	185.11

5.3.3.1a.5 Effect of pH and temperature on the inclusion complex

Effect of pH and temperature on inclusion complex is depicted in Table 5.67 and it is seen that optimal yield of active protein was obtained between 25°C- 37°C at pH 8.5 [47].

Table 5.67 Effect pH and temperature on inclusion complex

pH	Temp.(°C)	Initial % Activity	% Recovery
8.5	15	100.11	107.16
8.5	25	100.08	170.23
8.5	37	100.05	185.19
8.5	50	99.98	71.30
5.0	37	100.06	80.12
6.0	37	99.97	115.03
7.0	37	100.03	155.28
8.0	37	100.10	165.22
9.0	37	100.01	160.58

5.3.3.1a.6 Effect of ionic strength on the inclusion complex

Fungal alpha amylase inclusion complex was carried out with beta-cyclodextrin with addition of sodium chloride 5 mM to 500 mM concentration, calcium chloride 0.6 mM to 4.5 mM concentration separately and results of the activity of fungal alpha amylase enzyme is given in Table 5.68. The data demonstrates that ionic strength decreases the recovery of alpha amylase. In order to achieve higher yield, other conditions have to be optimized. However, even under non-optimal conditions, refolding in the presence of beta-cyclodextrin significantly increases the amount of folded protein.

Table 5.68 Effect of ionic strength on inclusion complex

Sodium Chloride (mM)	% Recovery	Calcium Chloride (mM)	% Recovery
5	115.02	0.6	103.03
10	119.12	0.9	109.14
25	124.14	1.1	124.39
50	120.56	1.8	96.23
250	118.18	2.7	100.17
500	108.14	4.5	106.43

5.4.3a.2 Characterization of inclusion complex

Inclusion complex of fungal alpha amylase at 9.8 μM concentration was carried out with beta-cyclodextrin at molecular ratio 3:1 and stirred for 2 hours at 37 °C in buffer (50 mM Tris, pH 8.5) and the solution was subjected to Ultraviolet spectroscopy, Fluorescence spectroscopy, Infrared spectroscopy, proton-Nuclear Magnetic Resonance Spectroscopy and Differential Scanning Calorimetry.

5.4.3a.2a UV spectroscopy studies

UV absorbance spectrum of fungal alpha amylase in Tris buffer (Figure 5.36) shows the λ max at 278 nm and UV absorbance spectrum of fungal alpha amylase with beta-cyclodextrin (Figure 5.37) also shows the λ max at 278 nm.

This data shows that there are no alterations found in the aromatic residues of the fungal alpha amylase when interacted with beta-cyclodextrin.

UV spectroscopy of inclusion complex of different molecular ratio also shows that molecular ratio 3:1 gives the hypochromic shift in UV absorbance which confirms the formation of complex. Graph of absorbance for different molecular ratios is shown in Figure 5.38.

5.4.3a.2b Fluorescence spectroscopy studies

A fluorescence spectrum (Figure 5.39) was recorded for inclusion complex of fungal alpha amylase with beta-cyclodextrin at 425 nm excitation wavelength and emission wavelength in the range 200- 500 nm. It is observed it shows λ max at about 275 nm and which is identical to fluorescence spectra of fungal alpha amylase (Figure 5.9), and it is confirm that there is no change in the structure of fungal alpha amylase after interacting with beta-cyclodextrin. Similar results are reported [47] where alpha –cyclodextrin renatured carbonic anhydrase shown identical fluorescence spectra as that of native enzyme.

5.4.3a.2c FTIR spectroscopy

The FTIR spectrum for inclusion complex (Figure 5.40) shows the characteristic absorption bands of a protein. The absorption band at 3447.10 cm^{-1} is due to the N-H stretching, and the band at 1637.71 cm^{-1} is due to N-H bending. Similarly the FTIR spectrum for fungal alpha amylase solution (Figure 5.11) shows the absorption band at 3447.10 cm^{-1} is due to the N-H stretching and the band at 1637.71 cm^{-1} is due to N-H bending. The similar FTIR spectrum obtained confirms that there is no chemical interaction between fungal alpha amylase and beta-cyclodextrin.

5.4.3a.2d Proton-NMR spectroscopy

Figure 5.41 shows H1-NMR spectrum of fungal alpha amylase and figure 5.42 shows H1-NMR spectrum of inclusion complex. H1-NMR spectrum of fungal alpha amylase shows only the multiplet signals (4.653 to 4.825 ppm) whereas H1-NMR spectrum of inclusion complex shows triplet signals (4.708 to 4.75 ppm) and shows two singlet signals (3.526 ppm and 1.818 ppm). This H1-NMR data indicates the formation of inclusion complex.

5.4.3a.2e Differential scanning calorimetry (DSC)

DSC thermograph of fungal alpha amylase and inclusion complex are presented in figure 5.43 & 5.44. Fungal alpha amylase shows the endothermic peak at 112.8 °C and inclusion complex shows the endothermic peak at 111.9°C. This DSC data indicates the formation of inclusion complex.

Figure 5.36 UV spectrum of fungal alpha amylase in Tris buffer

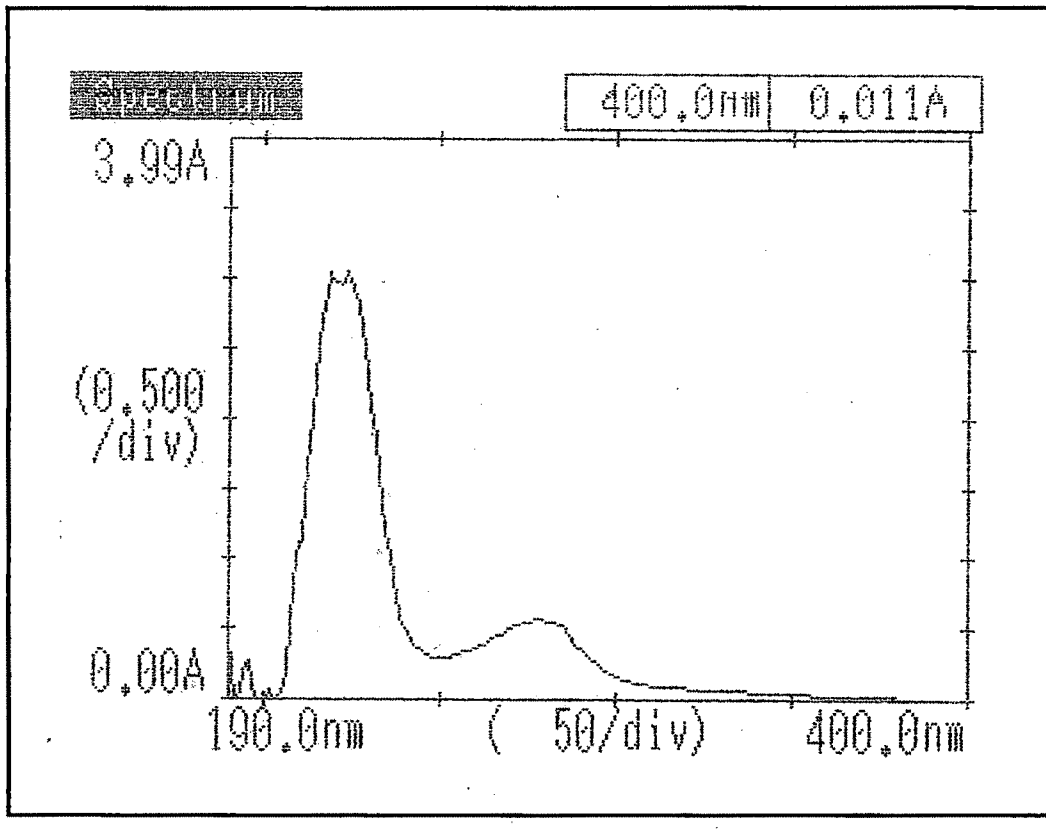


Figure 5.37 UV spectrum of inclusion complex of fungal alpha amylase with beta-cyclodextrin

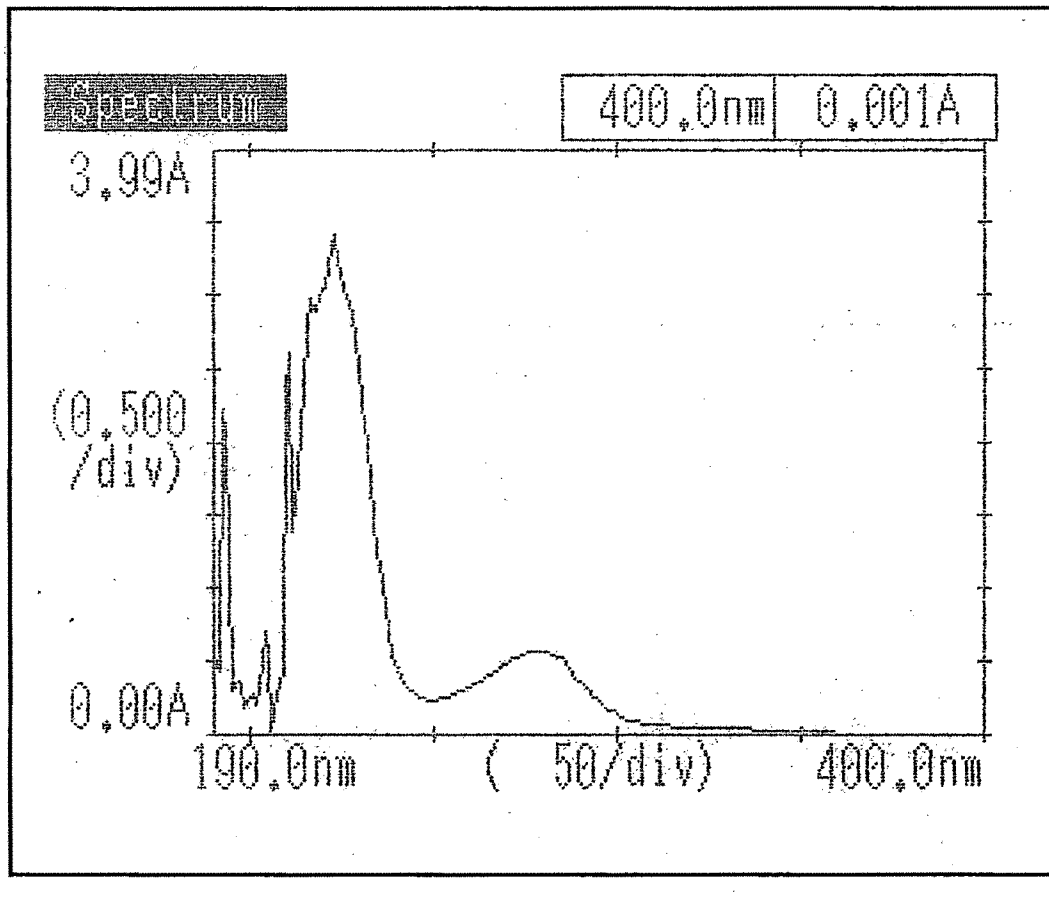


Figure 5.38 UV absorbance of inclusion complex of fungal alpha amylase with beta-cyclodextrin at different molecular ratio

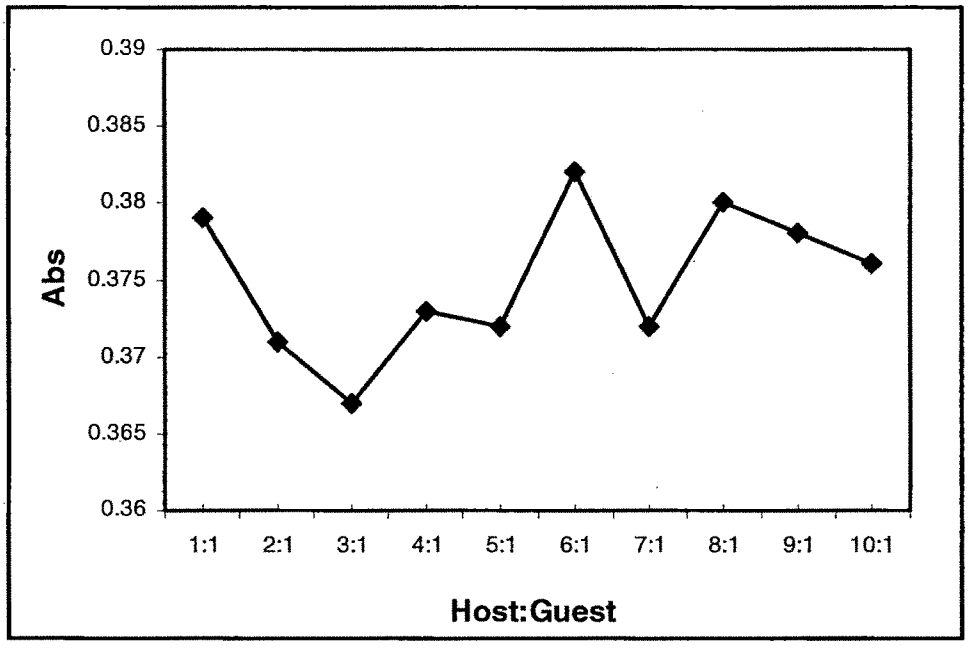


Figure 5.39 Fluorescence spectrum of inclusion complex of fungal alpha amylase with beta-cyclodextrin

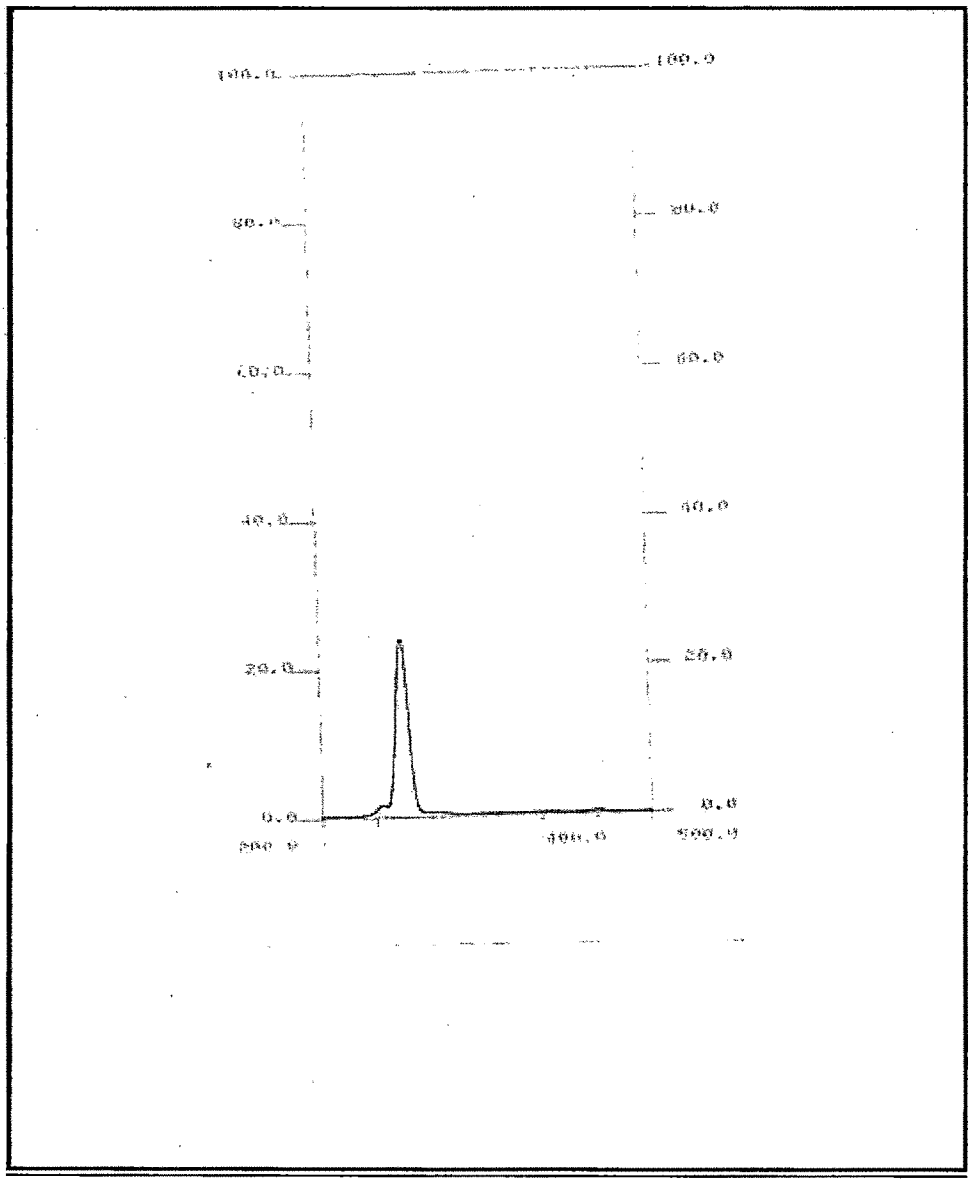


Figure 5.40 FTIR spectrum of inclusion complex of fungal alpha amylase with beta-cyclodextrin

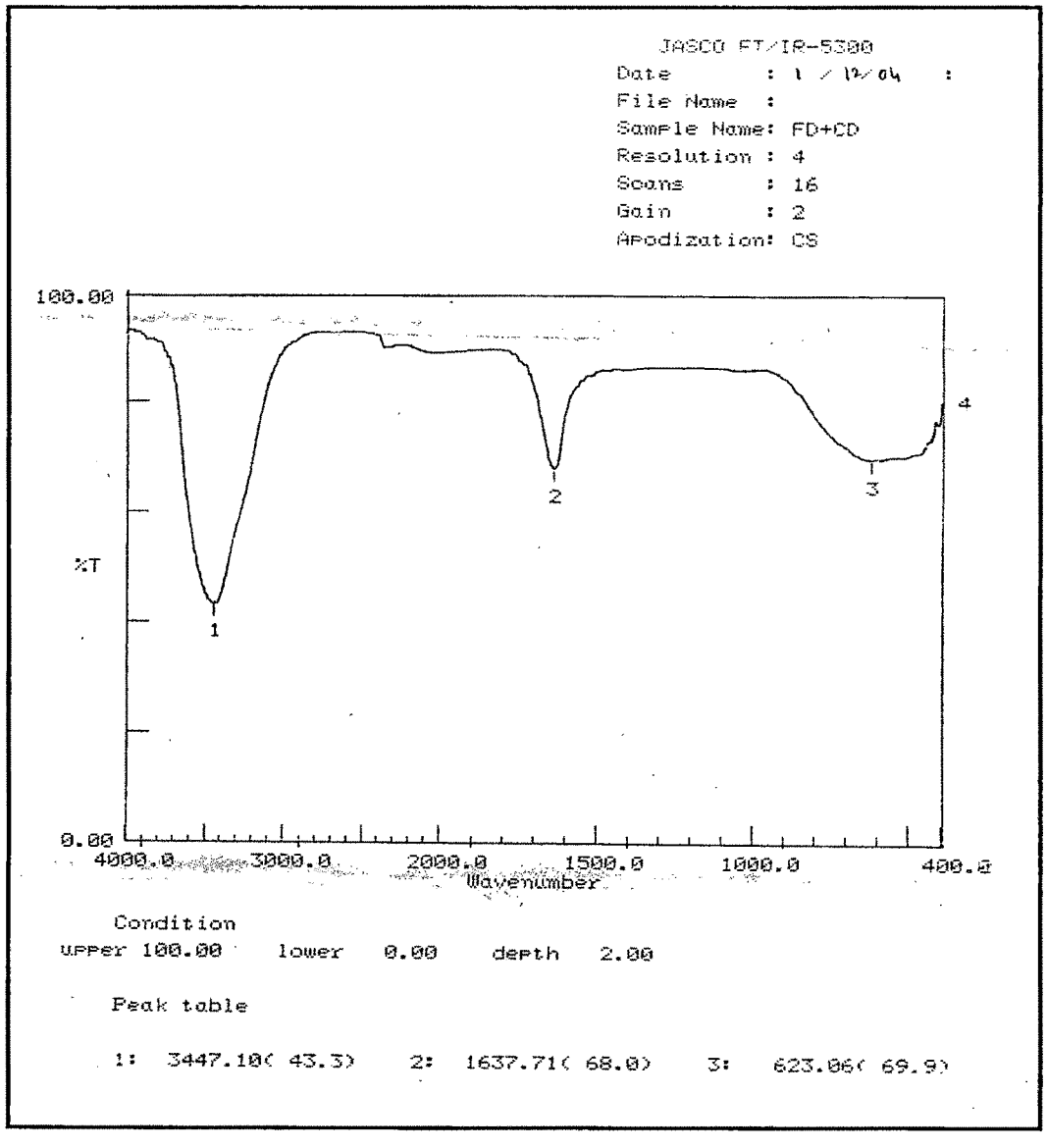


Figure 5.41 NMR spectrum of fungal alpha amylase

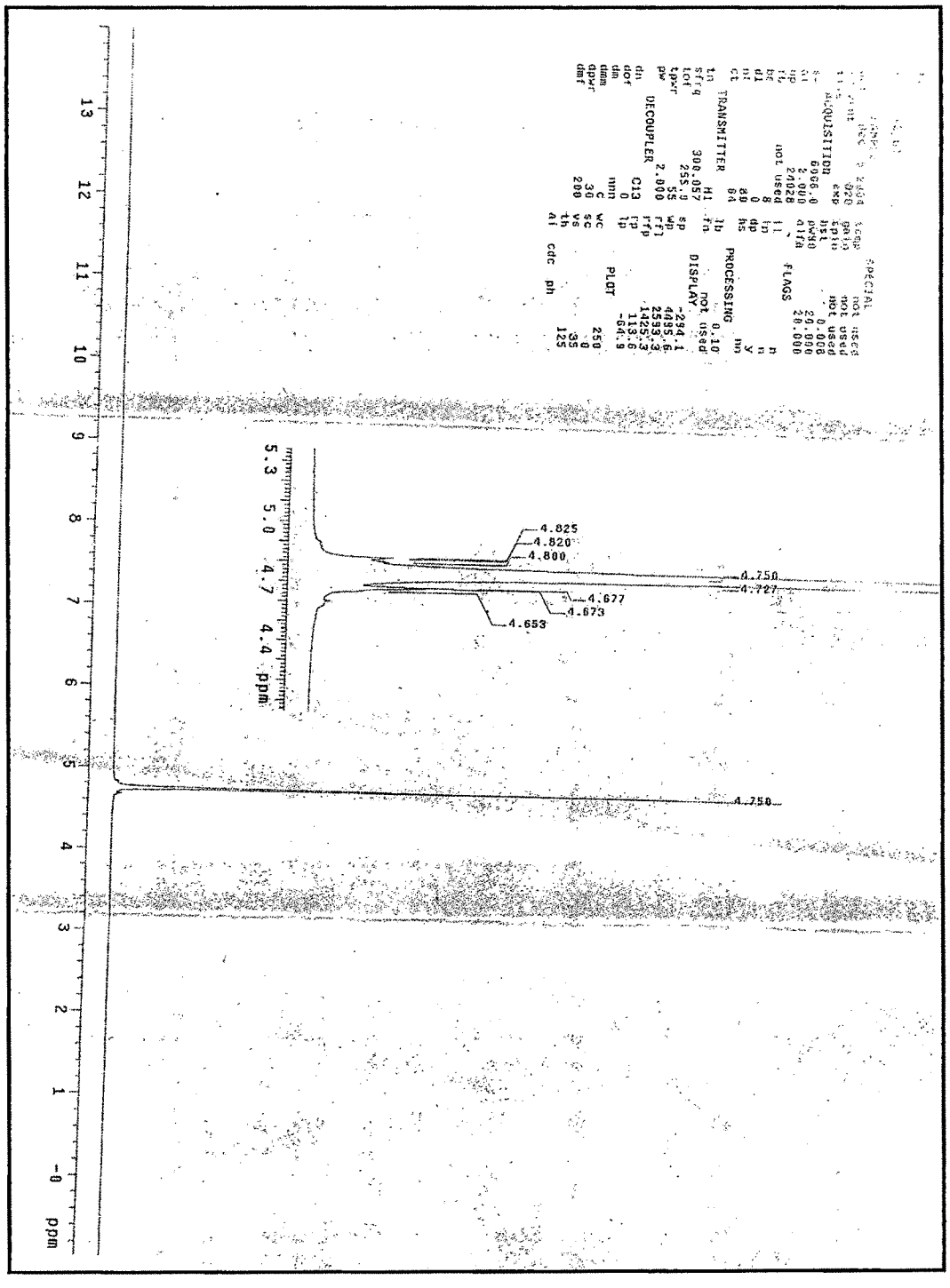


Figure 5.42 NMR spectrum of inclusion complex of fungal alpha amylase with beta-cyclodextrin

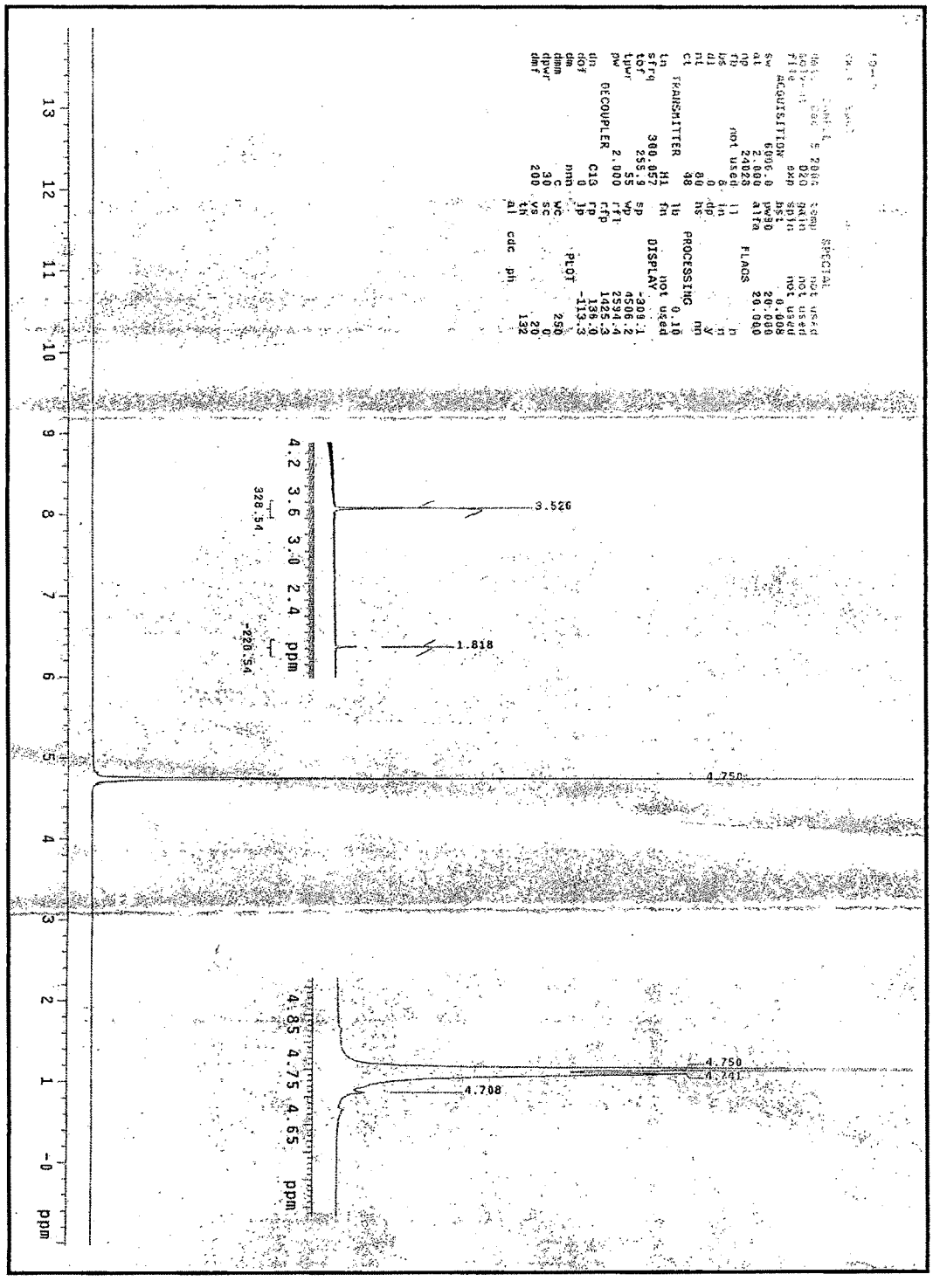


Figure 5.43 DSC thermograph of fungal alpha amylase

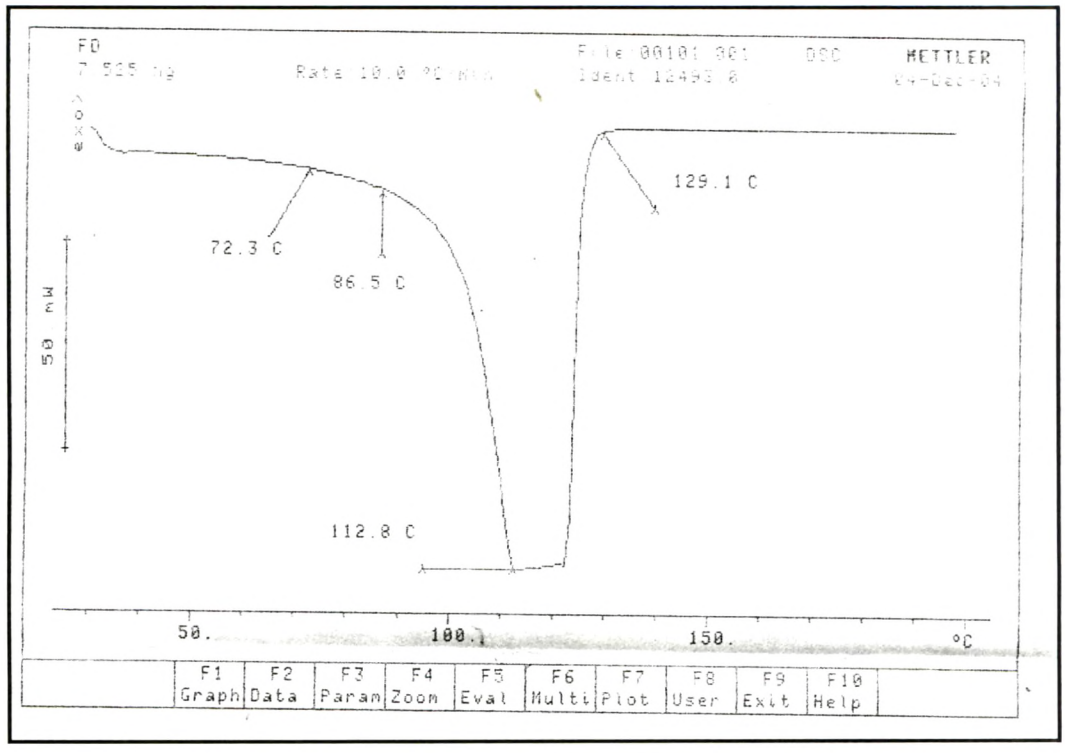
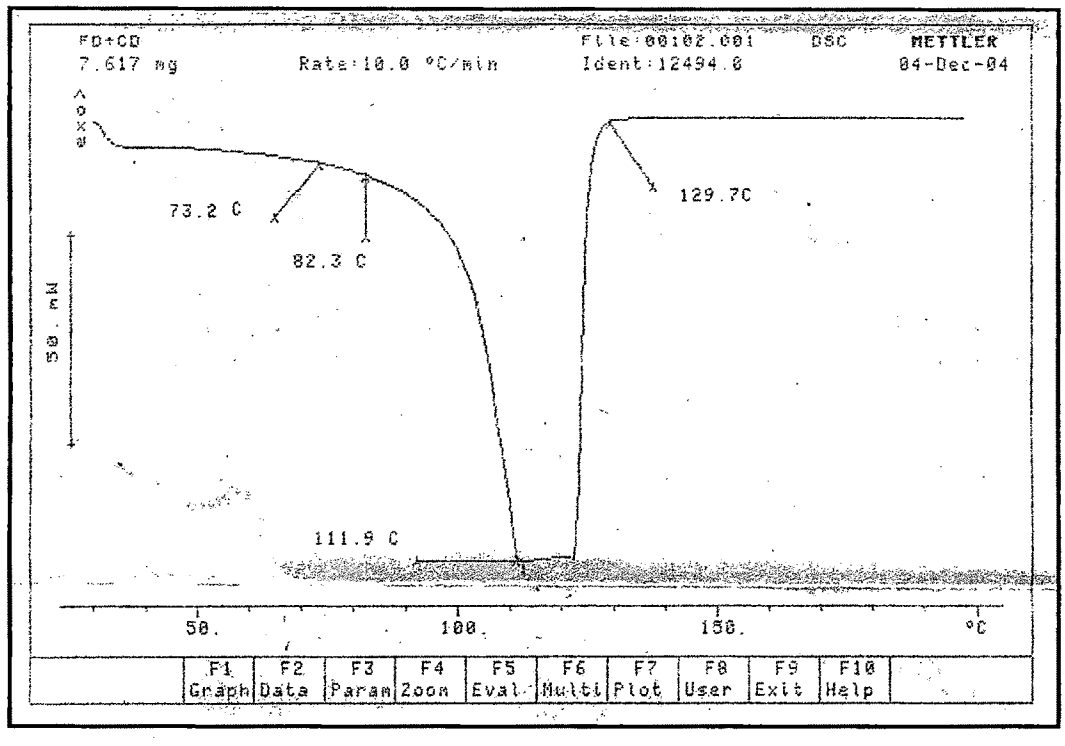


Figure 5.44 DSC thermograph of inclusion complex of fungal alpha amylase with beta-cyclodextrin



5.3.3.1b Oral liquid formulation

Characterization and stability data of oral liquid formulation batches IC-L-1 to Batch IC-L-4 prepared with inclusion complex are given in Table 5.69 and 5.70. Batch IC-L-1 and IC-L-2 were prepared with sugar syrup at pH 7.0 and 8.5 respectively. Batch IC-L-1 was found to be more unstable than Batch IC-L-2. Batch IC-L-1 at 45°C, 32.32 % of fungal alpha amylase was remained in 3rd month and Batch IC-L-2 shown 73.75 % of fungal alpha amylase remained. It seems Batch IC-L-3 and IC-L-4 was prepared with sorbitol base at pH 7.0 and 8.5 respectively. Batch IC-L-3 was found to be more unstable and at 45°C, 59.62 % of fungal alpha amylase was remained in 3rd month. Batch IC-L-4 was found to be most stable and from Figure 5.45, expected shelf life was estimated to be up to 26 months. The batch IC-L-4 may be stable due to final pH 8.5 where inclusion complex is stable.

Table 5.69 Stability cum characterization of oral liquid prepared with inclusion complex of fungal alpha amylase with beta-cyclodextrin Batch IC-L-1 and IC-L-2

Condition	Parameter	Initial	1st Month	2nd Month	3rd Month
Batch IC-L-1					
45°C	Description	A	A	A	A
	pH	7.002	6.967	6.936	6.901
	Sp.Gravity	1.225	1.226	1.228	1.229
	% Assay FAA	176.32	125.80	72.51	32.32
	% Assay PEP	148.97	135.25	118.97	103.88
30°C	Description	A	A	A	A
	pH	7.002	6.997	6.992	6.988
	Sp.Gravity	1.225	1.225	1.226	1.226
	% Assay FAA	176.32	170.16	163.88	157.85
	% Assay PEP	148.97	146.92	144.88	142.76
Refrigerated Condition [2°-8°C]	Description	A	A	A	A
	pH	7.002	7.002	7.000	6.999
	Sp.Gravity	1.225	1.225	1.224	1.225
	% Assay FAA	176.32	173.23	170.16	167.10
	% Assay PEP	148.97	147.68	146.37	144.85
Batch IC-L-2					
45°C	Description	A	A	A	A
	pH	8.502	8.470	8.439	8.402
	Sp.Gravity	1.228	1.229	1.230	1.231
	% Assay FAA	177.33	142.74	108.25	73.75
	% Assay PEP	149.10	134.06	118.28	102.78
30°C	Description	A	A	A	A
	pH	8.502	8.498	8.491	8.489
	Sp.Gravity	1.228	1.228	1.229	1.229
	% Assay FAA	177.33	172.96	168.56	164.12
	% Assay PEP	149.10	147.09	145.23	143.17
Refrigerated Condition [2°-8°C]	Description	A	A	A	A
	pH	8.502	8.500	8.500	8.499
	Sp.Gravity	1.228	1.228	1.228	1.227
	% Assay FAA	177.33	175.10	172.86	170.69
	% Assay PEP	149.10	148.41	147.25	145.87

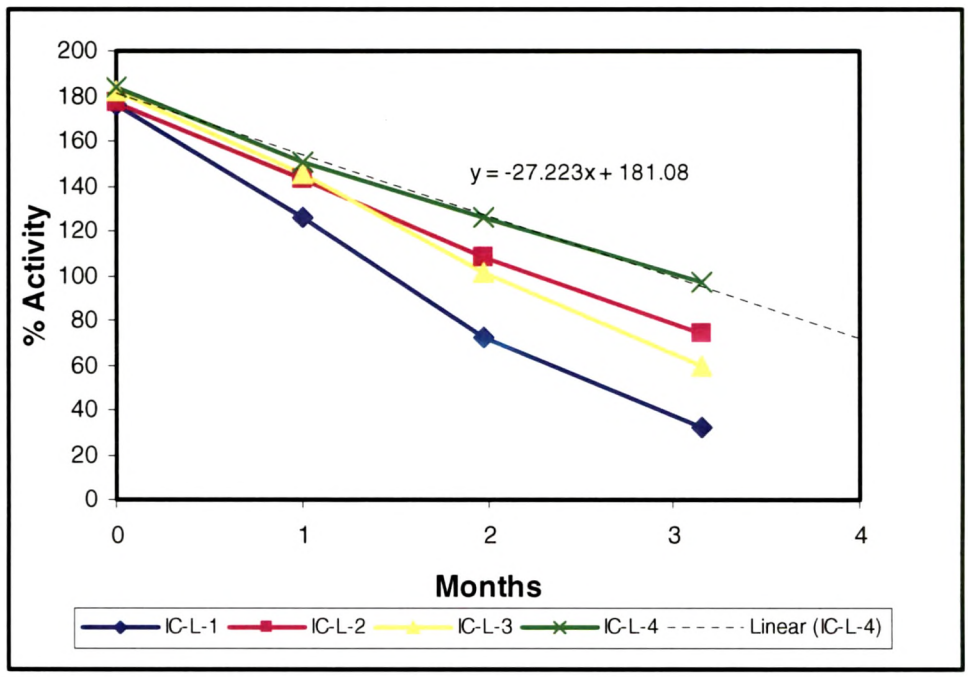
A- Clear light yellow syrupy liquid with cardamom flavour, FAA- Fungal alpha amylase, PEP- Pepsin

Table 5.70 Stability cum characterization of oral liquid prepared with inclusion complex of fungal alpha amylase with beta-cyclodextrin Batch IC-L-3 and IC-L-4

Condition	Parameter	Initial	1st Month	2nd Month	3rd Month
Batch IC-L-3					
45°C	Description	A	A	A	A
	pH	6.998	6.968	6.936	6.897
	Sp.Gravity	1.210	1.211	1.213	1.214
	% Assay FAA	182.36	145.20	101.15	59.62
	% Assay PEP	149.13	133.58	117.69	102.37
30°C	Description	A	A	A	A
	pH	6.998	6.992	6.989	6.985
	Sp.Gravity	1.210	1.210	1.211	1.213
	% Assay FAA	182.36	177.25	172.20	167.03
	% Assay PEP	149.13	147.10	145.00	142.74
Refrigerated Condition [2°-8°C]	Description	A	A	A	A
	pH	6.998	6.997	6.996	6.996
	Sp.Gravity	1.210	1.210	1.210	1.218
	% Assay FAA	182.36	179.53	177.22	174.08
	% Assay PEP	149.13	148.25	147.36	145.98
Batch IC-L-4					
45°C	Description	A	A	A	A
	pH	8.502	8.489	8.467	8.438
	Sp.Gravity	1.218	1.219	1.220	1.220
	% Assay FAA	183.50	151.13	125.96	97.08
	% Assay PEP	148.96	133.54	116.34	102.41
30°C	Description	A	A	A	A
	pH	8.502	8.499	8.492	8.489
	Sp.Gravity	1.218	1.218	1.219	1.220
	% Assay FAA	183.50	179.82	176.31	172.68
	% Assay PEP	148.96	146.58	142.47	140.23
Refrigerated Condition [2°-8°C]	Description	A	A	A	A
	pH	8.502	8.501	8.499	8.499
	Sp.Gravity	1.218	1.218	1.217	1.218
	% Assay FAA	183.50	181.20	179.55	177.85
	% Assay PEP	148.96	147.85	146.74	144.98

A- Clear light yellow syrupy liquid with cardamom flavour, FAA- Fungal alpha amylase, PEP- Pepsin

Figure 5.45 Stability data of oral liquid prepared with inclusion complex of fungal alpha amylase with beta-cyclodextrin of Batch IC-L-1 to IC-L-4 at 45°C



5.3.3.2 COMPUTER AIDED MOLECULAR MODELING

5.3.3.2a Docking results

The starting structure of fungal alpha amylase for simulations was taken from the 2.10Å resolution refined x-ray crystal structure from Brookhaven Protein Data Bank (PDB code 6taa) which is shown in Figure 5.46. Structure of beta-cyclodextrin is shown in Figure 5.47 which was taken from Brookhaven Protein Data Bank (PDB code GLC).

Using Catalytic Site Atlas (CSA) version 2.0.10 entry for 6taa, binding pocket residues His 122, Arg 204, Asp 206, Glu 230, His 296 & Asp 297 were defined as binding site subset. This binding subset is shown in Figure 5.48. These residues were confirmed by castP pocket information for 6taa molecule.

Fifty energy minimized docked structures were obtained by Monte Carlo search from Insight II software and the energy values of top 50 structures were ranging from -324.8 Kcal to -395.5 Kcal/mol. These structures were subjected to Molecular –dynamics simulated annealing from 500 K initial temperature to 300 K final temperature. The energy values of top 50 energy minimized structures were ranging from -323.8 Kcal/mol to - 412.3 Kcal/mol.

Four final structure's RMS deviations (RMSD) from fungal alpha amylase X-ray crystal structure were given in Table 5.71.

Figure 5.46 Structure of fungal alpha amylase taken from Brookhaven Protein Data Bank and structure was made by using Raswin Program

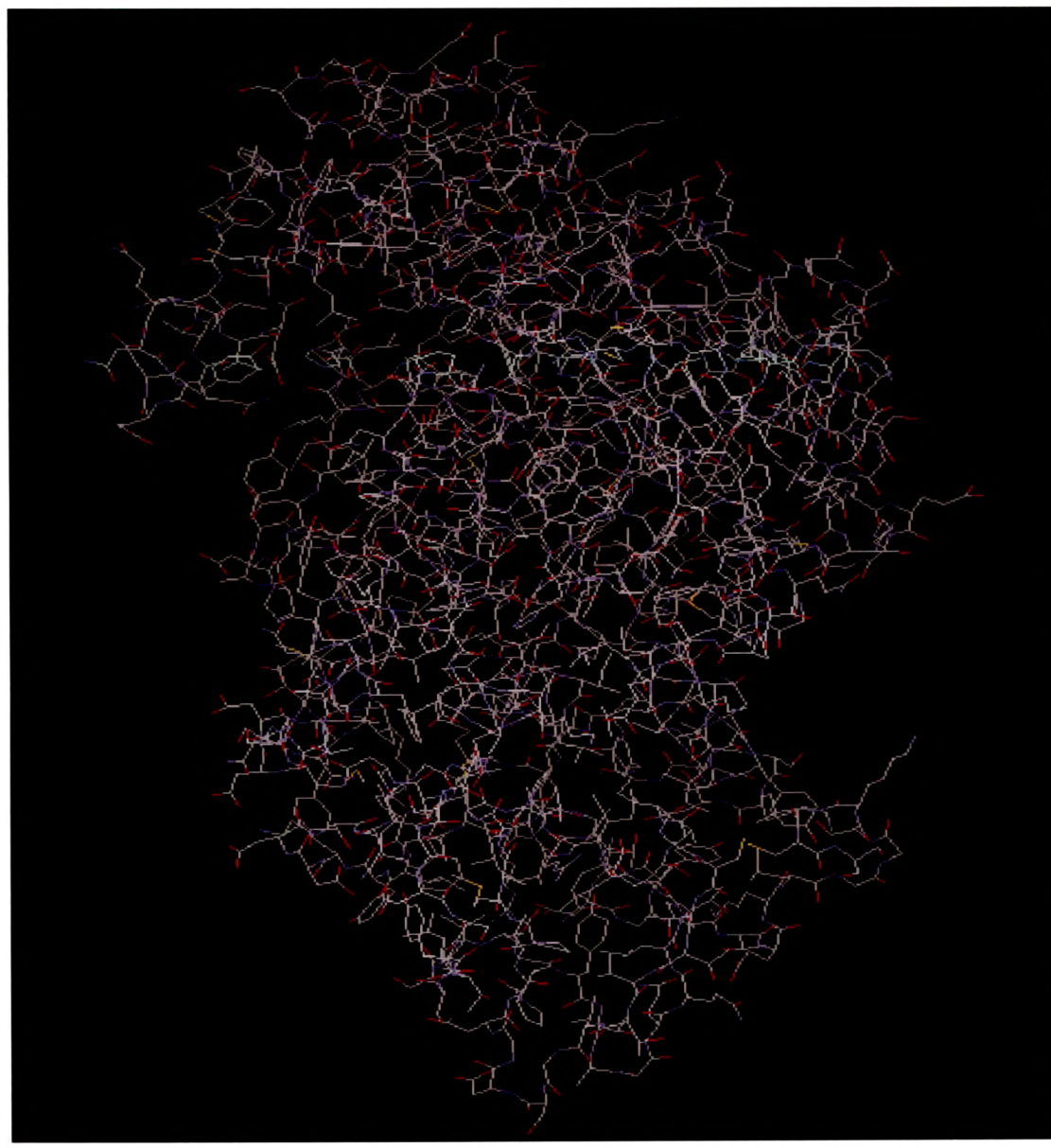


Figure 5.47 Structure of beta-cyclodextrin taken from Brookhaven Protein Data Bank and structure was made by using VMD Program

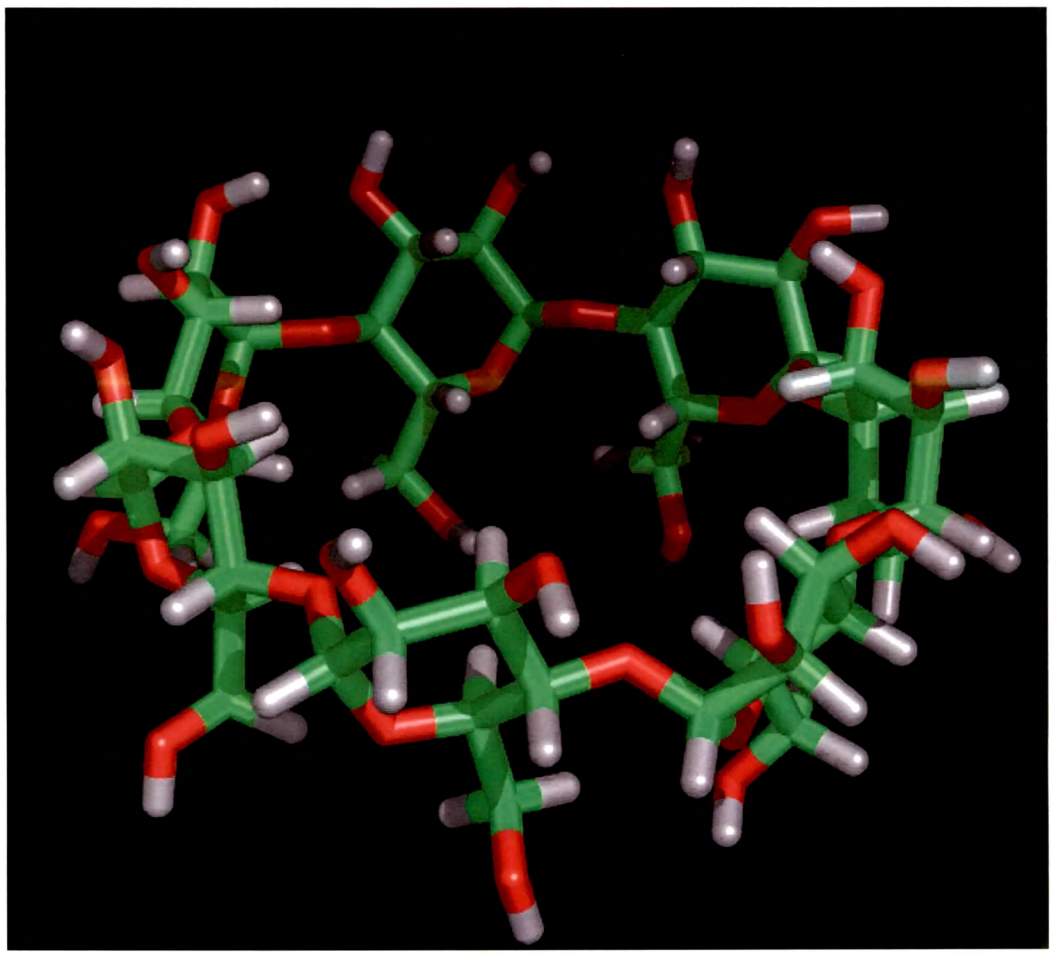


Figure 5.48 Binding pocket residues selected for docking are shown in Figure. Figure shows selected residues Arg 204, Glu 230, His 296 and Asp 297 from active site of fungal alpha amylase in magenta stick representation and fungal alpha amylase in orange space fill representation. Here remainder selected binding pocket residue His 122, Asp 206 are inside the pocket. The figure was made using Insight II program.

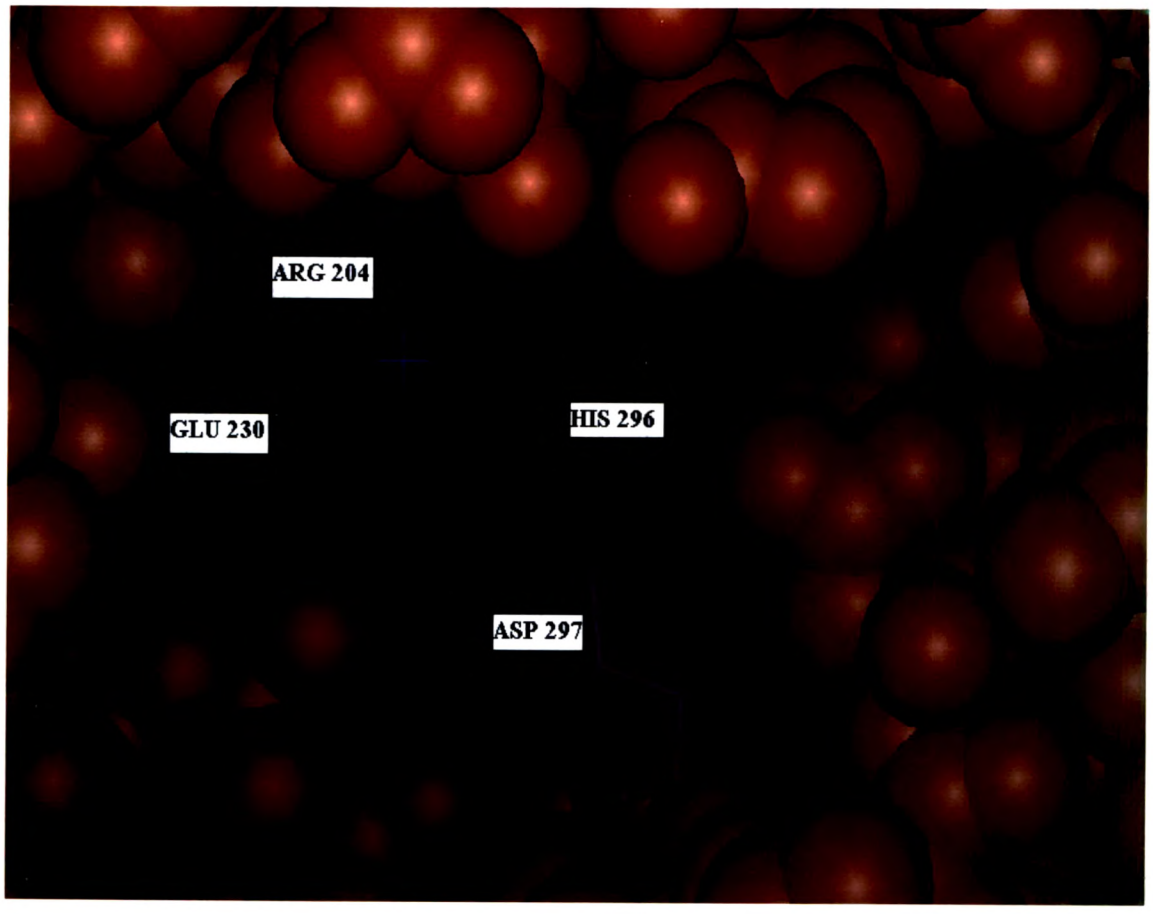


Table 5.71 RMS deviation data for lowest energy minimized structures

Structure	Energy	Heavy atom RMSD	Backbone RMSD
1	-412.311	0.078656	0.021613
2	-399.755	0.097920	0.022815
3	-398.121	0.078676	0.022083
4	-397.882	0.084554	0.023017

MOLMOL version 2K.2 software was used for calculating the Ramachandran plot values for structures of fungal alpha amylase and final docked inclusion complex. The number of residues in allowed region is found to be same for both structures 94.15 %. Ramachandran plot are given in Figure 5.49 and 5.50.

Ligand Protein Contacts (LPC) analyses were performed on lowest energy structure no.1 using LPC Software and data given in Table 5.72 & 5.73.

Hydrogen bond formed by beta-cyclodextrin with residues of fungal alpha amylase is shown in Figure 5.51.

The values of the accessible surface area for both fungal alpha amylase (6taa) and beta-cyclodextrin bound fungal alpha amylase were calculated using MOLMOL version 2K.2 software. The solvent accessible surface area for fungal alpha amylase is 17069.9 $A^{\circ 2}$ and for the complex is 16694.6 $A^{\circ 2}$. The plots of total solvent accessible surface area are shown in Figure 5.52 and 5.53.

Figure 5.49 Ramachandran plot for fungal alpha amylase

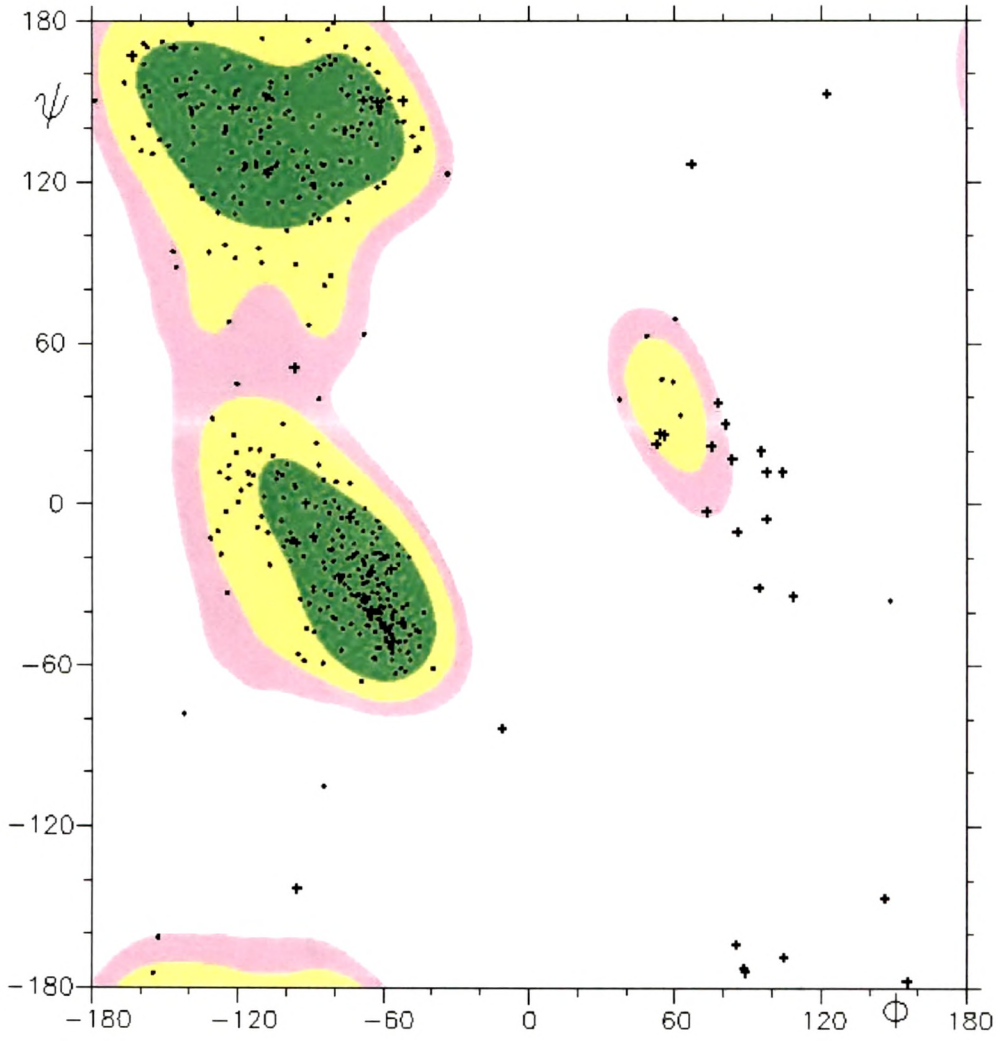


Figure 5.50 Ramachandran plot for inclusion complex of fungal alpha amylase with beta-cyclodextrin

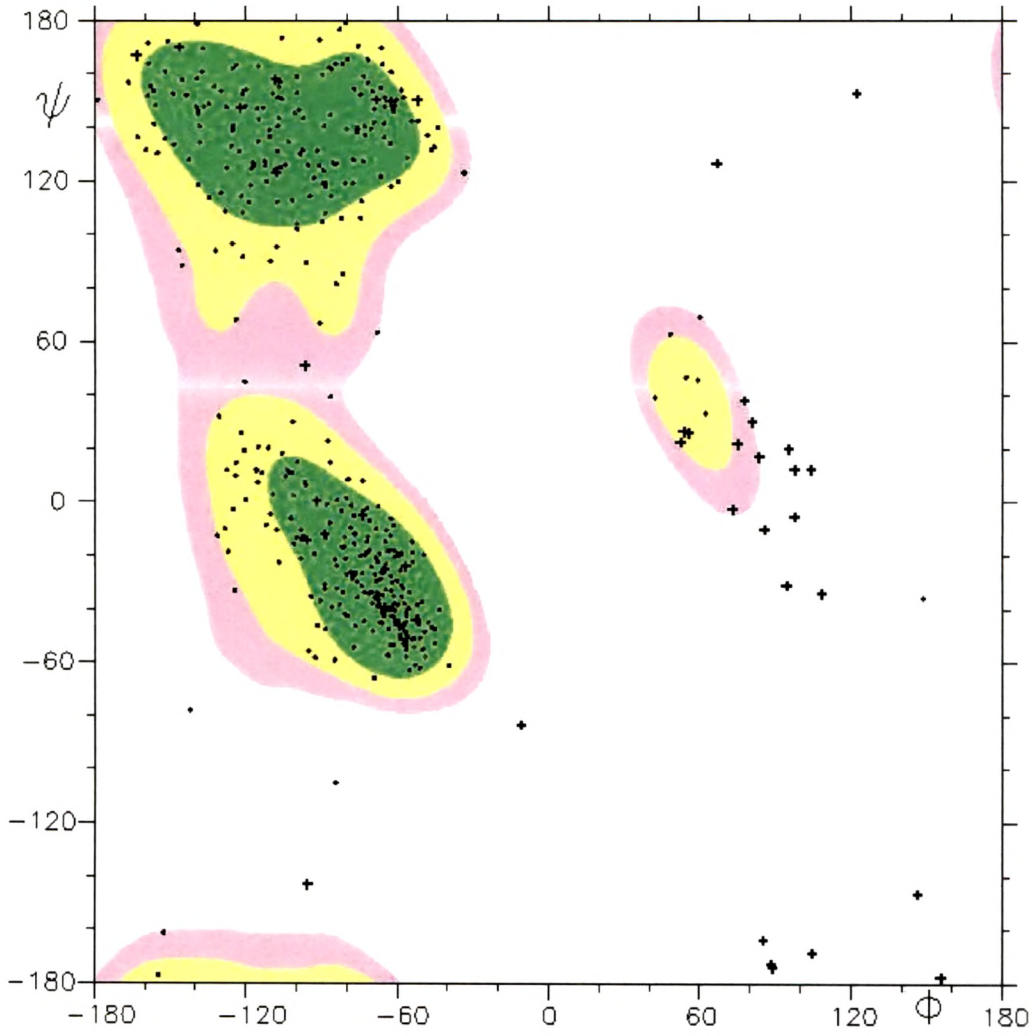


Table 5.72 Residues of fungal alpha amylase in contact with beta-cyclodextrin found out by Ligand Protein Contacts (LPC) analysis

				Specific contacts			
Residue		Dist	Surf	HB	Arom	Phob	DC
35	GLN*	3.3	29.2	+	-	-	-
75	TYR*	3.3	70.8	+	-	-	-
79	TYR*	5.5	1.7	+	-	-	-
80	HIS*	4.1	20.1	+	-	-	-
82	TYR*	4.2	23.2	-	-	-	+
83	TRP*	3.2	42.0	+	-	-	-
122	HIS*	4.9	3.8	-	-	-	-
155	TYR*	4.4	29.6	+	-	-	-
166	LEU*	3.6	26.5	-	-	-	+
167	GLY*	3.4	51.8	+	-	-	-
168	ASP*	4.8	5.2	+	-	-	-
171	VAL*	4.1	7.6	+	-	-	-
173	LEU*	3.9	15.6	-	-	-	+
206	ASP*	3.9	19.4	+	-	-	-
207	THR*	3.7	24.7	-	-	-	+
209	LYS*	3.5	32.3	-	-	-	-
210	HIS*	3.7	11.0	+	-	-	-
230	GLU*	4.6	6.2	+	-	-	-
232	LEU*	3.6	49.7	-	-	-	+
256	TYR*	4.7	9.4	+	-	-	-
296	HIS*	5.0	2.3	-	-	-	-
297	ASP*	3.3	54.3	+	-	-	+
298	ASN*	3.9	16.9	+	-	-	-
339	ASN*	3.3	48.3	+	-	-	+
340	ASP*	3.0	82.9	+	-	-	+
341	PRO*	3.8	2.7	-	-	-	+
344	ARG*	3.9	20.8	+	-	-	-

Legend:
Dist - nearest distance (Å) between atoms of the ligand and the residue
Surf - contact surface area (Å^2) between the ligand and the residue
HB - hydrophilic-hydrophilic contact (hydrogen bond)
Arom - aromatic-aromatic contact
Phob - hydrophobic-hydrophobic contact
DC - hydrophobic-hydrophilic contact
+/- - indicates presence/absence of a specific contacts
* - indicates residues contacting ligand by their side chain (including CA atoms)

Table 5.73 List of putative hydrogen bonds between beta-cyclodextrin and fungal alpha amylase given by Ligand Protein Contacts (LPC) analysis

Ligand atom			Protein atom					
N	Name	Class	Residue		Name	Class	Dist	Surf
43	O	I	ASP	340	O	II	3.0	11.6
43	O	I	ASP	340	OD2	II	3.3	3.5
44	O1	I	ASN	339	ND2	III	3.9	2.3
48	O5	I	GLN	35	NE2	III	3.3	11.3
48	O5	I	ASP	340	OD1	II	3.6	4.7
48	O5	I	TYR	75	O	II	4.6	2.3
48	O5	I	HIS	80	NE2	I	5.1	3.3
48	O5	I	ARG	344	NH1	III	5.2	0.5
48	O5	I	TYR	79	OH	I	5.5	1.2
49	O6	I	ASP	340	O	II	3.4	10.2
49	O6	I	ASP	340	OD1	II	3.4	4.7
49	O6	I	GLN	35	NE2	III	3.6	8.5
52	O9	I	TYR	75	OH	I	3.4	1.6
53	O10	I	ARG	344	NH2	III	4.5	7.3
53	O10	I	ASP	297	OD1	II	4.6	5.2
53	O10	I	HIS	80	ND1	I	4.9	0.2
54	O11	I	ASP	340	OD2	II	3.4	9.4
54	O11	I	ARG	344	NH1	III	3.9	12.6
54	O11	I	ARG	344	NH2	III	4.1	0.2
56	O13	II	TRP	83	NE1	III	3.2	6.9
57	O14	I	VAL	171	O	II	4.1	4.0
57	O14	I	ASP	168	N	III	4.8	0.2
58	O15	I	GLU	230	OE1	II	4.6	6.2
58	O15	I	ASP	297	OD2	II	4.8	1.0
59	O16	I	ASP	206	OD2	II	3.9	16.1
59	O16	I	ASP	206	OD1	II	4.3	3.3
59	O16	I	ASP	297	OD1	II	5.3	0.5
59	O16	I	ASP	297	OD2	II	5.3	0.3
61	O18	II	TYR	155	OH	I	5.1	0.3
62	O19	I	GLY	167	N	III	3.4	12.3
62	O19	I	TYR	155	OH	I	5.3	1.6
63	O20	I	ASP	297	O	II	3.3	19.1
63	O20	I	ASN	298	OD1	II	5.1	0.2
63	O20	I	ASN	339	ND2	III	5.6	0.3
64	O21	I	ASP	297	OD1	II	3.5	19.1
64	O21	I	ASP	297	OD2	II	3.8	0.3
67	O24	I	HIS	210	NE2	I	3.7	5.0
68	O25	I	ASN	298	OD1	II	5.1	1.9
68	O25	I	ASN	339	OD1	II	5.2	0.5
68	O25	I	ASN	339	ND2	III	5.6	1.6
69	O26	I	ASN	298	OD1	II	3.9	14.6
69	O26	I	ASP	297	O	II	4.6	0.2
69	O26	I	TYR	256	OH	I	4.7	8.5
73	O30	I	ASN	339	OD1	II	3.5	7.8
74	O31	I	ASN	339	OD1	II	3.3	11.8

Legend:
N - ligand atom number in PDB entry
Dist - distance (Å) between the ligand and protein atoms
Surf - contact surface area (Å^2) between the ligand and protein atoms

Figure 5.51 Fungal alpha amylase residues that were found to form hydrogen bonding with beta-cyclodextrin are shown in stick representation and beta-cyclodextrin shown in line representation using VMD program

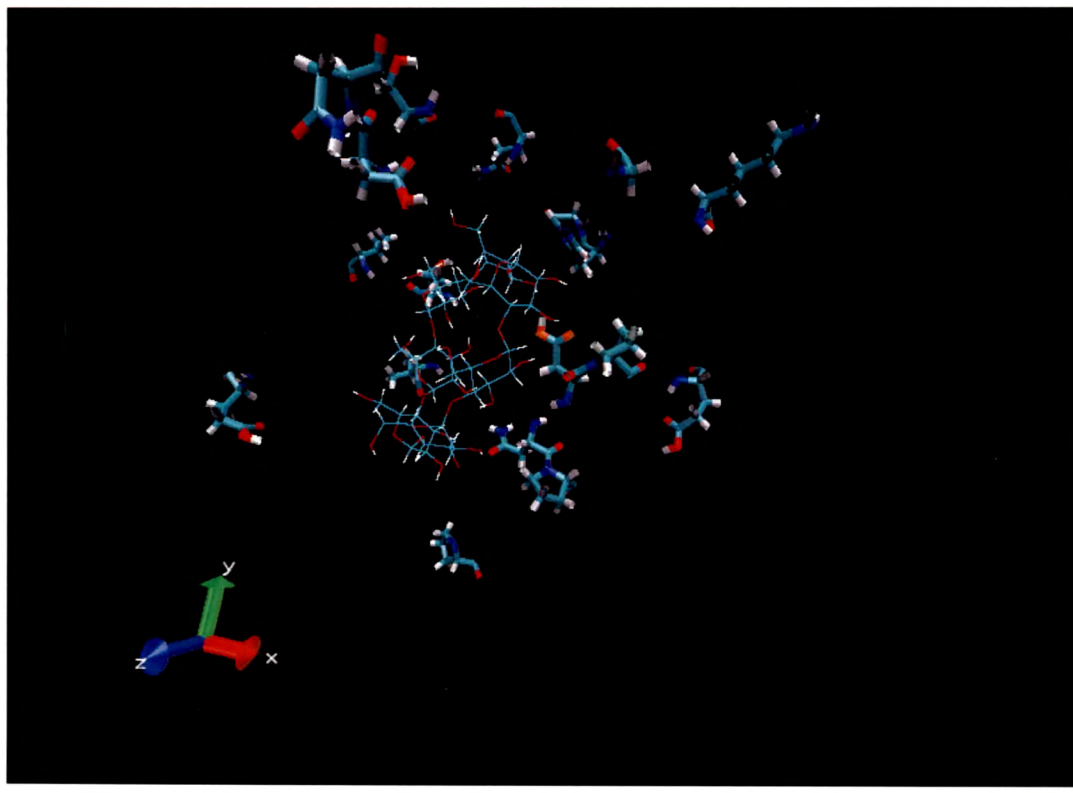


Figure 5.52 The plot of total solvent accessible surface area for fungal alpha amylase

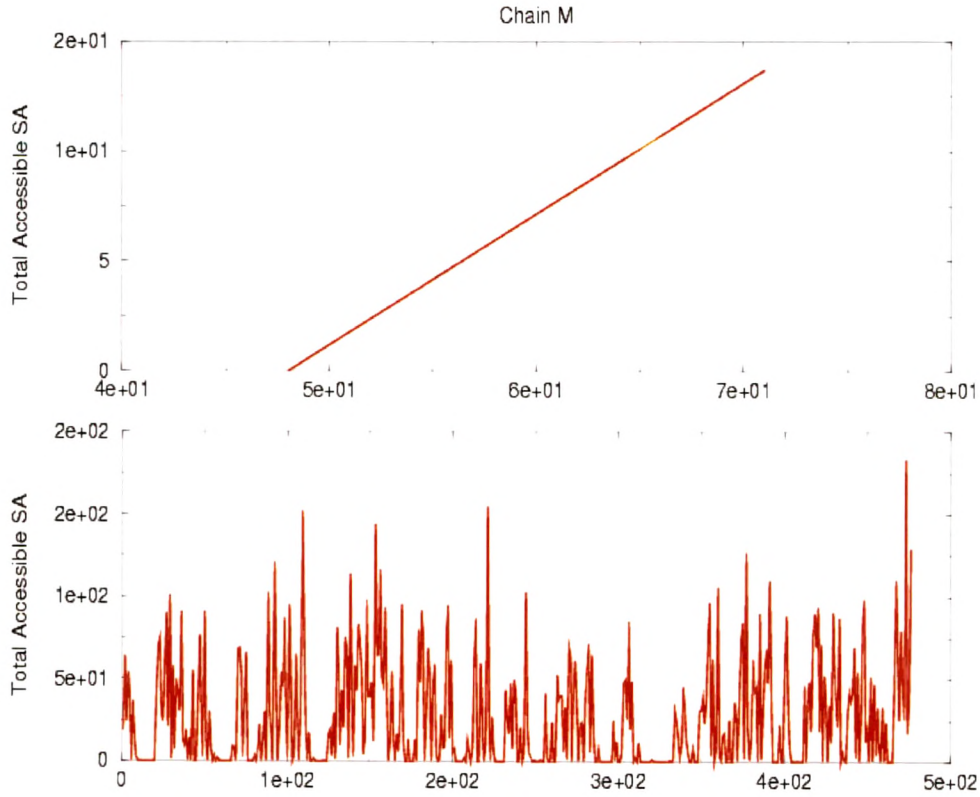
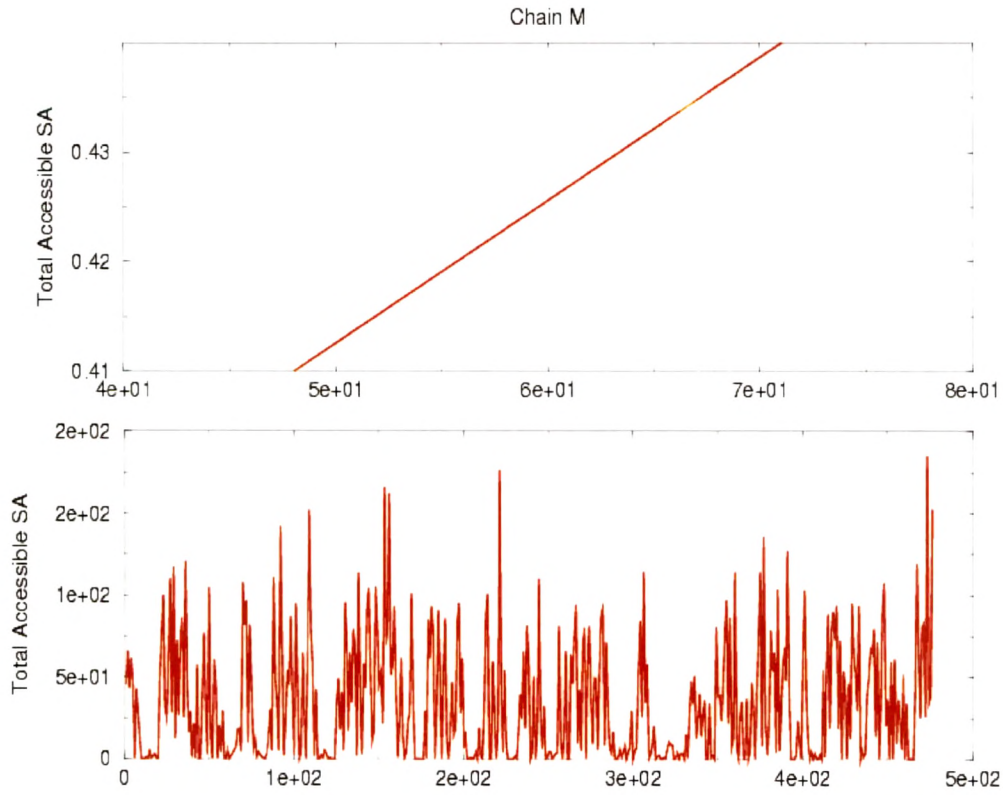


Figure 5.53 The plot of total solvent accessible surface area for beta-cyclodextrin- fungal alpha amylase inclusion complex is shown.



This docking work has been carried out for studying the interaction between beta-cyclodextrin and fungal alpha amylase. It has been demonstrated that the interaction between beta-cyclodextrin and fungal alpha amylase takes place at specific locations on the enzyme. If these findings can be generalized, they might explain the wide variety of effects of cyclodextrin on protein preparations that have been observed.

Enzymes assume many conformations. However, the native structure is the most energetically preferred. Any alteration to the native structure may lead to loss of an enzyme's activity, a phenomenon called denaturation. Since the native structure is maintained mostly by the weak forces of hydrogen bonding and electrostatic and hydrophobic interactions, enzymes can easily be denatured during purification, storage, transport and use. This novel method uses beta-cyclodextrin to renature or refolds a solution of partially denatured or inactive enzymes. The experimental results show the molecular ratio 3:1 gives maximum recovery yield up to 185 % over initial 100 % activity (Table 5.64). This study is supported by numerous reports of cyclodextrin inhibiting or slowing down the aggregation of proteins has appeared in the literature [48-50]. Cyclodextrin have also suggested acting as 'chaperone mimics' by enhancing protein refolding from denatured or even aggregated states [46, 51, 52].

After docking, top scoring docked conformations were found to be lowest energy structures with low RMS deviations (Table 5.71) from crystal structure of fungal alpha amylase which shows that the better performance of selected docking method. Ramachandran plot shows that values are in the allowed region which shows that quality of model is quite acceptable. Also Ramachandran plot (Figure 5.49 and 5.50) is found to be same for fungal alpha amylase and complex which suggest that there is not much of alterations in the beta-cyclodextrin bound fungal alpha amylase.

The changes in the accessibility of the residues in the fungal alpha amylase before and after binding with beta-cyclodextrin were calculated using MOLMOL version 2K.2 software. It can be seen that the accessible area

(Figure 5.52 and 5.53) remains almost same. This indicates that the structure was very stable during the whole simulation.

Ligand Protein Contacts (LPC) analyses of the lowest energy structure no.1 using LPC Software gives the residues in contact with beta-cyclodextrin (Table 5.72). The list of all hydrogen bonds and their distances and contact surface area that the beta-cyclodextrin makes in the binding site is given in Table 5.73. In addition, LPC analysis indicates that beta-cyclodextrin also form hydrophobic- hydrophilic contacts and does not form any aromatic-aromatic contacts and hydrophobic-hydrophobic contacts with fungal alpha amylase. Figure 5.54 shows the final docking results of beta-cyclodextrin. Different representations of final docked structure are given in Figure 5.55 to 5.58.

Figure 5.54 Solid surface diagram of the fungal alpha amylase pocket with final docking results of the beta-cyclodextrin (Pymol program used for structural presentation)

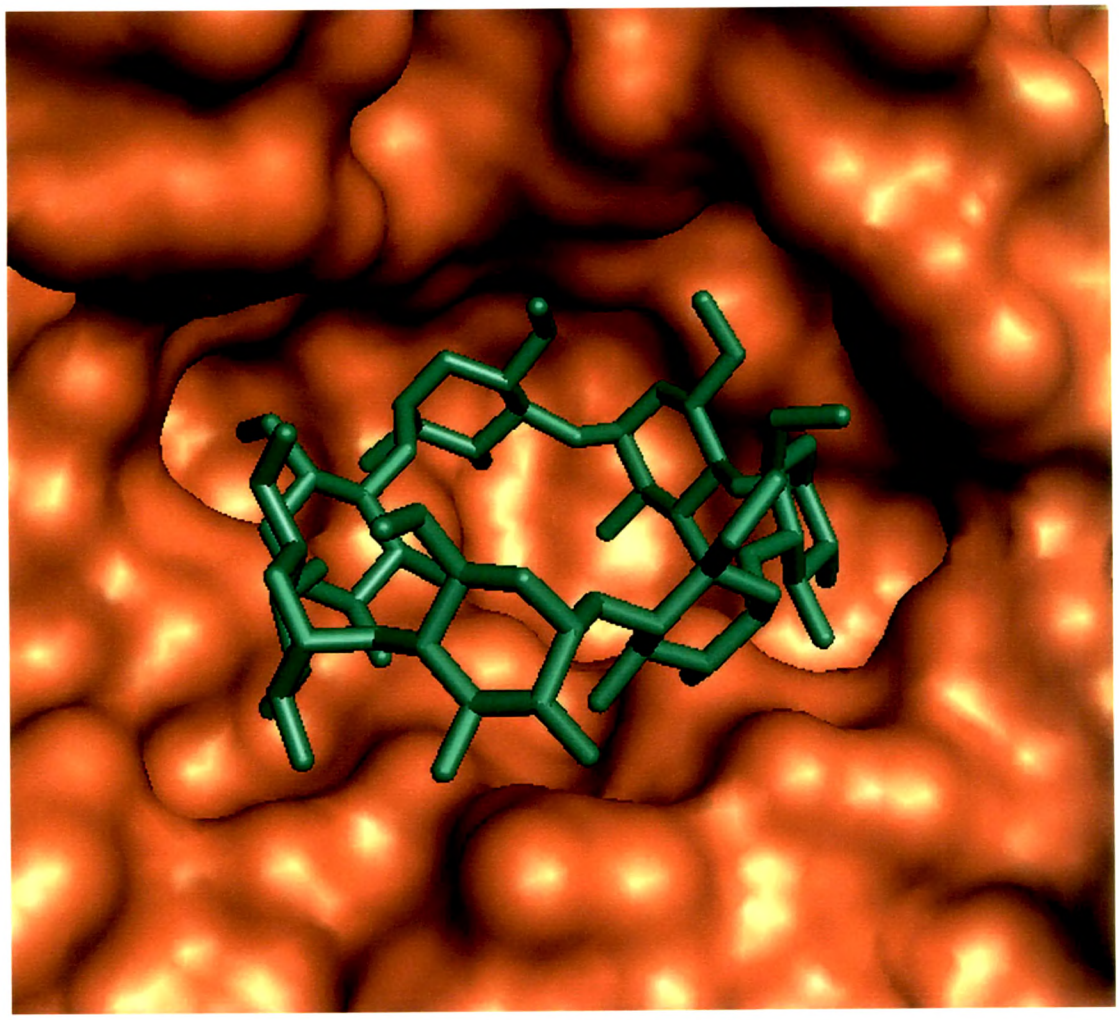
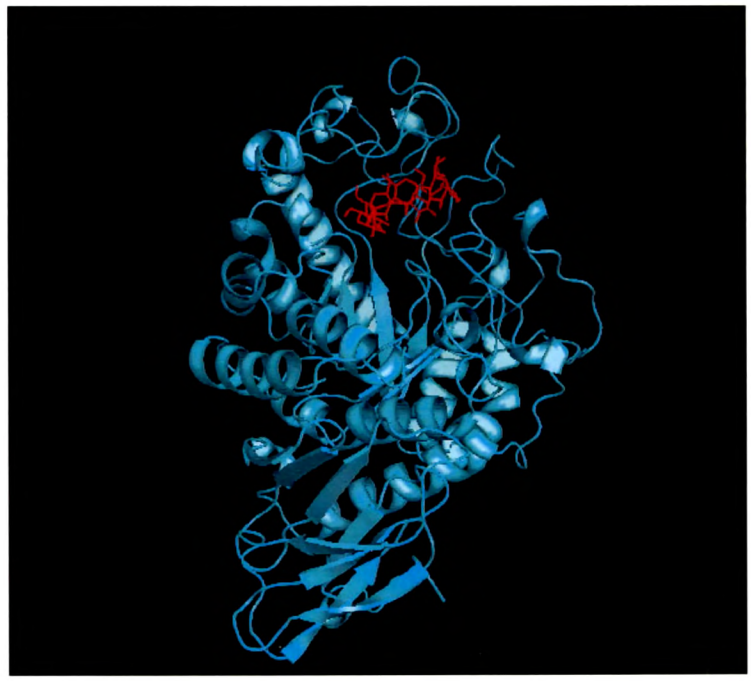
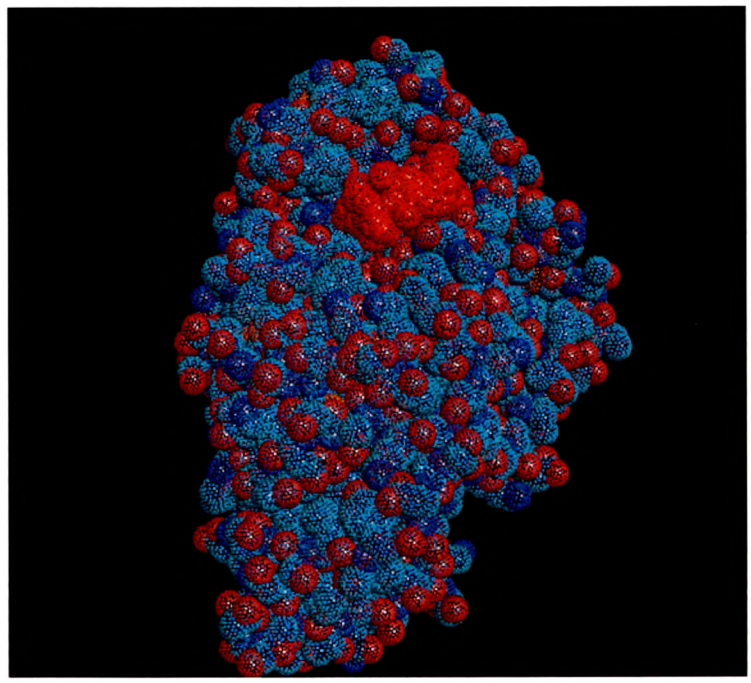


Figure 5.55 Final docked structure represented in form A) cartoon and B) dots using VMD program

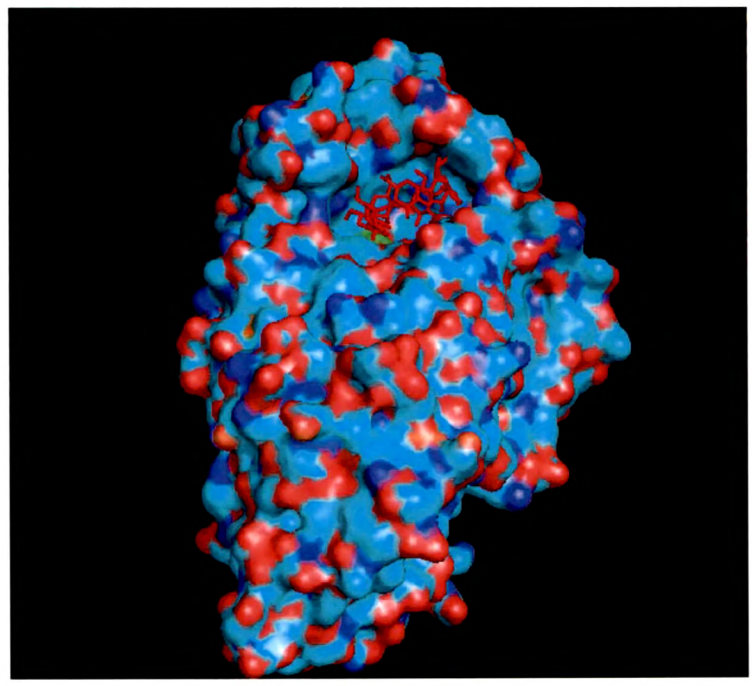


A

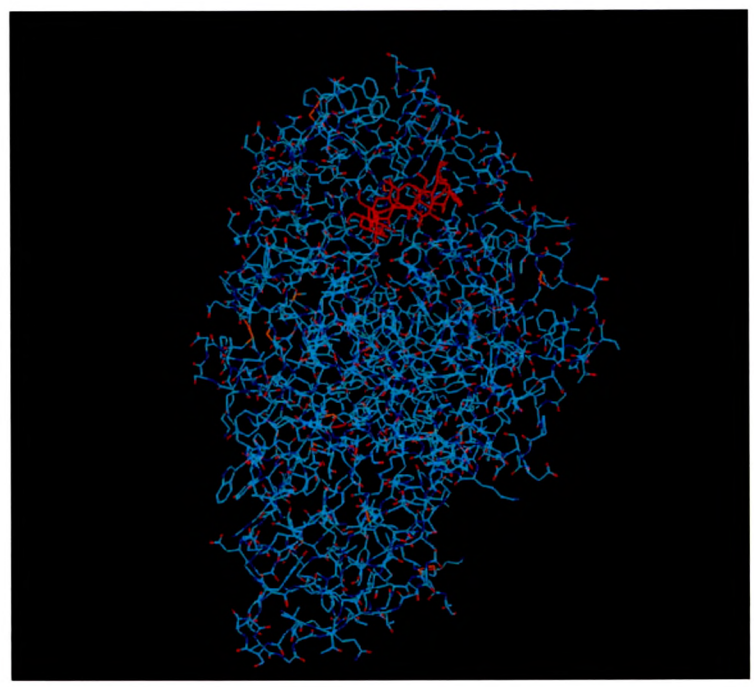


B

Figure 5.56 Final docked structure represented in form C) surface and D) line using VMD program

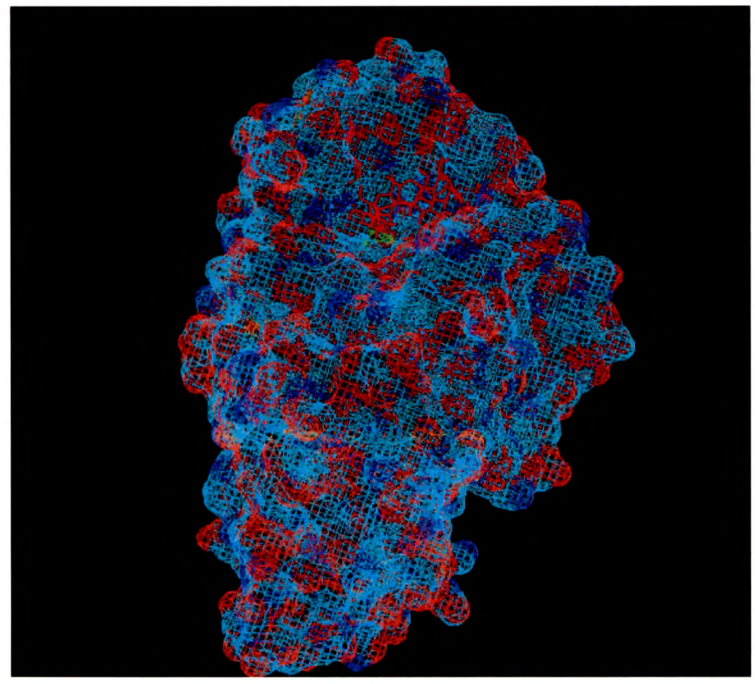


C

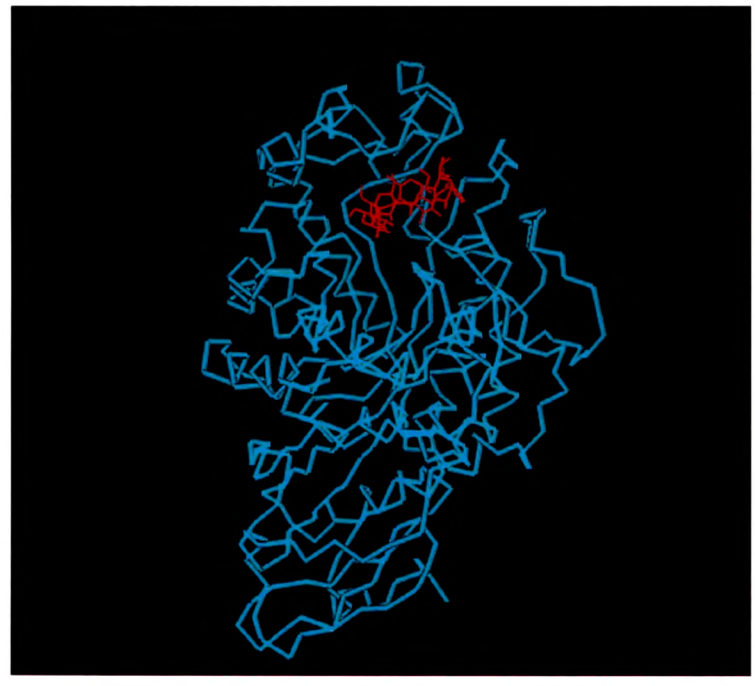


D

Figure 5.57 Final docked structure represented in form E) mesh and F) ribbon using VMD program

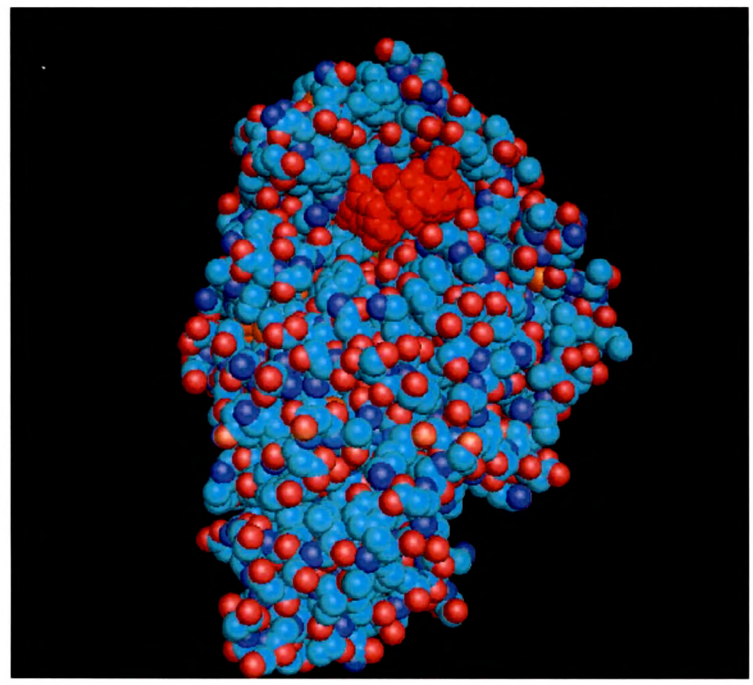


E

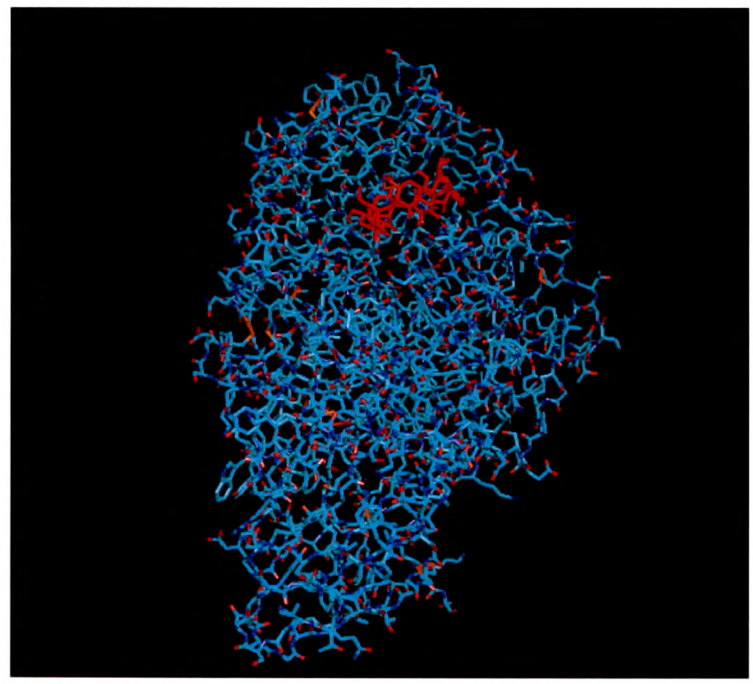


F

Figure 5.58 Final docked structure represented in form G) sphere and H) stick using VMD program



G



H

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