SECTION II: MATERIALS & METHODS

6. ALGINATE BEADS

6.1. Alginate-Amylase Beads

6.1.1. MATERIALS

Potassium dihydrogen phosphate, sodium hydroxide, hydrochloric acid (Qualigens Fine Chemicals, Mumbai, India) and soluble starch (Himedia Laboratories Pvt. Ltd., Mumbai, India) were used as received. Fungal α-amylase, sodium alginate, calcium chloride dihydrate, iodine, and potassium iodide were purchased from S. D. Fine-Chem Ltd., Mumbai, India. All the other chemicals and solvents were of analytical grade and were used without further purification. Deionized double-distilled water was used through out the study.

6.1.2. PREPARATION OF BEADS

Concentrated sodium alginate solution in distilled water was prepared well before required. Required quantity of enzyme (200 mg α-amylase in 50 ml of final sodium alginate solution) was dissolved in small quantity of water and mixed with concentrated sodium alginate solution. Final concentration of sodium alginate was adjusted in the range of 1-2% w/v and was used after being degassed under a vacuum. The beads were prepared by dropping the sodium alginate solution (10 ml) containing α-amylase from the dropping device such as syringe with 26G×½" flat-tip hypodermic needle to a magnetically stirred calcium chloride solution (40 ml) at a rate of 5 ml/min and were

allowed to harden for specific time. Different levels (Table 6.1) of sodium alginate, calcium chloride and hardening time were selected. The beads were collected by decanting calcium chloride solution, washed with deionized water and dried to a constant weight in vacuum desiccator (Tarsons Products Pvt. Ltd., Kolkata, India) at room temperature for 36 hours.

Table 6.1: Process variables and their levels for Doehlert shell design.

Factors	Coded levels	Actual levels
A: Sodium Alginate Concentration (%w/v)	-1	1.0 % w/v
	-0.5	1.3 % w/v
	0	1.5 % w/v
	0.5	1.8 % w/v
	1	2.0 % w/v
B: Calcium Chloride Concentration (M)	-0.866	0.0567 M
	-0.577	0.0712 M
	-0.289	0.0856 M
	0	0.1000 M
	0.289	0.1145
	0.577	0.1289
	0.866	0.1433
C: Hardening Time (min)	-0.816	20.9 min
	0	25.0 min
	0.816	29.1 min

6.1.3. CHARACTERIZATION OF BEADS

6.1.3.1. Estimation of α-Amylase (Dextrinogenic Assay)

The iodine test of Smith and Roe (Smith and Roe, 1957; Hsiu et al., 1964) was modified as follows: Two ml of a 0.2% starch solution was added to 1.0 ml of enzyme diluted in 0.05 M phosphate buffer pH 6.8. The mixture was incubated for 3 minutes at 25° and then reaction was stopped with 1 ml of 1 N HCl. Finally, 20 ml of water and 0.5 ml of 0.01 N iodine solution prepared according to Rice (Rice, 1959) were added and the absorbance *A*

was recorded on a spectrophotometer (Shimadzu UV-1601, Japan) at 660 nm. The instrument was adjusted to zero reading with an iodine blank containing neither enzyme nor substrate. The dextrinogenic activity is expressed in arbitrary units as follows:

Eq. 6.1 $$D = [(A_B - A)/A_B] \cdot E$$

Where A_B is the absorbance of the starch-iodine complex in the absence of enzyme and E is the enzyme dilution. Best results were obtained when the enzyme solution was diluted in such a manner as to make the ratio $(A_B - A)/A_B$, approach 0.20-0.25.

6.1.3.2. Determination of Entrapment Efficiency

Entrapment efficiency was determined by dissolving the enzyme loaded beads in a magnetically stirred simulated intestinal fluid without enzyme (USP XXVI) for about 45 min. The resulting solution was centrifuged at 2500 rpm for 10 min (Remi Instruments Ltd, Mumbai, India) and supernatant was assayed (n=3) for enzyme content by dextrinogenic assay as above. Entrapment efficiency was calculated as:

Eq. 6.2 $$\text{Entrapment efficiency} = \frac{\text{Enzyme loaded}}{\text{Theoratical enzyme loading}} \times 100$$

6.1.3.3. Determination of T_{50} and T_{90}

Time required for 50 (T_{50}) and 90 (T_{90}) percent of enzyme release were used to evaluate the onset of action and duration of action respectively. For optimization purpose, dissolution study of all batches was carried out in 500 ml of simulated intestinal fluid without enzyme using the USP XXVI dissolution apparatus 2 (TDT-60T, Electrolab, Mumbai, India) at 37±0.5°C with paddle speed of 75 rpm. Accurately weighed samples (n=3) equivalent to about 40 mg of α-amylase were subjected to dissolution and aliquots

of 2 ml were assayed at 0, 5, 10, 15, 20, 30, 45, 60, 90 and 120 min. T_{50} and T_{90} were found by extrapolating the % enzyme released versus time plot.

6.1.3.4. Particle Size Measurements

Particle size is an important parameter for the formulation development. Optimized batch of the beads was filled in the capsules during which the particle size was the evolutionary parameter. Larger particles show higher weight variation during capsule filling, hence the experimental conditions results in smaller particles are preferable. Particle size was determined with the laser diffraction particle size analyzer (MAN 0244/ HYDRO 2000 SM, Malvern Instruments Ltd., UK) using isopropyl alcohol as a vehicle.

6.1.3.5. Angle of Repose Measurements

Angle of repose was measured for estimating flowability of the beads. If the angle exceeds 50°, the material will not flow satisfactorily while materials having values near the minimum, flow easily and well. The rougher and more irregular the surface of the particles, the higher will be the angle of repose. The angle also increases with decrease in particle size. Angle of repose was measured by passing beads through a funnel on the horizontal surface. The height (h) of the heap formed was measured with a cathetometer and the radius (r) of the cone base was also determined. The angle of repose (Φ) was calculated from:

Eq. 6.3

$$\tan \Phi = \frac{h}{r}$$

6.1.3.6. Effect of pH on Release Profile

To study the effect of pH on α-amylase release profile, *'in vitro'* dissolution study was carried out as before using 500 ml of different pH media (simulated gastric juce pH 1.2, phosphate buffer pH 4.0, neutralized phthalate buffer pH 5.4, simulated intestinal fluid without enzyme pH 6.8, phosphate buffer pH 7.4, and phosphate buffer pH 8.0) on the optimized batch. Accurately weighed samples (n=3) equivalent to about 40 mg of α-amylase were introduced to dissolution media and samples of 2 ml were collected at 0, 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0 hr. Samples were filtered through 0.4 μm whatman® membrane filter and assayed for enzyme content as before.

6.1.3.7. Fourier Transform Infra-red Spectroscopy (FTIR)

IR transmission spectra were obtained using a FTIR spectrophotometer (FTIR-8300, Shimadzu, Japan). A total of 2% (w/w) of sample, with respect to the potassium bromide (KBr) disc, was mixed with dry KBr (S. D. Fine Chem Ltd., Mumbai, India). The mixture was ground into a fine powder using an agate mortar before compressing into KBr disc under a hydraulic press at 10,000 psi. Each KBr disc was scanned at 4 mm/s at a resolution of 2 cm over a wavenumber region of 400–4500 cm^{-1}. The characteristic peaks were recorded.

6.1.3.8. Differential Scanning Calorimetry (DSC)

Differential scanning calorimetric analysis was used to characterize the thermal behaviour of the isolated substances, their physical mixture and empty and enzyme loaded beads. DSC thermograms were obtained using an automatic thermal analyzer system (DSC-60, Shimadzu, Japan). Temperature calibration was performed using indium as a standard. Samples were crimped in a standard aluminium pan and heated from 40–400°C at a heating rate of 10°C/min under constant purging of dry nitrogen at 30 ml/min. An empty pan, sealed in the same way as the sample, was used as a reference.

6.1.3.9. Scanning Electron Microscopy (SEM)

The purpose of SEM study was to obtain a topographical characterization of beads. The beads were mounted on brass stubs using carbon paste. SEM photographs were taken with scanning electron microscope (JSM-5610LV, Jeol Ltd., Japan) at the required magnification at room temperature. The working distance of 39 mm was maintained and acceleration voltage used was 15 kV, with the secondary electron image (SEI) as a detector.

6.1.4. CURVE FITTING

The release pattern was evaluated to check the goodness of fit to the zero-order release kinetics (Eq. 6.4), first-order release kinetics (Gibaldi and Feldman, 1967; Wagner, 1969) (Eq. 6.5), Higuchi's square root of time equation (Higuchi, 1961; 1963) (Eq. 6.6), Korsmeyer-Peppas' power law equation (Korsmeyer et al., 1983; Peppas, 1985;

Spiepmann and Peppas, 2001) (Eq. 6.7), and Hixson-Crowell's cube root of time equation (Hixson and Crowell, 1931) (Eq. 6.8). The goodness of fit was evaluated by r (correlation coefficient) values.

Eq. 6.4 $$Q_t = Q_0 + K_0 t$$

where Q_t is the amount of drug dissolved in time t, Q_0 is the initial amount of drug in the solution (most times, Q_0=0), K_0 is the zero order release constant and t is release time.

Eq. 6.5 $$Q_t = Q_0 e^{-K_1 t}$$

Where Q_t is the amount of drug dissolved in time t, Q_0 is the initial amount of drug in the solution, K_1 is the first order release constant and t is release time.

Eq. 6.6 $$Q_t = K_H \sqrt{t}$$

where Q_t is the amount of drug dissolved in time t, Q_H is the Higuchi dissolution constant and t is release time.

Eq. 6.7 $$Q_t / Q_\infty = K_k t^n$$

where Q_t is the amount of drug dissolved in time t, Q_∞ is the amount of drug dissolved in ∞ time (the drug loaded in the formulation), Q_t / Q_∞ is the fractional release of the drug in time t, K_k is a constant incorporating structural and geometric characteristic of drug dosage form, n is the release (diffusional) exponent that depends on the release mechanism and the shape of the matrix tested (Ritger and Peppas, 1987) and t is release time. Interpretation of diffusional exponent is given in Table 6.2.

Table 6.2: Interpretation of Korsmeyer-Peppas power law release exponent.

Release exponent (n)	Drug transport mechanism	Rate as a function of time
0.5	Fickian diffusion	$t^{-0.5}$
0.5<n<1.0	Anomalous transport	t^{n-1}
1.0	Case-II transport	Zero order release
Higher than 1.0	Super Case-II transport	t^{n-1}

Eq. 6.8 $$Q_0^{1/3} - Q_t^{1/3} = K_s t$$

where Q_0 is the initial amount of drug in the pharmaceutical dosage form, Q_t is the remaining amount of drug in pharmaceutical dosage form at time t, K_s is a constant incorporating the surface-volume relation and t is release time.

In order to understand the release mechanism, the release data of the optimized batch was fitted to empirical equations proposed by Kopcha (Kopcha et al., 1991) (Eq. 6.9),

Eq. 6.9 $$M = At^{1/2} + Bt$$

In the above equations, M ($\leq$ 70%) is the percentage of drug released at time t, while A and B are, respectively, diffusion and erosion terms. According to this equation, if diffusion and erosion ratio, A/B=1, then the release mechanism includes both diffusion and erosion equally. If A/B>1, then diffusion prevails, while for A/B<1, erosion predominates (Ratsimbazafy et al., 1996).

6.1.5. FACTORIAL DESIGN

In order to attain the optimum response, three-level full factorial designs can be employed. However, these designs involve a very great number of runs ($N=3^f$, where f is the number of the independent variables) compared with the total quadratic coefficients to be determined, m. To alleviate this situation, central composite designs (CCD) can alternatively be used. The introduction of extra runs (star points and centre of design) to augment two-level factorial designs is to estimate the coefficients of the model equation in an efficient way. The number of runs to be made in both orthogonal or rotatable CCD are $N=2^f+2f+1$ (without replications), sensibly lower than three level factorial designs. For this reason, CCD are widely used in experimental optimization. However, a more efficient design was devised by Doehlert in 1970 (Doehlert, 1970), who proposed uniform

shell designs. These designs being more uniform than any other, show excellent interpolation features. The total number of runs without replication needed for applying Doehlert shell (DS) design is $N=f^2+f+1$, even lesser than in CCD. Thus DS design lead to the maximum efficiency (ratio between the number of coefficients to be evaluated, m, and the number of runs, N, in the design). For this reason, DS designs are highly applied in very different scientific frames. (Vojnovic et al., 1996; Martin et al., 1997; El Hajjaji et al., 1998; Geze et al., 1999; Gustavo Gonzalez and Gonzalez-Arjona, 1999; Voinovich et al., 2000) Before the application of the design, a number of preliminary trials were conducted to determine the conditions at which the process resulted to beads. In the present study the DS matrix contains 13 points (with f=3). To determine the experimental error, the experiment at the centre point was repeated three times at different days. The mean % entrapment, T_{50}, T_{90}, particle size and angle of repose at the center-replicated points were 82.85±0.50%, 15.61±0.15 min, 79.88±0.70 min, 261.46±3.64 μm and 20.56±0.14° respectively and showed good reproducibility of the process. The quadratic coefficients were estimated by using the least-squares multiple regression to the observed response. The analysis of variance (ANOVA) was performed in order to determine significance of the fitted equation. All analytical treatments were supported by NCSS software. (Hintze, 2003) The process variables with their coded experimental values are reported in Table 6.3, while three-dimensional representation of DS matrix is shown in Figure 6.1.

Table 6.3: Distribution of Doehlert shell design experiments in the space of three process variables.

Factors/ levels			
ES[a]	Na. alginate (% w/v)	Calcium chloride (M)	Hardening time (min)
11	0	0	0
9	1	0	0
8	0.5	0.866	0
2	-0.5	0.866	0
16	-1	0	0
7	-0.5	-0.866	0
14	0.5	-0.866	0
10	0.5	0.289	0.816
3	-0.5	0.289	0.816
13	0	-0.577	0.816
5	0.5	-0.289	-0.816
4	**-0.5**	**-0.289**	**-0.816**
15	0	0.577	-0.816
12	0	0	0
6	0	0	0
1	0	0	0

[a] ES, experimental sequence.

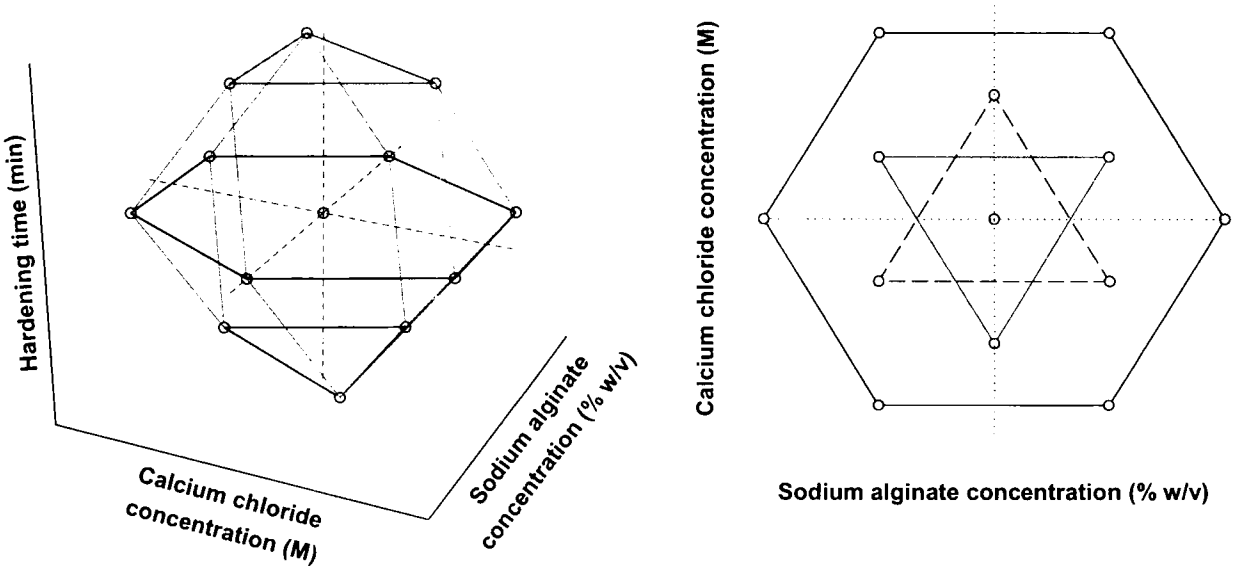

Figure 6.1: Doehlert matrix-design and distribution of the experimental points in the space of three variables. (A) three-dimensional and (B) two-dimensional view.

6.1.6. DESIRABILITY FUNCTION

Finally the desirability function (Derringer and Suich, 1980) was used for the optimization process. The application of the desirability function combines all the responses in one measurement, and gives the possibility to predict the optimum levels for the independent variables. The combination of the responses in one desirability function requires the calculation of the individual desirability. In this particular study % entrapment and T_{50} was selected for calculating the overall desirability. The individual desirability for each response was calculated using the following methods. (Lewis et al., 1999)

The % entrapment value was maximized in the optimization procedure, as the higher values of this parameter are desirable. The desirability function of this parameter was calculated by using the following equation:

Eq. 6.10

$$d_1 = \frac{Y_i - Y_{min}}{Y_{max} - Y_{min}}$$

where d_1 is the individual desirability of % entrapment and Y_i is the experimental result. The values of Y_{max} and Y_{min} for % entrapment were 88.34 and 70.99 respectively.

The T_{50} value was minimized in the optimization procedure, as lower values of this parameter give quicker and complete release of enzyme from the beads. The calculation of the desirability function was carried out using the equation:

Eq. 6.11

$$d_2 = \frac{Y_{max} - Y_i}{Y_{max} - Y_{min}}$$

where d_2 is the individual desirability of T_{50} and Y_i is the experimental result. The values of Y_{max} and Y_{min} were 19.24 and 9.01 min for T_{50} respectively.

The overall desirability values were calculated from the individual values by using the following equation (Eq. 6.12):

Eq. 6.12

$$D = (d_1 \times d_2)^{1/2} = \left[\prod_{i=1}^{2} d_i \right]^{1/2}$$

6.1.7. PREPARATION OF CAPSULE FORMULATION, PACKAGING, AND STABILITY STUDY

Accurately weighed alginate beads equivalent to 40 mg of α-amylase were filled into a hard gelatin capsule manually. The joint of the capsule body and cap was carefully sealed by pressing them to fit in the lock mechanism. The capsules were packaged in high density polyethylene (HDPE) bottles with polypropylene (PP) caps (foamed polyethylene and pressure sensitive liner). The capsules were subjected to stability testing according to the International Conference on Harmonization guidelines for zone III and IV. The packed containers of prepared capsules along with marketed formulation and bulk α-amylase were kept for accelerated (40±2°C/75±5% relative humidity) and long term (30±2°C/65±5% relative humidity) stability in desiccators with saturated salt solution for up to 12 months. A visual inspection (for discoloration of capsule content), dissolution testing and α-amylase content estimation was carried out every 15 days for the entire period of stability study.

6.2. Alginate-Papain Beads

6.2.1. MATERIALS

Purified papain, sodium alginate, calcium chloride dihydrate, dibasic sodium phosphate and citric acid were purchased from S. D. Fine-Chem Ltd., Mumbai, India. Hammersten type casein (Himedia Laboratories Pvt. Ltd., Mumbai, India) and trichloroacetic acid (Qualigens Fine Chemicals, Mumbai, India) were used as received. All the other chemicals and solvents were of analytical grade and were used without further purification. Deionized double-distilled water was used through out the study.

6.2.2. PREPARATION OF BEADS

Concentrated sodium alginate solution was prepared well before required by dissolving sodium alginate in distilled water. Required quantity of enzyme (200 mg papain in 50 ml of final sodium alginate solution) was dissolved in small quantity of water and mixed with concentrated sodium alginate solution. Final concentration of sodium alginate was adjusted in the range of 1-2% w/v and was used after being degassed under a vacuum. The beads were prepared by dropping of the sodium alginate solution (50 ml) containing enzyme from the dropping device such as syringe with 26G×½" flat-tip hypodermic needle to a magnetically stirred calcium chloride solution (200 ml) and were allowed to harden for specific time. Different levels (Table 6.4) of sodium alginate, calcium chloride and hardening time were selected. The beads were collected by decanting calcium chloride solution, washed with deionized water and dried in vacuum desiccator (Tarsons

Products Pvt. Ltd., Kolkata, India) for 36 hours. Above process was carried out at controlled room temperature (20°C).

Table 6.4: Factorial 3^3: factors and their levels.

Factors	Low level (-1)	Middle level (0)	High level (1)
A: Sodium alginate (% w/v)	1.0	1.5	2.0
B: Calcium chloride (M)	0.05	0.10	0.15
C: Hardening time (min)	20	25	30

6.2.3. EVALUATION OF BEADS

6.2.3.1. Determination of Entrapment Efficiency

Entrapment efficiency was the important evolutionary parameter for optimization of entrapment. Total amount of enzyme entrapped in beads was determined by dissolving the beads in magnetically stirred simulated intestinal fluid without enzyme for about 45 min. The solution was centrifuged at 2500 rpm for 10 min (Remi Instruments Ltd, Mumbai, India) and supernatant was assayed (n=3) for enzyme content by casein digestion method of USP XXVI. Entrapment efficiency was calculated as:

Eq. 6.13

$$\text{Entrapment efficiency} = \frac{\text{Enzyme loading}}{\text{Theoratical enzyme loading}} \times 100$$

6.2.3.2. Determination of pH Dependent Release Profile

'In vitro' dissolution studies were carried out using the USP XXVI dissolution apparatus 2 (TDT-60T, Electrolab, Mumbai, India) in 500 ml of different pH media ranging from 1.2 to 8.0 on one experimental batch at 37±0.5°C with paddle speed of 50 rpm.

Accurately weighed samples (n=3) equivalent to about 40 mg of papain were introduced to dissolution media and samples of 2 ml were collected at 0, 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0 hr. Samples were filtered through 0.4 μm membrane filter and assayed for enzyme content as before. Alginate beads showed pH dependant release profile and it was maximum in simulated intestinal fluid without enzyme.

6.2.3.3. Determination of T_{50} and T_{90}

Time required for 50 (T_{50}) and 90 (T_{90}) percent of enzyme release are important parameters for enzyme release study. For optimization purpose, dissolution study of all batches was carried out in 500 ml of simulated intestinal fluid without enzyme as before. Accurately weighed samples (n=3) equivalent to about 40 mg of papain were subjected to dissolution and aliquots of 2 ml were assayed at 0, 5, 10, 15, 20, 30, 45, 60, 90 and 120 min. T_{50} and T_{90} were found from % enzyme released versus time plot by drawing a projection line on the time axes at 50% and 90% release respectively.

6.2.3.4. Particle Size Measurements

Particle size was determined with the laser diffraction particle size analyzer (MAN 0244/ HYDRO 2000 SM, Malvern Instruments Ltd., UK) using isopropyl alcohol as a vehicle.

6.2.3.5. Angle of Repose Measurements

Angle of repose was measured by passing beads through a funnel on the horizontal surface. The height (h) of the heap formed was measured with a cathetometer and the radius (r) of the cone base was also determined. The angle of repose (Φ) was calculated from:

Eq. 6.14

$$\tan \Phi = \frac{h}{r}$$

6.2.3.6. Differential Scanning Calorimetry (DSC)

Differential scanning calorimetric analysis was used to characterize the thermal behaviour of the isolated substances, empty and loaded beads. DSC thermograms were obtained using an automatic thermal analyzer system (DSC-60, Shimadzu, Japan). Temperature calibrations were performed using indium as a standard. Samples were crimped in a standard aluminium pan and heated from 30–400°C at 10°C/min under constant purging of dry nitrogen at 30 ml/min. An empty pan, sealed in the same way as the sample, was used as a reference.

6.2.3.7. Scanning Electron Microscopy

The purpose of SEM study was to obtain a topographical characterization of beads. The beads were mounted on brass stubs using double-sided adhesive tape. SEM photographs were taken with scanning electron microscope (JSM-5610LV, Jeol Ltd., Japan) at the required magnification at room temperature. The working distance of 39 mm was

maintained and acceleration voltage used was 15 kV, with the secondary electron image (SEI) as a detector.

6.2.4. FACTORIAL DESIGN AND THE DESIRABILITY FUNCTION

In this study a 3^3 full factorial design was used to determine the effect of the concentration of sodium alginate (% w/v), the concentration of calcium chloride (M) and the hardening time (min). Before the application of the design a number of preliminary trials were conducted to determine the conditions at which the process resulted to beads. The factors and their levels are shown in Table 6.4.

The matrix of the experiments and the results of the responses are listed in Table 6.5. To determine the experimental error, the experiment at the centre point was replicated five times at different days. The mean % entrapment, T_{50}, T_{90}, particle size and angle of repose of these experiments were 85.88±1.02, 15.57±0.28, 82.43±0.33, 261.130±0.868 and 20.61±0.29 respectively. The above-mentioned values showed good reproducibility of the process. The statistical evaluation of the results was carried out by analysis of variance (ANOVA) using a commercially available statistical software package (DESIGN EXPERT V 6.0.10, Minneapolis, USA). The quadratic model was selected for this analysis. Finally the desirability function was used for the optimization process. The application of the desirability function combines all the responses in one measurement, and gives the possibility to predict the optimum levels for the independent variables. The combination of the responses in one desirability function requires the calculation of the individual desirability function. In this particular study there were not special requirements for the particle size of the optimum formulation, so the range of the values of the produced formulations was selected. The optimum formulation of this study should have a particle

size ranging between 169.737 and 715.268 μm, with maximum entrapment and minimum T_{50}, T_{90} and angle of repose. The individual desirability for each response was calculated using the following methods (Lewis et al., 1999).

Table 6.5: Factorial 3^3: matrix of the experiments and results for the measured responses and the desirability.

Factors/ levels				Responses					
ES[a]	Na. Alginate (% w/v)	Calcium chloride (M)	Hardening Time (min)	% Entrapment	T_{50}	T_{90}	Size (μm)	Angle of repose	Overall desirability
1	1.0	0.05	20	91.80	6.50	23.80	211.136	22.62	0.962
10	1.0	0.05	25	89.00	8.30	25.70	207.319	22.88	0.913
19	1.0	0.05	30	86.20	9.35	28.40	202.628	23.27	0.868
4	1.0	0.10	20	85.00	7.80	27.50	184.327	23.39	0.878
13	1.0	0.10	25	82.60	9.10	30.55	181.059	23.75	0.831
22	1.0	0.10	30	80.71	10.70	34.00	178.289	24.15	0.781
7	1.0	0.15	20	68.80	8.60	34.60	175.983	24.07	0.599
16	1.0	0.15	25	67.79	10.30	39.00	171.384	24.94	0.533
25	1.0	0.15	30	65.28	12.05	59.25	169.737	26.33	0.000
2	1.5	0.05	20	93.40	13.35	48.00	297.039	19.50	0.815
11	1.5	0.05	25	90.80	14.45	57.40	291.606	19.76	0.747
20	1.5	0.05	30	88.00	16.30	57.40	285.497	20.02	0.694
5	1.5	0.10	20	87.70	14.35	75.00	266.941	20.27	0.648
29	1.5	0.10	25	87.01	15.30	82.50	261.453	20.56	0.579
32	1.5	0.10	25	86.29	15.90	82.90	259.825	20.20	0.563
31	1.5	0.10	25	85.06	15.20	82.40	260.348	20.98	0.569
28	1.5	0.10	25	84.35	15.80	81.90	261.874	20.44	0.561
14	1.5	0.10	25	86.78	15.70	82.30	262.013	20.89	0.572
30	1.5	0.10	25	85.80	15.50	82.60	261.265	20.58	0.568
23	1.5	0.10	30	83.50	16.80	87.65	255.367	20.97	0.489
8	1.5	0.15	20	77.30	15.55	80.55	258.163	21.20	0.522
17	1.5	0.15	25	75.30	16.90	83.50	252.709	21.84	0.464
26	1.5	0.15	30	72.00	19.30	86.50	246.003	22.20	0.366
3	2.0	0.05	20	94.40	15.90	83.80	715.268	16.14	0.599
12	2.0	0.05	25	91.39	17.05	91.50	708.007	16.49	0.476
21	2.0	0.05	30	88.50	18.60	95.60	701.294	16.87	0.339
6	2.0	0.10	20	88.40	17.70	90.80	682.689	17.00	0.464
15	2.0	0.10	25	86.00	18.60	94.20	677.136	17.26	0.375
24	2.0	0.10	30	84.00	20.20	96.15	671.935	17.77	0.270
9	2.0	0.15	20	79.50	19.40	95.60	673.543	18.03	0.293
18	2.0	0.15	25	76.60	20.40	96.30	668.162	18.56	0.233
27	2.0	0.15	30	73.70	22.80	97.20	662.736	18.89	0.000

[a] ES, experimental sequence.

The % entrapment value was maximized in the optimization procedure, as the higher values of this parameter are desirable. The desirability function of this parameter was calculated by using the following equation:

Eq. 6.15
$$d_1 = \frac{Y_i - Y_{min}}{Y_{max} - Y_{min}}$$

where d_1 is the individual desirability of % entrapment and Y_i is the experimental result. The values of Y_{max} and Y_{min} for % entrapment were 94.40 and 65.28 respectively.

The T_{50} and T_{90} value were minimized in the optimization procedure, as lower values of these parameters give quicker and complete release of enzyme from the beads. The calculation of the desirability function was carried out using the equation:

Eq. 6.16
$$d_2 \text{ or } d_3 = \frac{Y_{max} - Y_i}{Y_{max} - Y_{min}}$$

where d_2 is the individual desirability of T_{50}, d_3 is individual desirability of T_{90} and Y_i is the experimental result. The values of Y_{max} and Y_{min} were 22.8 and 6.5 min for T_{50} and 97.2 and 23.8 min for T_{90} respectively.

Formulations that have a particle size within the range of 169.737-715.268 μm have a desirability function of 1, while the formulations that have values out of this range have a desirability value of 0. These can be described by the following equations:

Eq. 6.17
$$d_4 = 0 \quad \text{for} \quad Y_i < Y_{min}$$

Eq. 6.18
$$d_4 = 1 \quad \text{for} \quad Y_{min} < Y_i < Y_{max}$$

Eq. 6.19
$$d_4 = 0 \quad \text{for} \quad Y_i > Y_{max}$$

where d_4 is the individual desirability of the particle size and Y_i is the experimental result. Non-linear partial desirability function was selected for angle of repose which was less important in the optimization. The value was minimized as lower angle of repose was desirable. In this case all the experimental values were acceptable, however, the values far

from the target, are little penalized, by choosing 0<s<1 (0.1 in this case) in the following equations:

Eq. 6.20 $$d_5 = 1 \quad \text{if} \quad Y_i \le Y_{\min}$$

Eq. 6.21 $$d_5 = \left(\frac{Y_{\max} - Y_i}{Y_{\max} - Y_{\min}} \right)^s \quad \text{if} \quad Y_{\min} \le Y_i \le Y_{\max}$$

Eq. 6.22 $$d_5 = 0 \quad \text{if} \quad Y_{\max} \le Y_i$$

where d_5 is the individual desirability of angle of repose and Y_i is the experimental result. The values of $Y_{\max}$ and $Y_{\min}$ for angle of repose were 26.326 and 16.137 respectively.

The overall desirability values were calculated from the individual values by using the following equation:

Eq. 6.23 $$D = (d_1 \times d_2 \times d_3 \times d_4 \times d_5)^{1/5} = \left[\prod_{i=1}^{5} d_i \right]^{1/5}$$

6.3. Alginate-Papain Beads (Neu. Net.)

6.3.1. MATERIALS

Hammersten type casein USP (Himedia Laboratories Pvt. Ltd., Mumbai, India) and trichloroacetic acid (98.0%, Qualigens Fine Chemicals, Mumbai, India) were used as received. Purified papain IP, sodium alginate IP, calcium chloride dihydrate (98.0%), dibasic sodium phosphate (99.5%), disodium ethylenediaminetetraacetate (99.5%), cystein hydrochloride (99.0%), and citric acid (98.0%) were purchased from S. D. Fine-Chem Ltd., Mumbai, India. All the other chemicals and solvents were of analytical grade and were used without further purification. Deionized double-distilled water was used through out the study.

6.3.2. PREPARATION OF BEADS

Concentrated sodium alginate solution in distilled water was prepared well before required. Required quantity of enzyme (200 mg papain in 50 ml of final sodium alginate solution) was dissolved in small quantity of water and mixed with concentrated sodium alginate solution. Final concentration of sodium alginate was adjusted in the range of 1-2% w/v and was used after being degassed under a vacuum. The beads were prepared by dropping the sodium alginate solution (10 ml) containing papain from the dropping device such as syringe with 26G×½" flat-tip hypodermic needle to a magnetically stirred calcium chloride solution (40 ml) at a rate of 5 ml/min and were allowed to harden for specific time. Different levels (Table 6.6) of sodium alginate, calcium chloride and

hardening time were selected. The beads were collected by decanting calcium chloride solution, washed with deionized water and dried to a constant weight in vacuum desiccator (Tarsons Products Pvt. Ltd., Kolkata, India) at room temperature for 36 hours.

Table 6.6: Factorial 3^3: factors and their levels.

Factors	Low level (-1)	Middle level (0)	High level (1)
A: Sodium alginate (% w/v)	1.0	1.5	2.0
B: Calcium chloride (M)	0.05	0.10	0.15
C: Hardening time (min)	20	25	30

6.3.3. FACTORIAL DESIGN

In this study a 3^3 full factorial design was used to determine the effect of the sodium alginate concentration, the calcium chloride concentration, and the hardening time. Before the application of the design, a number of preliminary trials were conducted to determine the conditions at which the process resulted to beads. The matrix of the experiments and the results of the responses are listed in Table 6.7. To determine the experimental error, the experiment at the centre point was repeated five times at different days. The mean % entrapment, T_{50}, T_{90}, particle size and angle of repose of these experiments were 85.88±1.02%, 15.57±0.28 min, 82.43±0.33 min, 261.1±0.9 μm and 20.61±0.29° respectively. The above-mentioned values showed good reproducibility of the process.

Table 6.7: Matrix of the experiments and results for the measured responses.

Factors/ levels				Responses				
ES[a]	Na. alginate (% w/v)	Calcium chloride (M)	Harde-ning time (min)	% Entrap-ment	T_{50}	T_{90}	Particle size (μm)	Angle of repose
1	1.0	0.05	20	91.80	6.50	23.80	211.1	22.62
10	1.0	0.05	25	89.00	8.30	25.70	207.3	22.88
19	1.0	0.05	30	86.20	9.35	28.40	202.6	23.27
4	1.0	0.10	20	85.00	7.80	27.50	184.3	23.39
13	1.0	0.10	25	82.60	9.10	30.55	181.1	23.75
22	1.0	0.10	30	80.71	10.70	34.00	178.3	24.15
7	1.0	0.15	20	68.80	8.60	34.60	176.0	24.07
16	1.0	0.15	25	67.79	10.30	39.00	171.4	24.94
25	1.0	0.15	30	65.28	12.05	59.25	169.7	26.33
2	1.5	0.05	20	93.40	13.35	48.00	297.0	19.50
11	1.5	0.05	25	90.80	14.45	57.40	291.6	19.76
20	1.5	0.05	30	88.00	16.30	57.40	285.5	20.02
5	1.5	0.10	20	87.70	14.35	75.00	266.9	20.27
29	1.5	0.10	25	87.01	15.30	82.50	261.5	20.56
32	1.5	0.10	25	86.29	15.90	82.90	259.8	20.20
31	1.5	0.10	25	85.06	15.20	82.40	260.3	20.98
28	1.5	0.10	25	84.35	15.80	81.90	261.9	20.44
14	1.5	0.10	25	86.78	15.70	82.30	262.0	20.89
30	1.5	0.10	25	85.80	15.50	82.60	261.3	20.58
23	1.5	0.10	30	83.50	16.80	87.65	255.4	20.97
8	1.5	0.15	20	77.30	15.55	80.55	258.2	21.20
17	1.5	0.15	25	75.30	16.90	83.50	252.7	21.84
26	1.5	0.15	30	72.00	19.30	86.50	246.0	22.20
3	2.0	0.05	20	94.40	15.90	83.80	715.3	16.14
12	2.0	0.05	25	91.39	17.05	91.50	708.0	16.49
21	2.0	0.05	30	88.50	18.60	95.60	701.3	16.87
6	2.0	0.10	20	88.40	17.70	90.80	682.7	17.00
15	2.0	0.10	25	86.00	18.60	94.20	677.1	17.26
24	2.0	0.10	30	84.00	20.20	96.15	671.9	17.77
9	2.0	0.15	20	79.50	19.40	95.60	673.5	18.03
18	2.0	0.15	25	76.60	20.40	96.30	668.2	18.56
27	2.0	0.15	30	73.70	22.80	97.20	662.7	18.89

[a] ES, experimental sequence.

6.3.4. NEURAL NETWORK SOFTWARE AND NETWORK TOPOLOGY

The Microsoft®-Windows® based neural network software; NeuroSolutions® Version 4.24 (NeuroDimension, Inc.) was used. A multilayer perceptron (MLP) with single

hidden layer architecture was chosen. The experimental matrix of 32 input:desired output data sets (Table 6.7) was inserted in to the model, with three input neuron (sodium alginate concentration, calcium chloride concentration and hardening time), one hidden layer and six output neuron (% entrapment, T_{50}, T_{90}, particle size, angle of repose and overall desirability) as shown in Figure 6.2. Various adjustable parameters like number of neurons in hidden layer, step size and momentum of hidden layer and output layer, etc. were optimized.

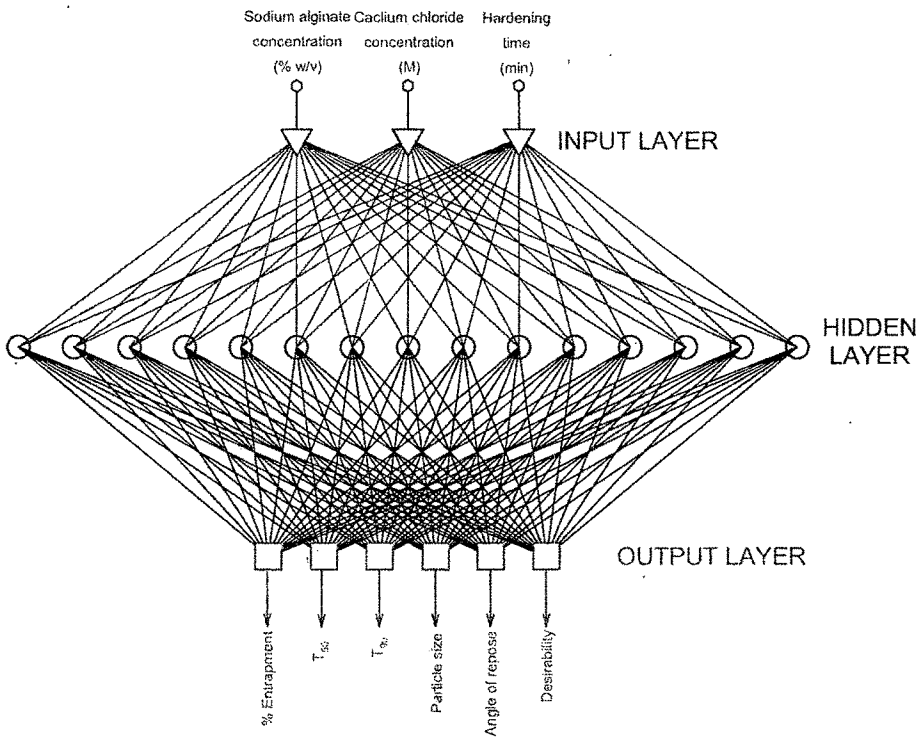

Figure 6.2: Architecture of three-layer neural network.

The neural network was trained with 5 to 35 hidden neurons with 2000 training epochs and performance was tested after each one addition of neurons. Training was repeated for 3 times for optimization of all parameters. At the start of the training run, weights were

initialized with random values. During training, 5 additional data sets of input:desired output were used for the cross-validation and was back-propagated through the network to evaluate the trained network. A mean square error (MSE) termination criterion was based on the cross validation set. By selecting this option training stops when the MSE of the cross validation set begins to increase, a sign of network overtraining when network simply memorizes the training set and is unable to generalize the problem. The network trained under optimum conditions was used to predict responses at different factor values and response surface were generated for interpretation.

6.3.5. CHARACTERIZATION OF BEADS

6.3.5.1. Estimation of Papain

Papain was estimated by modified casein digestion method of USP XXVI in presence of cysteine hydrochloride. Different aliquots of standard papain solution in phosphate-cysteine disodium ethylenediaminetetraacetate buffer were added to 5 ml of buffered substrate (hammersten-type casein 10 mg/ml, pH 6.0±0.1) and incubated for 60 min at 40°C. Digestion process of casein was stopped by adding 3 ml of 30% w/v trichloroacetic acid solution and allowed to stand for 30 – 40 min at 40°C. Digested amino acids were filtered through whatman® filter paper no. 42 by discarding first 3 ml of filtrate and absorbance was measured at 280 nm against their respective blanks. The method was found to be linear over an analytical range of 3 – 100 µg/ml with correlation coefficient (r) of 0.9996. Limit of detection, limit of quantitation, and regression equation were found to be 0.77 µg/ml, 2.57 µg/ml, and $y = 0.0042x - 0.0033$ respectively.

6.3.5.2. Determination of Entrapment Efficiency

Entrapment efficiency was determined by dissolving the enzyme loaded beads in a magnetically stirred simulated intestinal fluid without enzyme (USP XXVI) for about 45 min. The resulting solution was centrifuged at 2500 rpm for 10 min (Remi Instruments Ltd, Mumbai, India) and supernatant was assayed (n=3) for enzyme content by modified casein digestion method of USP XXVI. Entrapment efficiency was calculated as:

Eq. 6.24

$$\text{Entrapment efficiency} = \frac{\text{Enzyme loaded}}{\text{Theoratical enzyme loading}} \times 100$$

6.3.5.3. Effect of pH on Release Profile

To study the effect of pH on papain release profile, *'in vitro'* dissolution study was carried out using the USP XXVI dissolution apparatus 2 (TDT-60T, Electrolab, Mumbai, India) in 500 ml of different pH media (simulated gastric juce pH 1.2 (USP), phosphate buffer pH 4.0 (IP), neutralized phthalate buffer pH 5.4 (IP), simulated intestinal fluid without enzyme pH 6.8 (USP), phosphate buffer pH 7.4 (IP), and phosphate buffer pH 8.0 (IP)) on the optimized batch at 37±0.5°C with paddle speed of 75 rpm. Accurately weighed samples (n=3) equivalent to about 40 mg of papain were introduced to dissolution media and samples of 2 ml were collected at 0, 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0 hr. Samples were filtered through 0.4 µm whatman® membrane filter and assayed for enzyme content as before.

6.3.5.4. Determination of T_{50} and T_{90}

Time required for 50 (T_{50}) and 90 (T_{90}) percent of enzyme release are important parameters for enzyme release study and were used to evaluate the onset of action and duration of action respectively. For optimization purpose, dissolution study of all batches was carried out in 500 ml of simulated intestinal fluid without enzyme as before. Accurately weighed samples (n=3) equivalent to about 40 mg of papain were subjected to dissolution and aliquots of 2 ml were assayed at 0, 5, 10, 15, 20, 30, 45, 60, 90 and 120 min. T_{50} and T_{90} were found by extrapolating the % enzyme released versus time plot.

6.3.5.5. Particle Size Measurements

Particle size is an important parameter for the formulation development. Optimized batch of the beads was filled in the capsules during which the particle size was the evolutionary parameter. Larger particles show higher weight variation during capsule filling, hence the experimental conditions result in smaller particles is preferable. Particle size was determined with the laser diffraction particle size analyzer (MAN 0244/ HYDRO 2000 SM, Malvern Instruments Ltd., UK) using isopropyl alcohol as a vehicle.

6.3.5.6. Angle of Repose Measurements

Angle of repose was measured for estimating flowability of the beads. If the angle exceeds 50°, the material will not flow satisfactorily while materials having values near the minimum, flow easily and well. The rougher and more irregular the surface of the particles, the higher will be the angle of repose. The angle also increases with decrease in

particle size. Angle of repose was measured by passing beads through a funnel on the horizontal surface. The height (h) of the heap formed was measured with a cathetometer and the radius (r) of the cone base was also determined. The angle of repose (Φ) was calculated from:

Eq. 6.25

$$\tan \Phi = \frac{h}{r}$$

6.3.5.7. Fourier Transform Infra-red Spectroscopy (FTIR)

IR transmission spectra were obtained using a FTIR spectrophotometer (FTIR-8300, Shimadzu, Japan). A total of 5% (w/w) of sample, with respect to the potassium bromide (KBr) disc, was mixed with dry KBr (S. D. Fine Chem Ltd., Mumbai, India). The mixture was ground into a fine powder using an agate mortar before compressing into KBr disc under a hydraulic press at 10,000 psi. Each KBr disc was scanned at 4 mm/s at a resolution of 2 cm over a wavenumber region of 400–4500 cm^{-1}. The characteristic peaks were recorded.

6.3.5.8. Differential Scanning Calorimetry (DSC)

Differential scanning calorimetric analysis was used to characterize the thermal behaviour of the isolated substances, their physical mixture and empty and loaded beads. DSC thermograms were obtained using an automatic thermal analyzer system (DSC-60, Shimadzu, Japan). Temperature calibration was performed using indium as a standard. Samples were crimped in a standard aluminium pan and heated from 40–400 °C at a heating rate of 10 °C/min under constant purging of dry nitrogen at 30 ml/min. An empty

pan, sealed in the same way as the sample, was used as a reference. The characteristic endothermic peaks and specific heat of the melting endotherm were recorded.

6.3.5.9. Scanning Electron Microscopy (SEM)

The purpose of SEM study was to obtain a topographical characterization of beads. The beads were mounted on brass stubs using double-sided adhesive tape. SEM photographs were taken with scanning electron microscope (JSM-5610LV, Jeol Ltd., Japan) at the required magnification at room temperature. The working distance of 39 mm was maintained and acceleration voltage used was 15 kV, with the secondary electron image (SEI) as a detector.

6.3.6. PREPARATION OF CAPSULE FORMULATION & STABILITY STUDY

Accurately weighed alginate beads equivalent to 40 mg of papain were filled into a hard gelatin capsule manually. The joint of the capsule body and cap was carefully sealed by pressing them to fit in the lock mechanism. The capsules were packaged in high density polyethylene (HDPE) bottles with polypropylene (PP) caps (foamed polyethylene and pressure sensitive liner). The capsules were subjected to stability testing according to the International Conference on Harmonization guidelines for zone III and IV. The packed containers of prepared capsules along with marketed formulation and bulk papain were kept for accelerated (40±2°C/75±5% relative humidity) and long term (30±2°C/65±5% relative humidity) stability in desiccators with saturated salt solution for up to 12 months. A visual inspection (for discoloration of capsule content), dissolution testing and papain content estimation was carried out every 15 days for the entire period of stability study.

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