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BIOCHEMISTRY

Improvement in Storage and Thermal Stability of Micro-structures of Amylase Entrapped in the Pectin Gel

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ABSTRACT

Mathematical models concerning the synthesis of micro structures, having amylase entrapped in pectin gel was established with response surface methodology. Pectin concentration, pH, temperature and incubation period were selected as the process variables for optimum entrapment of amylase. Sonication and calcium ions enhanced the residual activity of particles. Pectin entrapped amylase exhibited maximum catalysis at pH 6.5 and temperature 60°C. The kinetics of amylase in free and micro particles form was compatible. SEM micrographs showed spherical particles with an average size of 6.9 μm. Storage stability of pectin loaded amylase was enhanced in liquid medium when compared to free enzyme.

Highlights

- Storage stability of amylase micro-structure enhanced as proportional to free enzyme.
- Kinetic properties of amylase entrapped in micro-structure were compatible with free enzyme.

Keywords: α-amylase, micro particles, pectin, synthesis

Protein and polysaccharide mixtures can be used to fabricate biopolymer particles with a variety of different structures and applications (McClements 2006). Associative connections generally occur through electrostatic attraction between proteins and polysaccharides that have opposite electrical charges. Under controlled pH conditions, electrostatic interactions between proteins and polysaccharides may be manipulated to form a variety of biopolymer particles, such as soluble complexes, coacervates or precipitates. The biopolymer particles have good stability for the subsequent alterations in the surrounding aqueous conditions (Jones & McClements 2008; Yu *et al.* 2006). So these biopolymer particles are suitable in a variety of applications. Natural polymers such as cellulose, dextrin, gelatin, pectin etc. are widely available in nature and they constitute an important class of biopolymers for the synthesis of micro particles (Peltonen *et al.* 2012).

Biocatalysts are the key players in a variety of industrial processes. Constant efforts are being made to improve the functional properties of enzyme for industrial application (Wang *et al.* 2010). The potential of using various polysaccharides for the synthesis of micro-structures has stimulated interest in the exploration of biocatalysis with enhanced operational stability. Various types of carriers such as gelatin, alginate and polyacrylamide have been previously investigated for the effective binding of alpha amylase. Polysaccharides have been investigated for the synthesis of micro-structures because of their excellent physico-chemical and biocompatible properties. Polysaccharides have enhanced thermal resistance and catalytic properties of enzymes due to the formation of high stable polyelectrolyte complexes (Go´mez *et al.* 2006; Satar *et al.* 2008). Protein and polysaccharide mixtures can be used to fabricate biopolymer particles with a variety of different compositions, structures and

dimensions depending up on the nature of the biopolymers involved and the assembly principle used (McClements 2006; Tolstoguzov 2003). Properties of polysaccharide-protein complexes depend on various factors like charge, nature of biopolymers, pH, ionic strength and temperature of the medium (Ghosh & Bandyopadhyay 2011).

The aim of the present research was to optimize the process variable for the synthesis and the characterization of micro structures of amylase entrapped in the pectin gel. We hypothesized that the binding of two biopolymers enzyme and pectin would influence the functional properties of molecules.

MATERIALS AND METHODS

The enzyme used in the study was amylase from *Aspergillus oryzae*. All the chemicals of the analytical grade were purchased from Hi- media (Mumbai, India).

Biopolymer particle formation

A Central composite design, (CCD) (Box and Draper 1987) was applied to determine the optimum levels of the variables for the synthesis of pectin amylase-micro-structures. The central values (zero level) chosen for the experimental design for pectin concentration (A), pH (B); Temperature (C) and incubation Time (D) were as per CCD (Table 1). For the synthesis of pectin amylase biopolymer fusion, 5 ml of free amylase enzyme (1%) was mixed with pectin. pH was adjusted and the solution was incubated for a particular time.

Concentration of pectin, pH, temp, and the incubation time were adjusted as per RSM design (Table 2). Centrifugation was carried out at 10,000 *g* for 20 min (4°C) using a high speed centrifuge (Remi cooling centrifuge).The enzyme concentration in the particles was determined from the difference between the enzyme activity in total and pellet. Residual enzyme activity in pellet was determined by a standard procedure using the following method:

$$\% \text{ Residual Activity} = \frac{\text{Enzyme activity of micro-particles} \times 100}{\text{Enzyme activity of control}}$$

Variance analysis

The analysis of variance (ANOVA) was employed for the significant variables. ANOVA classifies spastically the results tested by the means of the specified classification difference which was carried out by Fisher's Statistical Test (F-test). The RSM design modeling and analysis were carried out using the Design expert Software version 9.0 (Der and Everitt, 2001).

Table 1: Different parameters for the syntheis of PAMs using Response Surface Methodology in CCD

CCD Design for amylase micro-structures synthesis					
Factor	**Name**	**Units**	**Low actual (-1)**	**Value (0)**	**High actual (+1)**
A	Pectin	%	0.1	1.000=2.2	2.2
B	pH	-	2	1.000=10	10
C	Temp	0 C	35	1.000=80	95
D	Time	Hrs	0.5	1.000=6.5	6.5

Amylase assay

The reaction mixture of amylase solution (0.5 ml) and 0.5 ml starch (0.5% starch dissolved in phosphate buffer pH 7) were incubated for 30 min at 60°C. The Amylase activity was assayed by measuring the production of reducing sugar from starch using 3, 5-dinitrosalicylic acid (DNS) (Miller, 1959). One international unit (IU) of enzyme activity was defined as the amount of enzyme that catalyzed the liberation of reducing sugar equivalent to 1.0 μM min^{-1} under assay conditions.

Effects of Sonication and metal ions on activity of PAMs

The enzyme micro particles were subjected to sonication with Pci Analyst Model 1.5LS0, at 50 Hz for different time intervals (5-30 min) and the residual activity was determined. Metal ions affect the gelling mechanism of pectin (Sungthongjeen *et al.* 2004). Effect of different metal ions: calcium carbonate, potassium chloride, sodium nitrite, nickel sulphate, mercuric chloride and magnesium sulphate was investigated to understand the formation of amylase pectin micro structures with maximumenzyme residual activity.

Table 2: Central composite designs (CCD) for the synthesis of PAMs

Design variables and Value				Response
Pectin (A, %)	pH (B)	Temperature (C,^{0}C)	Incubation period (D, h)	Residual activity (%)
0.1	8	50	2	5.12
1.5	8	50	2	15.9
0.8	6	95	3.5	33.9
0.1	8	80	5	0.1
1.5	8	80	2	8.97
0.8	6	65	0.5	0.02
0.8	6	65	6.5	18.49
0.1	4	50	2	10.9
1.5	4	50	2	15.92
0.1	4	50	5	0.14
0.1	8	50	5	0.12
1.5	8	80	5	34.1
0.1	4	80	2	12.39
1.5	4	80	5	28.7
0.1	8	80	2	11.11
1.5	4	50	5	40.1
0.8	6	35	3.5	44.6
0.8	2	65	3.5	24.52
0.8	10	65	3.5	22.64
0.8	6	65	3.5	55.97
1.5	8	50	5	25.64
0.8	6	65	3.5	55.97
0.1	4	80	5	0.05
1.5	4	80	2	31.3
2.2	**6**	**65**	**3.5**	**57.8**

Structural characterization of PAMs

Particle size in the solid state and the size distribution were measured by an ultraviolet laser diffraction nanoparticle size analyzer (SALD-7101; Shimadzu Corporation, Kyoto, Japan). Prior to each measurement, the background measurements were conceded by a blank cell filled with deionized water. Then, 1.5 mL of the sample was added to the blank for measurement. The refractive index of the measured samples was 1.60–0.10.

The shape and surface morphology of the micro-structures were investigated by scanning under an electron microscope (SEM) (S-800; Hitachi Ltd, Tokyo, Japan). The sample was fixed on an aluminum specimen stub covered with a double-sided adhesive carbon disc, and then sputter coated (Hummer VI Sputtering System; Technics, Anatech USA, Union City, CA, USA) with gold prior to imaging. Sputter coating was performed at 20 mA.

Functional characterization of free enzyme and PAMs

The effect of pH on the amylase micro-structures and free enzyme was studied using the buffers of different pH; 50 mM of citrate buffer pH 3–5 and sodium phosphate buffer pH 6–8 in the assay system. Effect of temperature on enzymatic activity was determined by incubating an assay mixture of the optimum pH at different temperatures ranging from 20 to 100^{0}C. Each assay experiment was performed in a triplicate. The residual activity was determined under optimum pH and temperature condition. The K_m and V_{max} values were computed by linear regression from Line weaver Burk plot (Lineweaver & Burk, 1934). The storage stability of the free enzyme and micro-structures was tested by determining the enzyme activity that retained after incubation at different temperatures (4 and 37°C) for a period of 15 d.

Substrate Specificity of the Enzyme

Substrate specificity of pectin loaded amylase and free enzyme for different starchy substrates: wheat, rice and potato starch in 50mM phosphate buffer (pH 6.5) at 60°C for 15 min were analysed. The amount of reducing sugar produced by the reaction was measured using an assay method as described earlier.

RESULTS AND DISCUSSION

Biopolymer particle formation

Extent of hydrogen bonding and electrostatic interaction between the biopolymer depends on the solutionpH, ionic strength and temperature. The response surface methodology with CCD was used to reveal the optimum levels of the parameters leading to a maximum % residual activity. Twenty five experiments (Table 2) designed with central composite design were conducted for the synthesis of PAMs. The highest residual activity 57.8% was achieved with; Pectin (A) conc. 22.2 g l^{-1}; pH (B) 6, temperature (C) 65°C and incubation period (D) 3.5 h. The statistical significance of the second order model equation was checked by an F-test (Table 3). The analysis gives the value of the model. The F

value is the test for comparing model variance with residual (error) variance. The regression model for PAMs synthesis was highly significant ($p<0.01$) with the satisfactory value of determination coefficient (0.9833) indicating that 95% variables in response could be explained by the second order model equation.

Table 3: Analysis of variance (ANOVA) for response surface central composite model obtained from experimental designs for synthesis of PAMs

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob> F
Model	7103.24	14	507.37	4.88	0.0080
A-Pectin	89.79	1	89.79	0.86	0.3746
B-pH	110.86	1	110.86	1.07	0.3261
C-Temp	2.98	1	2.98	0.029	0.8690
D-Incubation Periods	480.71	1	480.71	4.62	0.0571
AB	37.15	1	37.15	0.36	0.5633
AC	0.22	1	0.22	2.079E-003	0.9645
AD	570.73	1	570.73	5.49	0.0411
BC	0.28	1	0.28	2.701E-003	0.9596
BD	25.96	1	25.96	0.25	0.6281
CD	22.52	1	22.52	0.22	0.6517
A^2	908.80	1	908.80	8.74	0.0144
B^2	1630.56	1	1630.56	15.68	0.0027
C^2	583.72	1	583.72	5.61	0.0393
D^2	3047.92	1	3047.92	29.31	0.0003
Residual	1039.80	10	103.98		
Lack of Fit	1039.80	9	115.53		
Pure Error	0.000	1	0.000		
Cor Total	8143.04	24			

The Model F-value of 4.88 implies that the model is significant. There is only a 0.01% chance that F-value this large could occur due to noise. Values of "Prob> F" less than 0.0500 indicates that the model terms are significant. R-squared (coefficient of determination) value provides a measure of variability in the observed response values that can be explained by the experimental factor and their interaction. R^2 value (0.98 Quadrate cubic) closer to 1.0 shows that stronger the model is, better it predicts the response. (Kaushik *et al.* 2006) The 3D contours response surface graph based on dependent variables (Fig. 1) revealed the effect of variables on the residual activity. The iso-response contour plots of RSM as a function of two factors at a time, holding all other factors at fixed coded level, are helpful for understanding both the main and the interaction effects of these two factors. Validation of the predicted results was accomplished by performing additional experiments in triplicate with the parameters suggested by the numerical modeling. These three sets of experiment yielded an average residual activity of 57±2%.

Table 4: Statistical parameters obtained from Analysis of variance (ANOVA) for the central composite design PAMs synthesis

Lack of Fit Tests					
Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob> F**
Linear	5231.49	19	275.34	2.78	0.0548
2FI	4574.64	13	351.90	0.34	0.9071
Quadratic	1039.80	9	115.53	8.50	0.0030
Cubic	135.72	1	135.72	1.67	0.4285
Pure Error	0.000	1	0.000		

Model Summary Statistics					
Source	**Std. Dev.**	**R-Squared**	**Adjusted R-Squared**	**Predicted R-Squared**	**PRESS**
Linear	16.17	0.3576	0.2291	0.1206	7161.38
2FI	18.08	0.4382	0.0369	-0.2371	10073.44
Quadratic	10.20	0.8723	0.6935	0.0496	7738.78
Cubic	8.24	0.9833	0.8000		+

Effect of sonication time and metal ions

Increase in sonication time enhanced the catalysis activity initially, but after 25 min of treatment, the rate of reaction was constant (Fig. 3a). Shock waves produced by sonication reduces the size of particles, hence the surface area of micro-structure increases. As a consequence enzyme-substrate binding opportunity enhances which improves the rate of bio-catalysis. Most of the amylases are known to be metal ion-dependent (Panday *et al.* 2000), so the metal ions were used to test their effect on the residual activity. Calcium carbonate, potassium chloride and sodium nitrite nickel sulphate enhanced the activity by 14 and 13, 8 and 7% respectively. But mercuric chloride and magnesium sulphate inhibited the activity. The inhibitory effect of mercury has also been reported on glucoamylase (Selvakumar *et al.* 1998). Enhancement of amylase activity with metal ions could be based on its ability to interact with

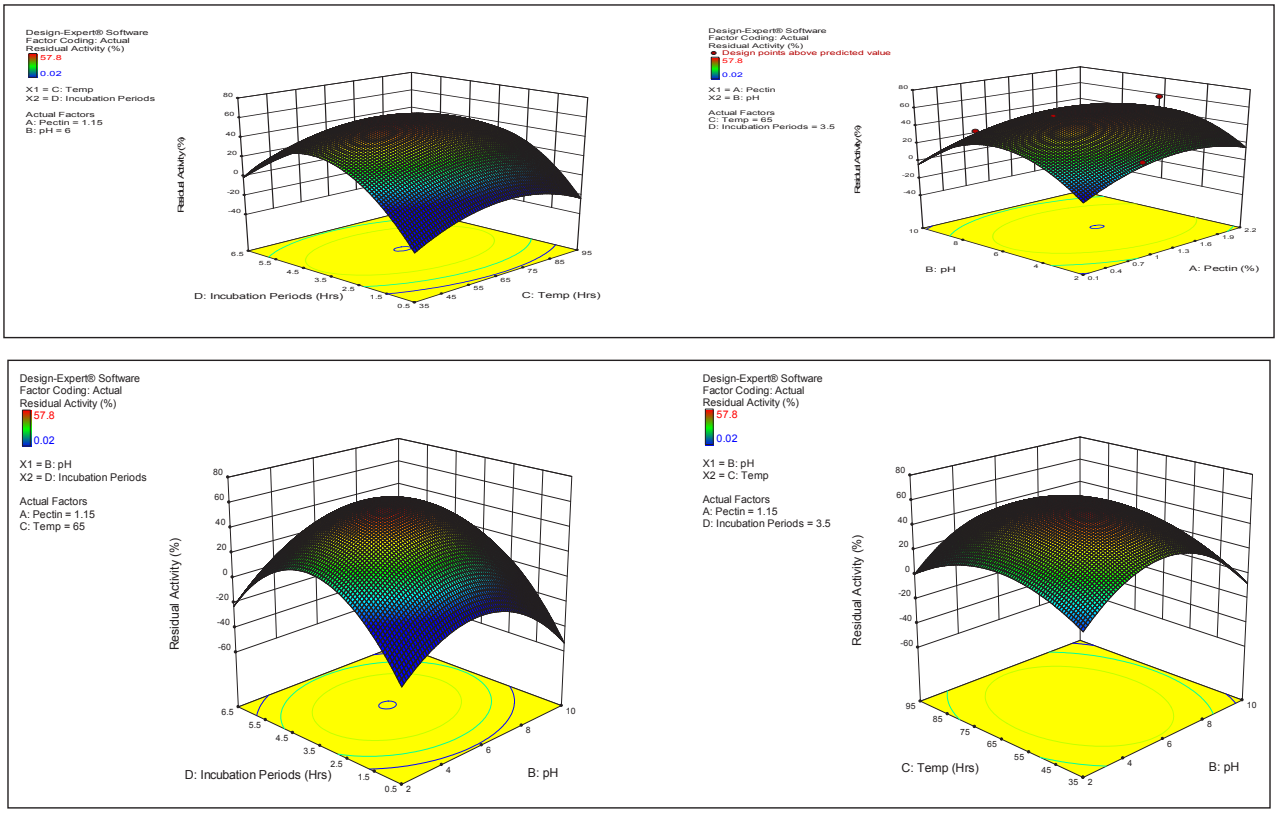

Fig. 1: Surface plot for the effect of **(a)** incubation and temp **(b)** pectin and pH; **(c)** incubation and pH **(d)** temperature and pH on PAMs synthesis with the central composite design

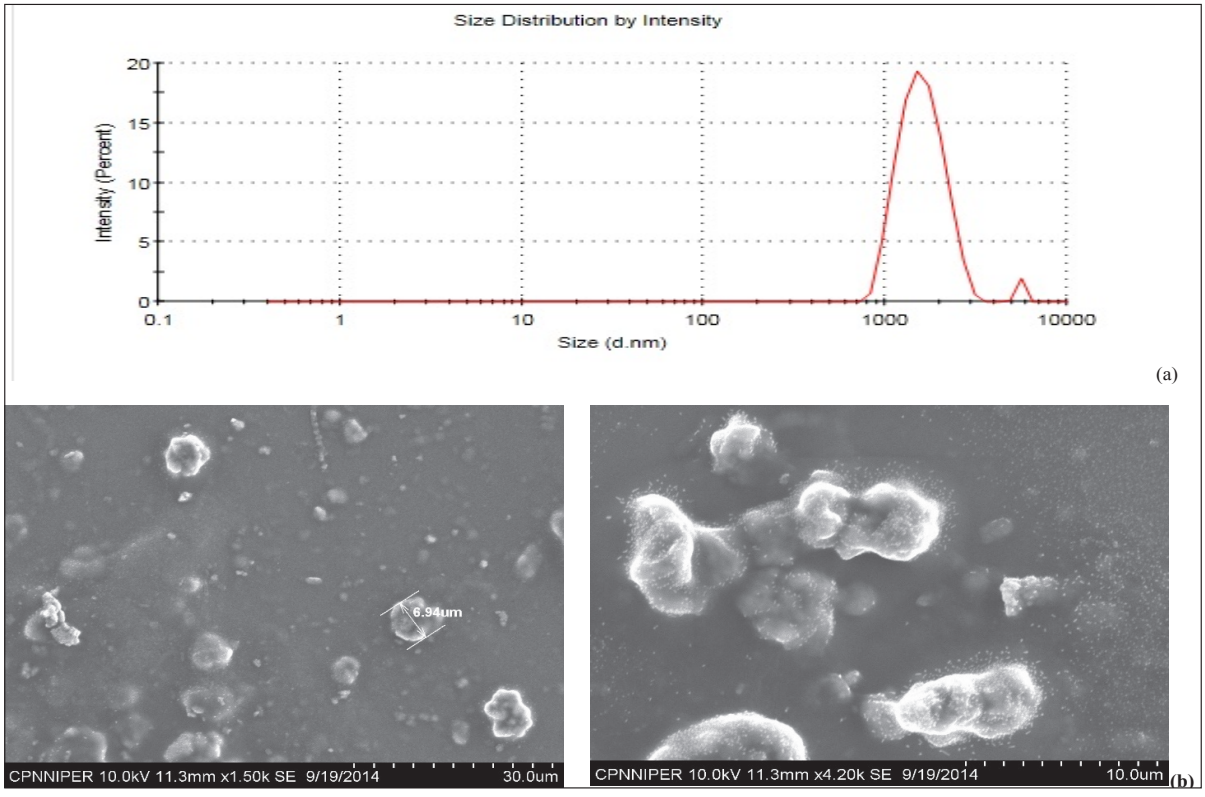

Fig. 2(a): Particle size distribution of amylase microstructures analyzed with laser diffractometer. **(b)** SEM image of pectin amylase microstructures synthesized by pectin binding at magnification of x4.06 K

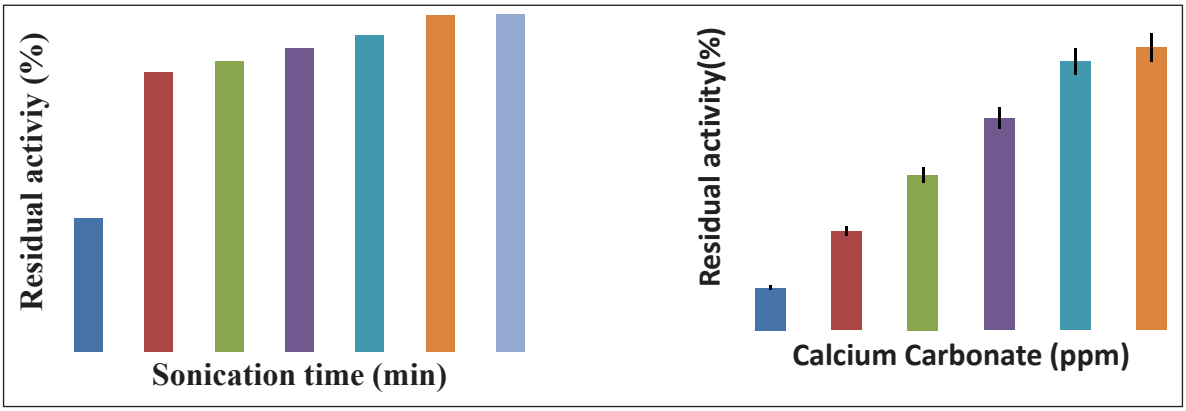

Fig. 3: Effect of (a) sonication time and (b) calcium carbonate conc. on the residual activity of PAMs in liquid medium. (a) The optimum sonication time for the PAMs residual activity was determined using starch substrate (0.5% starch in Phosphate buffer pH 6.5) and temperature60^{0}C. The optimum residual activity 66% was achieved after 25 min of sonication. (b) The optimum calcium carbonate concentration was determined at pH value in the range of 6.0–6.5 using starch as a substrate for 30 min at 60°C. The reported values are the mean values of the three independent experiments

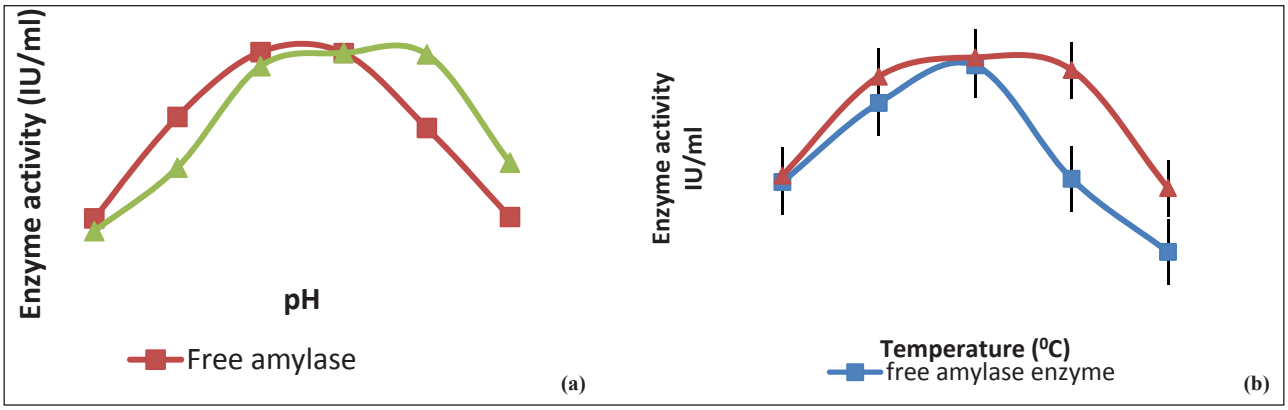

Fig. 4: pH (a) and temperature (b) optima for the biocatalysts for free enzyme and PAMs

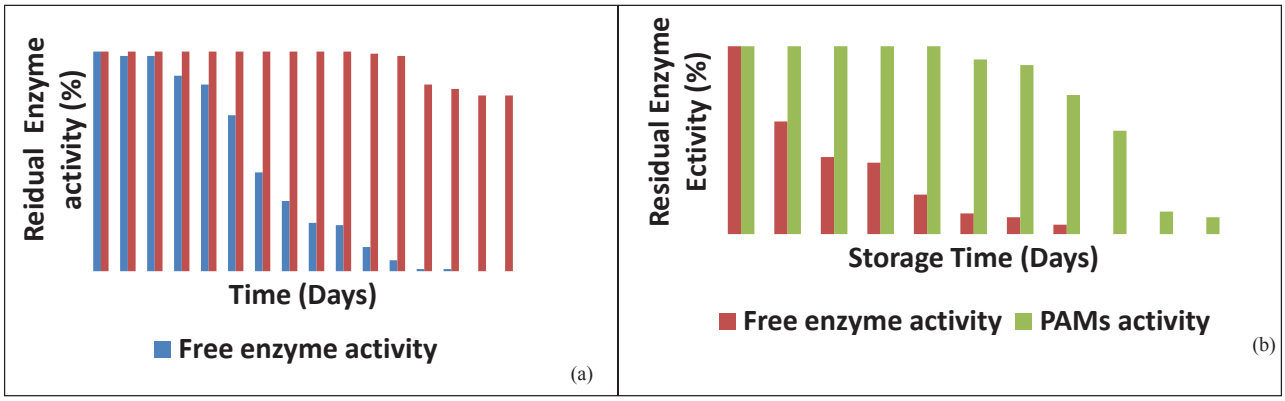

Fig. 5: Operational stability of free enzyme and PAMs at (a) 4 and (b) 37°C storage temperature in liquid medium at pH 6.5

the negatively charged amino acid residues such as aspartic and glutamic acid (Linden *et al.* 2003). Maximum residual activity (75%) was at 1000 mg L^{-1} calcium carbonate concentration in the liquid (Fig. 3b). The affinity of Ca^{2+} to amylase is much stronger than other ions because amylase is a metalloenzyme which contains at least one activating Ca^{2+} ion (Gupta *et al.* 2010).

Structural characterization of PAMs

The size and the shape of amylase micro-structures were revealed with the particle size analyzer based on laser diffractometer and SEM. The average particle size of free enzyme and micro-structures was1000-10000 d.nm (Fig. 2a). The micro-structures scanned using SEM showed relatively irregular

shapes in a range of 6.95 µm at a magnification of X 4.06 K (Fig. 2b). This confirms the binding of pectin with amylase to form amylase micro-structures.

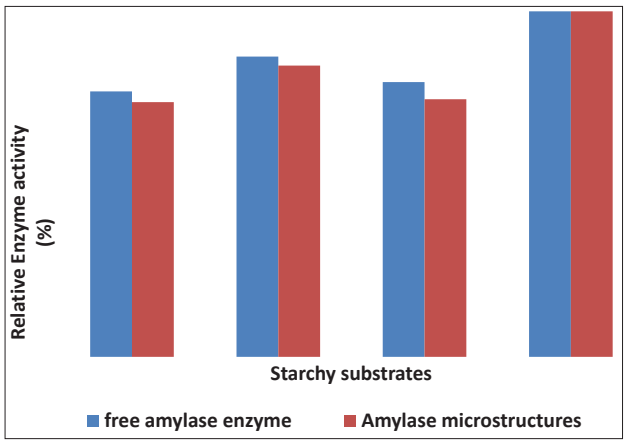

Fig. 6: Relative activity of free amylase and PAMs by utilizing different starch sources. The enzyme activity (amylase) was measured at pH 6.5 (60 ◦C) and the activities were expressed as percentage relative to the soluble starch which was considered 100%

Functional characterization of micro-structures

PAMs exhibited optimal activity in pH range of 5-7, while the free enzyme exhibited 5-6 with the highest activity of pH at 6.5 (Fig. 4a). pH activity profile of the micro-structures for starch hydrolysis was enhanced when compared to the free amylase. Optimum temperature for the free and PAMs was 60˚C. PAMs has an optimum range of 40-80°C which then increased with respect to an optimum range of 40-70°C for free enzyme (Fig. 4b). Kinetic variables K_m and V_{max} of enzyme were determined by Lineweaver-Burk double reciprocal plot of reaction rate vs. starch substrate concentration 2.5–10 mg ml^{-1}. Free enzyme and PAMs exhibited K_m of 1.5 and 1.7 mg ml^{-1}, respectively, for starch at pH 6.5 and 60°C. The V_{max} was 1.5 and 1.34 mmol glucose min^{-1} mg $protein^{-1}$, respectively for PAMs and free amylase. Storage stability of the micro-structures and free enzyme at 4 and 37°C storage conditions as function of time (days) is presented in Figure (Fig. 5 a, b). PAMs activity decreased slower and the residual activity was 99 and 11% after 10 days at 4 and 37°C, respectively in the liquid medium. However, under the same conditions, 11 and 1% residual activity was observed with free enzyme. Pectin amylase fusion enhanced the storage stability of amylase. Similarly, Dev *et al.* (2003) and Mukherjee *et al.* (2010) concluded that free enzyme loses its activity early when compared to the matrix bound enzyme. PAMs exhibited multi-substrate specificity for starch hydrolysis. Activities with rice starch, potato starch and wheat starch were 86, 79 and 76 % respectively. These values are compatible to the experimental value of free enzyme. (Fig. 6)

CONCLUSION

This study has shown that the biopolymer associative complex particles can be formed by the thermal treatment of amylase and pectin. Optimal conditions for the formation of these particles were optimized by response surface methodology. Pectin(A) conc. 22.2 g pectin l^{-1}; pH (B) 6, temperature (C) 65˚C and incubation period (D) 3.5 h was optimum to achieve the highest, 57.8% residual activity. Biopolymer particles formed appear tobe in a range of 6.95 µm. The physico-chemical mechanism for the development of these biopolymer particles is currently unknown. The PAMs has enhanced temperature and the storage stability when compared to the free enzyme.

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