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Immobilization of Protease on Synthesized Beads of K-Carrageenan by Entrapment: its use in Commercial Activity like Juice Purification

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Abstract: -

In the present work immobilization of Protease enzyme on synthesized k-carrageenan beads by Entrapment method was done. K-carrageenan beads were synthesized in by proper method. Then protease was immobilized by Entrapment method. After immobilization process for further study, various parameters were optimized and carried out the comparison studies by using protease loaded glass beads. Furthermore, FTIR and SEM analysis were done to prove that the enzyme was immobilized on the support bead. In application protease of it was applied to purified the fruit juice i.e. Pineapple juice, watermelon juice and sugarcane juice. In addition, this study introduces a new achievement of the eco-friendly effect of immobilized enzyme, due to reuse full function of immobilized enzyme.

Keywords: Protease, k-carrageenan beads, Enzyme Immobilization, FTIR & SEM study of protease.

1. Introduction:

Enzymes are biocatalysts as they serve as the key to technology for chemical synthesis. In green chemistry, they are widely accepted due to their ecofriendly nature. Protease is from the group of proteolytic enzymes. Which hydrolyze the peptide bonds presence in proteins to convert it to shorter polypeptides and amino acids.

Further consumption of alcohol often goes with food in living organisms. Fruits are an important nutritional resource for humans. It contains many natural antioxidants. Several fruits have been reported to have an inhibitory effect on ethanol. Thus, this study of the effect of fruits on ethanol metabolism work really becomes very interesting and also important as it is connected with human health. In this work effect of three selected fruit juices on ethanol, metabolism was investigated.

In addition, when the enzyme was immobilized on the support it will be used more than one time, but the free enzyme cannot be used again as it is discsolved in it. The immobilized enzyme used repeatedly also reduces the overall cost of the whole process and really proves eco-friendly.

2. Experimental

2.1 Materials

Protease **(200U/mg)** from sisco research laboratories Pvt. Ltd., Phenol, Hypo chloride solution from Qualikems Fine Chem. Pvt. Ltd., PEI polyethyleneimine and k-carrageenan from TCI.

2.2 Characterization

For FTIR analysis, some part of bead reactor was taken and KBr was used for preparing bead of support sample. In FTIR Spectrum Characteristic peak at 3447.60 cm^{-1} shows the Presence of –OH Stretching vibration. Pease peak pears at 1635.61 m^{-1} shows stretching of C=O and band stretching vibration of 1069.85 cm^{-1} proves that amino group is there. This starching show that Protease was immobilized on the support. FTIR analysis was done from Charuset University laboratory, Changa.

2.3.1 Effect of PEI concentration:

Hardening and retention of enzyme were checked by using 5.0 % PEI concentration. Solution of 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, 4.0%, 4.5% and 5.0% Concentration were used but, suitable required hardness was found at 5.0% of PEI solution. The protein content of the bead and supernatant liquid was also calculated. The activity of the bounded enzyme was checked as explained in the literature.

2.3.2 pH activity profile

As enzymes consist of protein, the catalytic activity is markedly affected by environmental conditions, especially the pH of the aqueous medium. Thus, information on changes in pH-activity behavior caused by the immobilization of enzymes is useful for an understanding of the structure-function relationship of enzyme protein. Hence, the activity of the free and immobilized protease has been measured by incubating free and immobilized enzymes at 35 °C for 30 min in the 50 mM phosphate buffers of different pH ranging from 4 to 11 and using casein as a substrate. The absorbance of the reaction mixture was measured at 420 nm and correlated to the concentration of the enzyme. From the calibration, plot activity of the enzyme was determined.

2.3.3 Thermal stability

As a result of the immobilization of enzyme the heat stability is enhanced, it is advantageous for the industrial application of immobilized enzymes and is thus important in determining the feasibility of immobilized enzymes for a particular application. Therefore, the thermal stability of free and immobilized enzymes was investigated. Free and immobilized enzymes were placed in the optimum pH buffer and incubated at different temperatures (30 to 60 °C) for different time intervals, activity of the enzyme was then determined as described earlier Thermal deactivation constant (Kd) was calculated by using the the following equation :

$\ln At = \ln Ao - Kd(t)$

where ‘Ao’ is the initial activity and ‘At’ is the activity after heat treat for minutes**.**

2.3.4 Depository Steadiness

The residual activities of the free and immobilized enzymes stored at room temperature (35°C) were determined and the activities were expressed as percentage retention of their residual activities at different times.

2.3.5 Reusable capacity of bounded Enzyme

The reusable capacity of bound enzymes are the most important factors affecting the success of industrialization of an immobilized system. To evaluate the reusable capacity of the bounded protease it was washed with water and buffered after each use and then suspended again in a fresh reaction mixture to measure the enzymatic activity. This procedure was repeated for ten cycles. The reusable capacity of bounded protease was examined by using ethanol as a substrate. Leakage of the enzyme if any was determined by measuring the enzyme activity in the washings.

2.3.6 Determination of kinetic constants

The Michael’s constant (***Km)*** and maximum reaction velocity constant (Vmax) for the free and immobilized protease were determined by measuring the velocity of the reaction.Free and immobilized enzymes in optimum pH buffer were incubated with substrates for 25 min at 30 °C. From the activity of the enzymes, ***Km*** and ***Vmax*** were calculated using the Line weaver-Burk plot of 1/s vs. 1/v.

3. Result and Discussion:

3.1 PEI concentration:

In many research as hardening agent for k-carrageenan have used KCl. But the preservation activity of enzymes and hardening was not found to be desirable. Devi found that PEI is a good hardening agent it was also found that it is superior then KCl. Hence we have used a 1.0%- 5.0 % solution of PEI for hardening of support beads. It seen that maximum hardening is achieved at 1.5% PEI concentration and beyond that, there was no further increase in hardening as well as enzyme activity.

3.2 pH Outline

Every enzyme has an optimum pH at which it shows optimum activity. **Figure 1**shows the pH activity of free and bounded . We have observed that free and entrapped enzyme was showing a maximum of 4 to 11 pH. During immobilization, results shows that there is no conformational change.

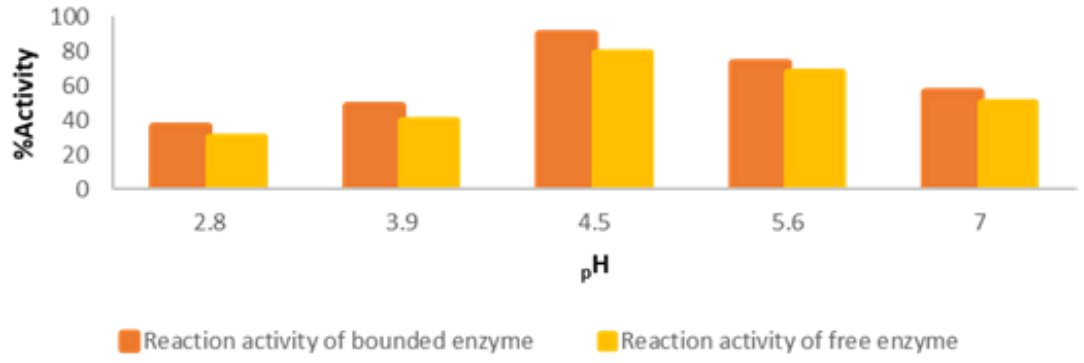


Fig:- 1 pH activity comparison graph of Protease

3.3 Thermal Stability:

Enzymes is temperature-dependent. When the temperature increases enzyme reactivity increases and beyond a definite limit the enzyme, gets deactivated. **Figure 2**shows that entrapped enzyme show better thermal stability compared to free enzyme. The entrapped enzyme showed better thermal stability as they are encapsulated within the beads.

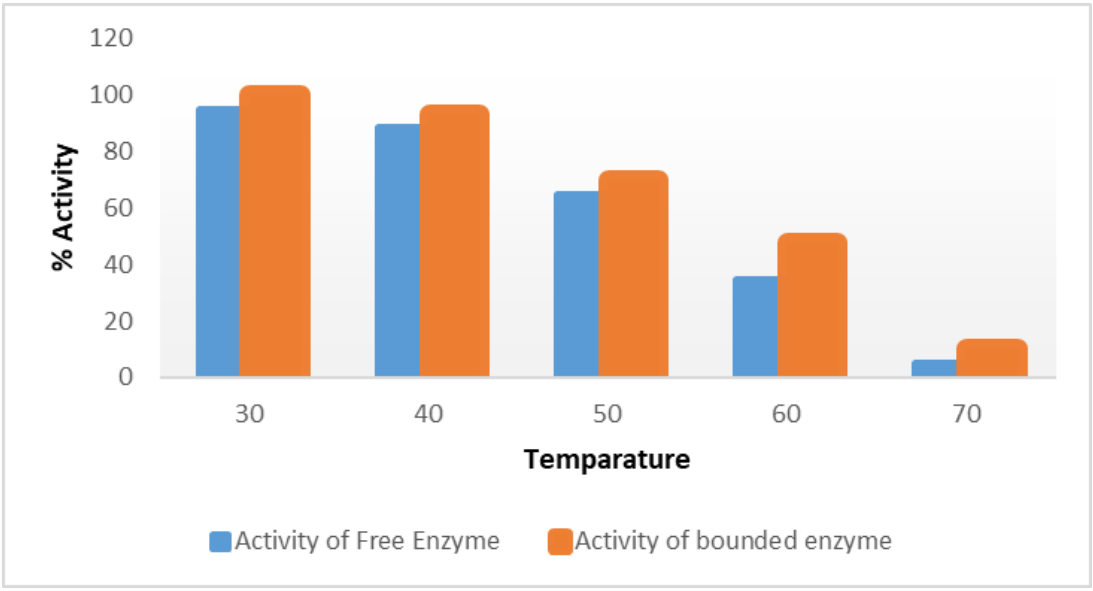


Fig:-2 Thermal stability graph of protease enzyme

3.4 Reusability of bead:

In industries and bio-chemical reaction, reusability of entrapped enzyme has great importance. It was checked by using beads in the assay method in place of free enzyme solution. The reusability of entrapped enzyme beads was checked and it was founded as shown in. bounded enzyme employed 55% of its enzyme reactivity after 5 or 6 rotations and 24 % activity after 8 cycles showing the advantage of immobilized enzyme and which increases its applicability.

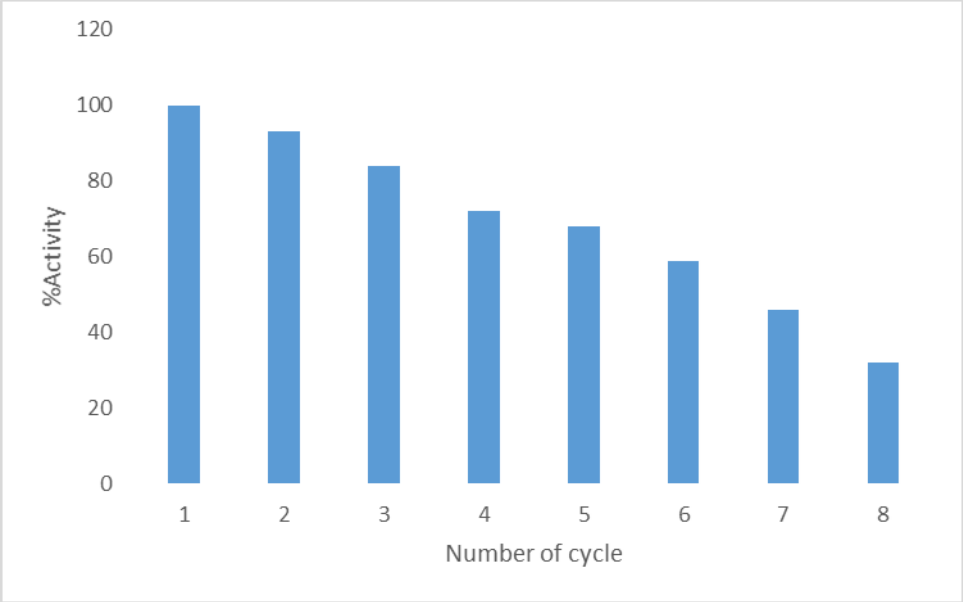


Fig:-3 Reusability graph protease

3.4 FTIR analysis

FTIR analysis of bead was done. FTIR analysis shows the chemical presence of specific chemical groups of enzyme. Spectra were recorded in the spectral range of 4000 – 500 cm-1.α-amylase have amide group in its structure. Pease peak pears at 1635.61 m^{-1} shows stretching of C=O and 1067.85 cm-1 show the bond starching vibration of amide. 3447.60 cm-1 shows OH starching vibration.

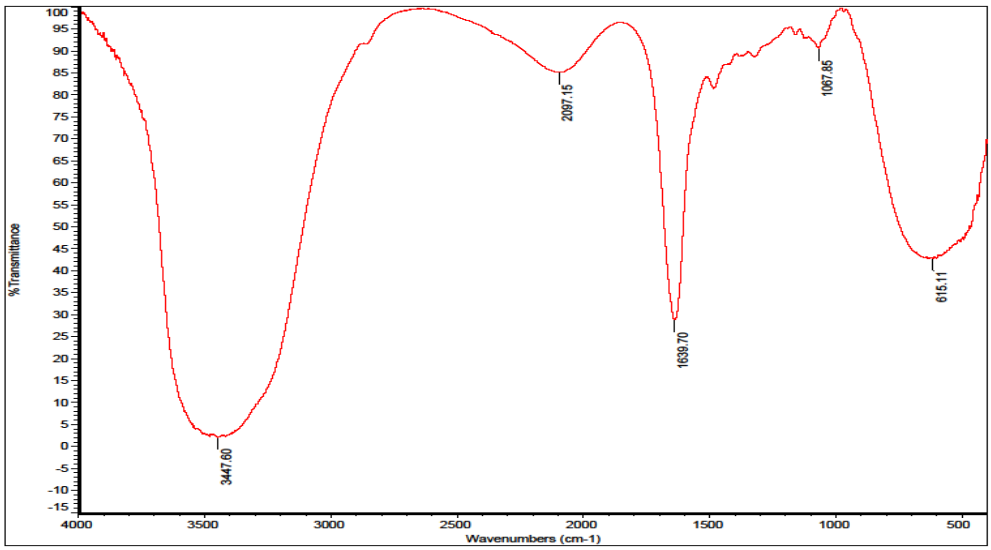


Fig:-4 FTIR of immobilized protease.

3.5 SEM analysis (Scanning electron microscopy)

SEM analysis of bead in which have enzyme was entrapped, purpose of this study was to obtain a topographical Characterization of the support beads. SEM photographs were taken using a scanning electron microscope, at required magnification at room temperature. Distance of working 9.5 mm was maintained, and the acceleration voltage used was 20.00 KV

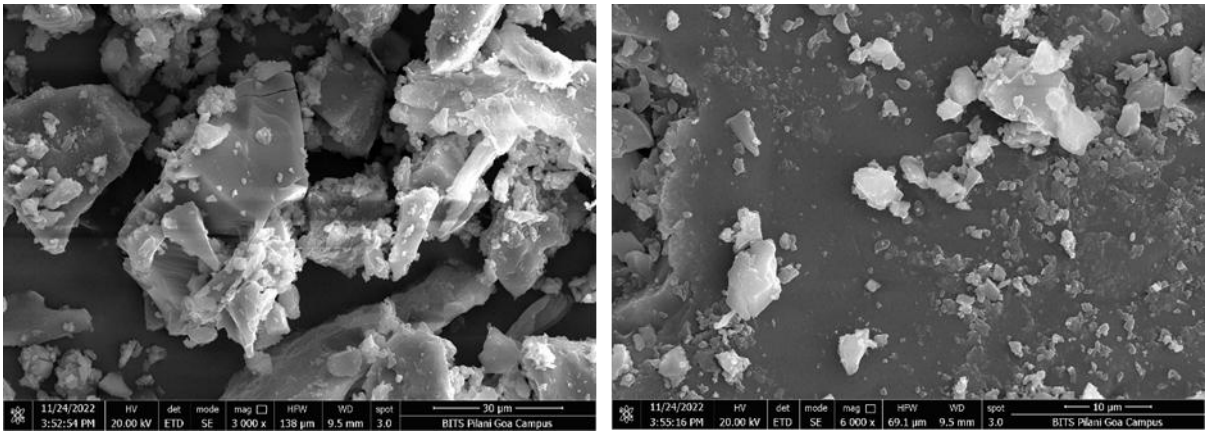


Fig:-5 SEM images of immobilized protease.

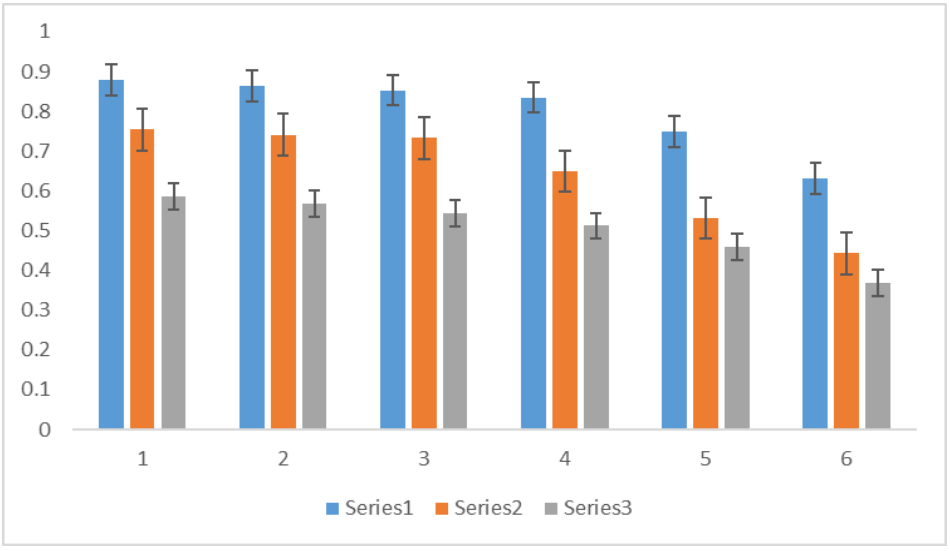
3.6 Kinetic variables:

The kinetic variables govern the reaction rate of the reaction. The following table showed the kinetic variables Kmand *Vmax*. In this research after completing all process it observed that there is not much change in the value of *Km* and *Vmax* for the unbounded and bounded enzymes.

Properties	***Free***	***Entrapped***	***Covalently bound***
Optimum pH	*8*	*7.5*	*7.5*
Optimum temperature	*60 ˚*	*65 ˚*	*65˚*
Storage stability at RT (room temperature)	*10*	*24*	*35*
Thermal deactivation constant (K_d) at 50°C	2.13×10^{-2}	1.85×10^{-2}	1.72×10^{-2}
Durability till it looses 50% activity (Cycles)	-	*7*	*6*
Michaelis Constant(Km) mm	2.59×10^{-2}	1.86×10^{-2}	1.65×10^{-2}
Maximum velocity (V_{max})(mM/min)	4.36×10^{-4}	3.69×10^{-4}	3.26×10^{-4}

4. Use of immobilized Protease in Juice purification.

Fresh pineapple juice, watermelon juice, sugarcane juice weaken. For this 5ml of juice was taken and added to the vial. Then entrapped enzymes beads were added in it after this in next step 5 ml of buffer solution was added in the test vial, and 1 ml of casein solution was added. Then vial was incubated in a shaker incubator at 35 ˚C for 30 min. after incubation 5 ml of carbonate and 1 ml of folin's reagent were added. And again kept the test vial for 30 min at 35 ˚C. Practical was performed with a free enzyme and also with entrapped enzyme. OD (optical density) was carried out for further calculation at 420 nm.



Series 1-sugarcane juice, 2-Pineapple juice , 3- watermelon juice

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6. Conclusion: -

The technology of immobilized enzymes is still going through a phase of evolution and maturation. Evolution is reflected in the ever-broadening range of applications of immobilized enzymes. Maturation is mirrored in the development of the theory of how immobilized enzymes function and how the technique of immobilization is related to their primary structure through the formation and configuration of their three dimensional structure. There remains much room for the development of useful processes and materials based on this hard-won understanding. Immobilized enzymes will clearly be more widely used in the future. This is just the beginning of the enzyme technology era.

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