International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2022): 7.942

Isolation, Characterization of Chitosan Polymer from Mulberry Cocoons for Enzyme Immobilization

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Abstract: ***Chitin is one of the world's most abundant, renewable organic resources to be synthesized and removed as by-product from various industries to the environment every year. Silk pupae are a by-product of sericulture industry which can be used in multiple industries as it is rich in oils, protein and chitin. Chitin, along with its deacetylated derivative chitosan, can be utilised to immobilise enzymes. Because chitin-chitosan has good carrier qualities, it was utilised to immobilise cellulase, a significant biodegrader of lignocellulosic materials. The immobilisation of cellulose on chitin-chitosan carrier yield was determined to be over 60% in this study, which is superior to previous immobilisation techniques. Characterization of immobilised cellulase also shows better stability over a wide range of pH (3.5 to 5.0), temperature (50 to 60°C) and reusability (up to 8 cycles) on substrate CMC. Capability of immobilised cellulase was checked on various agricultural wastes such as rice husk, wheat husk and corn cob and it was observed that the immobilised enzyme breaks down the waste to a considerable extent. Hence in comparison to native cellulase, immobilised cellulase on stable chitin-chitosan carrier seems to be a promising tool for bioremediation and large-scale application.***

Keywords: Chitosan, Cellulase, Immobilisation

1. Introduction

Chitin is a natural polyaminosaccharide that is one of the most abundant and renewable organic substances on the planet. It is a major component of crustacean shells, insect exoskeletons, and fungal cell walls, where it gives strength and stability. Chitin is expected to be synthesised and degraded in the biosphere at a rate of at least 10 metric tonnes per year. Chitin is a very firm material and it help protect an insect against harm and pressure. Depending on its thickness, chitin can be rigid and stiff in nature.

CHITOSAN

Chitosan is a natural biocompatible polymer derived from naturally-occurring bio- polymer, chitin, by deacetylation with an alkali leaving behind a free amino group (-NH_2) (. Kumar, M.N.V.R, 2000). Chitosan naturally exists only in few species of fungi but it is mainly extracted from the cuticular and exoskeletons of invertebrates like crustaceans, molluscs, crabs and shrimp. Chitosan has reactive amino and hydroxyl groups that can be chemically modified. Chitosan has unique features due to its basicity: it is soluble in aqueous acidic environments at pH of <6.5.

Chitosan is a partially deacetylated derivative of chitin, a second largest polymer next to cellulose, has emerged as biomaterial for food, pharmaceutical, waste water treatments, textile and other industries (Choi YJ, Eun jung kim et al, 2004).

Silk industry is an important agro based industry; India is the second largest producer of silk next to China (http://www.csb.gov.in/about-us/mandate/).Silkworm pupae are by-products of reeling industry, it is estimated that annually 1.5 lakh metric tons of pupae are produced which is considered as waste material. India is being one of the leading silk producers in the world which produced 14, 048 tons of mulberry raw silk, with about 10, 000 tons of silk waste during 1997-98. India produces, 1, 16, 672 tons of green mulberry cocoons out of which 20 per centis dry pupae weight.

Chitin and chitosan in combination have immense applications in various fields such as food industry, cosmetics, agriculture, water treatment, biomedicine, textile, biotechnology, paper industry; wound healing agents etc., (Kattiet al, 1996). & Chen et al, 2002).

Textile, pulp, and paper industries use hydrolytic enzymes extensively. Immobilized enzymes are defined as "enzymes that are physically restricted or localised in a particular region of space and retain their catalytic activity, allowing them to be employed repeatedly and continuously" (Katchalski-Katzir.E.1993). Immobilization allows for continued cost-effective operation, automation, and the recovery of high-purity products (D'Souza, S.F.1998). As a result, industrial demand for immobilised biocatalysts is increasing. Because of their different chemical characteristics and composition of enzymes there is no single method and support is best for all enzymes, different properties of substrates and products and different uses to which the product can be applied. Besides, every method has its own advantages and disadvantages.

Because of its inherent advantages, such as simplicity, low cost, and effectiveness, adsorption immobilisation has the upper hand over other approaches. Covalent attachment and crosslinking are effective and long-lasting, but they are costly and can readily degrade enzyme activity. Entrapment and micro encapsulation are fundamental issues in membrane reactor confinement. Therefore an optimal immobilization condition for a chosen enzyme is a critical criterion for its optimal application (Bullock C. 1995& Tischer W, Wedekind f.1999).

Chitin-Chitosan Based Immobilization

The qualities of both the enzyme and the support material influence the properties of immobilised enzymes (Kennedy JF et al, 1983). The interaction between the two lends an immobilised enzyme specific physico-chemical and kinetic properties that may be crucial for its practical application, and so a support that is carefully chosen can significantly improve the immobilised system's operational performance. Although it is acknowledged that there is no universal support for all enzymes and their applications, any material considered for immobilising enzymes should have a number of desired features.

High affinity for proteins, availability of reactive functional groups for direct reactions with enzymes and chemical modifications, hydrophilicity, mechanical stability and rigidity, regenerability and ease of preparation in various geometrical configurations that provide the system with permeability and surface area suitable for a chosen biotransformation are just a few of them. Nontoxicity and biocompatibility of materials are, of course, necessary for food, pharmaceutical, medical, and agricultural uses. Furthermore, the materials should be biodegradable and affordable in order to respond to increased public health and environmental consciousness. Chitin and chitosan are of interest since they offer most of the above qualities among the various carriers that have been examined and tested for immobilising enzymes, organic or inorganic, natural or synthetic.

Cellulase

Cellulases are refers to a group of enzymes which, acting together, hydrolyze cellulose.

Native crystalline cellulose is insoluble and occurs as fibres of densely packed hydrogen bonded anhydro-glucose chains of 15 to 10, 000 glucose units. Its density and complexity make it very resistant to hydrolysis without preliminary chemical or mechanical degradation or swelling. In nature cellulose is usually associated with other polysaccharides such as xylan or lignin. It is the skeletal basis of plant cell walls and most abundant organic source of feed, fuel and chemicals (Spano, L; Medeiros et al, 1975).

Widely used in the biotechnology industry, among other things for clarifying juices and wines, for extracting plant oils and coffee, for the bioconversion of agricultural waste (Pi, skin AK. et al, 1993) and for improving the digestibility of animal feed ingredients (Butt, M.S., et al, 2008). A major application at present is in the biodegradation or bioconversion of cellulose- and hemicellulose-containing materials to monomeric sugars (Simon, et al, 1993). Agricultural waste rich in lignocellulosic material could be used in manufacturing a whole range of commercial products including ethanol (Liu et al, 2006), Organic acids (Qu et al, 2006) and if the process were economically competitive, other chemical products (Shen et al, 2006).

Chitosan has unique chemical and biological characteristics. Chitosan has amino and hydroxyl groups that can be chemically modified in its linear polyglucosamine chains of high molecular weight. Chitosan has good gel-forming characteristics due to its solubility in acidic liquids and aggregation with polyanions (Cao et al, 1997& Dutta PK, et al, 2002). Along with unique biological properties such as biocompatibility, biodegradability to harmless products, nontoxicity, physiological inertness, remarkable affinity for proteins, haemostatic, fungi static, antitumoral, and anticholesteremic properties, chitin and chitosan, both of which are currently underutilised, have enormous potential in a wide range of applications that are expected to growrapidly.

Chitin - chitosan based materials have been used to develop numerous products for various research areas like, (Peter et al, 1995). heavy metal ions removal, dyes removal by flocculation/coagulation, membrane purification processes in waste water treatment, preservative for fat binding, animal feed additive, packaging material in food industry, fertilizer coating, controlled agrochemical release, seed in agricultural field, photographic paper in paper industry, body creams lotions, moisturizer in cosmetics and toiletries. However, because of their superior biological qualities, the most intriguing applications of chitin/chitosan-based materials are in medicine and biotechnology. They are used as bacteriostatic and fungi static agents, drug delivery vehicles, drug-controlled release systems, artificial cells, wound healing ointments/ dressings, haemodialysis membranes, contact lenses, and artificial corneas in medicine (Khor E. Chitin et al, 2002). In biotechnology, however, they can be used as chromatographic matrices, membranes for membrane separations, and, most importantly, enzyme/cell immobilisation supports.. Chitosan gels can be made in the shape of beads, membranes, coatings, capsules, fibres, hollow fibres, and sponges in this fashion.

The study was performed with the aim -Isolation of chitin & chitosan from pupae of *Bombyxmori*, Formation of chitin-chitosan enzyme carrier, Immobilization of enzyme on chitin-chitosancarrier and to characterisethe Immobilized enzyme.

2. Materials and Methods

Extraction of chitin & chitosan from *Bombyxmori*

Cocoon was collected from local market of Anekal, Bangalore, India. Pupae were separated from cocoon using sharp knife. The collected pupae wastes were then kept for drying at 70 °C for 48 hours in hot air oven. The dried pupae were than crushed to powdered form to facilitate chemical extraction of chitin and chitosan. Extraction of chitin was carried out using a sequential three step process namely Demineralization, Deproteinization andDecolouration.

Qualitative analysis ofchitosan

Bromocresol purple was used for the colorimetric assay and prepared at a concentration of 20 mg/mL in water. Chitosan samples were weighed accurately and added directly into small sintered glass funnels packed at the bottom with glass wool. The porosity of the filter pad was secured when glass wool holds chitosan. The powdered sample in each tube was then wetted with 0.2 mL of deionised water and allowed to soak for 15 minutes to allow swelling the matrix. Then approximately 0.3 mL of the dye solution was slowly passed through the sintered funnels. Each tube was then loaded with 0.2 ml of the dye solution.

Excess dye solution was drained out and washed out with 0.5 mL of deionised water followed by 95% ethanol till complete removal of all color in the wash solution.

The tube packed with chitosan-dye complex is then transferred in clean 20 mL volumetric flask. The chitosan-bound dye was then stripped off the bed by 20 ml of 1N HCl solution and completed to volume. The acid solution was filtered through a 0.45 µ membrane filter. A 5ml of aliquot was withdrawn from each sample concentration into a separate 50 ml volumetric flask and completed to volume with 1N sodium hydroxide solution. The developed blue color for each sample was measured at 589 nm(Mohamed abou-shoer et al, 2010).

Enzyme activityassay

0.5 ml enzyme was diluted in citrate buffer and added to a 25 ml test tube and pre incubated at 50°C temperature for 5 mins. 0.5 ml substrate solution was added to the enzyme, mixed well and incubated at 50°C for 30 minutes. The reaction was arrested by adding 3.0 ml DNS and kept in a boiling water bath for 5 mins. A separate test tube contains the same content without enzyme used as control. After boiling, tubes were transferred immediately to a cold-water bath and allowed to cool room temperature. The colour formed was measured at 540 nm. The absorbance of the sample was translated into glucose production using a glucose standard curve (T.K. Ghose et al, 2010).

One unit of enzyme activity (U) was defined as the amount of enzyme required to release one µmol of glucose per minute under standard assay conditions.

3. Results and Discussion

Extraction of chitosan from *Bombyxmori*

One kg of wet pupae was dried in oven at 80 °C and then powdered using mixer grinder. From the powder obtained chitin was extracted by different methods demineralization, deproteinization and decolourization. Further from the sample chitosan was extracted bydeacetylation.

Figure 1

(a) **(b)** **(c)** **(d)**

Figure 1a) Silk cocoons b) Dried pupae c) Powdered pupae d) Chitosan

Figure 2: shows the chitin-chitosan carrier obtained by adsorption process

Immobilizedenzyme

After carrier formation, enzyme was immobilized on the carrier using adsorption. The enzyme activity was performed after immobilization and was found to be 60% yield.

Characterization of Immobilized enzymes

Effect of pH on Immobilizedenzyme

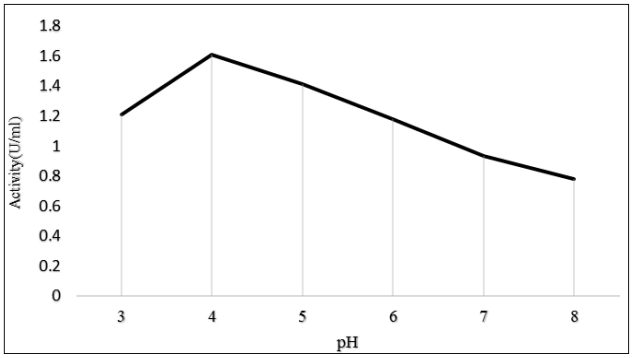

Figure 3: Activity of immobilised cellulase at different pH

Different pH range 3.0-8.0 was selected for the present study to check its stability. Results obtained showed that the enzyme was stable at acidic pH 4 (figure 3). Increasing the pH to higher range showed decreased activity. Previous studies showed that native cellulase shows higher activity at 5.0, after immobilization the activity was stable up to pH 4 which may helpful in industries. Therefore, the immobilised enzyme can be used at a lesser pH than for the native enzymes giving a wider scope for usage of the enzyme.

The optimum pH for cellulase was discovered to be 4.0, reflecting the fact that polyanionic matrices cause protons to partition between the bulk pH and the enzyme microenvironment, causing fluctuations in the optimal pH value. Changes are dependent on the immobilisation method

utilised, as well as the matrix's structure and charge (Busto et al, 1997).

Effect of temperature of Immobilizedenzyme

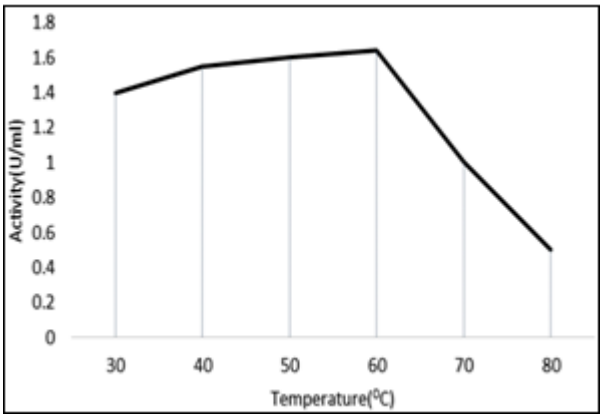

Figure 4 (a): Activity of immobilised cellulase at different temperature

Figure 4 (b): Colour development at different temperature

Different temperature range from 30-80^{0}C was selected, it was seen that the immobilised enzymes were stable from temperature 30-70^{0}C (figure 4 a). From previous studies, it was proved that native cellulase enzyme has maximum activity at 50^{0}C but after immobilisation the enzyme showed the activity highest at 60^{0}C. Therefore, the immobilised enzyme can be used at different temperatures.

This improved thermostability proved useful in later study, as enzymes could not remain stable across such a wide temperature range when immobilised in other matrices (Chen et al, 2003). According to Akkaya et al., the ideal temperature for immobilised enzyme could be higher, lower, or the same as the original enzyme.

Reusability of immobilized enzyme

The reusability of cellulase immobilise on chitin-chitosan carrier is shown in figure 5.5, it can be inferred that immobilised enzyme was found to be stable and reusable up to 8 cycles.

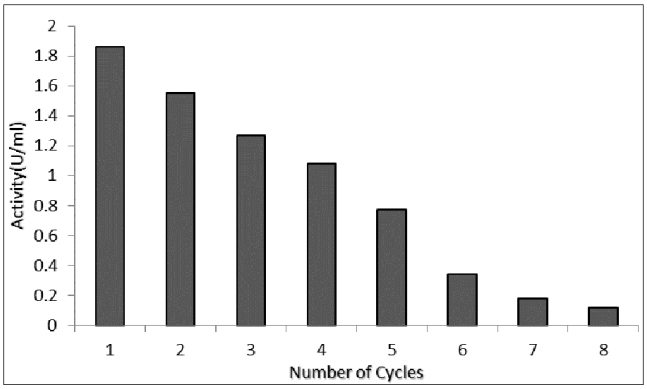

Figure 5: Activity of immobilised cellulase at different cycles.

In order to reduce the costs associated with enzymatic digestion of cellulosic material, we investigated whether immobilized cellulase could be reused for several successive rounds of hydrolysis, which would be extremely advantageous in an industrial setting The activity of immobilised enzyme was assayed for different cycles with Carboxymethylcellulase (CMC) as substrate, in order to find out the reusability of the immobilised enzyme. It is observed that enzyme loses most of its activity after the 6th cycle. This decrease in activity was due to the leakage of enzyme from the chitin-chitosan carrier, which was due to washing of the carrier at the end of each cycle.

The reusability of the chitin-chitosan carrier at different cycles using CMC as a substrate retains activity up to 6 cycles. Results of the present study shows the efficiency of adsorbed enzyme was good compared with other studies. The highest activity of enzyme immobilisation proves that there is sufficient amount of enzyme available for activity.

Wu et al, 2005recorded only 36% residual activities after 6 reuses. Cellulase entrapped in a MeTMOS/TMOS (3:1 molar ratio) made sol–gel matrix can be reused 6 times with 20 % initial activity retained. Ungurea M et al, 2013, Mubarak et al. 2014 reported that cellulase immobilized on acid pretreated MWCNTs by physical adsorption retained 26 % initial activity for the 8th recycle. In another study, 55 % initial activity was retained after 4 recycles when cellulase covalently bound to magnetic graphene nanoparticles (Gokhale et al, 2013). Cellulase immobilized on magnetic nanoparticles via covalent binding can be reused 6 recycles with 40 % initial activity retained (Abraham et al, 2014). The possible reasons of activity loss after each cycle might be immobilized enzyme loss during separation and washing processes after each cycle, enzyme denaturation, and enzyme leak (desorption)(Zangl et al, 2014).

Application of immobilisedcellulase

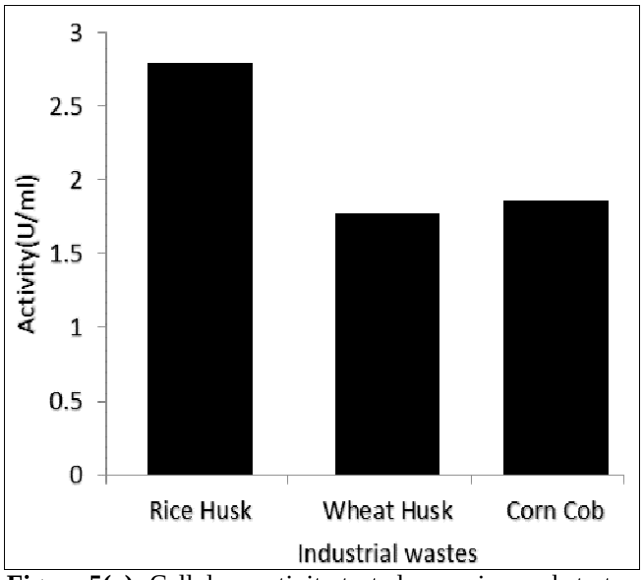

Figure 5(a): Cellulase activity tested on various substrates

Figure 5 (b): Colour development for various substrate.

The effect of immobilization on the hydrolysis of lignocellulosic biomass was tested to check its practical application. The agricultural wastes used were rice husk, wheat husk and corn cob. All three substrates were hydrolysed to glucose using immobilised cellulase. Out of all selected substrates rice husk showed highest activity followed by corn cob & wheat husk. The converted glucose can be further used for fermentation or enzyme production figure 5.

Sutarlie and Yang hydrolyzed palm fiber using hybrid cellulase aggregates with silica gel and achieved a 28% retained enzyme activity (Sutarlie l et al, 2013). Xu et al., 2011. immobilized a cellulase cocktail on magnetic nanoparticles and applied them to steam-explode corn stover. This group obtained 33% retained enzymatic activity. Additionally, Mandali and Dalay immobilized cellulases and hemicellulases on porous glass beads and hydrolyzed corn stover (Mandali p et al, 2010). The authors achieved 7-14% retained enzyme activities.

4. Summary and Conclusion

Chitin extracted from the silk pupae was converted to chitosan and used as carrier for immobilization of enzyme cellulose. The chitin-chitosan carrier tagged enzyme was showing more activity after binding with the carrier. Activity of the immobilized enzyme shows the good residual activity after different cycles at different time intervals over a period of 2 hours.

In addition immobilized enzyme with the carrier at different pH shows an optimum pH of 5.0 with a wide stability range from 4.0 to 6.5. Similarly at different temperature enzyme shows an optimum temperature of 60 °C

Enzyme activity was checked with various environmental wastes as substrate like rice husk, wheat husk and corn cob instead of CMC as its application. The carrier bound immobilized enzyme shows good activity by degrading the substrate and releasing the glucose.

Therefore, results of the present study conclude that, cellulase shows good residual activity over a wide range of pH and temperature after immobilizing with the chitin-chitosan carrier. In addition stabilization of cellulase using immobilization on chitin carrier provides certain advantage as compared to normal enzyme. Immobilization allows for easy separation of enzyme from the reaction mixture and can significantly reduce the cost of enzymes. The immobilized enzyme provides well balanced overall performance, low mass transfer limitations, high operational stability and optimal reusability. Therefore, the present results indicate that the immobilized cellulase seems to be a promising procedure for bioremediation, industrial and large-scale applications.

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