Characterization of alginate lyase and its immobilization to hydrolyze alginate

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***Declaration**:*

I certify that this thesis does not contain material which has been accepted for the award of degree of Maters of Biotechnology and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text of this thesis.

Simranjeet Kaur

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List of keywords:

Alginate

Alginate lyase

Alginate oligosaccharides (AOS)

DNS (Dinitro salicylic acid) assay

Immobilized alginate lyase

Magnetic nanoparticle (MNP)

Glutaraldehyde cross-linker

List of Abbreviations:

µL, mL	microliter, milliliter
µM, mM	micromolar, millimolar
µg, mg	microgram, milligram
µg/L	microgram per litre
mg/L	milligram per litre
g/L	gram per liter
% hydrolysis	percentage hydrolysis
°C	degree Celsius
rpm	revolution per minute
min.	minutes
h	hours
w/v	weight by volume
std. dev.	standard deviation
HPLC	High performance liquid chromatography
IU	International units

ABSTRACT:

Alginate is the most abundant polysaccharide of brown algae, which consists of two monomeric units such as β-D mannuronic acid (M) and α-L-guluronic acid (G), and these uronic acids are arranged as M-, G-, MG blocks. Alginate lyase, a polysaccharide degrading enzyme can degrade alginate by hydrolyzing glycosidic bond and produce unsaturated oligosaccharides (AOS) that have wide application in food, nutraceuticals, and pharmaceutical industry. Immobilized enzymes facilitate industrial applications owing to their stability, reusability, and cost-effectiveness. This study investigated kinetic characterization of soluble and immobilized alginate lyase on magnetic nanoparticles. Alginate lyase immobilization was achieved using glutaraldehyde as cross-linker to a magnetic nanoparticle. Scanning electron microscopy (SEM) validated structure of nanomaterial and binding to alginate lyase. Free and immobilized enzyme exhibited different temperature optima at 37 °C and 45 °C respectively. The optimum pH was shifted from 7.4 for soluble to 9 for immobilized alginate lyase, which confirms the stability of the immobilized form. The immobilized alginate lyase exhibited thermal stability at 45 °C and retained more than 60% activity after 3 h. The reusability of immobilized alginate lyase was analyzed for 8 consecutive cycles and 50% activity was recorded till 6^{th} cycle. This study further compared the enzymatic activity of hydrolyzing alginate from various commercial sources. The soluble and immobilized enzyme demonstrated 76% and 63% of hydrolysis of raw seaweed biomass in 60 h. The immobilized enzyme demonstrated enhanced thermal and pH tolerance, extended storage stability, and reusability fulfilling proposed objectives of the study. HPLC profile exhibited monosaccharide yield with soluble (20.5%) and immobilized (17.6%) alginate lyase.

Chapter 1

INTRODUCTION

1. Introduction

Alginate lyase is the chief enzyme of interest found in this study, which follows β-elimination mechanism of hydrolyzing a hetero-polymer alginate. Thus, the enzyme is named as alginate lyase or alginases or alginate depolymerases (Wong *et al.*, 2000). The structure of the alginate comprises of two monomeric units such as β-D mannuronic acid (M) and α-L-guluronic acid (G), is arranged in specific configurations and form homo-oligomeric/hetero-oligomeric domains by the linkage between MM, GG and MG blocks and fabricate a linear polysaccharide (Peng *et al.*, 2018). The classification of alginate lyase is described below on the basis on selective hydrolysis of monomeric units and thus exhibit substrate specificity. They can be poly-Mannose lyase (M) or poly-Glucuronide lyase (G) as depolymerize specific subunits.

An important biotechnology application for alginate lyase is to obtain the alginate oligosaccharide (AO) by the depolymerization of alginate, In addition, alginate lyase could be used as (i) biochemical in biofilm degradation of mucoid *P. aeruginosa* for the treatment of cystic fibrosis patients, (ii) extraction of DNA like intracellular bioactive materials from the brown algal cells and (iii) for the structural and functional analysis of alginate and preparation of algal protoplasts. Alginate compounds being bioactive and profitable biomaterial, have significant applications in numerous industries (Batista *et al.*, 2019). Because of the spectacular physical properties of the compounds, such as mechanical stiffness, ability to form hydrogels, interfibre self-bonding, they are being used as an emulsifier, gelling agent and viscosifier in the food and beverage industry. Being functional bioactive compound, they are being used in pharmaceutical industries apart from printing and textile industries (Xing *et al.*, 2020). The expansion of the application of alginate lyase has been possible due to the recombination technology. Cloning of alginate lyase gene into the *E.coli* and other bacteria has facilitated the structural and functional analysis of this gene to utilize the advantageous

physicochemical properties (Jiang *et al.*, 2019). For the improvement of hydrolysis mechanism of alginate lyase, the hydrolysis of the polymer can be controlled, and the desired oligosaccharides can be obtained which can be potentially used as biologically active compound. There are numerous biological applications which are being exhibited by the oligosaccharide's products obtained by the depolymerization of alginate through alginate lyase. These active compounds can show the bacteriostatic, antioxidant activity, anti-tumor, immunomodulation properties and the promotion of plant growth, prevention of dental caries apart from food, agriculture and medical industries (Cheng *et al.*, 2020a). The low molecular weight oligosaccharides obtained by the depolymerization of alginate are functional carbohydrates of industrial interest. Because of the optimistic application of these compounds, they are significantly involved in the food, dietary supplements, personal care, beverage, and livestock feed products. Fibers and dietary ingredients are the components of functional foods with the market revenue of USD 1.42 billion. Moreover, the enhanced awareness about these functional carbohydrates has an anticipation of growth in the market up to USD 9.84 billion till 2024. However, there is the limited bioavailability of low molecular weight oligosaccharides of interest and the limitation for the commercial application is because of the high viscosity of the alginate. Thus the alginate polymer can be sliced into low molecular weight oligosaccharides (Peng *et al.*, 2018). Also, the production of defined product by controlling the hydrolysis of the product was the next step to the challenge (Kruschitz *et al.*, 2020).

The conducted study depicts the sources, structure, classification, functions, its biochemical properties and potential applications of enzyme. The study was conducted for the characterization of alginate lyase enzyme and its immobilization onto a nanoparticle. Then the hydrolysis of alginate was analyzed by both forms for the controlled hydrolysis of polymer to obtain the desired oligosaccharides and to investigate its biological application.

1.1 Significance of the study

Diverse applications of alginate-based products have necessitated the elucidation of hydrolysis pattern of alginate by the lyase enzyme, which directs for the expansion of its applications in varied fields. There are number of methods for the hydrolysis of alginate into oligosaccharides, however, physical and chemical methods are expensive and certain limitations are also associated (Liu *et al.*, 2019). Among the three, enzymatic method is opted to be the efficient due to accuracy and less consumption of energy, chemical and other inputs. Although, the major issue with the enzymatic hydrolysis of alginate is the high price of alginate lyase (Nair *et al.*, 2007). As 100 mg of commercial alginate lyase costs 300 AUD, it is highly expensive to obtain oligosaccharides by this method. Immobilization of the enzyme onto a nanoparticle is the promising approach for the revolution of enzymatic depolymerization of alginate (Li *et al.*, 2020b). This involves the attachment of enzyme on an inert surface to conduct multiple reactions thus facilitating its reuse. The properties of the nanoparticle enable the enzyme to be separated from the reaction mixture and ensures its reusability and storage. The immobilization of enzyme also strengthens the thermal stability of the enzyme and thus making it highly efficient and reliable (Jiang *et al.*, 2020). Using immobilized enzyme for the alginate hydrolysis will be cost-effective approach to fulfil the emerging demand of functional carbohydrates and oligosaccharides.

1.2 Objectives

- Biochemical characterization of soluble alginate lyase for the hydrolysis of alginate
- Immobilization and characterization of alginate lyase on a nanomaterial
- Use of soluble and immobilized alginate lyase for hydrolysis of raw seaweed biomass for alginate oligosaccharides (AOs) production.

Chapter 2

LITERATURE REVIEW

2. Literature review

2.1 Sources of alginate lyase

Alginate lyase can be obtained from naturally occurring bacteria, algae, molluscs and echinoderms in marine environment and to obtain nutrition by degrading alginate. Apart from that, alginate lyase was also obtained from hepatopancreas of certain marine species. *Klebsiella, Vibrio, Flavobacterium, Pseudomonas, Streptomyces* Nitrogen-fixing bacteria*, Enterobacter, Bacillus* etc. are the most widely used bacterial species for the alginate lyase study and to commercially obtain the enzyme (Fig. 2.1). Apart from all this, to obtain the higher yield of product, enzymatic method is proved as the most reliable, cost-effective and efficient technique (Cheng *et al.*, 2020a). There are number of companies across the world, where commercial alginate is available. SIGMA-Aldrich, Thermofisher are the companies located within Australia which extract enzyme from marine and terrestrial viruses, fungi and bacteria. International companies like Megazyme which supplies the alginate lyase (from *Sphigmomonas* sp.) in the form of powder (5000 units) costs €152.00. With the growth of the potential market of seaweeds derived products, seaweeds processing technologies are growing, yet the enzyme-cocktail for the hydrolysis of seaweed polymers into fermentable sugars is yet to be commercialized. Alginate lyase is the chief enzyme of that cocktail for complete depolymerization of complex polymer of alginate (Arntzen *et al.*, 2021).

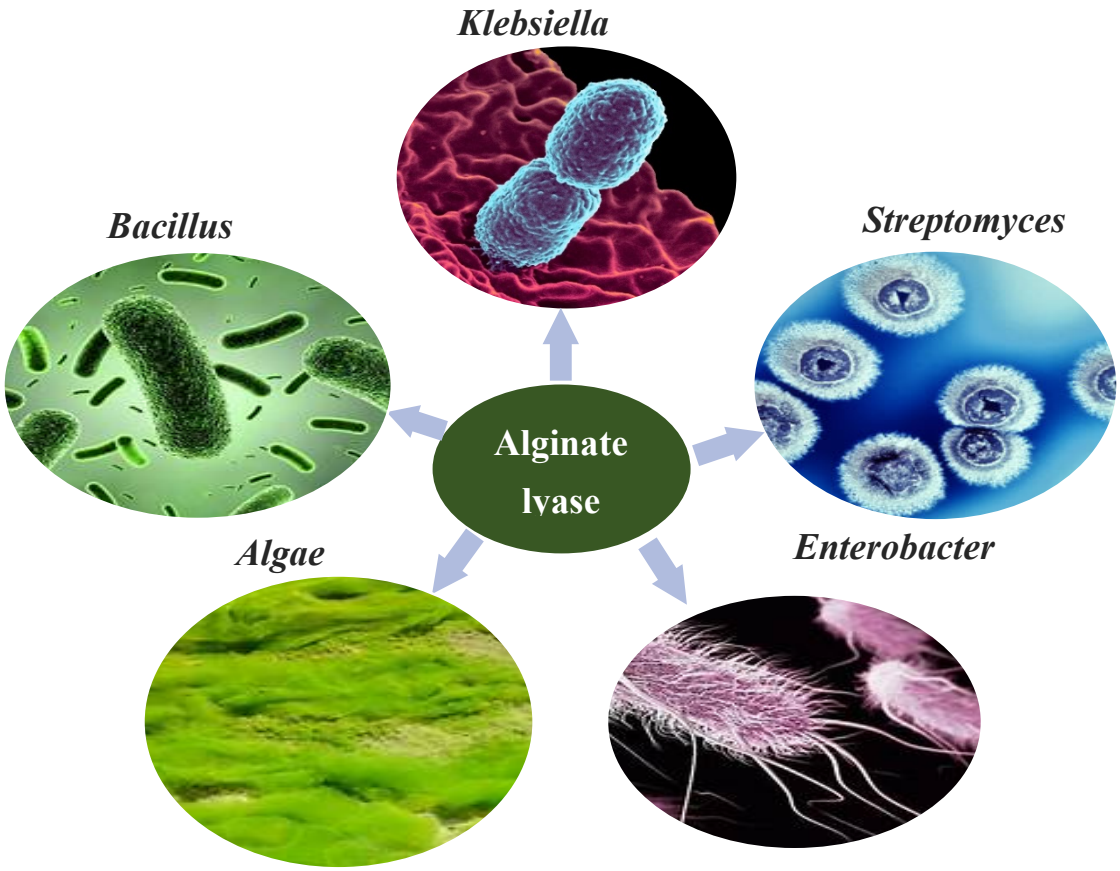

Fig. 2.1 Different sources of alginate lyase (Cooke et al., 2017; Yan et al., 2019)

2.2 Sources of alginate

The molecular weight of commercially available alginate ranges from 33,000 and 400,000 Da. Alginate is the structural component of cell wall in brown algae (*Phaeophyceae*) and intracellular gel matrix. Naturally, 40% of dry weight of brown seaweeds comprises alginate, thus abundantly present in marine biomass. *Laminaria* and *Microcystis* are the most significantly used marine algal species for the commercial production of alginate (Fig. 2.2). The different alginates obtained from various species or different tissues varies in terms of composition of monomers or the sequential structure of alginate. Alginate compounds can also be obtained from bacterial cells as these compounds present naturally to adapt in adverse environment. Particularly, acetylated (C2 /C3 hydroxyl group of M residue unit) alginate can be found in *Azotobacter* and *Pseudomonas* strains (Xue *et al.*, 2019)

However, production of alginate from these species is limited to small scale as this is the part of cyst formation. Alginate is the polymer which maintains the stability of biofilms and cell hydration in the bacterial cells apart from protecting the cells from the unfavorable conditions of the environment like toxicity of metals. As Alginate exhibit ion-exchange specifically with the Ca^{2+}, thus the Pseudomonas aeruginosa If there is the scarcity of the species for the alginate, apart from prior mentioned species, *Sargassum, Ecklonia, Lessonia* species can also be utilized for the alginate however, the quality will be compromised (Liu *et al.*, 2019) Commercial alginate and alginate based products are being supplied by companies like Dalchem and the price ranges as per the product type. For instance, chromatic Alginate, it costs $25.85 and for plaster bandage its $9.85. The alginate exists as the salt derivatives i.e. Sodium alginate, Calcium alginate, Potassium alginate, Ammonium alginate, Alginic acid (Gomes *et al.*, 2020). There is the involvement of seaweed and alginate derived products in plants-based food materials, cosmetics, pharmaceutical products, and personal care products. Thus seaweeds are considered as potential renewable resource for the production of biofuels and numerous bioactive compounds, the anticipation in the enhancement of global market of seaweed cultivation from 2020 (16.7 USD) to 2025 (30.2 USD) is almost double (Arntzen *et al.*, 2021).

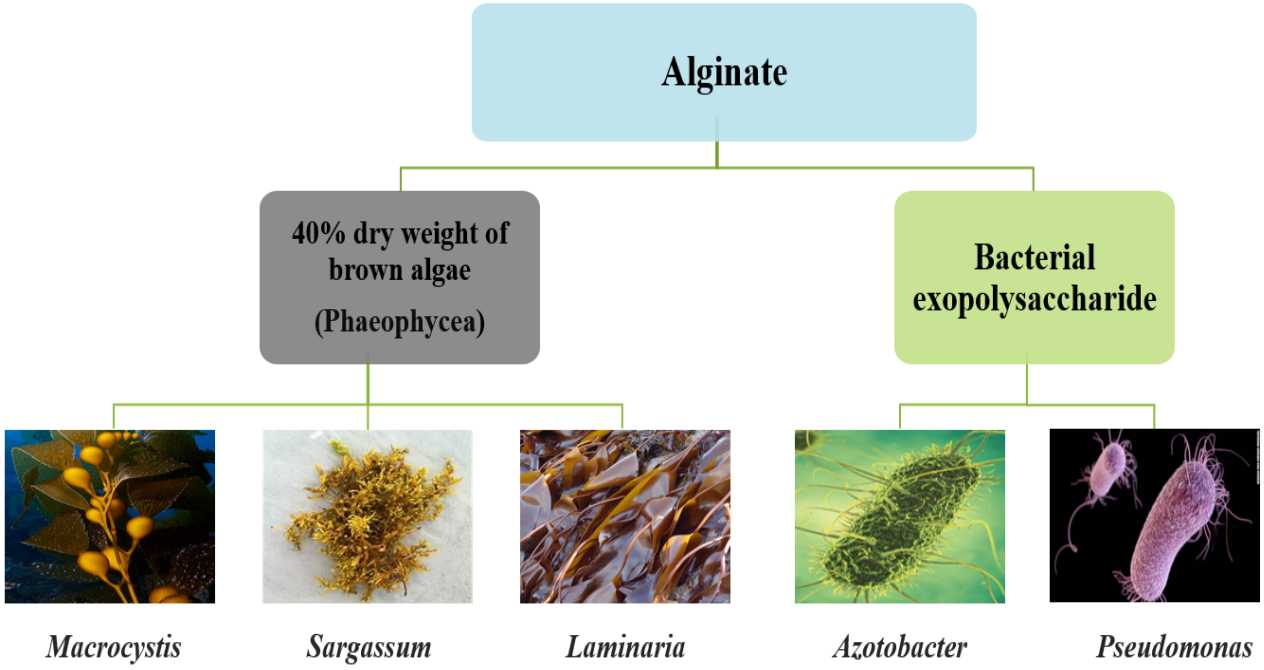

Fig. 2.2 Different sources of alginate (Brown algae: Macrocystis, Sargassum, Laminaria and Bactria: Azotobacter, Pseudomonas)(Belik et al., 2020; Kuznetsova et al., 2020; Littler et al., 2010)

2.3 Structure of alginate

The mannuronic acid (M) and glucuronic acid (G) linked in different specific manners to exhibit the complex polymeric configuration. The basic difference in mannuronic acid (M) and glucuronic acid (G) units is of conformation however they are epimers with difference on C5. Thus, the conformation of pG (polymer) is like an egg box and exhibits α-1,4-glycosidic linkage however, due to the β-1,4-glycosidic linkage in pM (polymer), the conformation resembles to that of belt chains due to the intramolecular hydrogen binding (Fig. 2.3). Among naturally occurring polysaccharides, only alginate is the marine biopolymer which possess carboxyl group in all sugar rings (Xing *et al.*, 2020). Hence, the degradation of the complex copolymer alginate is mediated by the alginate lyases by the

β-elimination of glycosidic bond mechanism (Fernando *et al.*, 2019) and the oligosaccharides can be obtained as products at non reducing end in the form of unsaturated sugar units (Peng *et al.*, 2018). Alginate form aggregates by interacting with ions in which both charge, and radius of the ion affect the structure of aggregates. More radius and valency of the cations exhibit greater strength and tends to arrange the alginate chains tightly (Wang *et al.*, 2021).

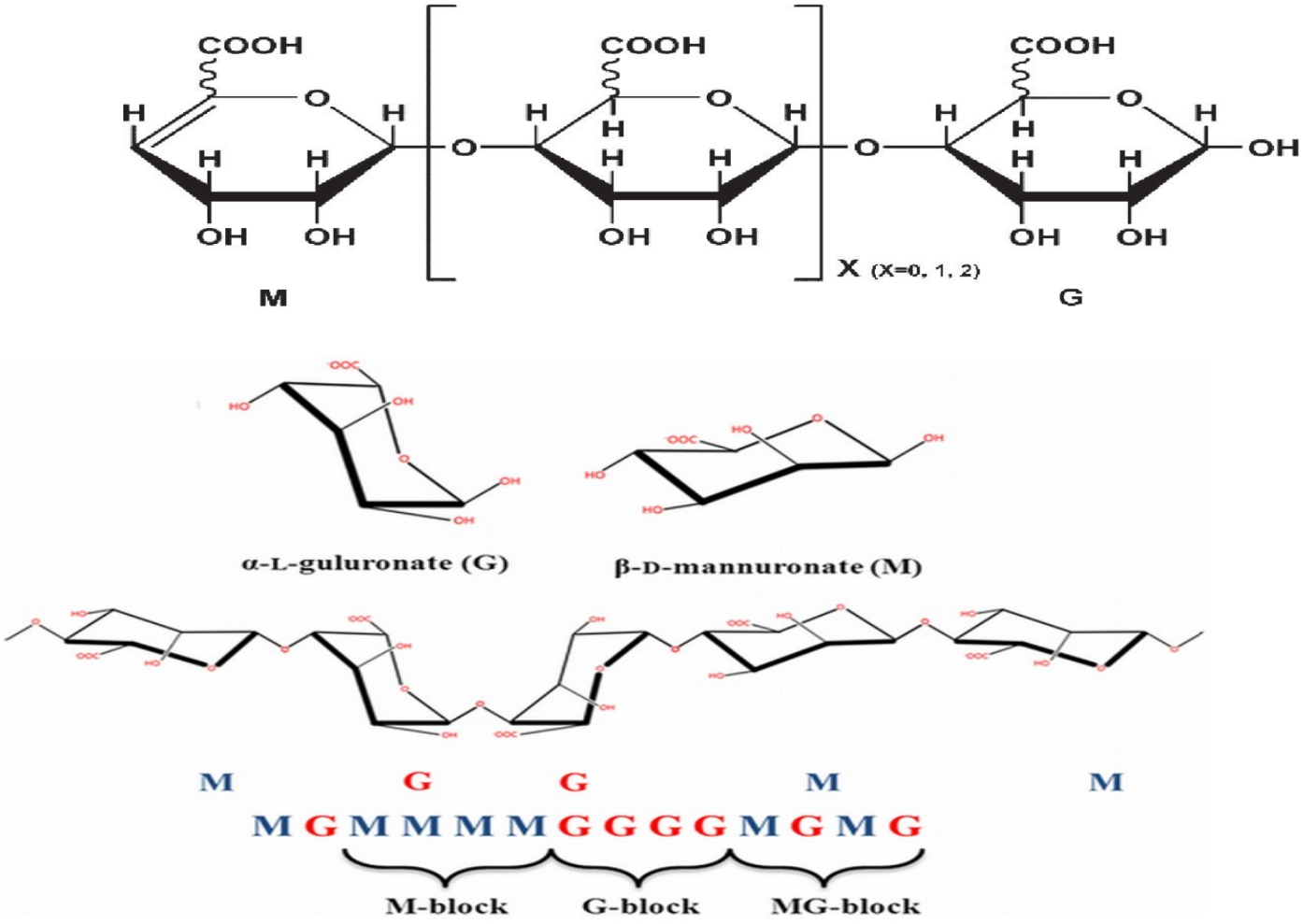

Fig. 2.3 Chemical structure of subunits of alginate: 1,4- α-Guluronic acid (monomer unit of G) and 1,4-β-D-mannuronic acid (monomer unit of G) linked by β-1,4 glycosidic bond (X= number of monomers) (Ming et al., 2021; Bhujbal et al., 2014; Eskens et al., 2021)

2.4 Methods to hydrolyze alginate

2.4.1 Chemical methods

In the chemical methods, various chemicals can be used to depolymerize the alginate polymer however, HCl and H_2O_2 are being used prominently for this purpose. Because of the resistance of acid hydrolysis of alginate, reaction is carried at very high temperature (95 to 121 °C) and for longer duration of time (80 to 180 min.). However, the source of alginate has a significant impact on the temperature and time to carry out the reaction. "Green process" is the depolymerization of alginate using H_2O_2, as the by-product produced will be water and synthesis of other unwanted impurities can be prevented (Ahmed *et al.*, 2016). However, the hydrolysis of polymers with the chemicals may give rise to unwanted by-products and may leads to impurities in the mixture. Moreover, it impacts the chemical composition of the substrate by breaking and forming bonds between various atoms.

2.4.2 Physical methods

Gamma, ultrasonic, and ultraviolet rays also used for the depolymerization of alginate out of which, gamma radiations are considered to be best in terms of efficiency of energy and oligosaccharides with various molecular weights can be thus obtained by this method (Liu *et al.*, 2019). However, radiation induced therapy is highly expensive (El-Mohdy, 2017).

Moreover, hydrothermal treatment, subcritical water hydrolysis, plasma treatment are effective methods to degrade alginate. Reactive species produced by the electrical discharge in a solution plasma process leads to the scission of the polysaccharide chain of alginate. In hydrothermal method, cleavage of glycosidic bond is the main target which give rise to lactic acid and glycolic acid (Jeon *et al.*, 2016). However, there may be the cracks in the structure due to the change in the tensile strength and physical pressure. Also, it can deliberately impact the molecular weight of the polymer and thus structure as well.

2.4.3 Enzymatic methods

The enzymatic method is the most significant method for the alginate depolymerization as compare to physical and chemical method. Due to the greater efficiency and less energy consumption, this method is more beneficial and doesn't utilize large amount of itself into the reaction for the hydrolysis of alginate (Liu *et al.*, 2019). Alginate lyase is the polysaccharide lyases used to depolymerize the alginate by the β-elimination of 1–4 O-linkages in alginate. As the enzymes are highly specific as per their mode of action so the product will be highly specific and accurate. Also, there is very less consumption of energy, chemicals and other related components associated with the reaction thus pocket friendly and reliable (Nair *et al.*, 2007). As this method does not produce any type of impurities or unwanted products, thus environment-friendly and reliable in terms of purification and downstream processing. There are no strict requirements of the chemicals and high energy consumption as the product can be obtained by maintaining the pH and temperature to carry out the normal enzymatic reaction (Liu *et al.*, 2019). This method is most effective as this can enhance the yield to a significant extent.

2.5 *Classification of alginate lyase*

To prepare the functional oligosaccharides of alginate, the alginate lyase is of greater interest as it can be obtained naturally and can be used in industry for the improved production of antibiotics with enhanced efficiency. Classification of enzyme depends on the factors to be considered i.e., depending upon substrate specificity, molecular weight, mode of action, hydrophobic clusters etc. Alginate lyase exhibit substrate specificity in the degradation pattern thus can be classified as poly-Mannose lyase (M) or poly-Glucuronide lyase (G) (Fig. 2.4). However, bifunctional lyases also exists which can potentially degrade both the polymers and thus possess more efficiency and applications. As per the

mode of action, the alginate lyase can be categorized as exo-alginate lyase and endo-alginate lyase. The exo-alginate lyase removes the monomer/ dimer or oligosaccharide from the terminal position at alginic acid and thus the reduction in the viscosity is slow and the rate for the synthesis of reducing sugar is opposite (faster) (Xue *et al.*, 2019). By the action of endo- alginate lyase, different range of polymers can be obtained as monomer, dimer, trimer, tetramer, pentamer etc. as this enzyme cleaves the 1,4-O glycosidic bond within the alginic acid linear polymer chain thus the viscosity of the solution decreases quickly but the reducing sugar synthesis rate is slow (Belik *et al.*, 2020). Based on the hydrophobic clusters of alginate lyase, it can be classified into seven families as PL-5, PL-6, PL-7, PL-14, PL-15, PL-17 and PL-18, out of which PL-5 and PL-7 are endo- alginate lyase (Zhu *et al.*, 2015a) and PL-15 and PL-17 families exhibit exo -alginate lyase properties. Apart from this, the classification can be based on the molecular weight of the protein of enzyme: the enzyme with molecular weight ranges between 20-35 kDa is categorized as small enzyme, with the weight more than 40 kDa is medium enzyme and more than 60 kDa is large enzyme (Xue *et al.*, 2019).

2.6 Enzymatic properties of alginate lyase

It has been revealed with the studies conducted, the alginate lyase varies in terms of products degradation, molecular size, substrate specificity with the unique enzymatic properties. In general, the molecular weight of the alginate lyase is 24 kDa –110 kDa (Zhu *et al.*, 2015a) and the optimum pH for the enzymatic activity is 7.0-7.6 however, the majority of them catalysis the reaction under acidic conditions (Gao *et al.*, 2018). They are some inducible alginate lyase detected in B. circulants, which exhibit hydrolysis of alginate however, constructive enzymes also exist (Wong *et al.*, 2000). Alginate lyase shows the maximum enzymatic activity between the temperature of 30 ºC to 50 ºC however, very less amount of alginate lyase depicts the optimum activity at temperature above 50 ºC

(Zhu *et al.*, 2015a). As the alginate lyase is not stable on high temperature, the catalysis carried out by this enzyme is not conducted at favorable higher temperature. There are metal ions, chelators and denaturants which exert some effects on the activity of alginate lyase as like other enzymes. Some metal ions like Ca^{2+}, Na^{+}, Mg^{2+}, K^{+} and lower concentration (<0.3mol/L) of sodium chloride intensify the enzymatic activity of alginate lyase however, Fe^{2+}, SDS, Mn^{2+}, Fe^{3+}, Cu^{2+}, EDTA Hg^{2+}, Ba^{2+}, $Zn^{2+}$and higher sodium chloride concentration (>1.0mo1/L) exhibits opposite effect and inhibit the alginate lyase activity differently (Gao *et al.*, 2018).

2.7 Mechanism of alginate lyase action

Alginate lyase follows the β-elimination reaction mechanism and leads to the cleavage of 1→4 glycosidic bond of alginate. With the depolymerization of alginate, between the non-reducing ends C4 and C5 of the product, there is the synthesis of uronic acid of 4-deoxy-L-erythro-hex-4-enopyranosyluronic oligosaccharide with unsaturated double bond. This oligosaccharide shows the absorption maxima at 230-240nm. The specificity of the enzyme catalyzed breakage is the double bond (Liu *et al.*, 2019).

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Original figure available online from the publisher:
https://www.tandfonline.com/doi/figure/10.1080/21655979.2015.1030543?scroll=top&needAccess=true

Fig. 2.4 The action of different lyases on the bases of substrate: Poly M lyase cleaves the bond between two M monomers, Poly MG lyase cleaves the bond between MG monomers, Poly G lyase cleaves the bond between two G monomers (Zhu et al., 2015b).

2.8 Detection of alginate lyase activity

Qualitative assay is required to determine the enzymatic activity of alginate lyase as this can be analyzed by digesting the substrate alginate through alginate lyase enzyme. Plate assay is one of the traditional methods which is conducted in order to determine the alginate lyase producing species along with the enzymatic activity analysis (Dharani *et al.*, 2020).

2.8.1 Assay for screening and detection of alginate lyase: Quantitative analysis

2.8.1.1 Plate Assay

The assay involves the culturing of the bacteria onto the solid medium containing alginate (substrate) and detection of the bacterial colonies producing alginate lyase enzyme. The cells which can digest alginate can survive and can be determined by the clear zones. The plates containing the alginate lyase producing cells can be flooded with CPC (Cetyl Pyridinium Chloride) which interacts with alginate. The clear zones will be produced by the alginate lyase producing cells due to the hydrolysis of alginate. Ruthenium dye can be used as an alternative as it indicates colonies of light pink color against dark red background (Dharani *et al.*, 2020). Thus, the native or heterologous test can be conducted for the presence of alginate lyase along with the presence of enzyme in soluble enzyme extract. It has been revealed in the experiments that instead of agar the better clarification and purification of the enzyme can be obtained in the agarose medium with the enhanced visualization of enzyme. The activity of alginate lyase can be detected and estimated by the potential of the bacteria in terms of release and secretion of the enzyme. To detect the alginate lyase activity and specificity of substrate, the alginate substrate can be substituted with poly (M) and poly (G) polymers (He *et al.*, 2018). An assay was conducted by using 1.5% agarose and 1% G rich sodium alginate obtained from *Laminaria hyperbola* for the detection of overexpression of activity of G specific lyase in *E. coli*. The

gene for lyase was cloned from *Klebsiella pneumoniae* into *E. coli.* 95% ethanol was used to flood the colonies and clear zones were observed around the bacterial colonies producing lyase against the milky opaque alginate precipitates. Diluted HCl or $CaCl_2$ can also be used to precipitate the undigested lyase however, for the better visualization of activity of alginate lyase, charcoal can be added into the culture medium tubes or plates (He *et al.*, 2018).

2.8.1.2 Turbidimetric assay

This is also for the qualitative assay of enzymatic activity which depends on the development of white turbidity in the acidic solution by the combination of acidic mucopolysaccharides with bovine albumin. However, plate method is for solid medium. The depolymerization of alginate by the alginate lyase inhibits the turbidity development. To detect the numbers of forms of lyase in crude bacterial extract, isoelectric focusing and substrate-overlay technique can be used based on which the identification of alginate lyase can be done (Chen *et al.*, 2020).

2.8.2 Quantitative analysis of activity of alginate lyase

This method is to determine the concentration of protein (enzyme) purified. The assay is based on the colorimetric method to estimate the protein concentration.

2.8.2.1 TBA assay

Numerous assays can be used for the qualitative assay of alginate lyase activity profitably. Thiobarbituric is one of the colorimetric assay based on the measurement of the absorbance of the product at 548nm (Hatch *et al.*, 1998). The action of alginate lyase on alginate produces unsaturated uronic acid and in this assay the enzyme units can be calculated as micromoles of β-formyl pyruvate produced per minute per milliliter of enzyme (Cho *et al.*, 2016).

2.8.2.2 BCA Assay

BCA assay is the enzymatic assay for the determination of concentration of protein (enzyme- alginate lyase). It is a colorimetric method to measure the concentration of protein as per the change in the color. The sample changes its color from green to purple and the concentration can be measured with the intensity of the color changed. The reduction of the copper ion takes place by the peptide bond of the protein. The amount of Cu^{2+} reduced is proportional to the amount of the protein (Fig. 2.5). Then the molecules of bicinchoninic acid will be chelated with the Cu^{+} ion and will synthesize a purple-colored complex which intensively absorb light at 562 nm. The presence of cysteine/ cystine, tyrosine, tryptophan side chains have a greater impact on the formation of bicinchoninic acid Cu^{+} complex. The quantitative analysis of the amount of protein present can be measured based on the absorption spectra and comparing with the proteins solution of known concentrations (Olson, 2016). BCA kit (Takara Bio) is available commercially which can be used for the qualitative determination of protein sample (Hatch *et al.*, 1998). Bovine Serum Albumin (BSA) is also the similar method used to plot the standard curve for the protein concentrations (with the different dilutions of BSA) and estimate the protein concentration (Li *et al.*, 2019).

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Fig. 2.5 Reaction involved in the BCA Assay for the estimation of Alginate lyase activity (www.quora.com)

2.8.2.3 Uronic acid assay test

Uronic acid enzyme assay is the spectrophotometric method to analyze the enzymatic activity. Hydrolysis of alginate by enzyme leads to the synthesis of unsaturated oligosaccharides. There is the detection of double bond formed in the C4 and C5 of reducing sugar which shows absorption maxima at 235 nm due to the unsaturated oligosaccharides. Thus by estimating the formation of double bonds, the synthesized product can be calculated by using spectrophotometric method (Jiang *et al.*, 2019; Ghose, 1987; Bisaria *et al.*, 1981). However, this method is not preferred because the number of double bonds formed are not directly proportional to the absorbance, thus the analysis of catalytic sites or the study of kinetics cannot be conducted (Inoue, 2018).

2.8.2.4 DNS Assay

DNS (Dinitro salicylic acid) assay is the most significantly and widely used assay for the analysis of enzymatic activity. In this assay, the DNS reagents reacts with the reducing sugar released as a product in a hydrolysis reaction which intensifies the yellow color of DNS reagent into orange and red and dark red as per the amount of sugar produced (Fig. 2.6). Thus, the standard curve can be prepared against glucose by measuring absorbance of glucose concentrations treated with DNS reagent at 540 nm. The enzyme can be incubated with the substrate and the enzyme activity can be determined by the estimation of saccharide molecules being produced by the hydrolysis of alginate through the alginate lyase. The sugar molecules being synthesized will interact with the DNS reagent to change the color and the enzyme activity can be calculated (Sun *et al.*, 2020b).

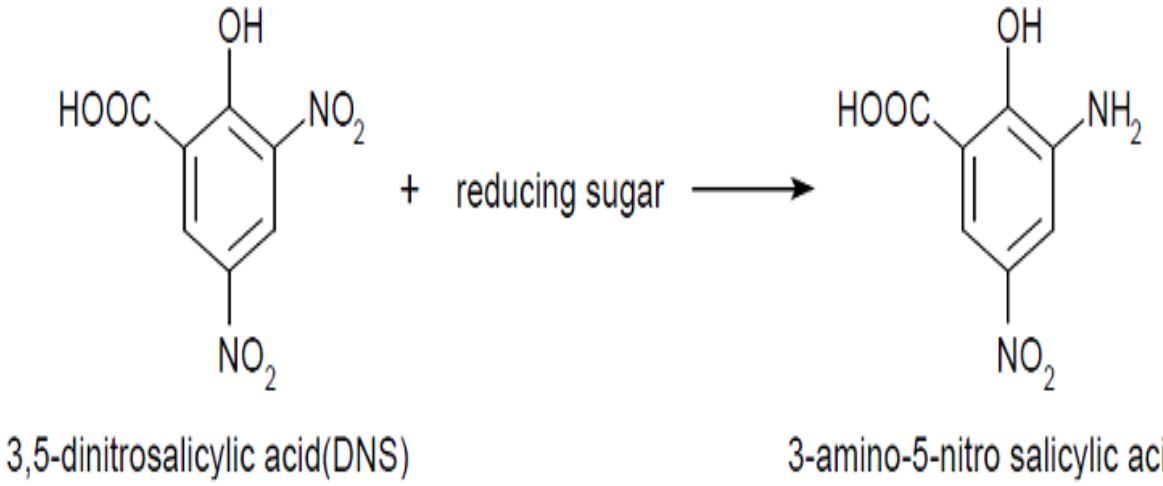

Fig. 2.6 Reaction involved in the DNS assay for the estimation of enzymatic activity (www.igem.org)

2.9 Immobilization of Alginate Lyase

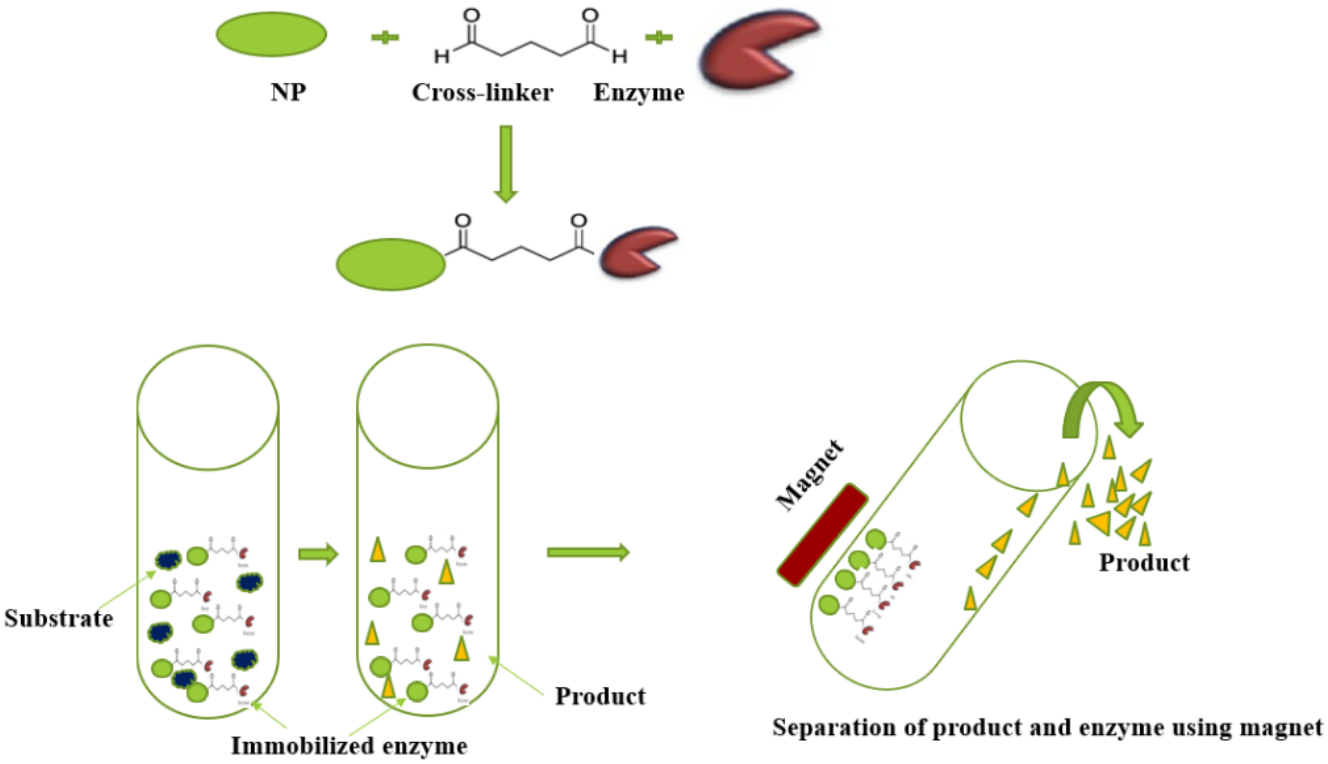

Fig. 2.7 Stepwise immobilization of alginate lyase and its use in alginate hydrolysis.

Because of the high content of polysaccharides in the seaweed, it is considered as the effective natural source to produce valuable bioactive compounds. Seaweeds are fastest growing organisms in nature without the input of fresh water, chemicals, land, fertilizers, and pesticides (Arntzen *et al.*, 2021). Due to the depletion of non-renewable sources, they are the future alternative source of chemicals, biofuels, and single cell proteins. The polymer alginate is the cellular structural component of these organisms which can produce fermentable sugars as bioactive products (Arntzen *et al.*, 2021).These bioactive compounds are extremely demanding in food, pharmaceutical, medical, clinical, drugs, textile, agricultural and many other industries. Thus, the alginate from the seaweed is hydrolyzed to produce functional desired oligosaccharides of interest. However, due to the heterogenous and unique carbohydrate structure of cell-wall of seaweed, it is complicated to hydrolyze the alginate which constitutes 40% of dry weight of brown seaweeds (Fauziee *et al.*, 2021). Hence, the production and harvesting cost of the alginate oligosaccharides is extremely high. Physical and chemical methods are highly energy intensive and leads to the production of lots of waste and toxic compounds thus not considered as environment friendly. Also, there is the adverse effect of these treatments on the nutritional value of the bioactive compounds (Liu *et al.*, 2019). Thus, the only method which is promising is the microbial enzymolysis. Because of the efficient depolymerization of alginate polymer using alginate lyase with the mild conditions is the significance reason of opting the enzymatic method. The major issue with the enzymatic method is about the cost as the commercially available enzyme is expensive (Costa *et al.*, 2021). To enhance the economic viability and reusability of the enzyme, immobilization revolutionized this aim. Immobilization enhanced its biochemical properties by providing reusability in multiple reactions and improved its resistance to towards temperature and thermal stability. The activated nanoparticle provides support by the covalent binding with the strong cross-linking. Various magnetic nanoparticles can be used for the

immobilization of the enzyme (Refer to Table 2.1). The activation of nanoparticle was achieved using the glutaraldehyde cross-linker which binds to the enzyme. Magnetic properties of the nanoparticle facilitate the separation of the immobilized enzyme from the reaction mixture for the reuse (Fig. 2.7).

Table 2.1 Different nanoparticles studied for the immobilization of alginate lyase

	Nanoparticles	**Efficiency**	**Applications**	**References**
1.(a)	Chitosan (glutaraldehyde cross-linked)	79.3% retainment of alginate lyase activity low K_m (1.21 mg/mL) and a high V_{max} (11.3 U/mg-protein	Pharmaceutical, food, agriculture, bioenergy, medical and diagnostic industries Antimicrobial therapy and cystic fibrosis	(Mohapatra *et al*., 2020)
1.(b)	Low molecular weight chitosan	Enhanced thermostability and retained activity of alginate lyase (80% till 6 cycles)	Antibiofilm activity	(Li *et al*., 2019b)
2	Iron-oxide (superparamagnetic material for recombinant alginate lyase)	Repeat 10 cycles	Bioenergy, diagnostic and medical industry	(Shin *et al*., 2011)
3	Silica (hybrid magnetic)	Repeat 10 cycles	Bioenergy, diagnostic and medical industry	(Shin *et al*., 2011)
4	Cellulose acetate membrane Ultrafiltration (dead end filtration)	80% retainment of activity after 21 days	Water filtration	(Meshram *et al*., 2016)
5	Ciprofloxacin (high methoxyl pectin as and alginate as matrix)	Stability of enzyme and antibiotic	Medical and pharmaceutical (cystic fibrosis)	(Islan *et al*., 2013)

	Nanoparticles	Efficiency	Applications	References
6	High-molecular-weight poly (L-lactide) (PLLA) and poly (D-lactide) (PDLA)	Operational stability on mechanical support, immobilizing efficiency	Medical and diagnostic and pharmaceutical industries	(Boi *et al*., 2019)
7	Polydopamine mediated (polycarbonate coated by polydopamine)	Impair adhesion of *P. aeruginosa*	Biomaterial, Medical, Interventional therapies	(Alves *et al*., 2016)
8	CLEA (cross linked enzyme aggregates) Using ammonium sulphate and alginate beads	Carrier free biocatalyst, very high catalytic activity per unit volume, enhanced volumetric productivity, versatile and reusable catalyst	Pharmacy, medicines, drug/protein delivery, tissue regeneration, cell encapsulation, surgery, and wound management	(Kunjukunju *et al*., 2018)
9	Mesoporous titanium oxide particles (MTOPs)	Retained 45.4% activity at 55.4 ºC and reused 10 times	Biomedical applications	(Li *et al*., 2020)

2.10 Applications of alginate derived products

Alginate lyase being able to degrade the alginate as a substrate, produces oligosaccharides which are the fragments of small sugar molecule chains. The reaction involves the acid hydrolysis, enzymatic mediated hydrolysis, and pyrolysis. Lyases enzyme so called alginate lyase participate in the catalysis of degradation reaction of long chain alginic acid into the smaller molecules in the enzymatic hydrolysis (Zhu *et al.*, 2020; Bambace *et al.*, 2019) .There are benefits of the enzymatic catalyzed breakdown of alginate into the oligomers (Zhu *et al.*, 2016a) as this is the highly efficient and there is requirement of mild conditions to conduct the reaction along with the strong controllability and this is the environment friendly approach without pollution (Mahajan *et al.*, 2020). Alginate and its oligosaccharides exhibit tremendous applications in countless fields. Biomaterial science and

nanomaterials based on alginate are the basis of the drug delivery and medicinal industry. The oligosaccharides of alginate show the versatility due to the structural and functional biocompatibility. Due to the properties like non-toxic, safe, inexpensive, high gelling and cross-linking properties, physicochemical properties of molecular weight and viscosity, it has necessitate the increasing demand of alginate and biocatalysts for its degradation (Abraham *et al.*, 2020).

2.10.1 Food Industry:

Fig. 2.8 Application of oligosaccharides in food industry: animal food, food additives (www.foodchem.com, www.21food.com)

The oligosaccharides (AO) obtained from the brown algae are being used a preservative and additive to fortify the nutritional value and to enhance the taste. This is used for the protection of food against

the physical, chemical, or biological adverse effects. As the alginate retains the beneficial effects of food quality and food safety for prolonged period by extending the shelf life. Currently, alginate is acting as an essential ingredient in the packaging industries as it can be used as a preservative (Bambace *et al.*, 2019). By considering alginate as a renewable and bio-degradable material for the packaging industry and environment friendly approach, this natural polymer is significant (Fig. 2.8). Alginate is coated on the edible material as an edible film or thin layer (0.3mm thick) to fortify the natural nutrients and is consumable with the food (Table 2.2) (Khalil *et al.*, 2017).

Table 2.2 Alginate based edible film and coatings EC: Edible (Coating, EF: Edible film).

Food material	Coating material	Antimicrobial	Outcome
Fresh-cut apple	alginate/$CaCl_2$ (EC)	thyme oil	Yeast and mold growth were inhibited by EC-thyme oil significantly
Strawberry	alginate	carvacrol, methyl cinnamat	Carvacrol inhibited both *E. coli* and *B. cinereal*, on the other hand, methyl cinnamate was effective only against B. cinerea
Capsicum	alginate/CaCl2 (EC)	pomegranate peel extract	Antimicrobial and antifungal activities were shown by EC-pomegranate peel.
Smoked salmon	alginate (EF)	sodium lactate, sodium diacetate, commercial formulation	The growth of L. monocytogenes during cold storage was inhibited by EC-antimicrobials and the microbial shelf life was prolonged during frozen storage.

2.10.2 Pharmaceutical industry

Fig. 2.9 Applications of oligosaccharides in Pharmaceutical and Medical industry (Gheorghita Puscaselu et al., 2020)

In the pharmaceutical and medical field, the oligosaccharides are being used as detoxifying agent, intestinal conditioning agent, blood and lipids control agent, anti-inflammatory, immune regulator, anti-bacterial, anti-diabetic, along with the properties to deal with rectal colon cancer, habitual constipation obesity (Fig. 2.9) (Xing *et al.*, 2020). Alginate beads are used as a carrier for the delivery of drug in the human body effectively. This enhances the surface area of the active compound by coating onto its surface and water holding capacity as well. This improves the specific activity of the

coated bioactive substance thus exhibit biomedical, biocatalytic and pharmaceutical fields (Kim *et al.*, 2017). Alginate oligosaccharides are nonimmunogenic, nontoxic and alginate is the biocompatible material and cannot be digested in the mammalian digestive tract due to the lack of alginate lyase enzyme (exist in prokaryotic organisms). Alginate derivatives possess antiviral, antibacterial, antifungal activities, thus extensively used in the pharmaceutical and medical industry (Wróblewska-Krepsztul *et al.*, 2019). Being negatively charged, alginate interacts with the outer bacterial membrane and disrupt the cell, causes the leakage of intracellular material out of the cell. It also inhibits the transportation of nutrients inside the cell by forming a viscous thick layer surrounding the cell. Alginate chelation causes the production of toxins inside the cell (Szekalska *et al.*, 2016). Sulphate derivatives of alginate polysaccharides and fractions containing alginic acid exhibit antiviral properties on numerous families of viruses-*Togaviridae, Herpesviridae, Flaviviridae, Rhabdoviridae* and thus beneficial to cure the viral infections. This can be linked to the interaction of sulphated alginate (negatively charged) with the cell of host (positively charged) and thus blocks the entry of virus unto the host cell and prevent viral infection. It was also revealed by a study that the macrophages efficacy can be enhanced for the production of cytotoxic molecules and cytokines by the polymer with more M content as they act as immunostimulant (Wróblewska-Krepsztul *et al.*, 2019). In wound dressing, calcium alginate is being used as it stimulates the platelets and thrombin production and function. Calcium acts as antagonist for the calcium channels and lowers the blood pressure down. Sodium alginate is used to control the blood pressure (to treat the hypertension). It also reduces the glomerular filtration rate thus prevent the early-stage injury to kidney. Potassium derivatives of alginate protects the human body from cardiovascular disorders (Szekalska *et al.*, 2016). The alginate oligosaccharides suppress the synthesis of Reactive Oxygen Species (ROS), nitric oxide, prostaglandin, and cyclooxygenase. This is by the secretion of anti-inflammatory cytokines by

monocytes stimulation exhibited by the alginate with high mannuronic content. Moreover, the chelating effect of the alginate enables the molecule to bind to the heavy metals and toxins and thus prevent the carcinogenesis process (Wróblewska-Krepsztul *et al.*, 2019).

2.10.3 Waste management

Alginate matrix is used for the removal of radioactive waste from the environment. Treatment of the waste with the alginate is a toxic free and inexpensive method. Because of the higher adsorption capacity, it is used to remove the heavy metals by the carboxylate function of the polymer. It makes the recovery and isolation highly favorable as it is a bio-sorbent and acts as photocatalyst as well. Apart from that, it is the valuable encapsulation material as used in the form of beads (Majidnia *et al.*, 2015).

Chapter 3:

MATERIALS AND METHODOLOGY

3. Materials and methodology

3.1 Materials

3.1.1 Chemicals, Buffers and Reagents

All the required components in this study such as Alginate lyase (*Flavobacterium sphingomonas*), Sodium alginate, Trizma® base, Protein assay kit (Folin & Ciocalteu's phenol reagent), 5-Dinitrosalicylic acid DNS, Calcium chloride, Cobalt bromide, Copper sulphate, Glucose, Iron chloride, Magnesium sulphate, Manganese chloride, Monopotassium phosphate, Potassium sodium tartrate tetrahydrate, Sodium chloride, Sodium Hydroxide, Zinc sulphate, Magnetic Nanoparticle were precured from Sigma Aldrich Pty. Ltd., Australia. Glutaraldehyde (Merck, VIC 3153) was used for immobilization.

For characterization of soluble enzyme, Incubator (Ratek Instruments PTY LTD, Victoria, Australia), Hot Plate (S.E.M. SA PTY LTD, Magill, South Australia), Vortex Mixer (Ratek Instruments PTY LTD, Victoria, Australia), Weighing balance (SB12001, Mettler Toledo, Victoria, Australia), Magnetic stirrer (IKA® RCT, Victoria, Australia), Omega Plate reader (Ω) (FLUOstar Omega, BMG Labtech, , Victoria, Australia), Laminar flow (Clyde- Apac air filtration, Minto, New South Wales) instruments were used.

For the immobilization of alginate lyase, instruments used were Freeze-drier (VirTis Benchtop K BTEKEL quantum Scientific, Kingston, New York), HPLC (UFLC XR SHIMADZU, Mundelein, USA), SEM Scanning electron microscope (FEI INSPECT F50, Bath, UK) and Sonicator (SONICS Vibra cell ™, Newtown, CT, USA).

3.1.2 Seaweed biomass

Dried *Bull Kelp* (*Durvillaea potatorum*) seaweed collected from Rivoli Bay, Beachport, South Australia (latitude: 37°30′55″S; longitude 140°4′17″E in March 2013) was provided by Dr Reinu E. Abraham. The samples were processed to produce a fine powder based on an optimized protocol (Abraham *et al.*, 2019).

3.2 Methodology followed during the proposed research work

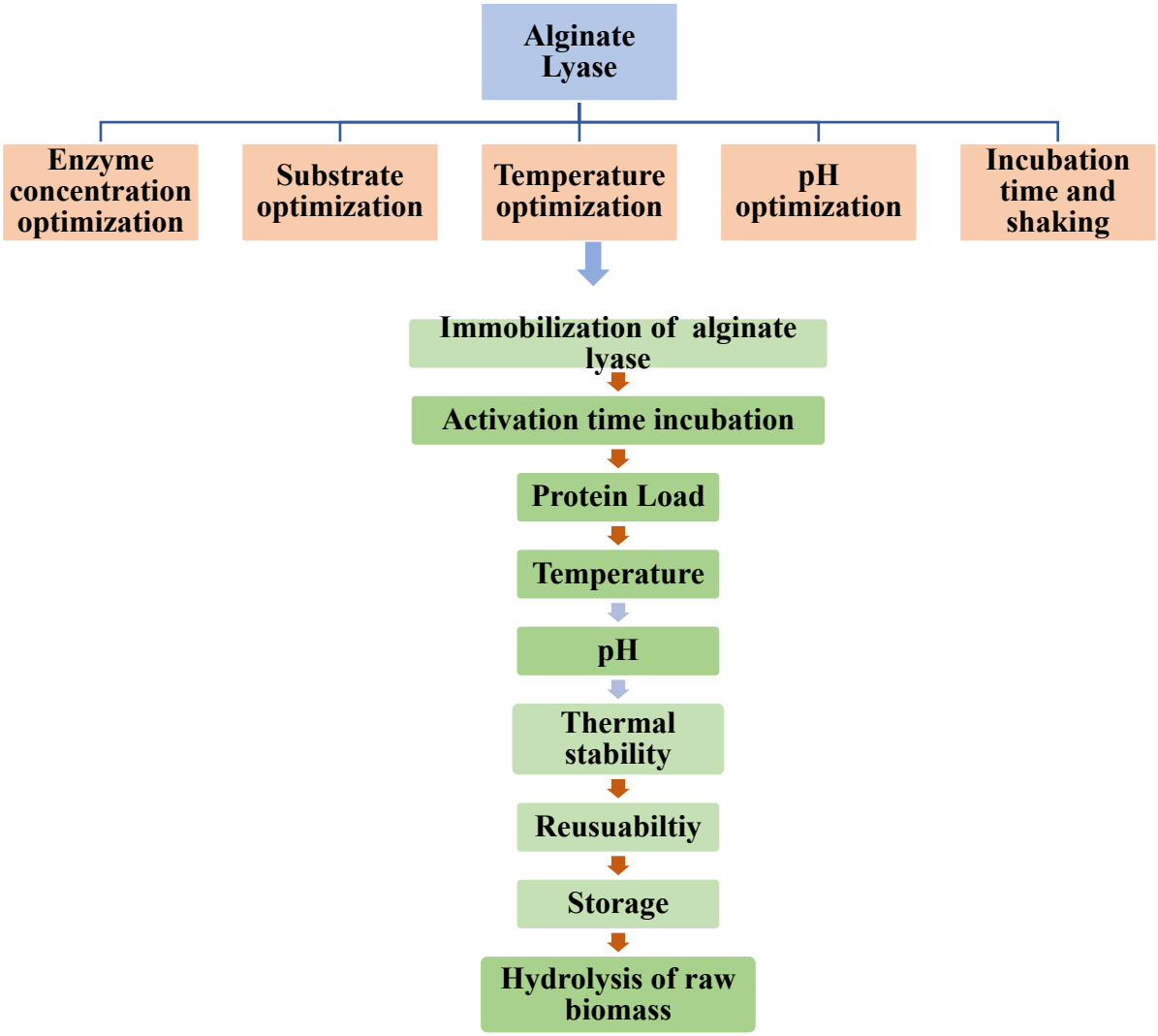

Fig 3.1 Outline of the methodology followed for alginate hydrolysis

3.2.1 Enzyme activity assay

Activity of alginate lyase was determined by 3,5- DNS (dinitro salicylic acid) (Sun *et al.*, 2020a). This is based on the principle of reduction of DNS to 3-amino 5-nitro salicylic acid by the reducing sugar released in the reaction or the oxidation of aldehyde/ ketone functional groups of reducing sugar which strongly absorbs light at 540 nm. Enzyme (25 IU) was incubated with the substrate (0.5% w/v) for 2 h under standard assay conditions. After incubation, DNS reagent was added, and reaction mixture was heated in boiling water for 10 min. It was cooled down to room temperature and the absorbance was recorded on a spectrophotometer at 540 nm (Yang *et al.*, 2019). The percentage hydrolysis was estimated by measuring the amount of reducing sugar produced per mg of sodium alginate added. For the immobilized alginate lyase (equivalent soluble enzyme units), the maximum substrate hydrolysis observed was considered as control based on which the relative activity was calculated. The values were expressed as representation of mean ± standard deviation (n = 3).

3.2.2 Reducing sugar determination

To determine the reducing sugar released by the hydrolysis of alginate, glucose standard curve was prepared, and the absorbance was recorded for the unknown to calculate the product formed. Percentage hydrolysis of sodium alginate was calculated (Bouhadir *et al.*, 2001).

$$\textit{Percentage hydrolysis (\%)} = \frac{\textit{Reducing sugar released (product in mg)} \times 100}{\textit{Sodium alginate added (substrate mg)}}$$

3.2.3 Enzyme Activity (IU/ml)

Activity of alginate lyase was calculated as the amount of reducing sugar released per mL of the reaction volume per unit time in min (Gudkov *et al.*, 2020).

$$\textit{Enzyme Activity (IU/ml):} \frac{\textit{Reducing sugar released (product) (µmoles/ml)}}{\textit{Time (min.)}}$$

3.3 Characterization of soluble Alginate Lyase

3.3.1 Effect of Enzyme concentration

Briefly, 100 µL enzyme with different concentrations i.e. 3, 5, 7, 10, 25, 50, 75 and 100 IU was incubated with 1 mL of sodium alginate (1% w/v) substrate (dissolved in 20 mM Tris buffer (20mM pH 7.4) for 2 h at 37 °C. DNS assay was performed and the absorbance was recorded at 540 nm (Zhu *et al.*, 2016b). The percentage of hydrolysis was estimated by the amount of reducing sugar released per mg of substrate added for different enzyme units.

3.3.2 Effect of substrate concentration

The substrate concentration was investigated by incubating 25 units of enzyme with different substrate concentrations i.e. 0.5% , 1%, 1.5%, 2%, 2.5% and 3% w/v (weight/ volume) at 37 °C for 120 min (Yang *et al.*, 2018; Zhu *et al.*, 2016b) and the reducing sugar released was determined by measuring the absorbance at 540 nm with DNS method.

3.3.3 Effect of temperature

The effect of temperature on the enzyme activity was anticipated for different temperatures i.e., 30, 35, 40, 45 and 50 °C. The activity of the enzyme was estimated by incubating 100 µL of enzyme (25 IU) and 1 mL (1.5% w/v) of substrate for the percentage of substrate hydrolysis at different temperatures (Manns *et al.*, 2016; Wang *et al.*, 2020) and it was calculated by recording the absorbance at 540 nm. After optimizing the wide range, the enzyme was also tested for narrow range of temperature i.e., 35, 36, 37, 38, 39 and 40 °C and DNS assay was performed to estimate reaction rate.

3.3.4 Effect of pH

The effect of pH for alginate lyase was investigated at various pH values i.e., 6, 7, 8, 9, 10 and 11 using 20 mM of Tris- base at 37 °C. The enzyme was tested for pH range of where maximum activity was observed. to be most active between the range of pH 7 to 8. The enzyme was further tested for narrow pH range 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9 and 8. (Wang *et al.*, 2019; Chen *et al.*, 2016; Zhuang *et al.*, 2018) DNS assay was performed, absorbance was recorded at 540 nm to calculate the percentage hydrolysis.

3.3.5 Effect of shaking and incubation time

To analyze the effect of shaking and incubation time, the enzyme 25 IU in tris base was incubated with 1mL of 1.5% (w/v) alginate, incubated with shaking speed of 0, 50 and 100 rpm to analyze its effect and for different incubation time periods (1h, 2h) (Li *et al.*, 2018).

3.3.6 Effect of metal ions

In order to determine the effect of different metal ions (monovalent, divalent and trivalent) (Yang *et al.*, 2020; Zhou *et al.*, 2020) on the activity of alginate lyase, 25 units of enzyme (50 µL in tris buffer) were incubated with 1 mL of substrate and the metal ions (50 µL, 1 mM) (Wang *et al.*, 2020) like K^{+}, Na^{+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Cu^{2+}, Mg^{2+}, Co^{3+}, Fe^{3+}, SDS and EDTA (Ma *et al.*, 2020), for 2 h. The DNS assay was performed to analyse the effect of these ions on the percantage of hydrolysis of alginate.

3.4 Immobilization of alginate lyase

3.4.1 Activation of nanoparticle

The nanoparticles (Iron oxide 8365W) for immobilization were sourced from Professor Youhong's Lab (Institute of Nanoscience Technology, Flinders University). Strong magnetic nanoparticle was used for the immobilization process (Abraham *et al.*, 2014). The nanoparticle was first suspended in milli-Q water and then sonicated for 30 min to homogenize the particles and to obtain a uniform size (Abraham *et al.*, 2014). To determine the incubation of glutaraldehyde with the nanoparticle, 1mL of glutaraldehyde was mixed per mL of nanoparticle (5 mg/mL in milli-Q water) and incubated for different time (30, 60, 90, 120 and 180 min). Then the enzyme was immobilized onto the nanoparticle and analyzed for the hydrolysis of alginate (Dal Magro *et al.*, 2020). The washing of the nanoparticle was done twice with the milli-Q water and once with the Tris buffer (20 mM, pH 7.4).

3.4.2 Immobilization of enzyme: Protein load

The immobilization of alginate lyase was followed (Abraham *et al.*, 2014). The activated nanoparticle and enzyme were incubated in the water bath for 2 h at 37 °C. The protein load was analyzed by

incubating different enzyme units (1, 10, 50, 100, 150, 200 and 250 IU) with 5 mg of nanoparticle. The enzyme linked to nanoparticle was washed twice with the water and once with the tris buffer to remove any unbound or loosely bound protein (Joseph *et al.*, 2020). To estimate the binding efficiency, the supernatant from the washing step was used for the estimation of unbound protein using Lowry method. The protein binding efficiency was estimated.

$$\textit{Binding Efficiency (\%)} = \frac{\textit{Total amount of protein bound} \times 100}{\textit{Total amount of protein added}}$$

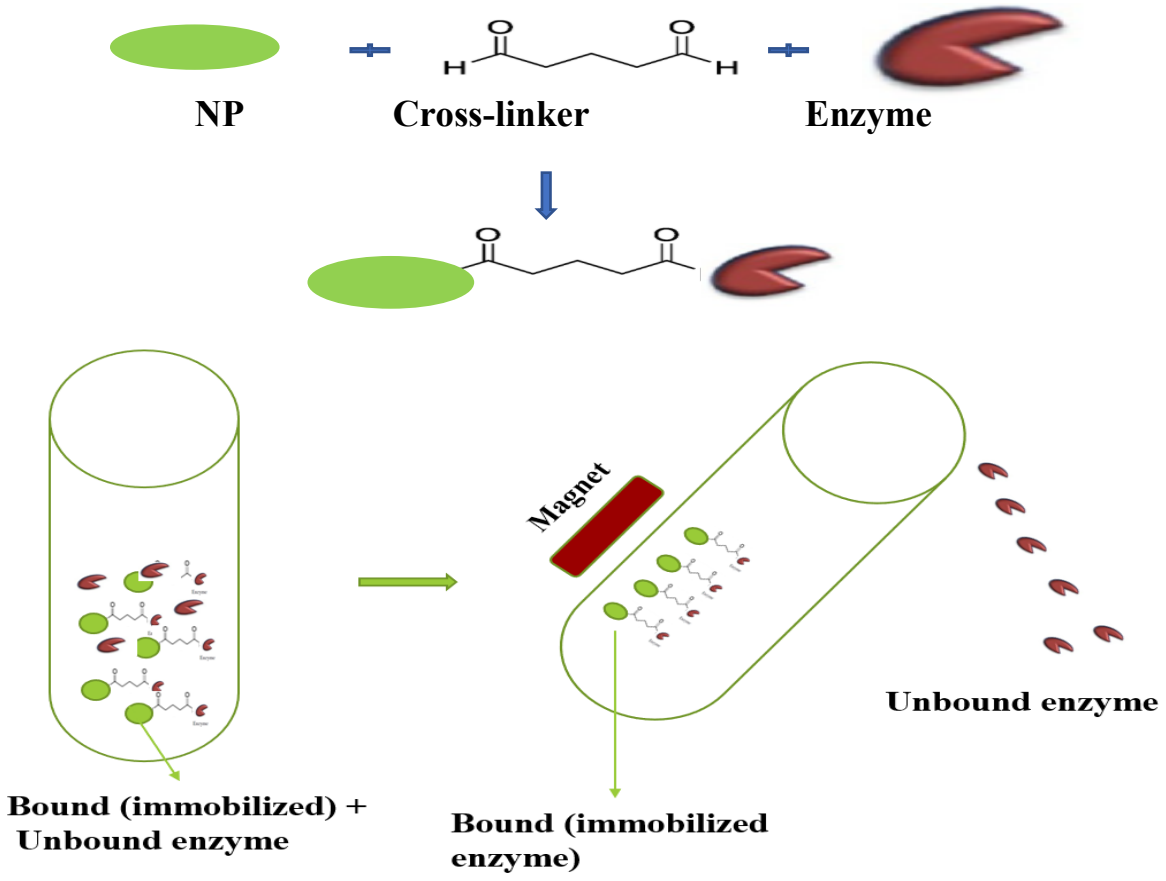

Fig 3.2 Schematic layout of immobilization process (Elution of unbound or loosely bound protein from the immobilized enzyme reaction mixture using magnetic properties of nanoparticle) (Abraham et al., 2014)

3.4.3 Characterization of Immobilized enzyme

SEM (Scanning Electron Microscopy)

The morphology and distribution of alginate lyase onto nanoparticle were determined by scanning electron microscope (FEI INSPECT F50) at the voltage of 5 kV. The samples were mounted on an aluminum stub, sputtered with 20 nm thickness platinum and observed under the microscope. The imaging was done at an accelerating voltage of 5 kV (Abraham *et al.*, 2014).

3.4.4 Effect of incubation time for activation of nanoparticle (with glutaraldehyde)

To optimize the incubation time for the activation of nanoparticle, 1 mL of glutaraldehyde was incubated with 1 mL of 5 mg/mL of magnetic nanoparticle in the incubator at 37 °C for different time periods (30, 60, 90, 120 and 180 min.). The relative activity of the immobilized enzyme was calculated for different treatments with respect to the soluble enzyme activity (Abraham *et al.*, 2014).

3.4.5 Effect of Protein Load

The binding efficiency of the protein to the nanoparticle was estimated by incubating 1 mL of nanoparticle (5 mg/mL) and different concentration of enzyme (protein) (1:1, 1:10, 1:50, 1:100, 1:150, 1:200 and 1:250) loading onto it. The percentage hydrolysis was estimated for different treatments using DNS assay and the optimum protein concentration was determined for the immobilization of alginate lyase (Joseph *et al.*, 2020). The binding of the enzyme to the nanoparticle was estimated by Lowry method. Thus the binding efficiency for the protein can be estimated for different protein loads (Abraham *et al.*, 2020).

3.4.6 Effect of temperature

The immobilized alginate lyase was incubated at different temperature (30, 35, 40, 45, 50 and 55 °C) and the activity was compared with the soluble alginate lyase. For the thermal stability, the assay for soluble and immobilized alginate lyase was conducted at 45 °C for different time intervals (30, 60, 90, 120, 150 and 180 min (Joseph *et al.*, 2020).

3.4.7 Effect of pH

For the optimum pH, the soluble and immobilized enzyme was incubated at 45 °C for 2 h at varying pH (6, 7, 8, 9, 10 and 11) using tris base. After incubation, DNS assay was performed to estimate the alginate lyase activity in two different forms and results were compared (Li *et al.*, 2020b).

3.4.8 Determination of Enzyme kinetics

The kinetic parameters were analyzed for the hydrolysis of sodium alginate by incubating increasing substrate concentration i.e. (0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5% and 4% w/v) with 25 IU of enzyme at 37 °C for 120 min. The reaction rate was determined by the substrate being utilized or the reducing sugar released per unit time. (Mohapatra, 2020; Nawawi *et al.*, 2020). The kinetics of the enzyme was studied to calculate the K_m and V_{max} values by plotting Lineweaver- Burk plot.

$$\textit{Reaction Rate (V)} = \frac{\textit{Reducing sugar released (µmoles/ml)}}{\textit{Time (min.)}}$$

3.4.9 Reusability, stability, and storage studies

For the determination of reusability, the enzyme assay for immobilized alginate lyase was conducted at 45 °C. The enzyme was washed with milli-Q water, resuspended in tris buffer reused for following cycle. The activity calculated for the first cycle was considered as 100% (control). To analyze the thermal stability, immobilized enzyme was incubated at 45 °C for different time periods (30, 60, 90, 120, 150 and 180 min.) without substrate and then tested for the hydrolysis of alginate. The immobilized enzyme was reused until 50% of the activity was retained which allowed calculation of the half-life of the enzyme (Jiang *et al.*, 2020). For the study of storage, immobilized enzyme was incubated at 4 °C in the refrigerator for one month and the activity was measured after 1, 3, 5, 7 days up to 21 days (Jiang *et al.*, 2020).

3.5 Effect of alginate lyase on raw seaweed biomass

Enzymatic hydrolysis of the dried raw biomass (0.5%, w/v of *Bull Kelp)* was performed by soluble and immobilized alginate lyase for 60 h (Abraham *et al.*, 2019). Amount of reducing sugars were measured after every 6 h by conducting DNS assay.

$$\textit{Alginate hydrolysis} = \frac{\textit{Reducing sugar released (mg)} \times \textit{0.9 correction factor} \times \textit{100}}{\textit{Substrate (mg)}}$$

3.6 HPLC analysis:

The reducing sugar released in the hydrolysate of the raw seaweed biomass was quantified using High Performance Liquid Chromatography (HPLC). HPLC (UFLC XR, Shimadzu, Australia) was

equipped with a Phenomenex column (Kinetex C18 2.6 um 3 x 100 mm 100A). HPLC was equipped with a solvent degasser, quaternary pump, auto-sampler, thermostat column compartment, and a refractive index detector. Glucose, cellobiose, arabinose, mannose, xylose and organic acids included succinic acid, oxalic acid, acetic acid, formic acid and ethanol were of HPLC grade and purchased from Sigma (St. Louis, MO, USA) and used for the preparation of standard curve. 2-DOG (2- deoxy glucose) was used as an internal standard and the concentration of each monosaccharide was estimated by extrapolating the standard curve.

After conducting the hydrolysis of raw seaweed biomass for 60 h using soluble and immobilized alginate lyase, the samples were analyzed using a HPLC. The column was washed with 30% v/v of acetonitrile for every set of experiment. The column was maintained at 30 °C at a flow rate of 0.8 mL/min, high pressure 600 bars and eluted with milli-Q water. The samples were filtered through a membrane filter unit (0.45 mm) purchased from Thermoscientific and the HPLC peaks were detected using a refractive index detector. All the chemicals used were of standard analytical grades and the run was performed in four replicates and represented with standard deviation (±SD) (Abraham, 2014). The hydrolysis of raw seaweed biomass was conducted for 60 h using soluble and immobilized alginate lyase and resulting samples were analyzed as mentioned above.

Chapter 4

RESULTS

4. Results

4.1 Soluble enzyme characterization

In this study, alginate lyase sourced from *Flavobacterium* sp was characterized with respect to alginate hydrolysis.

4.1.1 Enzyme units optimization

The enzyme units were optimized for the hydrolysis of alginate were investigated. When 3 IU of enzymes (in 100 µL of 20 mM Tris buffer pH 7.4) were used, 49.58 ± 0.02% of hydrolysis was observed, which incresed by 7 ± 0.03% by adding 5 units (Fig. 4.1). No considerable change in alginate hydrolysis was observed upon increasing enzyme units (7 or 10 IU). Significant increase (66 ± 0.05 %) in alginate hydrolysis was observed upon increasing the enzyme upto 25 units, therafter steady state attained.

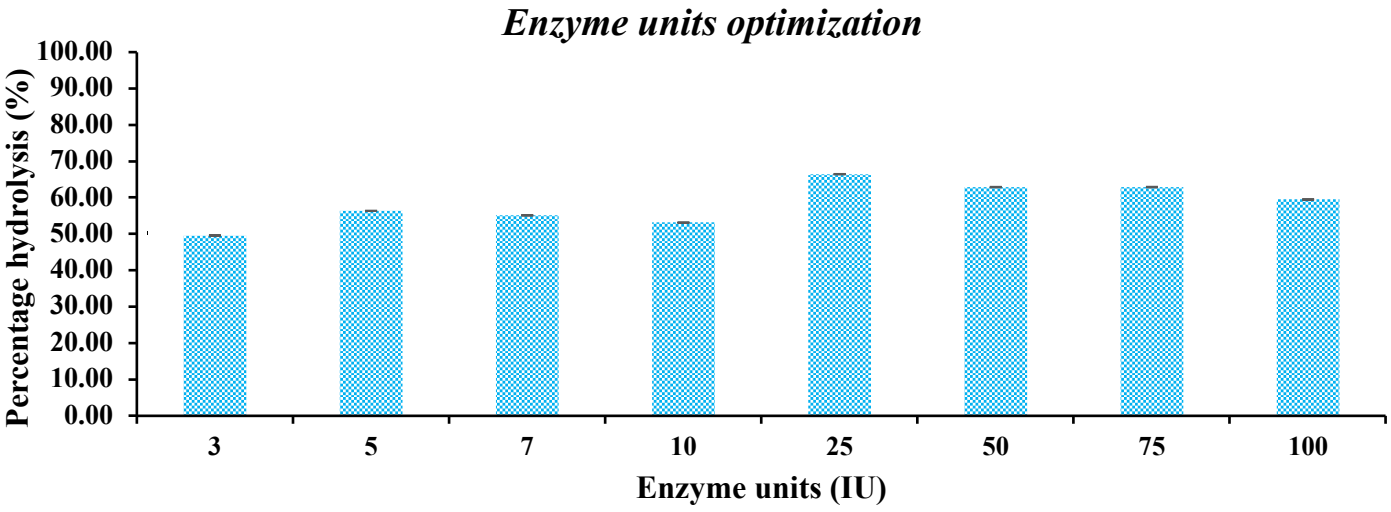

Fig 4.1 Percentage hydrolysis of sodium alginate as a function of enzyme optimization

4.1.2 Substrate concentration optimization

To investigate the substrate concentration, the percentage hydrolysis was compared for varying substrate concentrations (0.5%, 1%, 1.5%, 2%, 2.5% and 3% w/v). With 0.5% of substrate and 25 IU of alginate lyase, maximum hydrolysis (71.75 ± 0.02%) was observed. More sugars were released when alginate concentration increased. Upon using higher substrate (1% w/v) concentration, alginate (67.28 ± 0.01 %) hydrolysis was observed. Up to 1.5% (w/v) of sodium alginate concentration, hydrolysis of alginate (nearly 62 ± 0.02%) was observed (Fig. 4.2). With the further increase in substrate concentration (2% - 3%), percentage alginate hydrolysis declined (from 44.96 ± .02 to 37.80 ± 0.01 %). Thus, alginate concentration (1.5% w/v) was used for further experiments.

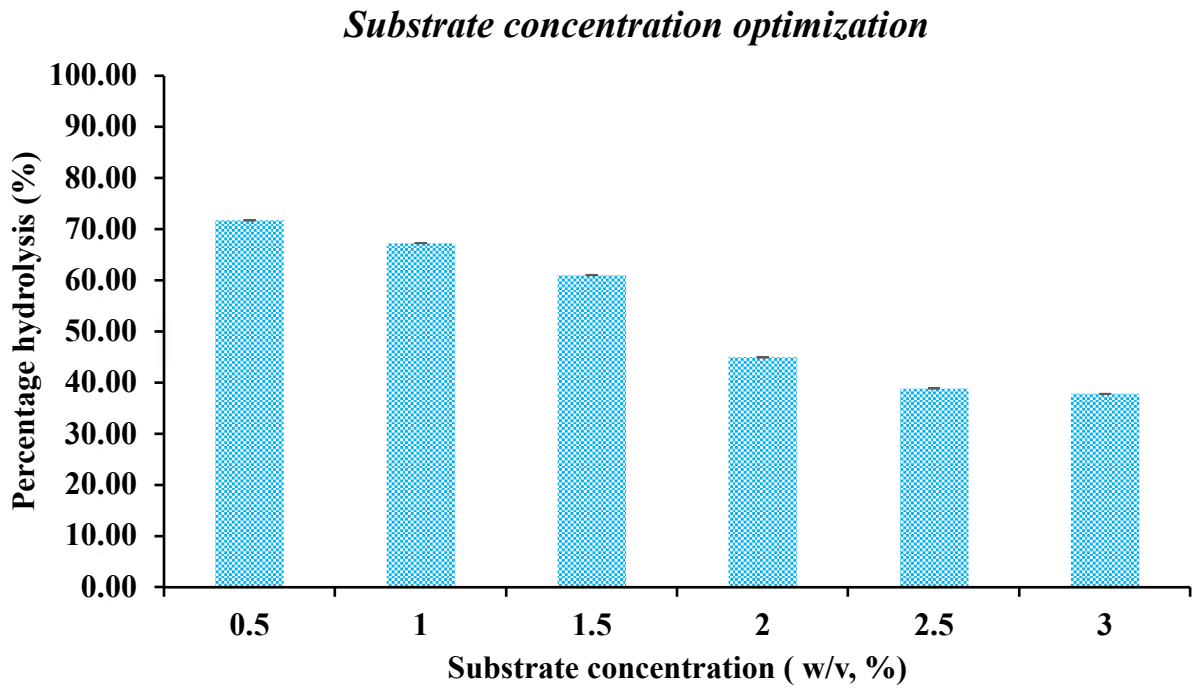

Fig 4.2 Substrate concentration optimization based on the percentage hydrolysis of alginate

4.1.3 Temperature optimization

Alginate lyase exhibited alginate hydrolysis (50.21 ± 0.06%) at 30 °C. With the increase in the temperature 35 °C to 40 °C, the substrate hydrolysis improved from 69 ± 0.03% to 73.48 % ± 0.06 %. However, upon further increasing temperature 40 °C to 45 °C and 50 °C, the substrate hydrolysis declined by 22 ± 0.05 % and 27 ± 0.05% (Fig. 4.3). Such results prompted to investigate narrow range of temperature (35, 36, 37, 38, 39, 40 °C) for substrate hydrolysis. At 37 °C, better substrate hydrolysis (76 ± 0.07 %) was observed. However, with the further rise in the temperature from 38 °C to 39 °C, the hydrolysis declined by 10 ± 0.04 % as shown in Fig. 4.4.

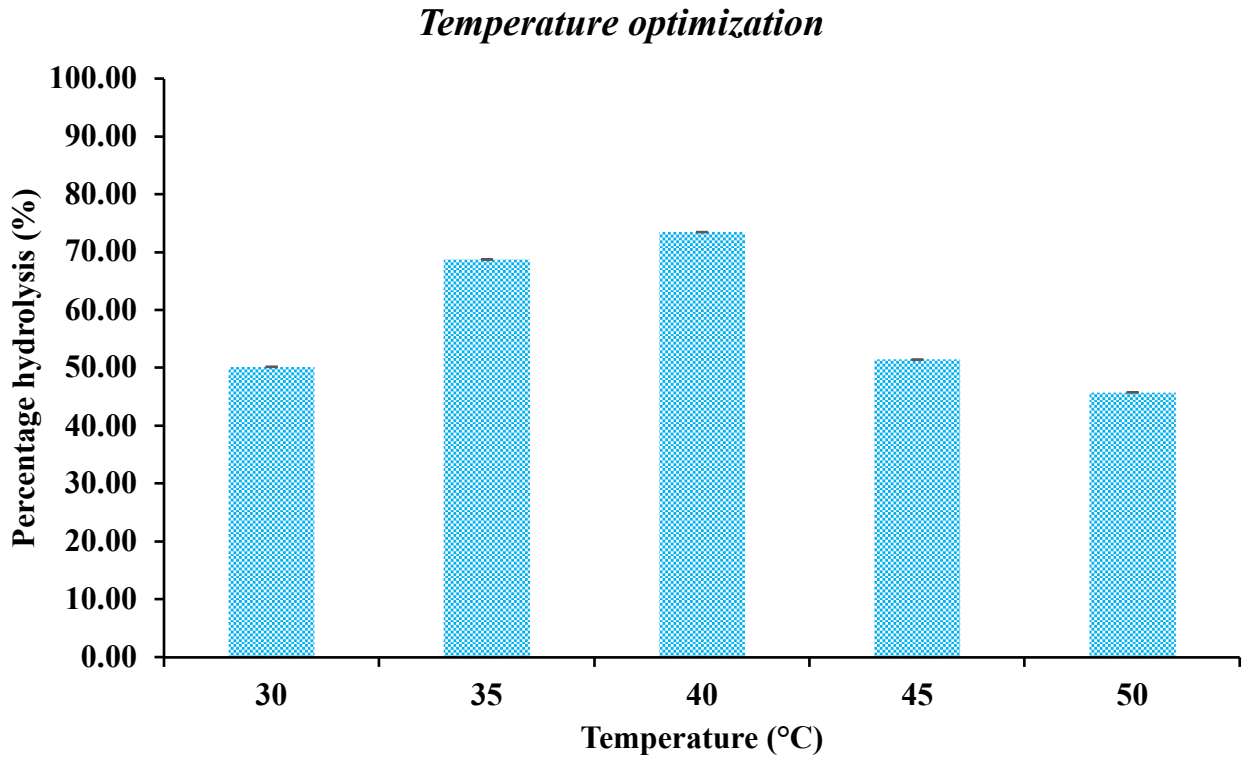

Fig 4.3 Effect of temperature on the percentage hydrolysis of alginate

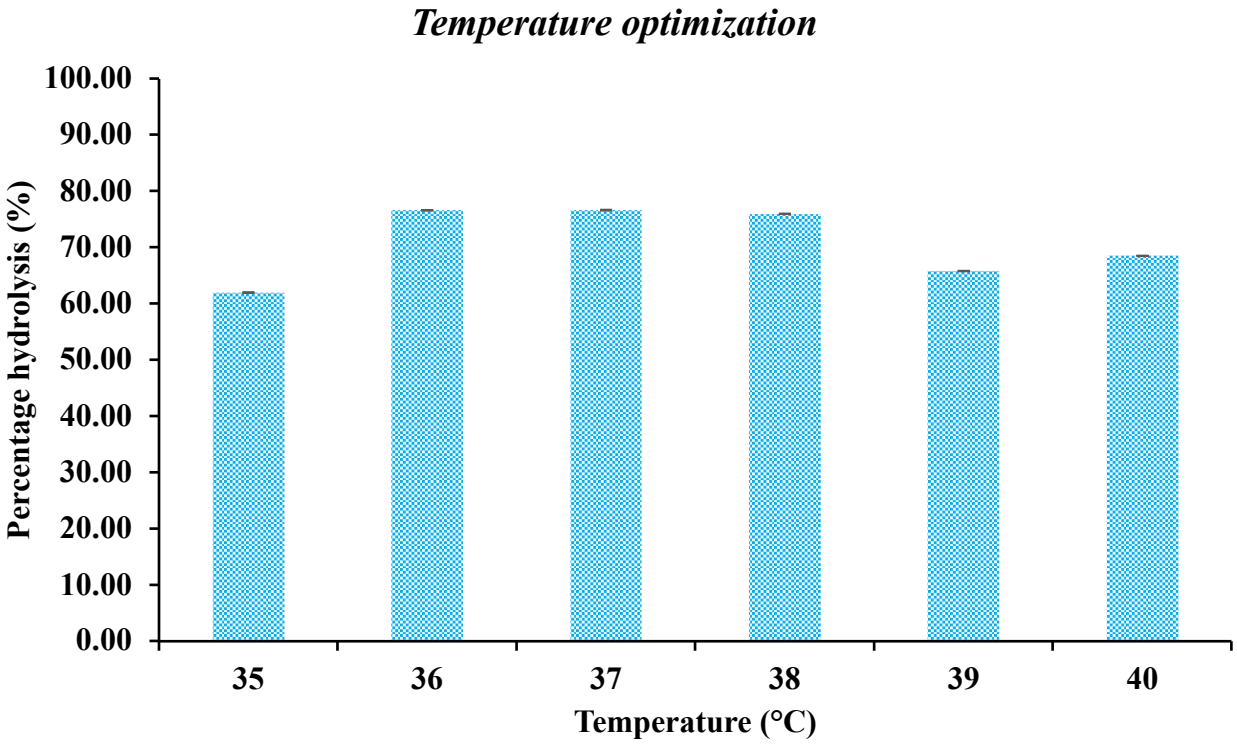

Fig 4.4 Optimization of narrow temperature range as a function of percentage alginate hydrolysis

4.1.4 pH optimization

The different pH values (6, 7, 8, 9, 10 and 11) were investigated for their effect on the activity of alginate lyase. Alginate lyase exhibited maximum activity (70 ± 0.01%) of depolymerization of alginate between pH 7 to 8 (Fig. 4.5). Further, the enzyme activity (66 ± 0.02% to 64 ± 0.04%) was observed to decrease at pH 9 and 10. Whereas, only 47.65 ± 0.01% enzyme activity was remained at pH 11. When enzyme activity analyzed for narrow pH range (between pH 6, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8 and 7.9), 66.66 ± 0.01% activity was observed at pH 6 (Fig. 4.6). Nearly 77 ± 4 % activity was reported for pH range of 7.1 to 7.5 and then the activity declined by 3-4 ± 0.06 % with the increase in the pH value from 7.7 to 7.9.

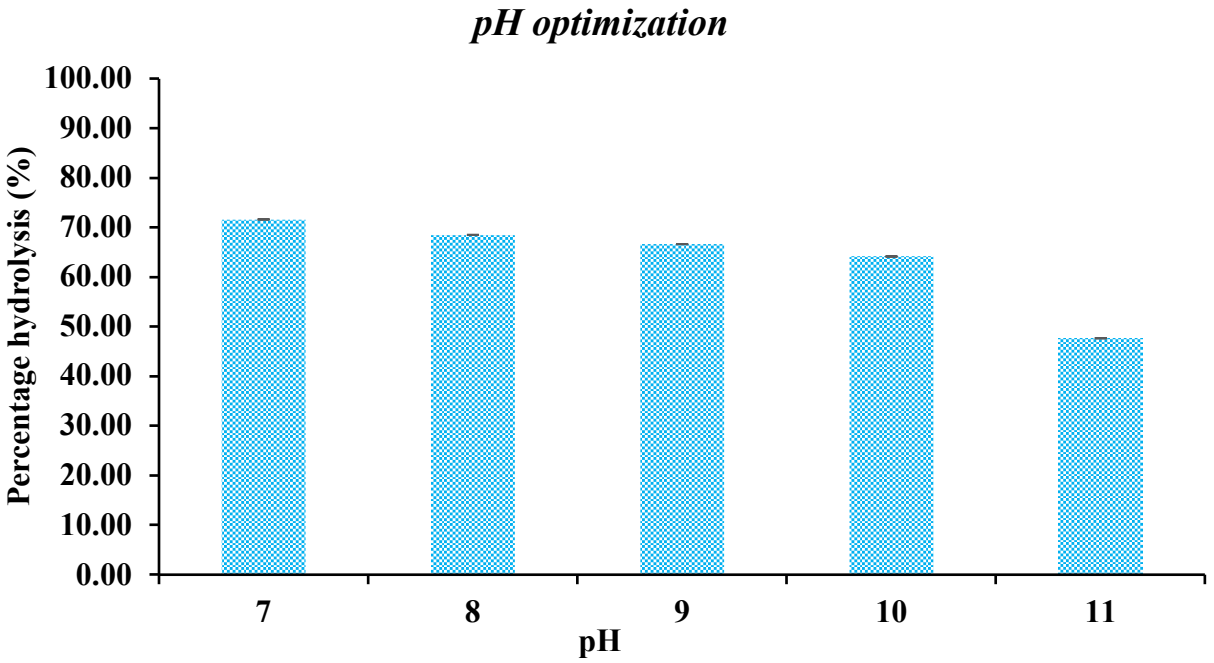

Fig 4.5 Effect of pH on the percentage hydrolysis of sodium alginate

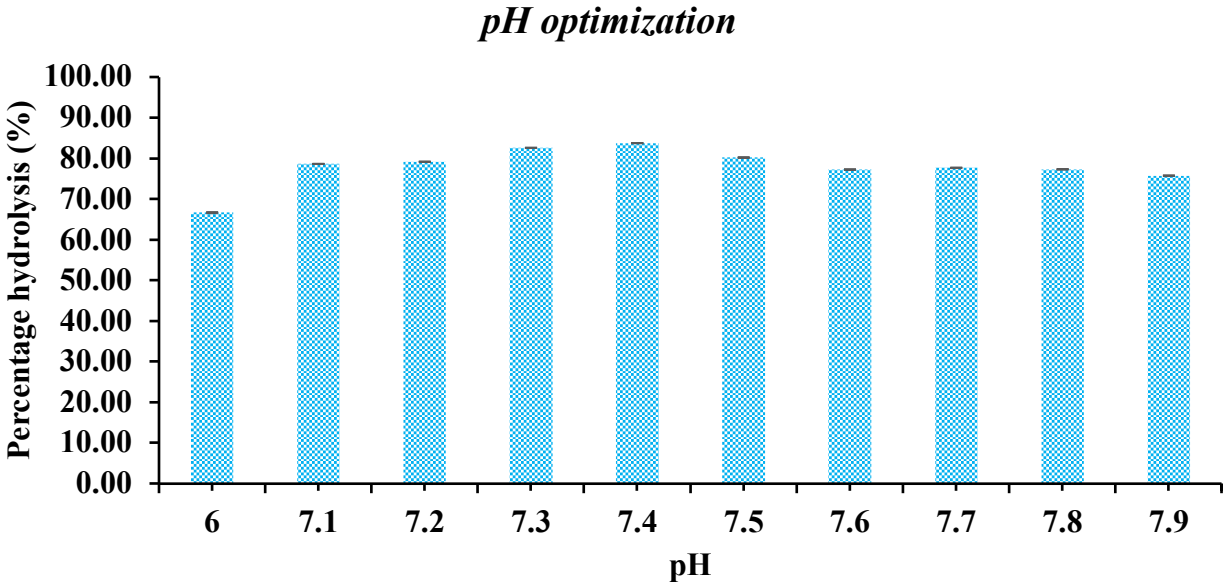

Fig 4.6 Effect of pH on the percentage hydrolysis of sodium alginate

4.1.5 Effect of Incubation time and shaking

The substrate hydrolysis increased by more than double when incubation time was increased from 1h (27.28 ± 0.01%) to 2 h (68.60 ± 0.02%). Thereafter, with the increase in the incubation time, no further improvement was in enzymatic hydrolysis was recorded. When reaction conducted under shaking condition, no improvement was observed (Fig. 4.7). Alginate hydrolysis was increased (3 ± 0.02%) when the reaction mixture was incubated for 2 h with the shaking at 50 rpm. Upon increasing shaking (100 rpm), it enhanced substrate hydrolysis (72.49 ± .04%).

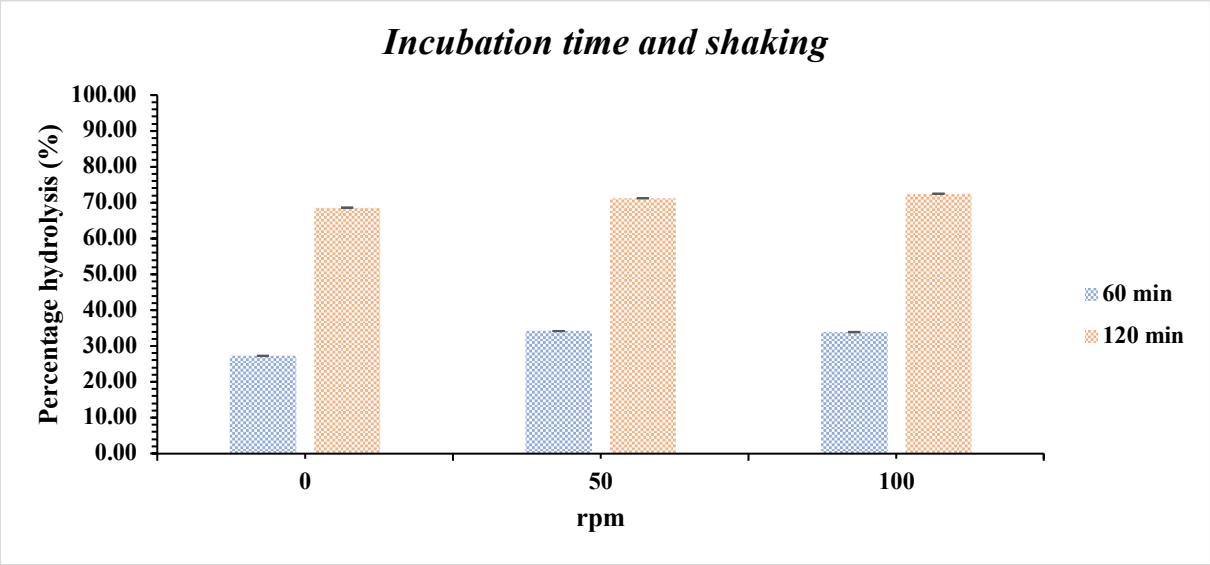

Fig 4.7 Effect of incubation time and shaking on the percentage hydrolysis of sodium alginate
(Incubation time- blue color: 1h, pink color: 2h, rpm: revolution per minute)

4.1.6 Effect of Metal ions

The effect of different metal ions, SDS, EDTA on the activity of alginate lyase was investigated by using metal ions solution of final concentration of 1mM (50 µL) and the effect was analyzed by comparing hydrolysis of alginate without any metal ion (control 100%). In the results recorded, most

of the ions enhanced the activity of alginate lyase however, Mn^{2+}(89.52 ± 3.48%), Zn^{2+} (87.85± 2.69%), Na^{+}(87.74 ± 1.53%), Fe^{3+}(85.94 ± 1.27%) enhanced the hydrolysis of alginate to more than 85% (by more than 15% than the control) (Fig. 4.8). Co^{3+} and Cu^{2+} did not show any reportable variation as there was negotiable increase by 3.5 ± 2.47 % and 2 ± 0.65%. respectively. The enzyme activity was increased by almost 8 % with Ca^{2+} (81.6 ± 4.92%), EDTA (81.43 ± 2.53%), KH_2PO_4 (82.88 ± 2.06%) and Mg^{2+} (82.17 ± 5.22%).

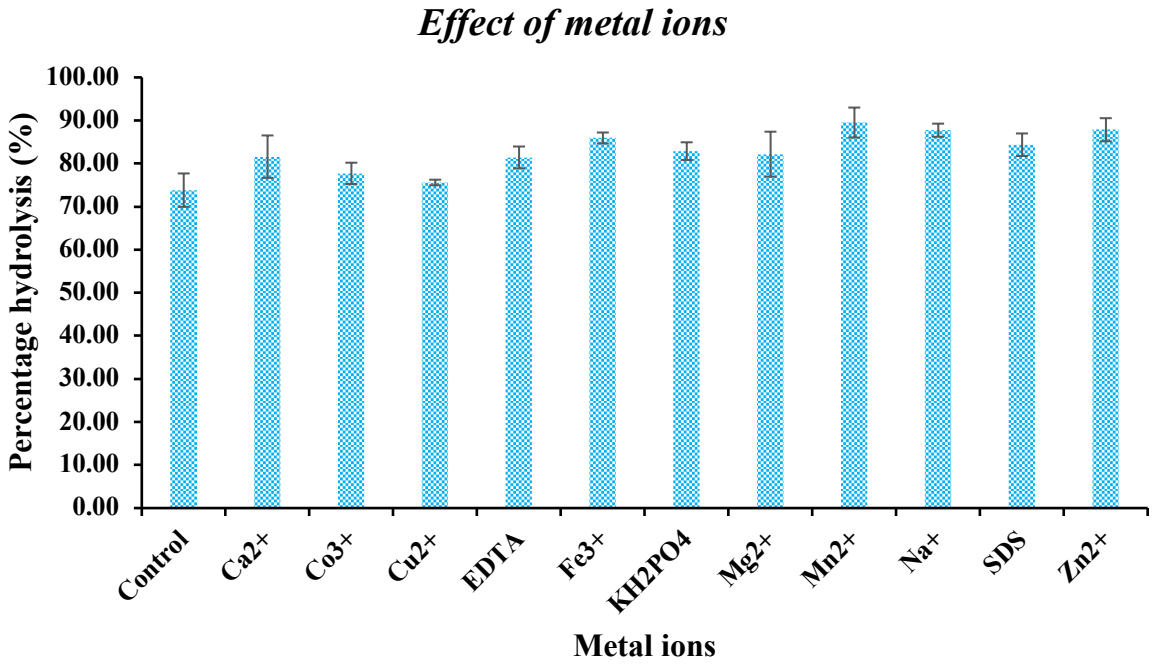

Fig 4.8 Effect of different metal on the percentage hydrolysis of sodium alginate

4.2 Immobilization of alginate lyase

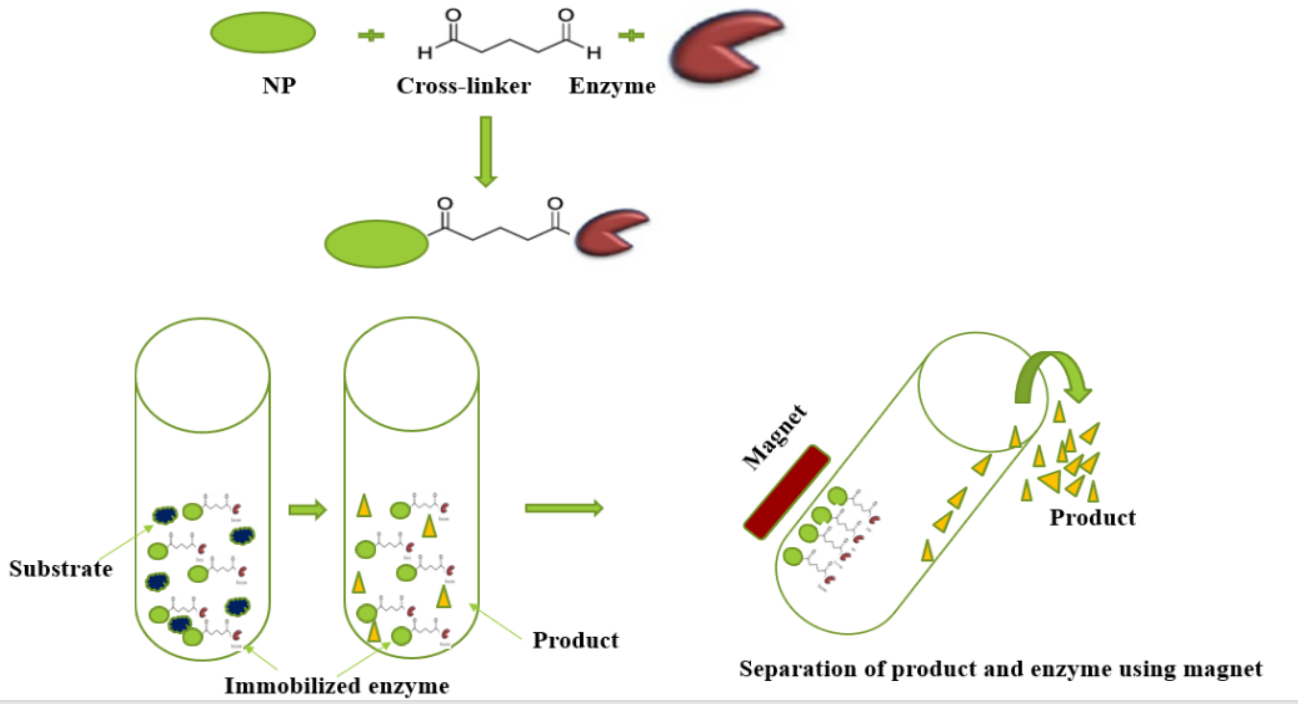

Fig 4.9 Stepwise immobilization of alginate lyase and its use in alginate hydrolysis

4.2.1 Optimization of glutaraldehyde assisted cross-linking

Glutaraldehyde molecule acts as a cross-linker for the attachment of enzyme with the nanoparticle. Fig. 4.9 demonstrates the maximum (59.18 ± 0.01%) activation of nanoparticle based on binding of glutaraldehyde molecule with the nanoparticle, that was achieved within one hour of incubation. However, in 30 min, only 51.57 ± 0.01% relative activity was recorded, and it increased by 9% by doubling the time. There was no considerable improvement observed (58.26 ± 0.01%) upon extending time to 180 min (Fig. 4.10).

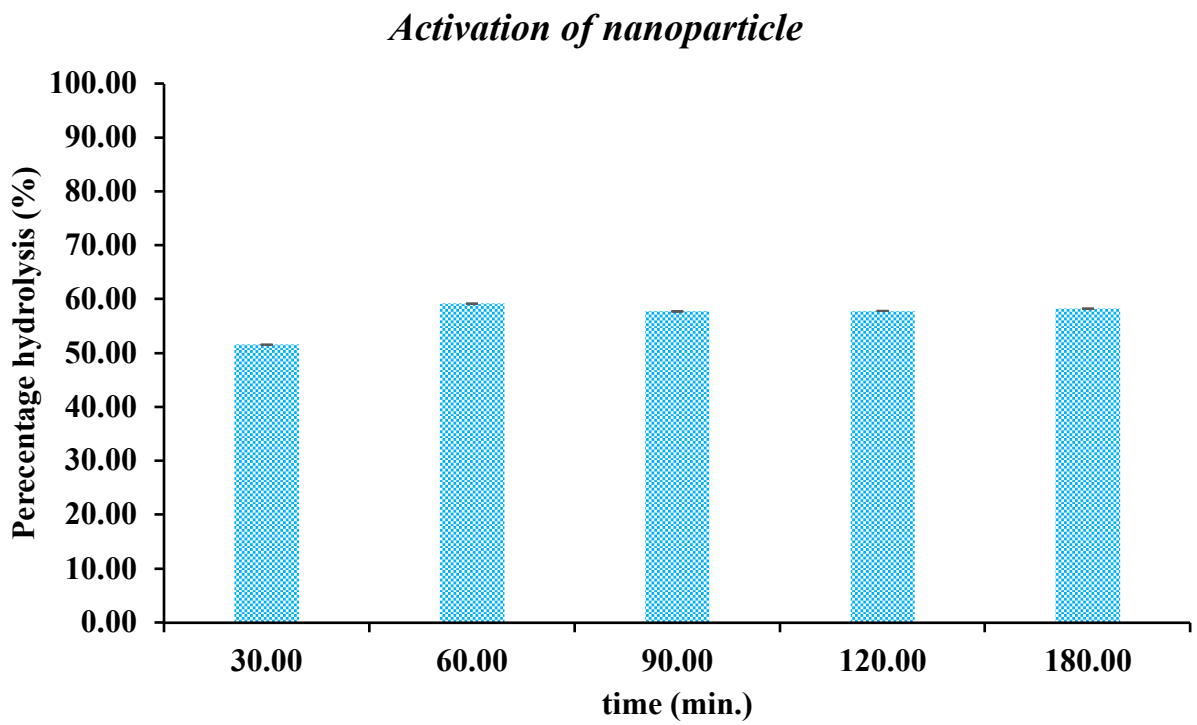

Fig 4.10 Activation of nanoparticle as a function of time

4.2.2 Optimization of protein load (nanoparticle: protein)

The optimization of protein load was assessed by loading different concentrations (units) of alginate lyase onto nanoparticle and the results were analyzed by determining the enzymatic activity for various nanoparticle:protein (1:1, 1: 10, 1: 50, 1: 100, 1: 150, 1: 200, 1: 250) ratios. There was a significant enhancement in the enzymatic activity with the increase in the ratio of the protein: nanoparticle. Upon increasing protein load from 1:10 to 1: 50 and 1: 100, a constant increase in the binding efficiency (55.4 ± 0.06%, 67.4 ± 0.038% and 68.8 ± 0.013%). Maximum protein binding (71.4 ± 0.001%) was observed with 150:1 (750 ug of protein: 5mg/mL of nanoparticle). With the further increment in the protein load (1: 250) no improvement in binding was observed (69.8 ± 0.003 %) (Fig. 4.11).

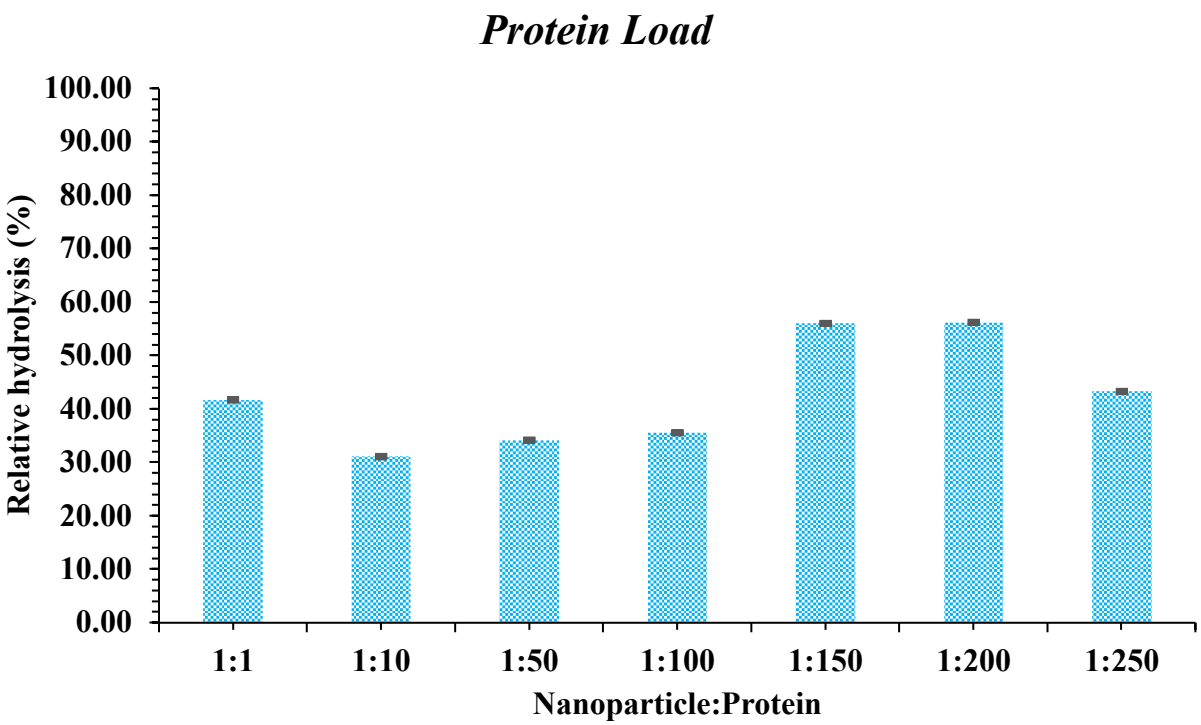

Fig 4.11 Effect of protein load (nanoparticle: protein) on the hydrolysis of sodium alginate by immobilized enzyme

4.2.3 Scanning Electron Microscope studies to support immobilization of Alginate lyase

The surface properties of nanoparticles were analyzed by SEM before and after immobilization. Images showed the clear view of spherical shaped nanoparticles with various size ranging from 2µm to 200nm. Before immobilization, the nanoparticle was observed to be smooth spherical, uniform, and spongy (Fig. 4.12) and the size of the nanoparticles tended to increase slightly after immobilization (Fig. 4.13).

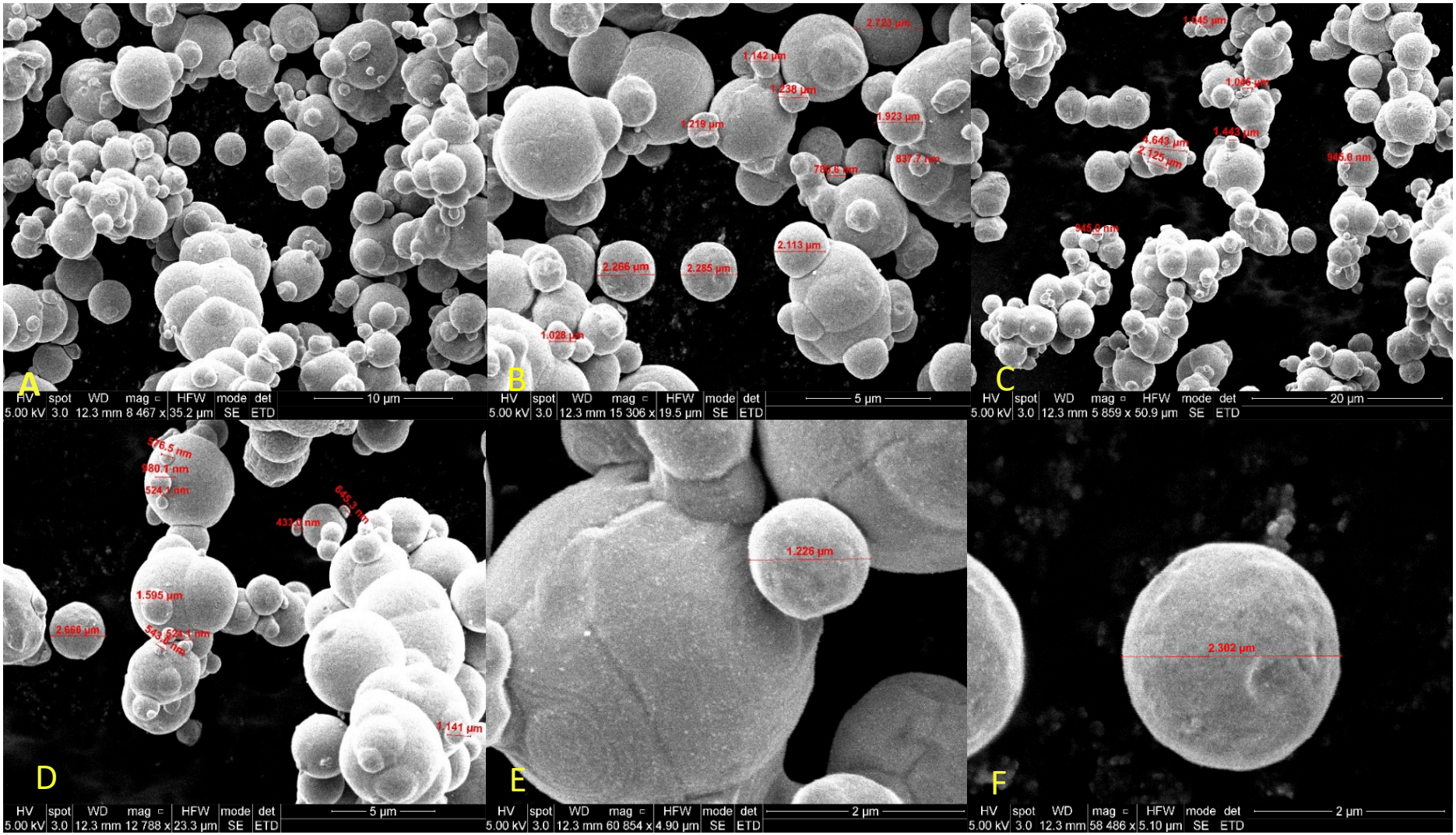

Fig 4.12 SEM images for nanoparticle at an accelerating voltage of 5 kV and magnification ranges from 500 nm to 300 µm (A: 200µm, B:20 µm, C: 10 µm, D: 5 µm, E: 3 µm, F: 500 nm)

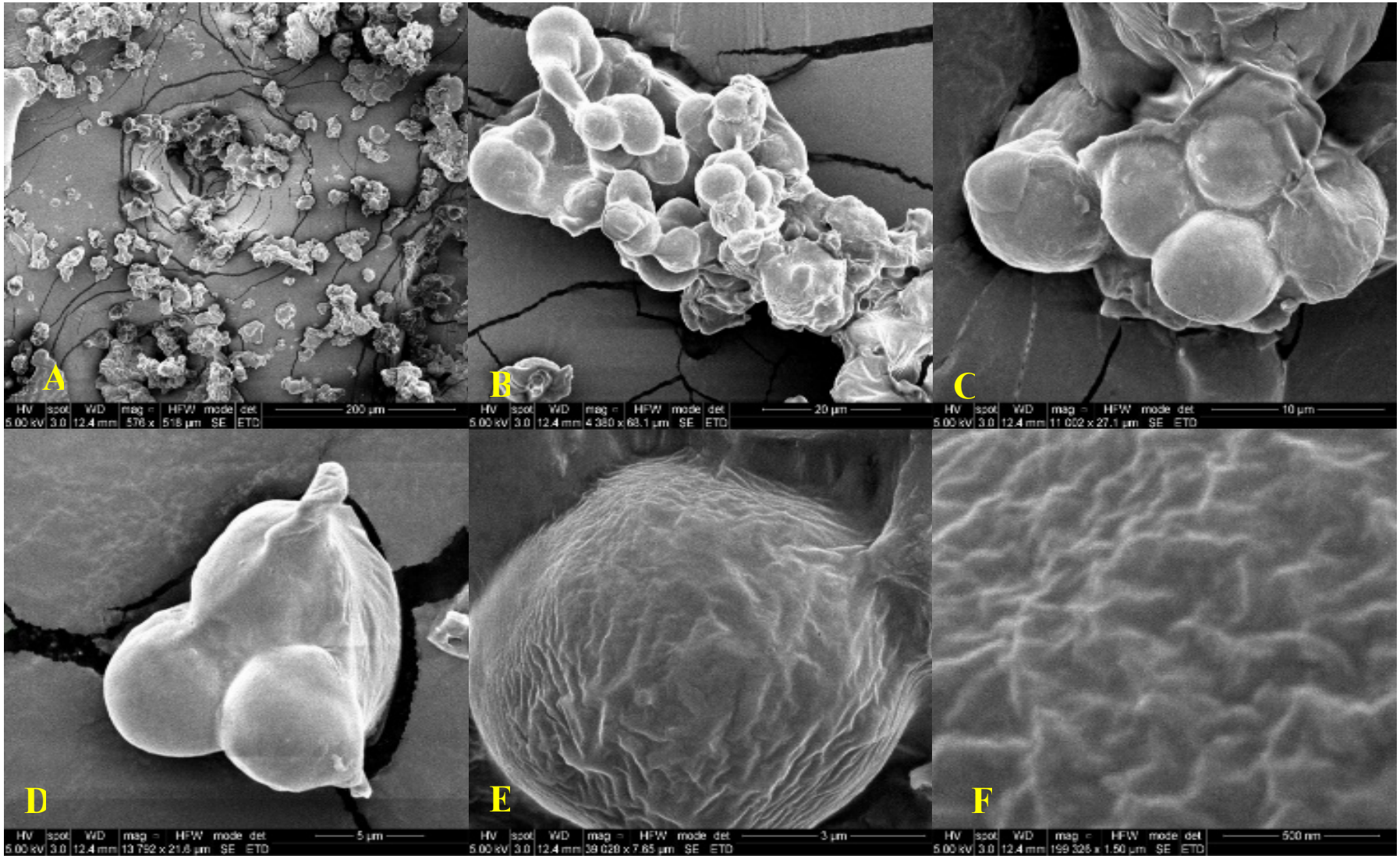

Fig 4.13 SEM images for immobilized alginate lyase at an accelerating voltage of 5 kV and magnification ranges from 500 nm to 300 µm showed variable size of the nanoparticle (A: 10µm, B:5 µm, C: 20 µm, D: 5 µm, E: 2 µm, F: 2 µm)

4.2.4 Temperature Optimization

Fig. 4.14 shows the thermal stability of the immobilized alginate lyase as compare to the soluble enzyme at various temperatures. At 30 °C, the activity for soluble and immobilized was observed to be 68.32 ± 0.06% and 61.59 ± 0.0% respectively. With the rise in temperature by 5 °C, further improvement in alginate hydrolysis (25 ± 0.06% 5 ± 0.0%) was observed. Notably, soluble alginate lyase showed maximum activity at 40 °C however, immobilized alginate lyase hydrolyzed alginate maximally at 45°C.The relative enzyme activity of soluble enzyme declined (70 ± 0.05%) at 45 °C. Whereas, immobilized enzyme presented 80 ± 0.01% activity at 50 °C (Fig. 4.14).

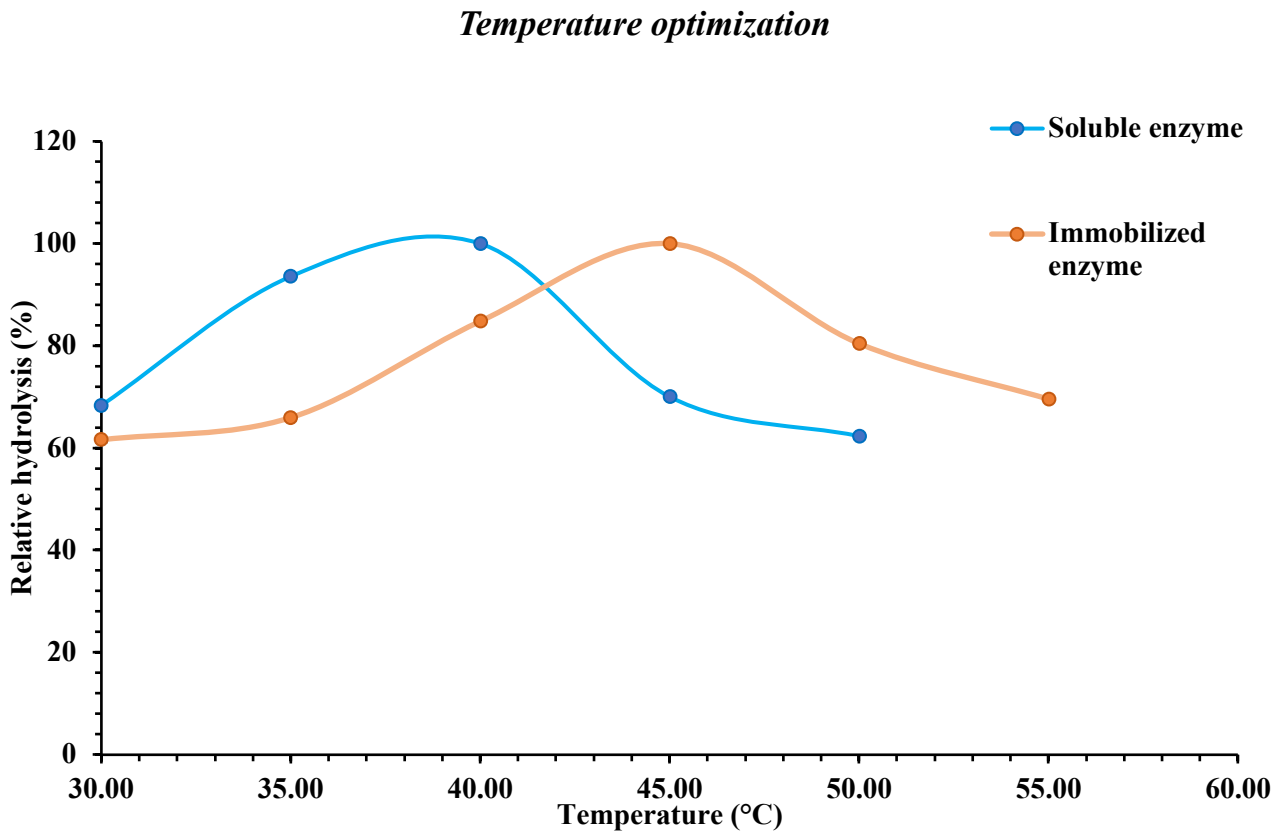

Fig 4.14 Effect of temperature on the hydrolysis of sodium alginate by soluble and immobilized enzyme

4.2.5 pH optimization

The enzyme activity was observed to increase when pH 6 raised to pH 7 (60.6 ± 0.01% to 89.3 ± 0.01%) for immobilized enzyme and from 88.7 ± 0.12% to 100% for soluble form. Maximum activity of the soluble enzyme was achieved at pH 7 (refer to previous Fig. 4.5) however, immobilized enzyme exhibited at pH 9. Beyond this pH, activity for both the soluble and immobilized declined (Fig. 4.15).

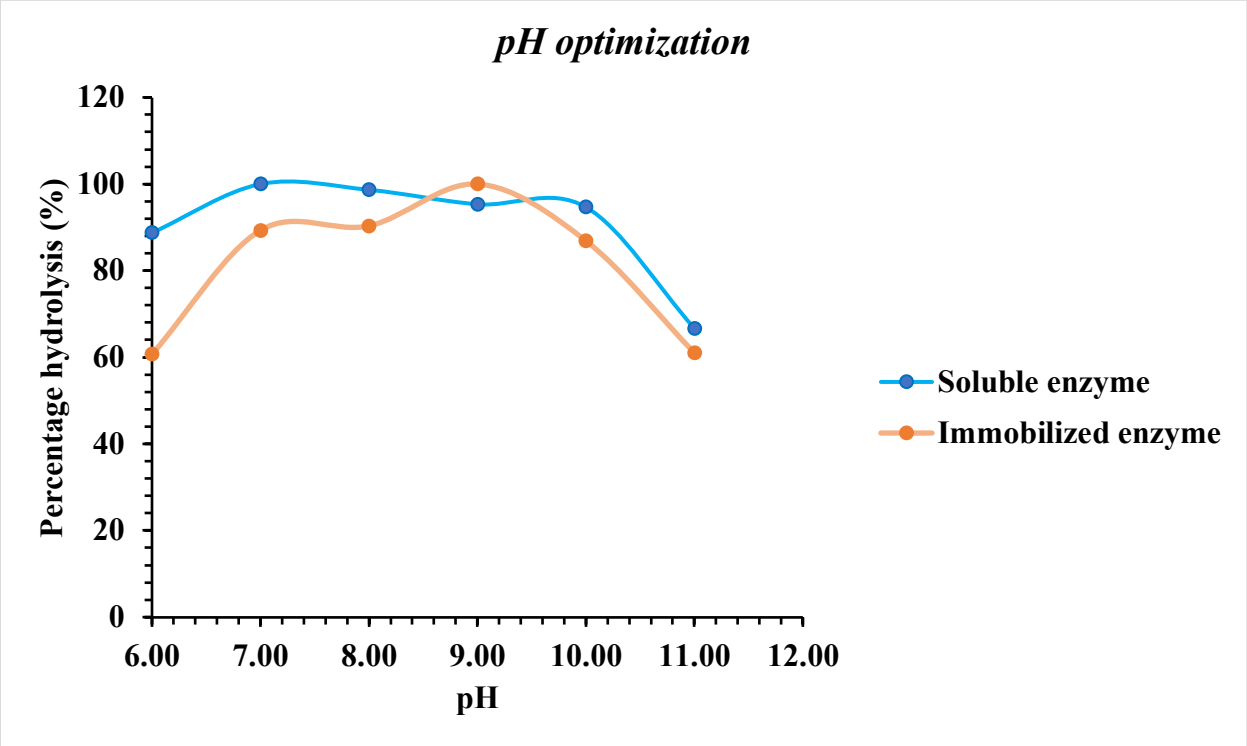

Fig 4.15 Effect of pH on the hydrolysis of sodium alginate by immobilized enzyme

4.2.6 Lineweaver-Burk Plot for determining kinetics parameters

Lineweaver- Burk plot was plotted to determine kinetic parameters. The rate of reaction elevated significantly with the increase in the substrate concentration from 0.5% to 1.5% (w/v), 0.23 ± 0.04

µmol/mL/min to 0.48 ± 0.05 µmol/mL/min for soluble enzyme. It was stable around 0.60 to 0.63 ± 0.02. In the kinetics parameters analysis for immobilized enzyme, K_m (20 mM) was calculated almost double than the soluble enzyme (10.10mM). However, V_{max} was found to be nearly same (0.2 mg/mL/min) for both (Fig. 4.16).

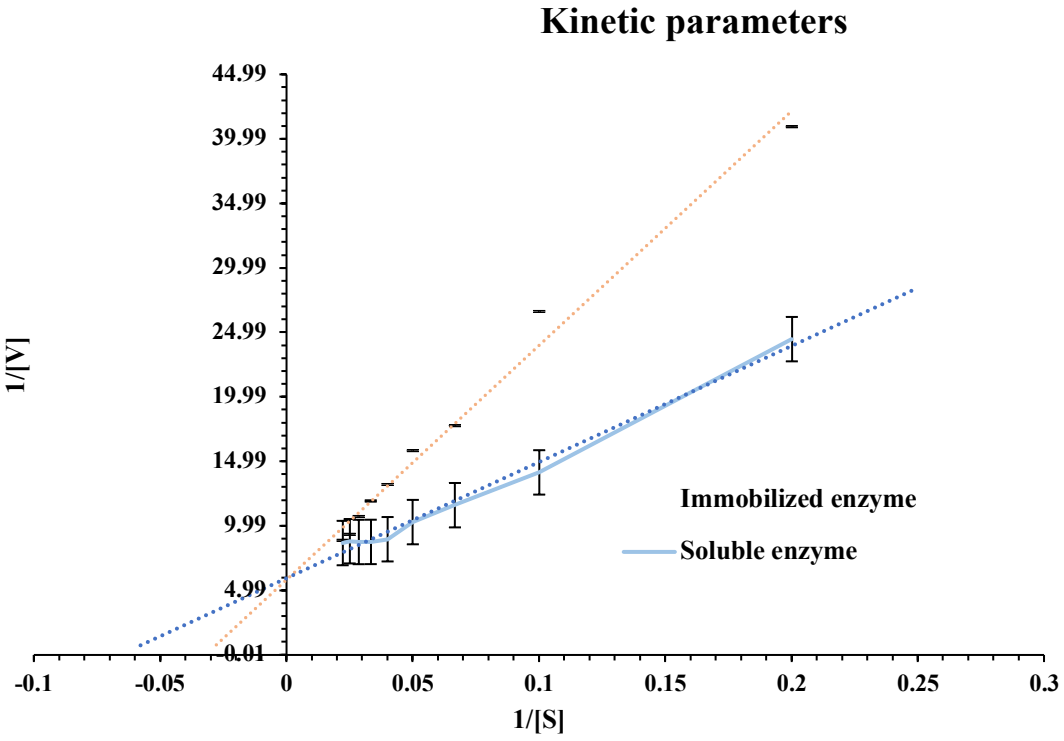

Fig 4.16 Lineweaver- Burk Plot for determining kinetic parameters of soluble and immobilized alginate lyase

4.2.7 Thermal stability

The immobilized alginate lyase retained activity (79.3 ± 0.01%) when kept at 45 °C for 60 mins. However, for soluble enzyme, the activity (64 ± 0.01%) was recorded. There was significant reduction in the activity of soluble enzyme from 60 min (44.2 ± 0.06%) to 90 min (33.6 ± 0.05%).

The immobilized alginate lyase activity reduced by (8 ± 0.001%) after 90 min. After incubation for 2 h, only 29.7 ± 0.06% of activity for soluble enzyme was retained however, immobilized enzyme preserved 68.9 ± 0.01% of its original activity. Constant reduction in the activity of soluble enzyme was observed as it was reduced to 15.2 ± 0.01% by 2.5 h and 7.4 ± 0.01% after 3 h. Immobilized enzyme retained activity (61.78 ± 0.01%) up to 3 h (Fig. 4.17).

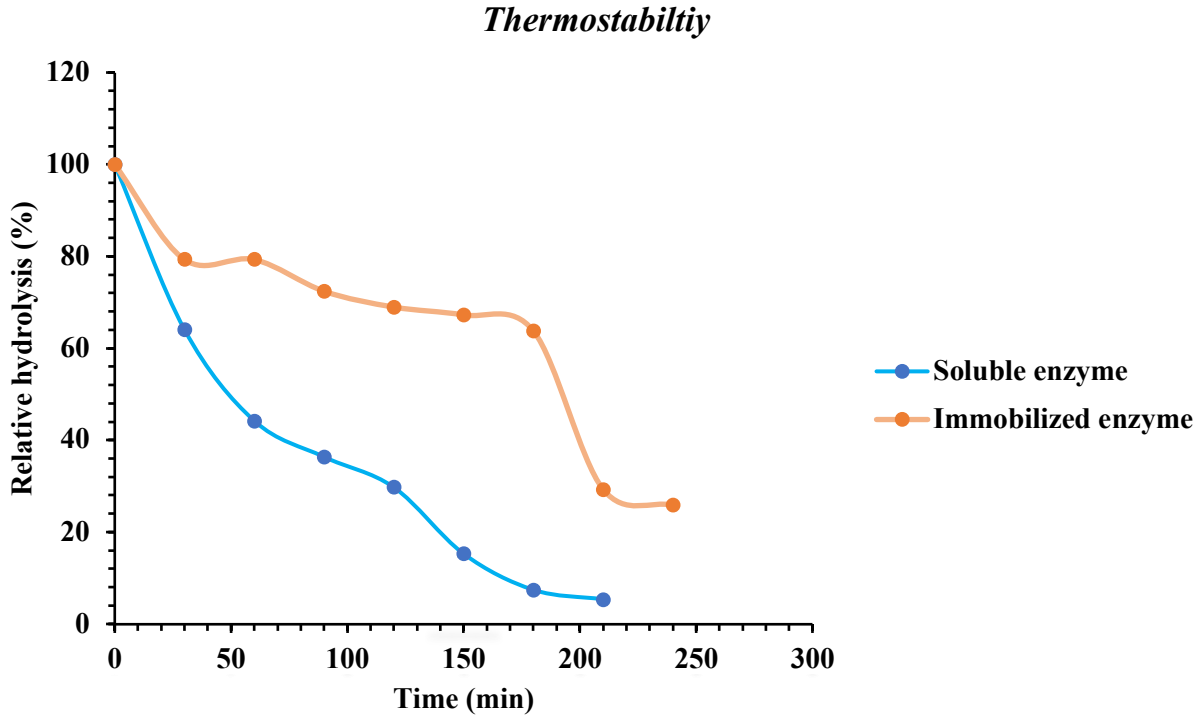

Fig 4.17 Thermostability studies of soluble and immobilized enzyme (45° C)

4.2.8 Reusability of immobilized alginate lyase

Immobilized alginate lyase retained 90.84 ± 0.002% of its activity when it was reused second time. When it was reused for third cycle, it showed 79.06 ± 0.003% activity, followed by 65.9 ± 0.001% activity after 4th cycle. In 5th cycle, ~59 ± 0.001% activity was retained. The activity of enzyme was reduced to 25.4 ± 0.001% when reused for 8th cycle (Fig. 4.18).

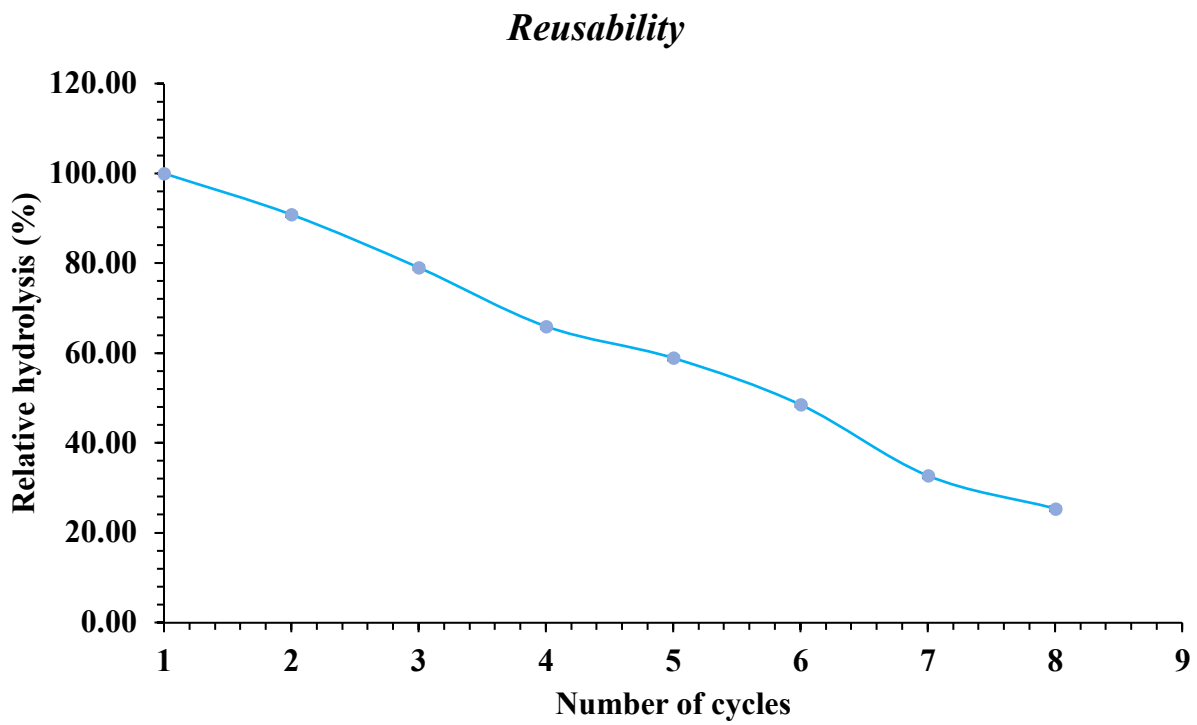

Fig 4.18 Reusability studies of immobilized alginate lyase for 8 cycles

4.2.9 Storage studies

It was observed that immobilized alginate lyase exhibited (83.9 ± 0.01%) when stored at 4°C for 3 days. On day 5, the activity was reduced to 77.57 ± 0.01% but remained stable until 11th days. On 15th day, the hydrolysis rate was reduced (67 ± 0.04%), followed by further decline in hydrolysis (45.5 %) on 17th day. About 50% activity of immobilized alginate lyase was retained for more than 16 days when it was stored at 4°C. Immobilized enzyme exhibited modest activity when stored continuously for 21 days (Fig. 4.19).

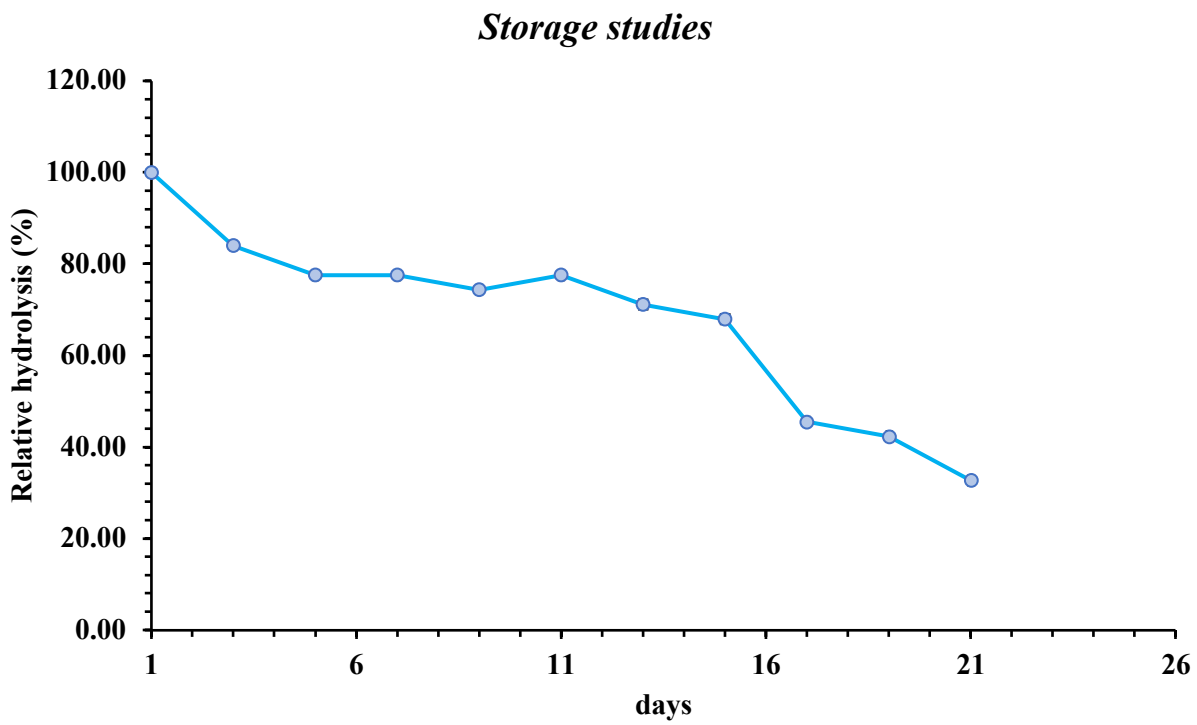

Fig 4.19 Storage of immobilized alginate lyase

4.3 Hydrolysis of raw seaweed biomass

The soluble and immobilized alginate lyase was used for hydrolyzing commercial alginate (sourced from an industrial partner Australian Kelp Industries, SA). Within 6 h of incubation, the rate of hydrolysis improved by about 5 times for soluble (22.27 ± 0.003%) and immobilized alginate lyase (10.1± 0.001%). The rate of hydrolysis was observed to be increasing constantly with increase in time duration for both forms of the enzymes however, it was higher for the soluble enzyme (29.02 ± 0.005% on 30 h) then immobilized enzyme (24.9 ± 0.001%). After incubating for 48 h, there was 17 ± 0.001% % rise in hydrolysis of alginate by soluble and 15 ± 0.001% by the immobilized enzyme, respectively. The maximum hydrolysis (76.9 ± 0.02% and 62.7 ±

0.010%) i.e., depolymerization of substrate was observed after 60 h by the soluble and immobilized enzyme, respectively (Fig. 4.20).

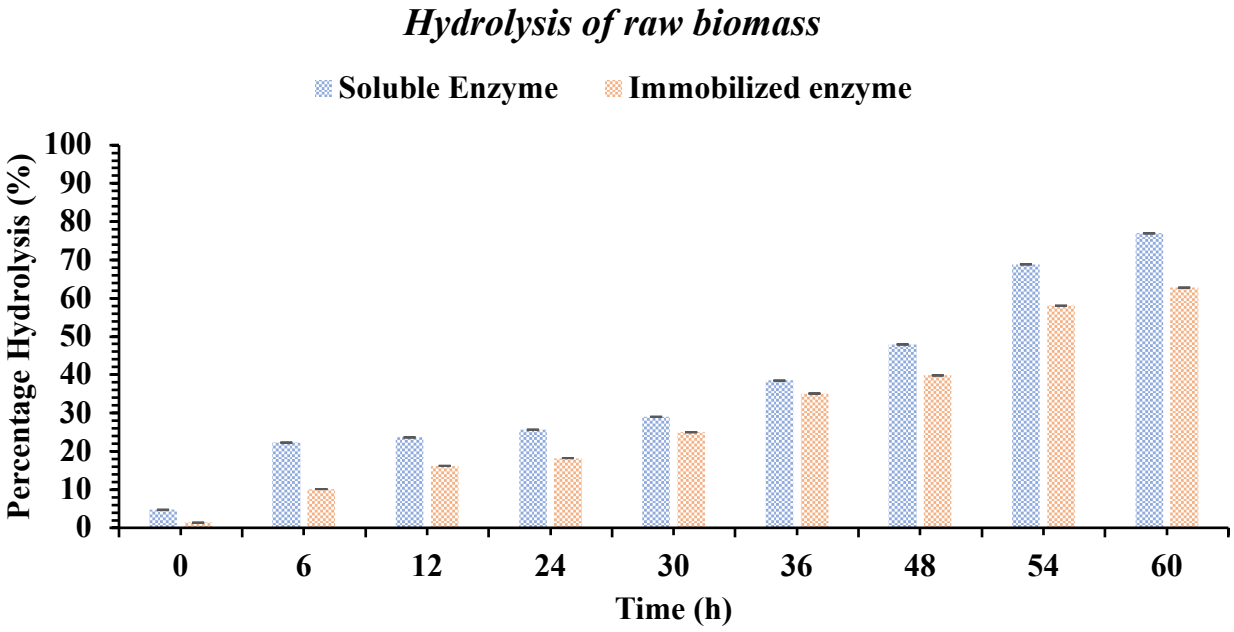

Fig 4.20 Hydrolysis of alginate sourced from a raw seaweed (Bull Kelp) by immobilized and soluble form of enzyme

4.4 HPLC analysis:

HPLC profile exhibited similar monosaccharide yield with soluble (20.5%) and immobilized (17.6%) alginate lyase. HPLC results confirmed that sugar hydrolysate contained insignificant variable quantities of monosaccharide sugars such as guluronic acid, mannuronic acid, mannose, galactose, xylose and fucose (Fig.4.22), however, glucose was significantly higher for hydrolyzed alginate by the soluble enzyme than the immobilized enzyme (Fig. 4.23). Immobilized alginate lyase led to less monosaccharide yield by 3% versus soluble form, however, immobilization can bring cost- effectivity by reusability.

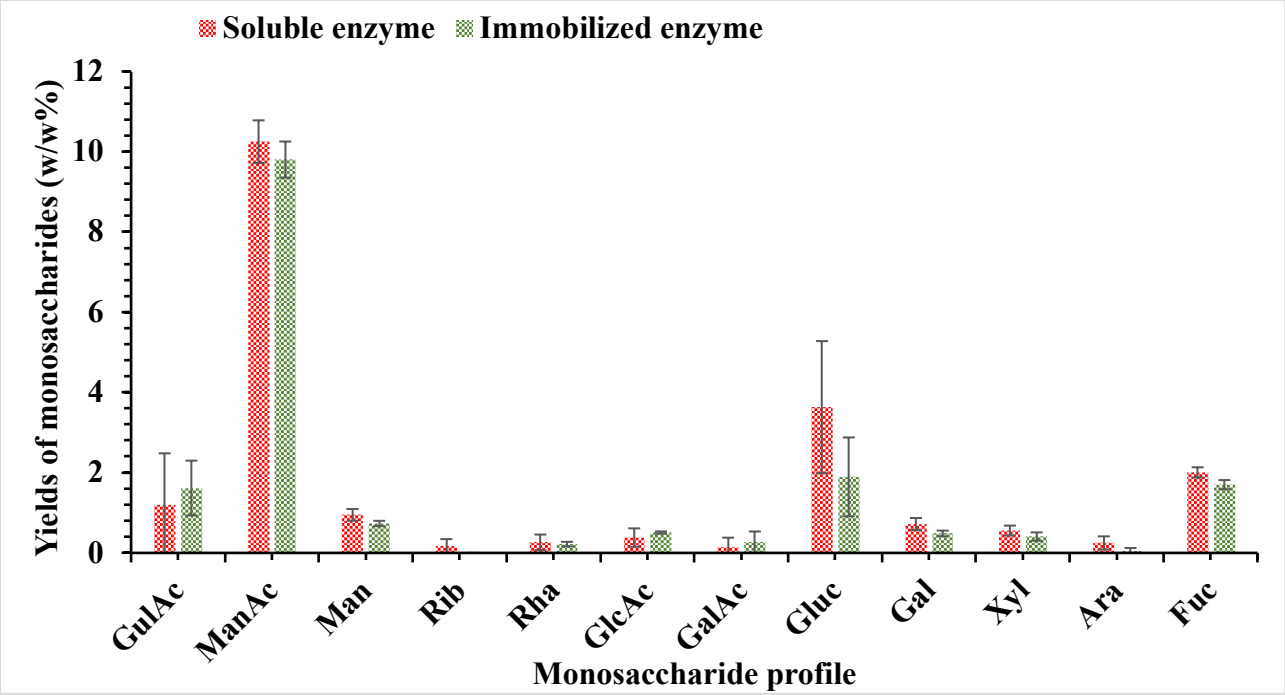

Fig 4.21 Hydrolysis of alginate using free and immobilized enzyme for 60 h (This experiment was conducted using soluble and immobilized alginate lyase. The hydrolysis was conducted for 60 h at 37 °C (free enzyme) and 45 °C (immobilized enzyme)

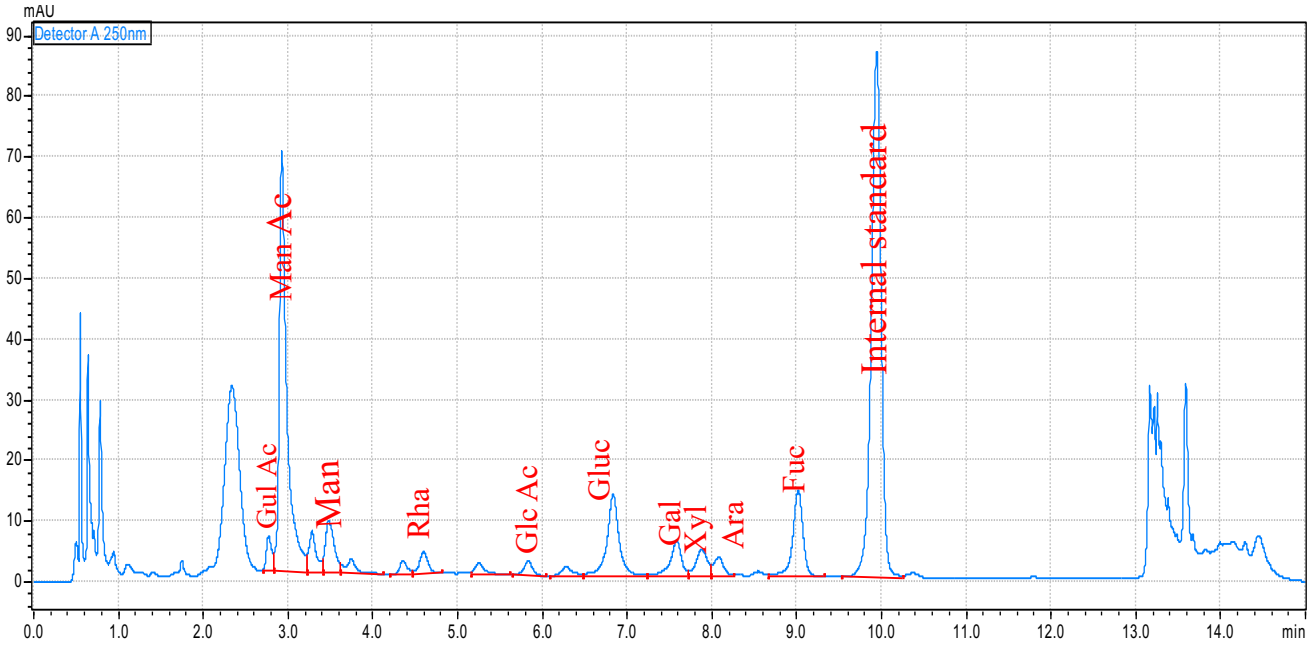

Fig 4.22 HPLC chromatograms: Quantitative analysis of hydrolysis of alginate with soluble enzyme

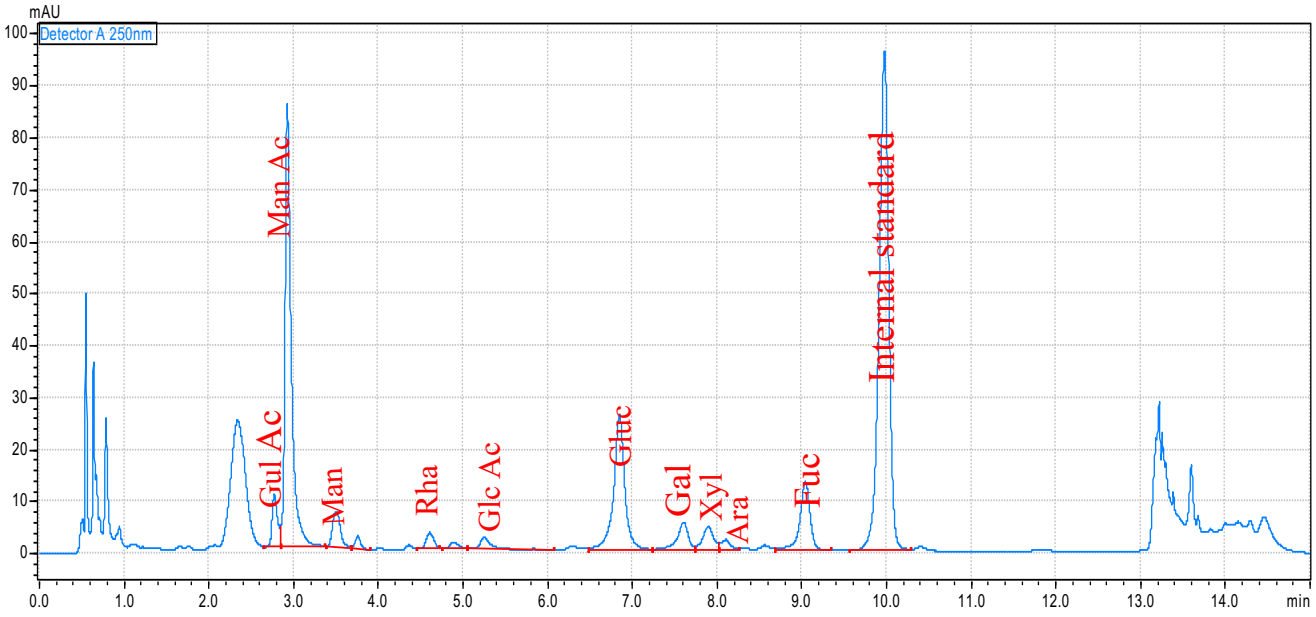

Fig 4.23 HPLC chromatograms: Quantitative analysis of hydrolysis of alginate with soluble enzyme

(Footnote: GulAc - guluronic acid; ManAc - mannuronic acid; Man - mannose; Rib - ribose; Rha - Rhamnose, GlcAc - glucuronic acid; GalAc - galacturonic acid; Gluc - glucose; Gal - Galactose, Xyl - Xylose, Ara - Arabinose, Fuc – Fucose)

Low hydrolysis of seaweed biomass was due to the intact structure of seaweed, as alginate is rigid and compactly packed, and the alginate lyase cannot penetrate in cellular components to hydrolyze alginate. We propose that a “pretreatment of alginate” with the physical aids such as temperature, and pressure can break the structure of raw biomass to smaller fragments (Maneein *et al.*, 2018). This will enhance the accessibility of the enzyme to the alginate and thus can facilitate hydrolysis of raw seaweed. Moreover, the hydrolysis of biomass can also be improved by using chemical methods such as acid or alkali treatments to open the kelp structure and improve reducing sugars yields (Abraham, 2014).

Chapter 5

Discussions and Conclusion

5. Discussions

Efficient and cost-effective controlled hydrolysis of alginate, a complex polymer has become a major challenge considering the excellent properties of resulting alginate oligosaccharides (AO). Biocatalytic hydrolysis has received much attention due to number of advantages and its considered environment- friendly benefits. Additionally, to cut down the cost associated, there was a call to reuse the enzyme by the immobilization (Arntzen *et al.*, 2021). In this study, the objective was to characterize the alginate lyase and to immobilize it onto a nanocarrier for improving its reusability and storage. To achieve the proposed objective, alginate lyase was covalently bound on a magnetic nanoparticle using glutaraldehyde as a cross-linker.

In the present study, various parameters were optimized for the hydrolysis of alginate using alginate lyase. The optimum enzyme concentration was observed to be 25 IU which was in agreement with a previous study, where 400 µL (Jiang *et al.*, 2019) achieved the reaction rate. Morover, a study reported the use of 2.3 and 3.3 IU of commercial enzyme from same source and observed nearly 20% of yield in 5-25 h (Kim *et al.*, 2012). As compared to the past studies, 0.2% (Li *et al.*, 2020b), 0.5% (Zhang *et al.*, 2020; Sun *et al.*, 2020a) to 1.5% (Gomaa *et al.*, 2019) substrate concentration were utilized for optimizing substrate hydrolysis. In one of the studies, less sodium alginate (0.3%w/v) was used for the characterization of novel alginate lyases (Lu *et al.*, 2019). However, 1% alginate was used for the characterization of new endo type alginate lyase (Zhu *et al.*, 2018b). This study was observed to be superior where 1.5% substrate was used to secure depolymerization by using low amount of alginate lyase. Enzyme showed maximum activity at 37 °C based on which, maximum hydrolysis (76.62%) of alginate was observed. Enzyme showed less activity at temperature 45 °C whereas one of the previous studies indicated, this temperature was optimum for maximum substrate degradation (Ma *et al.*, 2020) and it was stable with the maximum activity below this

temperature as in Fig. 4.3. While comparing with cold-adapted alginate lyases from *Vibrio* sp., which showed thermal stability below 35 °C, the alginate lyase showed similar activity (Ma *et al.*, 2020). However, most of the earlier reported enzymes were basophilic lyases and showed pH stability at higher salt concentrations (Ma *et al.*, 2020; Jiang *et al.*, 2019; Huang *et al.*, 2013). With the increase in the temperature beyond 40 °C, the reaction rate was reduced as it caused the denaturation of enzyme and pH 7.4 was observed to be maximum activity of the enzyme. As cited in earlier studies, alginate lyase exhibited optimum activity at acidic pH 6 (Li *et al.*, 2020a) and basic pH between 8 to 10 (Sun *et al.*, 2020b; Li *et al.*, 2011; Blanco-Cabra *et al.*, 2020; Hu *et al.*, 2020). In this study, optimum pH was at 7.4 and the further increase or decrease in the pH value made the enzyme unstable and thus hydrolysis of alginate declined. The maximum hydrolysis of the alginate was achieved within two hours and no obvious effect of shaking was observed. According to previous studies, the recorded incubation period was for an hour however, in the conducted study, it took 2 hours to attain maximum alginate hydrolysis (Li *et al.*, 2020b; Liu *et al.*, 2019; Lu *et al.*, 2019; Sun *et al.*, 2020a). Similar results for temperature and pH were observed from the previous study conducted by Cao *et al.* (2007).

Fe^{3+}, Mn^{2+}, Zn^{2+}, Na^{+} showed the enhancement in the activity of the enzyme however, different metal ions exhibit different effects on the activity of alginate lyase as reported previously (Lu *et al.*, 2019; Zhou *et al.*, 2020; Chen *et al.*, 2016). For instance, Sun *et al.* (2020b) and Zhou *et al.* (2020) accounted Fe^{3+} as an inhibitor for the alginate lyase however, in another study, Fe^{3+} enhanced the enzymatic activity (Yang *et al.*, 2019). Cu^{2+} was accounted as an inhibitor for the enzyme. Comparable effects of few metal ions were observed in the conducted study with the results reported by Cheng *et al.* (2020b).

The value of V_{max} was found to be same for both (0.2 mg/mL/min.) and K_m (10.10 mM) was low for soluble than the immobilized enzyme (20 mM). Previous studies indicated the high value of V_{max} 28.99 U/mg and K_m to be 5.5 mg/ml (Jiang *et al.*, 2020). Another study by Zhu *et al.* (2018b), the value for V_{max} was calculated to be 1.6 nmol/s. The results obtained for this study were less comparable with the results obtained from previous reports since the origin and source of the alginate lyase was different (Zhu *et al.*, 2018a).

During immobilization of the alginate lyase, the activation of the nanoparticle with the glutaraldehyde cross-linker was achieved in 60 min. As compare to other hydrolytic enzymes, for example, pectinase was incubated overnight to achieve the activation with the glutaraldehyde, (Dal Magro *et al.*, 2020). With the chitosan nanoparticle, the incubation was achieved in two hours, for the activation of magnetic nanoparticle (Dal Magro *et al.*, 2020). The observed Scanning Electron Microscope images confirmed immobilization of alginate lyase. Small spherical shaped nanoparticles with various size ranging from 500 nm to 300 μm (Fig. 4.12) were clearly observed under SEM. The formation of clusters of the nanoparticle confirmed the cross-linking of the nanoparticle to the enzyme (Fig. 4.13). At optimum temperature and pH, enhanced stability was recorded in the immobilized form of the enzyme than the soluble form. Similarly, studies demonstrated the stability of alginate lyase immobilized on chitosan nanoparticle at 45°C (Li *et al.*, 2019). Results showed the enhanced stability of immobilized alginate lyase at 45 °C and pH 9 as compare to soluble form (37 °C and pH 7.4) might be due to the solid matrix of nanoparticle which provide the buffering capacity to the enzyme and alteration in the protonation pattern. Activity of immobilized enzyme was retained (76%) when it was incubated for 1h at 45°C with magnetic nanoparticle and tannic acid (Jiang *et al.*, 2020). Another study demonstrated 83.7% activity of the enzyme immobilized on mesoporous titanium oxide particle, when incubated at same temperature and same period of time (Li *et al.*, 2020b). The

activity was insignificantly higher for the immobilized enzyme as compared to soluble. Higher pH leads to the enzyme-substrate complex distortion due to inactivation of enzyme (Mohapatra, 2020).

The operational stability of the magnetic nanoparticle- immobilized alginate lyase was analyzed by reusing the biocatalyst for eight consecutive batches. It was observed to be intact by 50% up to 5th cycle. Our results were in agreement with a study that reported the use of Fe_3O_4 nanoparticle for immobilized alginate lyase where immobilized enzyme exhibited 70% activity till 5th cycle and it declined to one third residual activity (Jiang *et al.*, 2020). Other reports also demonstrated the retainment of 60% of the activity of the immobilized enzyme by the end of 6th cycle which is highly significant (Li *et al.*, 2019). More than 66% of the activity of the immobilized enzyme was observed after using it for seven cycles when chitosan nanoparticle were used (Mohapatra, 2020).

The stability of immobilized enzyme was observed (68%) when stored at low temperature for 15 days. After incubation for 21 days, the activity of alginate lyase was recorded as 33%. However, for the alginate lyase (AlgL17 from recombinant *E. coli*) immobilized on Fe_3O_4, 44% activity was retained after 30 days. Thus comparing the studies, it showed the enhanced durability of the storage of immobilized enzyme than the soluble form (Jiang *et al.*, 2020). Upon translating alginate lyase activity to real substrate, 77% hydrolysis of seaweed biomass was observed by soluble enzyme, however, immobilized enzyme exhibited substrate hydrolysis (63%) in 60 h. Previous studies indicated hydrolysis (60.5%) of the biomass in 12 h when alginate lyase was isolated from microbes (*Pseudoalteromonas* sp) (Sun *et al.*, 2020a). The present study demonstrated improvement in the hydrolysis of biomass by using immobilized alginate lyase.

6. Conclusion

The significance of catalytic hydrolysis of alginate to produce value- added oligosaccharides proposed the requirement of biocatalyst that can endure harsh conditions for the industrial use. In the current study, the alginate lyase was characterized for all the factors associated with its enzymatic activity and immobilized onto a nanocarrier with the magnetic property to facilitates its separation from the reaction mixture and reuse. There was more than 75% of the hydrolysis of the alginate with the soluble enzyme which was attained after the optimization of all the factors such as enzyme concentration, pH and temperature along with the effect of metal ions and kinetic study. Results observed for immobilized enzyme confirmed the activity and thermal stability (for 3h) at higher temperature correspond to the soluble enzyme. Furthermore, immobilization enhances the stability of enzyme with elevated salts (from 7 to 9) and broad variations of pH with the improvement of reusability for six consecutive batches with 50% retained activity. Immobilized alginate lyase activity can be reused by storing it at 4°C for several days.

This confirmed the long-term reusability and storage of immobilized alginate lyase which suggests the cost effective and environmentally sustainable approach for the bioprocessing of seaweed.

7. Limitations and Future directions

Reports are available for number of studies conducted to enhance the thermal stability of the enzyme by physical, chemical, and biological methods. However, none of them was recorded to be efficient because of the limitations associated. Yang *et al.* (2018) studied the increase in the stability of enzyme for two hours by adding glycerol stabilizer (30%) which in turn raises the complications to purify and lyophilize the enzyme. The major challenge is to maintain the integrity of secondary structure of the protein and the activity of the enzyme along with the thermal stability. Due to the efficiency and various extraordinary properties of nanoparticles, immobilization technique is the best approach to significantly enhance thermal stability for different times, for the upholding of enzymatic activity and its applications in various fields (Li *et al.*, 2019). Although the hydrolysis rate of alginate by the soluble enzyme was higher than the immobilized enzyme, the factors like reusability of immobilized enzyme for 6 cycle with more than 50% relative activity, thermal and pH stability confirms the desirable and satisfactory use for the biocatalytic degradation of polymer. Moreover, the storage of immobilized enzyme fortifies its demand for industrial use.

Almost 50 alginate lyases are being identified and extracted from the different sources and the genes are being sequenced. Because of the valuable production of enzyme and the alginate products, the microorganisms are being focused to optimize the extraction and to obtain maximum yield. Extensively, the research on the micro-organisms is of greater importance for the expected alginate lyase production and to meet the requirements (Szekalska *et al.*, 2016). It is hard to realize the production of this enzyme at industrial level due to the limitations associated like: (i) tedious and time-consuming production process of alginate lyase, (ii) difficulty in downstream processing, iii) difficult to separate out the enzyme from the substrate due to the greater substrate specificity, and iv) weaker enzymatic activity, however, some microorganisms which produce alginate lyase also exhibit

pathogenicity- so risky to cultivate. Moreover, the commercially available enzyme is expensive (Ming *et al.*, 2021). There are some methods available for the commercial production of alginate lyase with high yields, but there are the boundaries with the practical applications. So the biological engineering of the cell at genetic and cellular level is the upcoming trend to revolutionize the technology of commercial production of enzyme and to improve the limitations (Szekalska *et al.*, 2016; Hu *et al.*, 2021).

Alginate can be extracted from the brown seaweeds and can be used for the synthesis of Algi-Matrix (a lyophilized sponge). The cells get entrapped inside the pores after the hydration of lyophilizate. Moreover, the foam structure of alginate can be synthesized to initiate gelation of polymer and the vial of alginate can be dissolved in the medium of culture. This is called as Nova-Matrix 3D and the pores once filled with the solution of alginate and this facilitates the quick cell immobilization in the physiological conditions (Cho *et al.*, 2016). Alginate-encapsulation technology is the advanced research for the cell transplantation therapy for the treatment of neurodegenerative diseases and diabetes. The most interesting polymers are the alginate oligosaccharides obtained by depolymerization of the alginate (Vidallon *et al.*, 2020). They have attracted greater attention because of the spectacular properties and being a drug vehicle in the treatment therapies. The oligosaccharides obtained with the higher G content is the novel therapeutic to exhibit the mucolytic activity and to deteriorate the biofilm synthesized by the bacterial colonization (Szekalska *et al.*, 2016).

Considering the well-documented applications of alginate based bioactive compounds in various industrial fields, the requirement of pocket friendly approach to generate oligosaccharides can be fulfilled by reusing same enzyme for multiple times by immobilizing it. This study confirms the improvement of enzyme activity through this technique however, further fortification to depolymerize alginate (more efficiently) is still a concern. So by analyzing the use various other

nanocarriers or particles for the immobilization can be done to amplify the seaweed alginate depolymerization.

8. References

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9. Appendices:

9.1 Figures:

Fig. 2.1: Different sources of alginate lyase

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http://nutrawiki.org/wp-content/uploads/2015/07/Laminaria-ochroleuca.jpg

https://upload.wikimedia.org/wikipedia/commons/thumb/b/b2/Kelp_300.jpg/397px-Kelp_300.jpg

Chemical-structure-of-alginate-Linear-block-polymers-of-b-d-mannuronate-M-and.png (784×545) (researchgate.net)

Fig. 2.3: Structure of subunits of alginate: 1,4- α-Guluronic acid (monomer unit of G) and 1,4-β-D-mannuronic acid (monomer unit of G)

https://www.mdpi.com/microarrays/microarrays-04-00133/article_deploy/htmL/images/microarrays-04-00133-g001.png

7http://2011.igem.org/wiki/images/2/27/Kyoto-digestion-DNSassay1.jpg

Fig. 2.6: Reaction involved in the DNS assay for the estimation of estimation of enzymatic activity (https://ars.els-cdn.com/content/image/1-s2.0-S0008621520301592-fx1.jpg)

Fig. 2.8: Application of oligosaccharides in food industry: animal food, food additives

https://cpimg.tistatic.com/04766098/b/4/extra-04766098.jpg

https://www.hpcimedia.com/images/website/ManChemNews/DIR_28/F_27153.jpg

https://cdn.shopify.com/s/files/1/1711/7499/products/sodium-alginate-cape-crystal-2-oz_800x.jpg?v=1537781228

https://www.artmolds.com/pub/media/wysiwyg/food-groups.png

http://www.shreejifood.com/products/alginate.jpg

http://www.food-chem.com/images/attributes/Sodium%20Alginate2.jpg

Fig. 2.9: Applications of oligosaccharides in Pharmaceutical and Medical industry

-https://medimart.com/wp-content/uploads/2020/02/00028069-280x280.jpg

https://renata-ltd.com/wp-content/uploads/2015/03/Pharmaceuticals-Algin-Syrup.jpg

https://ik.imagekit.io/Mc121541/pub/media/wysiwyg/unprocessed-supplements_1.png

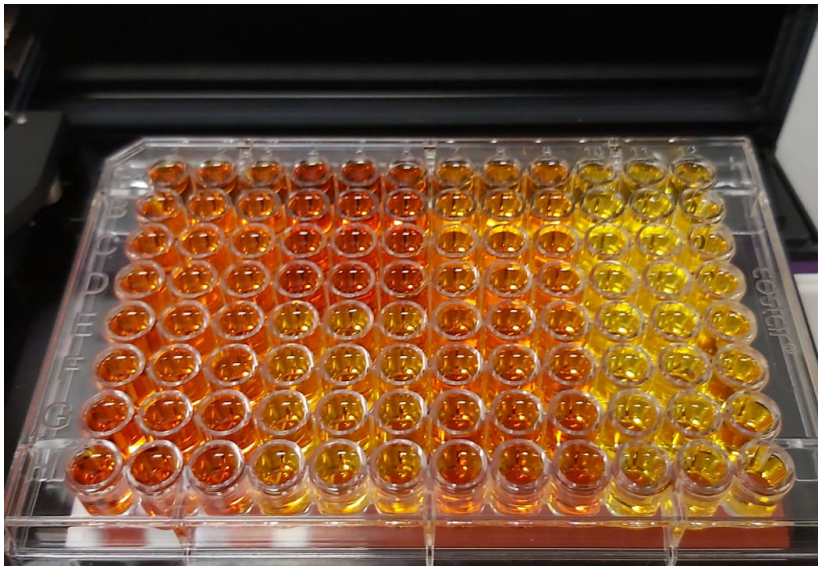

Fig 9.1 DNS assay for the estimation of reducing sugar released (product produced)

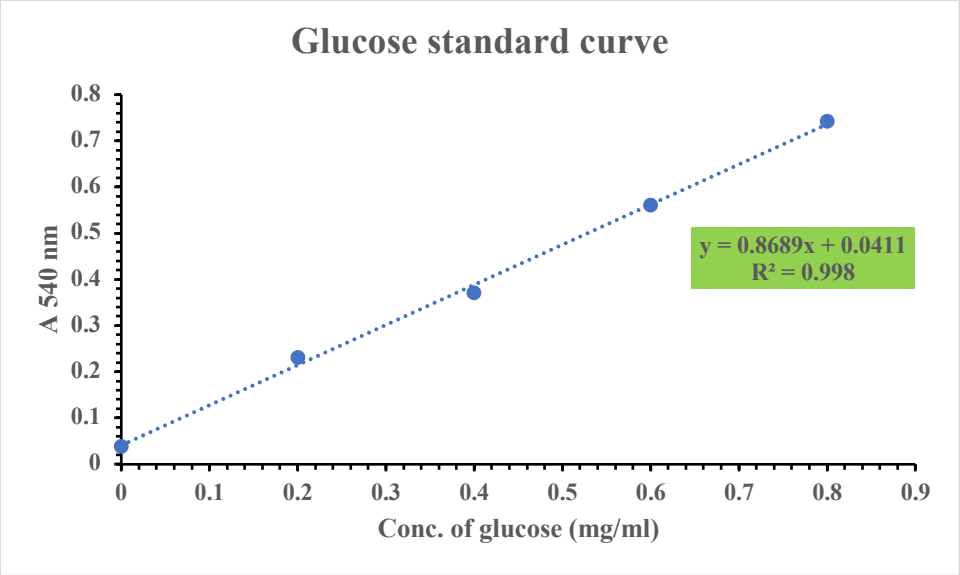

Fig 9.2 Glucose standard curve (A 540 nm vs glucose concentration)

9.2 FTIR (Fourier Transformation Infrared Spectroscopy) analysis:

FTIR spectroscopy was performed to assess the interaction between nanoparticle and enzyme using Shaimadzu Infrared Spectrophotometer. The diamond crystal was used to derive the spectra. Samples were scanned over the range of 400-4000 cm^{-1} wave number with 32 scans resolution. Finally, the spectra were obtained and analyzed (Patel *et al.*, 2019).

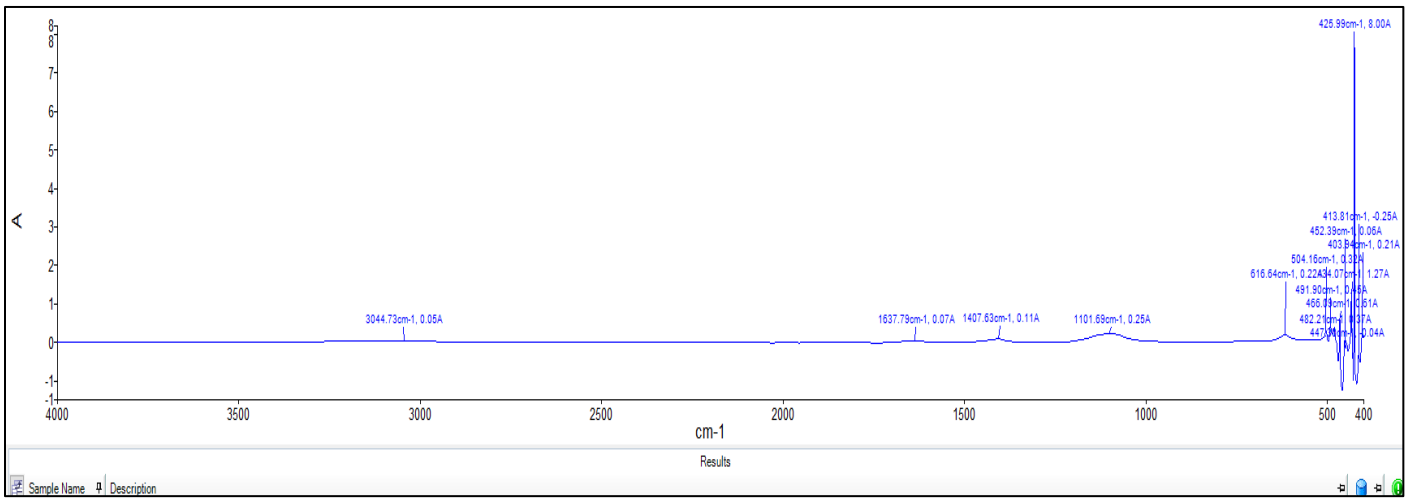

Fig 9.3 FTIR spectrum of alginate lyase

As shown in Fig. 9.3 of FTIR spectra of pure alginate lyase, absorption peak was observed between the range of 1101 to 1637 cm^{-1} which were reported for the vibration of C-H bond and C-O-H bond of 1° alcohol of the enzyme in previous studies (Mohapatra, 2020). However, most of the peaks appeared were in the range of 400- 500 cm^{-1} and no previous data was recorded in this range (Abraham, 2014)

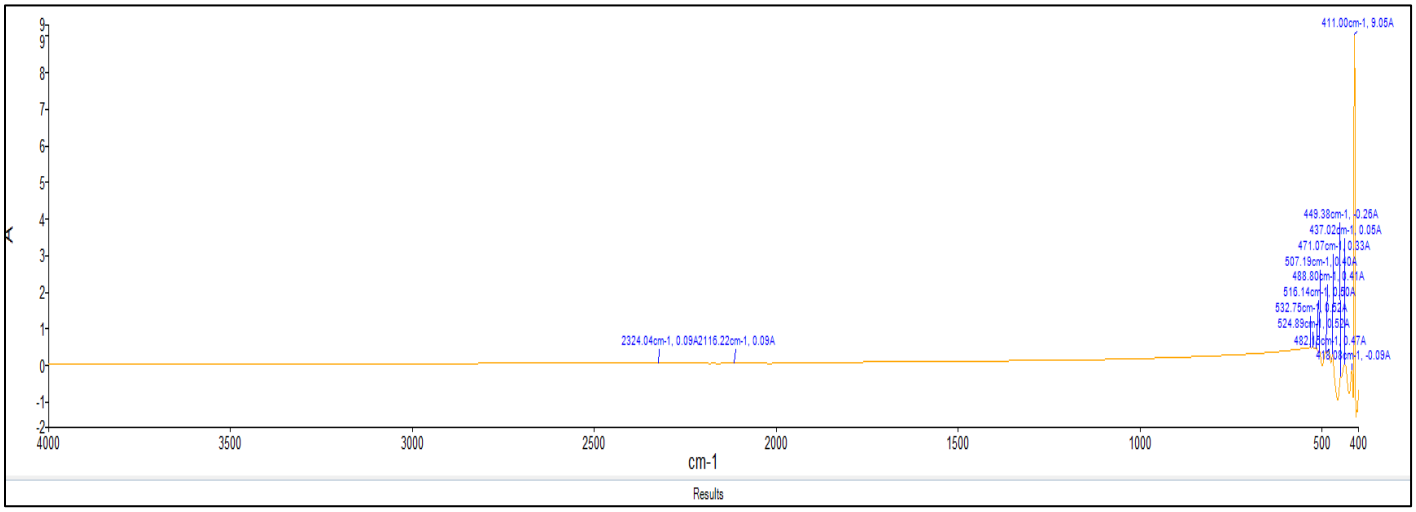

Fig 9.4 FTIR spectrum of magnetic nanoparticle

As shown in Fig. 9.4, two peaks were observed in the range between 2000 to 2500 cm^{-1}, and no similar results were obtained from previous reports.

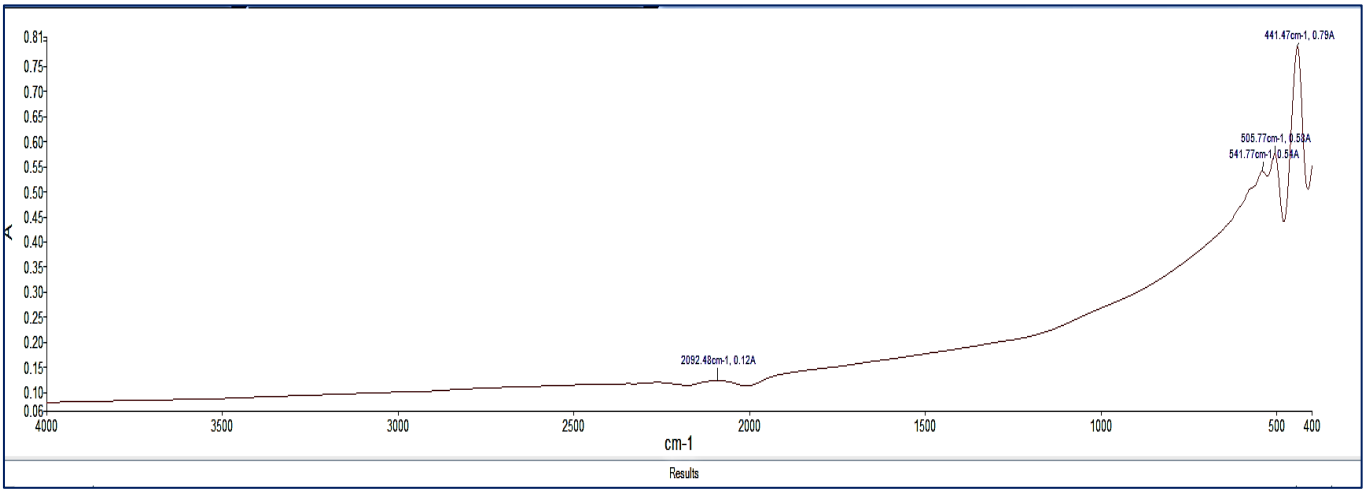

Fig 9.5 FTIR spectrum of immobilized enzyme

Results in Fig. 9.5 confirmed the stretching in one of the peaks from the nanoparticle spectrum (Fig. 9.4). The shift of the peak can be either due to the stretching of the bonds by the immobilization and binding of nanoparticle to the enzyme. The data obtained from the FTIR results was significantly variable from the previous reports due to the use of different nanoparticle for immobilization. As chitosan was used by Patel et al (2020) for the immobilization of same enzyme, uncommon peaks were observed in the FTIR spectrum (Patel et al., 2019).