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Review Article

Pectinase: Immobilization and Applications (A Review)

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HIGHLIGHTS

- Pectinase is a complex enzyme with different catalytic units including polygalacturonase, pectin esterase and pectin lyase.
- Pectinase immobilization enhanced the stability of pectinase and made the enzyme reusable for continuous industrial processes.
- Entrapment, binding to a support and enzyme crosslinking are the common methods used for the immobilization of pectinase.

ABSTRACT

Pectinase or pectinolytic enzyme is a complex enzyme with different catalytic units including polygalacturonase, pectin esterase and pectin lyase to degrade pectin polymers into different end products. The pectinase with degrading capability of pectin can be utilized in various industrial applications, such as clarification of fruits and vegetable juices, degumming of plant bast fibers, textile industries for removing non-cellulosic impurities, papermaking industries, wine clarification, coffee and tea fermentation, wastewater treatment, as well as, it is also used for the isolation of protoplasm in plant science research. Pectin derived oligosaccharides (POS) produced by pectinase are considered as prebiotic molecules. However, the low operational stability in harsh industrial conditions confines the utilization of pectinase in industrial processes. Immobilization is the technique, which not only enhanced the stability of pectinase, but also, made the enzyme reusable for continuous industrial processes. The current review shares information about the pectinase and how the immobilization technology can enhance the industrial application of pectinase. Furthermore, various supports such as sodium alginate, agar-agar, polyacrylamide, aminated silica gel, nanocomposite microspheres, silica coated chitosan, nylon-6, porous glass etc. have been tested for the immobilization of pectinase through different methods, including entrapment, binding to a support and enzyme crosslinking.

Keywords:
Degradation
Enzyme
Immobilization
Pectin
Pectinase

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Pectin

The cell wall surrounds the plant cell to protect the cell and its distinctive components from different environmental

stresses. The basic structure of plants cell wall composed of polysaccharides, protein, aromatic and aliphatic compounds and these compounds of cell wall make the plants to grow in various environmental positions (Fig. 1). Among these polysaccharides, pectin is the most interesting one, due to its structural organization and functionality. Pectin is present in the primary cell wall and middle lamellae of higher plants, and responsible for the structure integrity and cohesion of plant tissues (Rombouts and Pilnik, 1980; Chen, et al., 2015).

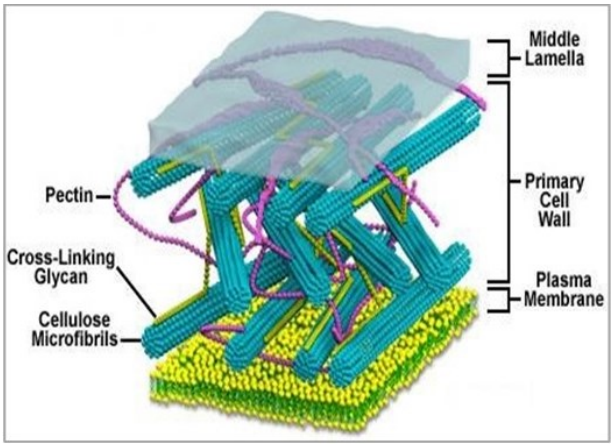

Figure 1. Plant cell wall structure.

The pectin was first discovered in eighteen century as a "peculiar substance" from tamarind fruit (Vauquelin, 1790), but at that time no specific name was given until nineteen century when the basic characterization of pectin exposed. It is one of active components of fruits and is responsible for gel formation in these product (Liang, et al., 2020). As an outcome, Nussinovitch suggested the word 'pectin' in reference to Greek work '*pektikos'*, which means congeal, solidify or curdle (Wang, et al., 2018). The actual chemistry of pectin was began when Ehrlich discovered that the D-galacturonic, an isomer of D-Glucuronic acid is the central constituent of pectin and the some of these D-galacturonic acid are partially esterified with methyl alcohol (Willats, et al., 2001). Pectin is composed of at least 17 kinds of different monosaccharides, in which D-galcturonic acid is usually the most abundant, followed by D-galactose or L-arabinose (Vincken et al., 2003; Yapo, 2011). The pectin is a complex carbohydrate polymer of galacturonic acid residues having esterified carboxylic groups with methanol. The degree of esterification of carboxylic groups of pectin is differing from source to source. Structurally, the pectin polymer is composed of four well characterized polysaccharide units, such as homogalacturonan, xylogalacturonan, rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII) (Fig. 2) (O'Neill et al., 1990; Jayani et al., 2005; Zhang, 2006). Homgalacturonan consists of α-1, 4 linked galacturonic acids molecules and makes the main chain of pectin polymer. RGI syntheses the branched area of pectin, consisting of various side chain of α-1, 2 linked rahmnopyranose residues. RGII contains side chain of D-apiose, 2-O-methyl-D-xylose-and 2-O-methyl-L- fructose (Chan, et al., 2017). The galacturonic residues are usually acetylated at the C-2 or C-3 position in rehmnogalacturonan 1. Most of the molecules in the chain are formed by D-galacturonic residues. The pectin polymer does not form a straight chain conformation in aqueous medium, but it is curved and extended with great flexibility. Variability in the composition of pectin was found from different sources and the properties of pectin are strongly depended on the methylation of galacturonic acids residues, which is usually 70 % (Thakur et al., 1997; Haas, et al., 2020).

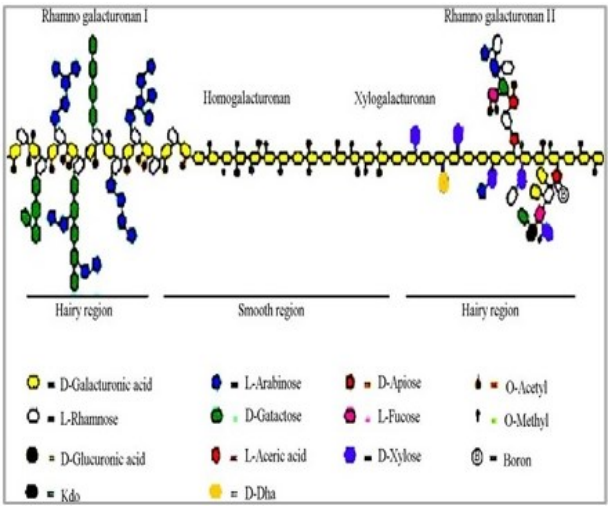

Figure 2. Basic structure of pectin (Zhang, 2006).

Classification of pectin

Pectin is classified into protopectin, pectic acid, pectinic acid and pectin due to some structural and composition changes (Valdés, et al., 2015).

A. Pectin

Pectin is terms used to illustrate the water insoluble pectic substances in plant tissues and from which soluble

pectin are produced. It is parent pectin and on restricted hydrolysis yields pectin or pectinic acid (Valdés, et al., 2015).

B. Pectic acid

Pectic acid is water soluble polymer of galacturonan that contain insignificant amount of methoxy groups (Valdés, et al., 2015).

C. Pectinic acid

Pectinic acid is the polygalacturonan that contain various amount of methoxy groups and also, has the property to form gel with sugar and acid (Valdés, et al., 2015).

D. Pectin (polymethylgalacturonate)

Pectin is the polygalacturonic acid in which the carboxyl groups of galacturonic acid residues are 75 % esterified with methanol (Valdés, et al., 2015).

Distribution of pectin in nature

Pectin is widely distributed in nature and mostly found in angiosperms and gymnosperms plants, along with pteridophytes, bryophytes, lycophytes and carophytes (Matsunaga et al., 2004; O'Neill et al., 2004). It is the main component of plant with high percent composition and it approximately covered 35% of a plant mass (Voragen et al., 2009). Based on environmental conditions, maturation time and type of plant, the content of pectin differs from plant to plant (Table 1). Apple pomaces and citrus peels are the commercial sources for pectin, which contain approximately up to 20 g of pectin per 100 g of source material (Fernandez, 2001).

Table 1. Pectin content in various fruits.

Fruits	Botanical name	Pectin content (%)	References
Apple	*Malus* spp	0.5-1.6	Karr, 1976
Apple pomace		1.5-2.5	Renard and Thibault, 1993
Banana	*Musa acuminate* L.	0.7-1.2	Karr, 1976
Beet pulp	*Beta vulgaris*	1.0	Renard and Thibault, 1993
Carrot	*Daucus carota*	0.2-0.5	Renard and Thibault, 1993
Giant granadilla	*Passiflora quandrangularis* L.	0.4	Hodgson and Kerr, 1991
Carambola	*Averrhoa carambola*	0.66	Hodgson and Kerr, 1991
Guava	*Psidium guajava* L.	0.77-0.99	Hodgson and Kerr, 1991
Lemon pulp	*Citrus limon*	2.5-4.0	Renard and Thibault, 1993
Lychee	*Litchi chinesis* S.	0.42	Karr, 1976
Mango	*Mangifera indica* L.	0.26-0.42	Hodgson and Kerr, 1991
Orange peel	*Citrus sinesis*	3.5-5.5	Renard and Thibault, 1993
Papaya	*Caricia papaya*	0.66-1.0	Hodgson and Karr, 1991
Passion fruit	*Passiflora edulis* L.		Hodgson and Karr, 1991
Passion fruit rind		2.1-3.0	Hodgson and Karr, 1991
Peaches	*Prunus persica*	0.1-0.9	Karr, 1976
Pineapple	*Ananas comosus* L.	0.04-0.13	Hodgson and Kerr, 1991
Strawberries	*Fragaria ananassa*	0.6-0.7	Hodgson and Kerr, 1991
Tamarind	*Tamarindus indica* L.	1.71	Hodgson and Kerr, 1991
Thimbleberry	*Rubus rosalfolius*	0.72	Hodgson and Kerr, 1991
Tomato fruit	*Lycopersicon esculentum*	0.2-0.6	Karr, 1976

Industrial uses of pectin

Pectins are biocompatible, inexpensive, and one of the most abundant natural compounds on earth having different industrial applications. The pectin have been used as food additive, thickening agent, gelling agent, cosmetic texturizing agent, a good source of dietary fiber and also, components of biodegradable films, adhesives, paper and recently developed antimicrobial food packaging (Mohnen, 2008). Pectin polymers are significantly important in different fields of industry and biomedicine. In order to increase pectin's usefulness, we must understand how the complex structures of pectin chains are enzymatically modified.

Pectinase

Sustainable methodologies in industrial processes for manufacturing of different important products, using enzyme, has much to offer as compared to chemical catalyst. Enzyme can perform its function under mild reaction conditions (physiological pH and temperature); it is a substrate specific and biodegradable. Enzymes affords a synthetic way that is shorter, generate less wastes and both environmentally and economically more feasible than conventional organic synthesis. Pectinase is an enzyme that catalyzes the degradation of pectin substances. Pectinase is widely distributed in nature and produced by different living organisms like fungi, bacteria, yeast, insects, nematodes and protozoa. Due to economic feasibility, microorganisms have been commonly used in many industries for commercial production of enzymes and microbial pectinases have tremendous applications in different industries (Hoondal et al., 2002). Pectinase is a generic term used for a group of enzymes that catalyzes the degradation of pectin substances by hydrolysis, trans-elimination, as well as, de-esterification reactions (Singh, et al., 2020). Pectinase is one of the most important industrial enzymes that have significance role in the current biotechnological era with wide range of applications in fruit juices extraction, textile processing, paper making, pectin containing waste water treatment, degumming of plants bast fibers, wine clarification, oil extraction, coffee and tea fermentation (Kashyap et al., 2001; Hoondal et al., 2002; Jayani et al., 2005).

Classification of pectinase

Pectinase have been classified into polygalacturonase, pectin lyase and pectin esterase (Fig. 3).

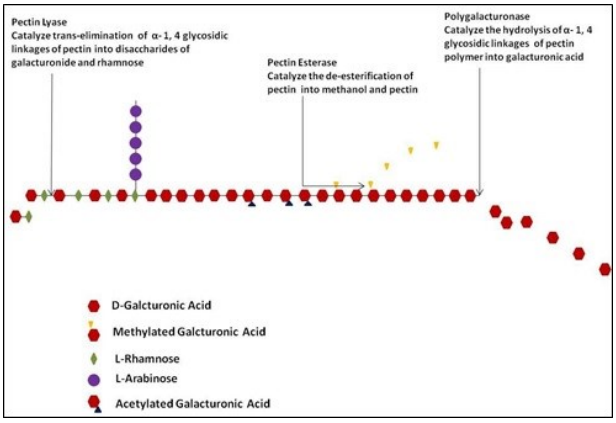

Figure 3. Mode of degradation of pectin by different pectinases.

A. Polygalacturonase

Polygalacturonase catalyzes the hydrolysis of pectin polymer into D- galacturonic acid monomer by addition of water molecules in α-1, 4 glycosidic linkages. Polygalacturonase is the most extensively studied pectinolytic enzyme and further classified into exo-polygalacturonase and endo-polygalacturonase by the breaking of external and internal α-1, 4 glycosidic linkages of pectin molecule, respectively (Satapathy, et al., 2020).

B. Pectin lyase

Pectin lyase catalyze the breaking of α- 1, 4 glycosidic linkages of pectin by trans-elimination and generate glacturonide with unsaturated bond between C-4 and C-5 at the non-reducing end of galacturonic acid. Pectin lyase is classified into exo pectin lyase and endo- lyase on the basis of catalyzing the cleavage of α- 1, 4 glycosidic linkages sequentially and randomly, respectively (Sangeeta, et al., 2020).

C. Pectin esterase

Pectin esterase is also known as pectin methyl hydrolase that catalyzes the de-esterification of pectin molecule into pectic acid and methanol. The pectin esterase preferentially acts on the methyl ester group next to non-esterified galacturonate unit end (Amin, et al., 2019).

Sources of pectinase

Pectinase plays a very significant role in various biological processes across the entire field of living

organism. The pectinase is widely distributed in nature and produced by different living organisms, such as plants, microorganisms, insects, nematodes and protozoa. Microorganisms are considered as primary source for the production of industrial important enzymes (Singh et al., 2019).

Microbial pectinases

Pectinase is one of important factor in the plant pathological process, in plant-microbe symbiosis and in the decomposition of plant decay matters. The microbial pectinase is playing important role in nature by contributing of natural recycling of carbon in the environment. *Aspergillus niger* is mostly used for the industrial production of pectinase (Maldonado et al., 2002). The pectinases from fungus sources are usually acidic in nature that only can work in acid conditions not in neutral or alkaline conditions, while the bacterial strains are known to produce alkaline pectinases which can be apply for alkaline preparation. *Bacillus licheniformis* KIBGE-IB21 isolated from rotten vegetable was found to produce pectinase at 37 °C and pH 7.0 after 48 hours of fermentation period (Rehman, et al., 2012).

Biochemical properties of pectinase

Biochemical properties of enzyme are very important for their commercialization. The characterization of pectinase from different microorganisms have been done previously, and the biochemical properties of enzyme was varied from source to source (Gummadi and Panda, 2003; Esquivel and Vogel, 2004; Kaur et al., 2004). The pectinase from different microorganism have optimum temperature in the range of 30 to 60 °C and pH from 3.5 to 9.0 for maximum enzymatic activities (Gummadi and Panda, 2003). They also have different molecular weight, thermal stability and kinetic parameters (Jayani, et al., 2005). The pectinase from *Bacillus sp.* (Kobayashi et. al., 2001), *Mucor flaves* (Gadre et al., 2003) and *Trichoderma reesei* (Mohamed et al., 2003) sources have optimum temperature for maximum enzymatic activity around 40 and 50°C. Mohamed et al. (2006) reported that pectinase from *Trichoderma harzianum* showed 100% stability at 30°C for 60 minutes. The stability of enzyme provides valuable information about its structure and function. The stability of pectinases is influenced by physical (pH and temperature) as well as chemical parameters (inhibitors and activators) conditions. Pectinase from *Acrophialophora nainiana* showed maximum activity at 60°C and pH 8.0, and stability at 50°C with half-life of 7 days (Celestino et al., 2006). *Bacillus sp.* MG-cp produced alkaline pectinase, which showed stability at broad pH range from 7.0 to 12.0 and it retained more than 80% of its initial activity at room temperature after 24 hours (Kapoor et al., 2000). The pectinase activity was enhanced in the presence of Fe^{2+}, Mn^{2+}, Ca^{2+} and Cu^{2+}, but strongly inhibited by Mg^{2+} (Kaur et al., 2004). Pectinase showed higher relative activity in the presence of Mg^{+2} and Ca^{+2} and the activity of reduced in the presence of Mn^{2+}. (Oumer and Abate, 2017). Calcium ions also improved the activity of pectinase from *Bacillus* MG-cp-2 (Kapoor et al., 2000).

Applications of pectinase

Pectinase has different applications in various industries. Following are some important applications of pectinase in different aspects.

A. Fruit and vegetable juices industries

Pectinase has been widely used in fruit and vegetable juices industries. These industries commercially produced different juices, including sparking clear juices, cloudy juices and unicellular product, where the objective is selectively hydrolyze the polysaccharides of middle lamella to protect the integrity of plant cells. The objectives of using pectinase are different in all these three types of fruit and vegetables juices. In sparking clear juices, pectinase is added to increase the yield during the pressing and straining of the juices, as well as, to remove suspended particles to produce sparkling clear juices. For example, apple juices are manufactured as natural, unfiltered, unclarified and pulp containing juice. Amber-colored and clarified juice is prepared by enzymatic treatment and pectinases are the major types of enzymes used in apple juice pressing because of depolymerising activity of highly esterified pectin (Kashyap et al., 2001). The uses of pectinases aid the production of French ciders with high and consistent quality from different varieties of cider apples (Jayani, et al., 2005). Therefore, the uses of pectinases encourage good fermentation to produce high quality of juices and aromatic cider. In case of cloudy juices, pectinases containing high levels of polygalacturonase activity are used to stabilize the cloud of citrus juices, purees and nectars. Unicellular product are formed through a

process called maceration, in which organized tissue transformed into suspension of intact cells that can be used as material for pulpy juices and nectars, as baby foods, an ingredients for the dairy products, such as puddings and yogurt. If the objective of maceration process is carried out to leave as many cells as possible and the transformation of tissues into a suspension of intact cells, then the enzymatic degradation of pectin after mild mechanical treatment is good method that often improves the properties of product (Amin et al., 2019).

B. Formation of protoplast

Two main methods including mechanical and enzymatic methods have been used for the formation of protoplast from plant cell. In mechanical method, the formation of protoplast involved cutting of plasmolysed tissue with sharp-edged knife and release of protoplast by deplasmolysis. Low yield of the protoplasts limit the uses mechanical method. Enzymatic method is easy to perform and gives higher yield of protoplast as compared to mechanical method. Therefore, enzymatic method was mostly used for this purpose. The pectinase with the combination of cellulase have been used for the formation of protoplasts from practically every plant tissue, in which cells have not acquired lignifications (Power and Cocking, 1969).

C. Textile industry

Pectinase with the combination of other enzymes such as amylase, lipase, cellulase and hemicellulase have been used to remove sizing agents from cotton replacing the uses of harsh chemical in textile industry. Uses of enzymes ensured the low discharge of waste chemical in environment and improved both the safety of textile workers with the quality of fabric. Traditionally, the scouring of cotton was done with uses caustic alkaline solution (3-6% aqueous sodium hydroxide) at high temperature to accomplish the consistent dyeing and finishing. The process requires huge amounts of water for rinsing once the process is complete, high energy requirement and yield toxic waste product that can damage the environment. Bioscouring is a novel process of using enzymes to specifically remove the non-cellulosic impurities, such as pectin, protein and fats from the fiber. For example, pectinases, proteases and lipases could be used for the degradation of pectinic substances, proteins and fats, respectively. More than energy conservation and environmental friendly bioscouring process do not affect the cellulose backbone, it significantly results limiting fiber damage (Hoondal et al., 2002).

D. Degumming of plants bast fibers

Pectinase has been used in degumming process of plant fibers such as ramie, sunn hemp, jute, flax and hemp to remove the gum before subjecting them for textile making (Kapoor et al., 2001; Rebello et al., 2017). The enzymatic degumming process using pectinase with combination of xylanase, which is environmentally friendly and economic, is excellent replacement of chemical degumming process, which is polluting, toxic and non-biodegradable (Kapoor et al., 2001).

E. Retting of plant fibers

In traditional retting process, a mixture of microbial cultures were used, which produced pectinase that release cellulosic fibers from fiber bundles. As compared to traditional retting enzymatic retting, it is faster, controlled and produces fewer odors. Pectinase has been used to separate the fibers from jute and flax by eliminating pectin (Henriksson et al., 1999; Hoondal et al., 2002).

F. Waste water treatment

Typically, multiples steps such as physical dewatering, spray irrigation, chemical coagulation and chemical hydrolysis are carried out for the treatment of wastewater from vegetable food industries that mostly contain pectinacious materials. The uses of pectinases are a good alternative, eco-friendly and cost effective method that selectively remove pectinacious material and make it feasible for decomposition by activated sludge treatment (Hoondal et al., 2002; Oumer, 2017).

G. Coffee and tea fermentation

Pectinase has a significant role in tea and coffee fermentation. In addition to increase tea fermentation, pectinase also destroy the foaming character of instant tea powder by degradation of pectin (Shet, et al., 2018). For the fermentation of coffee, pectinase has been used to remove the mucilage coat from coffee beans (Jayani et al., 2005).

Limitation of using native enzyme in industrial processes

Advancement of biotechnology over last three decades, enzyme have drawn considerable interest of many biotechnologist, because of having some excellent characteristics (high activity, selectivity and specificity). The stability of enzyme against extreme temperature, harsh pH environment and organic solvents is prime important for its commercialization. The low stability of enzymes against harsh industrial conditions limits their uses in commercial processes. Therefore, the engineering of enzymes from their native form to industrial bioreactor is very exciting object and many techniques such as protein engineering, chemical modification, adding additives and immobilization are available to improve catalytic properties of enzymes (Bilkova, et al., 1999; Khajeh, et al., 2001; Costa, et al., 2002; Minishull, et al., 2004). Immobilization is the technique that not only increases the catalytic properties of enzyme, but also makes them reusable for different batch of reactions. Immobilization also aids the efficient recovery of enzyme from the reaction mixture and the product is not contaminated with product. In addition to these, immobilization also enhanced the storage and operational stability of enzyme against denaturation by heat or organic solvents or by autolysis (Rehman, et al., 2014).

Enzyme immobilization

The immobilization of enzyme is the process to confine or localized the enzyme within/onto the carrier with the retention of its catalytic activity. Immobilization technology was first used for the production of pure amino acids and the hydrolysis of penicillin G (Nguyen and Kim, 2017). After that, a lot of research has been done for the immobilization of different enzymes. There are several reasons for using immobilized enzymes as compared with soluble enzymes; the immobilized enzymes have higher operational stability and easy to operate (Sheldon, 2007). The immobilized enzymes have different industrial applications including production of useful compounds, treatment of pollutants and continuous analysis (Poznansky, 1983).

History of enzyme immobilization

The history of immobilization can be visualize in three phases, the first phase was started with beginning of 19th century when immobilized microorganism were industrially used for production of vinegar and development of tricking filter for clarification of waste water. The enzyme immobilization history was goes back to the late 1940s, but much earlier work regarding the enzyme immobilization, was ignored by biotechnologist due to mostly published in journals of other disciplines. In 1960s, the second phase of history of enzyme immobilization was began that developed the base of present immobilization technology and the publications of immobilization of enzyme was largely increased in this phase of immobilization. Before 1970s, only single enzyme immobilized systems were used but after 1970s, more complex systems were developed including two-enzyme reactions with co-factor regeneration and living cells (Garcia-Galan, et al., 2011). The production of L – amino acid from keto – acid is good example of latter phase that involved two enzyme reactions procedure. In reaction involved in consumption of the NADH (nicotinamide adenine dinucleotide) and regeneration of the coenzyme by membrane reactor (Sheldon, 2007).

Properties of immobilized enzyme

Determination of kinetics properties of an immobilized enzyme is very important to evaluate the immobilization method. The properties of immobilized enzyme are based on the properties of both the enzyme and support material. The biochemical properties of enzyme and reactive molecules of carrier are very important for the properties of immobilized enzyme. The chemical structure of support determines the type of interaction between enzyme and support. If the support is porous in nature, then the pore size play crucial role on the catalytic properties of enzyme bioreactor. Small pore size limit the transportation of substrate and product in and out of the support ultimately reduce the immobilized enzyme activity. The procedure and parameters of immobilization, such as pH, temperature and time are also important for the catalytic properties of immobilized enzyme (Rehman, et al., 2013). Following are the some important characteristics of an enzyme that would be expected to change after immobilization.

A. Stability

The stability of an enzyme is very crucial for its commercialization. Both the storage, as well as, the operational stability of enzyme affects the usefulness of

enzyme-based products. The enzyme goes variety of denaturation reactions during production, storage and industrial processing. Denaturation is the unfolding of enzyme tertiary structure, so the intrinsic structure of an enzyme is the main factor of the stability of soluble enzyme during reaction processes. While after immobilization of enzyme onto the carrier, the stability of immobilized enzyme depended by many factors such as the nature of interactions of enzyme with the support, binding position and number of bonds, freedom of conformation change in the matrix, chemical and physical properties of supports. The stability of enzyme with respect to time, temperature and storage is expected to increase after immobilization and rate of deactivation of immobilized enzyme is often found to be reduced with the comparison of soluble enzyme. The shielding of enzyme within high molecular weight support also, protects the enzyme from microbial contamination and the attack of exogenous proteases (Qamar, et al., 2020).

B. Enzymatic activity

It has been observed that enzymatic activity of many enzymes have been increased after immobilization (Ursini et al., 1999; Goto et al., 2005). However, the immobilization reduced the inhibition effect of the substrate and therefore, the immobilized enzyme are often found to be more active than native enzyme. The immobilized glucoamylase showed higher enzymatic activity as compared to soluble enzyme, due to reduction of substrate inhibition effect after immobilization on to porous cellulose beads (Taheri-Kafrani et al., 2020). The penicillin G acylase activity was enhanced after immobilization on positive charged carrier might be due to positive partition effect (enrichment of substrate in the proximity of the enzyme) under controlled reaction of ampicillin synthesis (Blamey et al., 2017). The optimization of immobilization parameters, such as enzyme loading, pH, temperature, carrier and binding chemistry are required for the retention of high enzyme activity (Chapman, et al., 2018). However, increasing of enzymatic activity by immobilization is an addition advantage rather than rational objectives of immobilization.

Immobilization of pectinase

Different methods have been used for the immobilization of enzymes that can be categorized into three types such as binding of enzyme to a carrier, crosslinking of enzymes with each other and entrapment or encapsulation of enzymes within polymers (Fig. 4). The properties of both the enzyme and carrier are very important for the promising successful of immobilization method. The approach used for immobilization of enzyme is one of trial and error, until the satisfactory results have been obtained. For increasing the efficiency of immobilization, two or more methods can be combined for the immobilization of single enzyme (Sheldon, 2007).

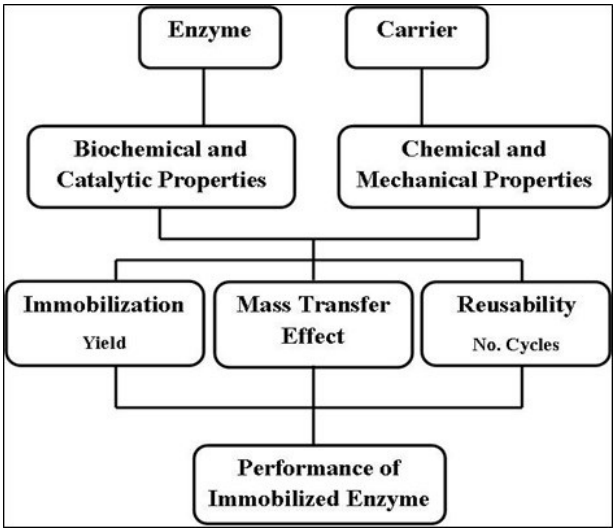

Figure 4. Methods for the immobilization of enzyme.

Entrapment

The entrapment of enzyme is the physical trapping of enzymes in three dimensional polymeric networks of polymers or matrices where the substrate and product can easily pass (Bahraman and Alemzadeh, 2017) (Fig. 5).

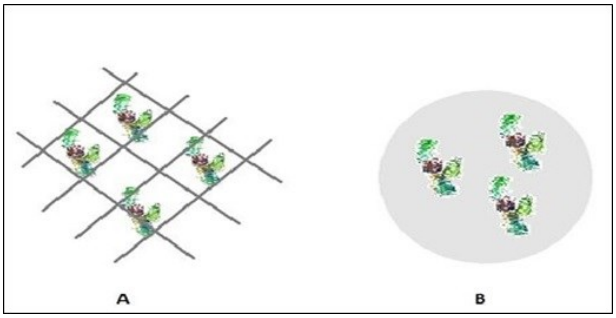

Figure 5. Entrapment of enzyme within a polymer. (A) Entrapment of enzyme into a porous structure of polymer matrix. (B) Encapsulation of enzyme within a polymer bead.

The entrapment of enzyme can be achieved by mixing of the enzyme with monomer solution and then subsequent polymerization of monomer keep the enzyme trap within the polymer matrix. The polymeric network of polymer should be rigorous enough to prevent the diffusion of enzymes, while allow the diffusion of substrate and product. Entrapment is simple and easy method of immobilization that induces no conformational modification on the biological molecule and the activity of enzymes retained during the immobilization process. However, leaching of enzyme from the support and substrate diffusion limitations can restrict the performances of entrapped enzymes. The internal surface areas of support play important role in the catalytic activity of enzyme and due to having high interval surface areas the porous polymeric materials have been used as solid supports for the immobilization of various enzymes (Blanco et al., 2004; Li et al., 2010). The pore sizes and specific surface area are important for enzyme loading efficiency and catalytic activity (Keeling and Brennan, 2001; Tsai and Doong, 2007; Das et al., 2010). However, a very high loading may cause diffusion limitation. Therefore, it is important to use support with large and specific surface areas. The polyacrylamide is one of those polymers, which cause less diffusion limitation for substrate and product to transport within the polymer. Polyacrylamide having various advantages such as availability of plentiful surface amino groups, strong mechanical strength, large surface area, adjustable particle size, easy regeneration, low operational cost and high stability were chosen as immobilization supports (Tang et al., 2001; Liu and Guo, 2006). These properties may provide advantages of the immobilization of pectinase and immobilized pectinase showed higher relative activity and stability as compared to native enzyme. Calcium alginate has been reported as an effective support for the immobilization of enzyme due to biocompatibility, low cost, easy availability and resistance to microbial contamination as compared to others (Dai, et al., 2019). Calcium alginate was tried as support for the immobilization of pectinase from *Bacillus licheniformis* KIBGE-IB21 and it retained 46% relative activity of pectinase after immobilization. The immobilized pectinase showed higher stability against various temperatures as compared to native pectinase (Rehman et al., 2013). The immobilized pectinase retained more than 60 % activity after 3rd cycle. Agar-agar is polymer of agarobiose with strong gelling ability, stability and has no reactivity with protein molecules (Prakash and Jaiswal, 2011). The agar-agar was used as a support for the pectinase immobilization and it showed better results as compared to calcium alginate (Rehman, et al., 2014). The agar-agar retained 80 % relative activity of pectinase after immobilization and immobilized pectinase showed more than 80% activity in 2nd cycle. The agar-agar showed promising results when it was activated by glutaraldehyde and it retained more than 80% activity even after repeating the experiment 10 times (Li et al., 2007).

The scanning electron microscopy (SEM) was used to study the morphological variation of supports, such as calcium alginate and agar-agar before and after immobilization of pectinase (Rehman, et al., 2013; Rehman, et al., 2014). The SEM was used for the surface morphologies of polymers and the surface of morphology was after immobilization of pectinase (Rehman, et. al., 2013). The surface of agar-agar matrix was appeared smooth before immobilization of pectinase but after entrapment of pectinase it became rough. Similar observation was seen in case of calcium alginate beads, various particles were seen on the surface of calcium alginate beads of immobilized pectinase that were not appeared on the surface of polymer without immobilized pectinase. Pectinase from *Aspergillus niger* NRC 1ami was entrapped within polyvinyl alcohol (PVA) sponge. PVA change the optimum temperature and pH from 40 °C and 4.0 to 50 °C to 6.0, respectively. The entrapment within PVA protects the pectinase from alkaline pHs (Esawy et al, 2013).

Binding to supports

Binding of enzyme to support is the classical technique for the immobilization of enzymes. This method is further classified into physical adsorption, ionic binding and covalent binding on the basis of binding of enzyme with carrier (Sheldon, 2019) (Fig. 6).

A. Physical adsorption

It is earliest and easiest method of enzyme immobilization. In this method, the enzyme is immobilized onto the carrier by van der Waals interactions, without any pre-activation of support. The binding of enzyme and support depend on chemical composition of support and enzyme molecule. Hydrophobic interaction, hydrogen bonding and Van

Deer Waals forces are involved to bind the enzyme molecules onto the carrier. The immobilization of enzyme is done by mixing of enzyme solution with suitable adsorbents under appropriate conditions of pH and ionic strength. The supports suits for immobilization of enzyme using physical adsorption have sufficient surface-charges properties (Sheldon, 2007). Adsorption is based on weak interaction and desorption can occur in the presence of higher binding affinity molecules, but the easy regeneration of the support and enzyme make this technique ideal for immobilization (Zdarta, et al., 2019). Ion exchange resin and Dowex Marathon WBA can be used to strongly bind the commercial pectinase for the immobilization without applying crosslinking agents such as glutaraldehyde, carbodiimide or cyanogens bromide (Chauhan, et al., 2013). Demir, et al., (2001) immobilized the pectinase on anion exchange St-DVB macroporous base resin through adsorption method. Pectinase from *Aspergillus niger* was immobilized on alginate-coated chitin support using adsorption method. The pectinase retained 60% of its initial activity after immobilization. The immobilization enhanced the thermal stability of pectinase and immobilized enzyme showed 10 fold more resistant at 50 °C than free enzyme (Hector, et al., 2013).

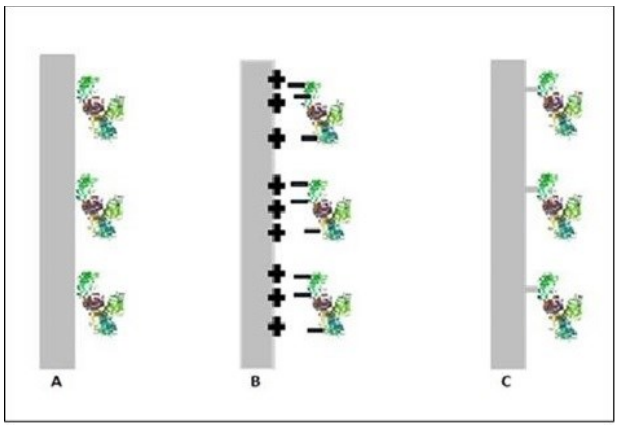

Figure 6. Binding of enzyme to a support. (A) Physical adsorption of enzyme onto a 517 support. (B) Ionic binding of enzyme onto a support. (C) Covalent binding of 518 enzyme onto a support.

B. Ionic binding

Ionic binding is another binding method of enzyme immobilization, which is mainly based on ionic interaction between enzyme and carrier (Brena and Batista-Viera, 2008). The difference between physical adsorption and ionic binding is the strength of interaction and ionic interaction is found to be stronger as compared to adsorption. The carrier in this technique can be carbohydrate polymers, synthetic polymers and inorganic compounds. The enzyme can be detached from the support due to presence of other ions and pH changes, so special care must be taken for the maintaining of ionic strength and pH of solution. Affinity layering is one of the techniques of ionic binding of enzyme with support materials. This technique has been used for immobilizing enzymes for biosensor designs (Farooqui, et al., 1997; Fritea, et al., 2018). The bio-affinity layering was used for the immobilization of tomato pectinase on agarose linked Concanavalin A–Seralose. The affinity layering enhanced the thermal, as well as, storage stability of enzyme and immobilized enzyme was able to retain its activity after repeatedly reusing in three reactions (Sardar and Gupta, 2005). Recently, magnetic nanoparticles (MNPs) of iron oxide have been taken into consideration for the immobilization of various enzymes, because MNPs could be easily separated from the reaction mixture and reused in continuous processes. The positively charged enzymes bound to negatively charged MNPs by electrostatic interactions (Bahrami and Hejazi, 2013). Docusate sodium salt (AOT) is an anionic biocompatible surfactant widely used in bioseparation systems. Pectinase has been immobilized on AOT using ionic binding. The AOT immobilized showed maximum 1.9 U/mg of specific activity and 610.5 mg enzyme/g support of loading efficiency. The immobilized enzyme only lost 10-20% activity after six cycles. The properties of MNPs were study by using Scanning Electron Microscopy (SEM), Fourier Transform Infrared (FT-IR) Spectroscopy and Dynamic Light Scattering analysis (Bahrami and Hejazi, 2013).

C. Covalent binding

The covalent binding of enzyme to the support is much stronger than physical adsorption and ionic binding. In this method, the enzyme is covalently attached to the reactive molecules present on the surface of carrier material. The reactive amino acids, which are mostly involved in covalent binding, are lysine, arginine, aspartic acid, glutamic acid, serine, threonine, and cysteine (Quirk et al., 2001; Connell, et al., 2017). The Covalent binding of enzyme to the support can be done either by direct linkage of enzyme to the support or through spacer arms. The main disadvantage of using

this method is the irreversible deactivation of enzyme after which, both the carrier and the enzyme become useless (Sheldon, 2007). Aminated silica gel was used for the immobilization of enzyme through covalent binding. The pectinase from *Aspergillus* was immobilized on silylated-montmorillonite clay using glutaraldehyde as a crosslinking agent and it retained 60% of its initial relative activity after 6 cycles (Mohammadi, et al., 2020). The thermal and pH stability of pectinase was increased after covalently immobilization on glass beads using polyaldehyde kefiran as a crosslinker (Hosseini, et al., 2020). Li, et al., (2007) used glutaraldehyde activated agar-agar gel for the immobilization of pectinase through multipoint attachment and maximum immobilization was achieved at 5°C, pH 3.6 with a 24 h reaction time. The activated agar-agar showed great reusability and retained 81 % activity after 10 batches of reactions. Nano, micro and macro Magnetic-Chitosan particles were tried for the immobilization of pectinase and it was found that the size of support material have a great effect on the performance of immobilized pectinase. The nano magnetic chitosan particle can performed catalytic activity with highest V_{max} value as compared to micro and macro because of the higher surface area for catalytic reactivity between enzyme and substrate (Dal Magro et al., 2019). The immobilization of pectinase on polyethylene glycol grafted magnetic nanoparticles by covalent bonding using trichlorotriazine crosslinker enhanced its catalytic properties with improved operational stability and reusability (Kharazmi et al., 2020). The industrial application of pectinase was enhanced to degrade the pectin effectively by covalently immobilizing it on chloride functionalized chitosan encapsulated magnetic nanoparticles (Soozanipour et al., 2019). Pectinase was immobilized on glass bead using polyaldehyde kefiran linking agent and after immobilization pectinase retained more 60% activity with higher thermal and pH stability (Hosseini et al., 2020).

Enzyme crosslinking

Enzyme crosslinking is a support free immobilization method that can be done by joining the individual enzyme units to one another using crosslinking agent such as glutardialdehyde (Fig. 7). The enzyme molecules can directly bind to each other using cross linker or non-reactive compounds are used between them to make the immobilized enzyme more stable. The main drawback of using crosslinking is the possibility of losing activity due to structural and chemical alterations of active site of enzyme during crosslinking process. Cross-linked enzyme crystals (CLECs), cross-linked enzymes, and cross-linked enzyme aggregates (CLEAs) are example of support free immobilization method (Sheldon, 2019; Yamaguchi, et al., 2018). In this method, the enzyme is precipitated from aqueous solution by the addition of a salt or an organic solvent or a polymer and these enzyme precipitates molecules are aggregates using bi-functional agent (Cao et al., 2001). CLEAs preparations do not need extensive purification of enzyme and it can be possible to form CLEAs, which has the ability to catalyze more than one reaction (Sheldon et al., 2005). CLEA having activities of pectinase, xylanase and cellulase was made and characterized for multipurpose applications. The half-lives of all three enzymes were increased after CLEA formation. The cellulase showed more thermal stability at 70 °C as compared to pectinase and xylanase. The half -life of pectinase was increased from 17 to 180 minutes after CLEA preparation (Dalal et al., 2007).

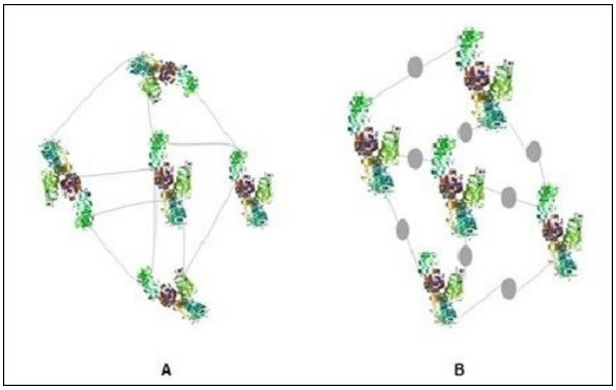

Figure 7. Immobilization of enzyme by means of crosslinking. (A) Direct crosslinking of enzymes. (B) Co -crosslinking of enzyme by incorporating inert molecule through using cross linker

Applications of immobilized pectinase

Clarification of fruit juices

The commercial pectinase that has been used in apple juice processing contain mixture of pectinesterase (PE), polygalacturonase (PG) and pectinlyase (PL) enzymes. In spite of proposing various support to immobilized pectinase for continuous degradation of pectin, the immobilization of pectinase on micro or ultrafiltration

membranes as seemed to be interesting alternative for treatment of fruit juices (Echavarría et al., 2012). The ultrafiltration membranes immobilized pectinase may efficiently degrade the high molecular weight pectin polymer into lower molecular weight of pectin oligosaccharides (POS) at the membrane permeate interface and extent the membrane operation without cleaning. The immobilized pectinase on different supports such as glass microspheres, Nylon pellets and PAN beads were used for the clarification of apple juice. The parameters such as rate of pectin degradation, time for 50% reduction of initial viscosity and the time require for complete depectinization were investigated (Diano et al., 2008). The commercial pectinase was immobilized on functionalized γ-alumina spheres and applied to hydrolyze pectin containing fruit juices. The activity of γ-alumina spheres immobilized pectinase was compared with the native enzyme and analyzed in continuous batch of several reactions. The apple juice was successfully depectinized using γ -alumina spheres immobilized pectinase bioreactor (Pagan, 2014).

Papermaking industries

The pectin is considered as a dominant troublesome substance that seriously decreased the run-ability of paper machine in process water closure. Cross-linked chitosan beads were prepared for finding a new way of lowering pectin concentration in papermaking industries. A sharp declined of cationic demand of pectin solution and decrease of molecular weight of pectin was after treatment of chitosan cross-linked immobilized pectinase (Liu et al., 2010).

Mash treatment

Uses of enzyme for mash treatment are a modern technique for gaining more juice from fruits and vegetables. Pectinase has been utilized for the degradation of cell wall and middle-lamina pectin of the fruits. Pectinase not only enhance the pressing capacity and juice yield but also has a positive effect for achieving high carotene and dry matter content of the product. A commercial pectinase known as Pectinex Ultra SP-L was immobilized and used for the preparation of carrot puree. The average yield of carrot juice was increased up to 30.23 % as compared from non-enzymatic treatment (Demir et al., 2001).

Production of pectinoligosaccharides (POS)

Pectin derived oligosaccharides (POS) have different biological activities including plant growth promotion and antimicrobial activity (Suziki, et al., 2002; Gullón, et al., 2013). The pectinoligosaccharides are not digestible by human and reach the colon where they are fermented by microbial flora such *as Bifidobacteria sp.*, and *Lactobacillus species* (Chung, et al., 2017). POS stimulate the growth and fermentation of microbial flora. POS are produced by chemical and enzymatic degradation of pectin polymer. In enzymatic method pectinase has been applied to degrade the α -1, 4 glycosidic linkage of pectin polymer into galacturonic acids and pectin oligosaccharides but due to problem in controlling of the process a large amounts of monosaccaharides were produced as a byproduct in batch production. Immobilized pectinase has been used for controlled enzymatic reaction to generate POS with defined range of degree of polymerization (DP) and specific characterization (Xue, et al., 2021).

Conclusion

Pectinase is an enzyme that catalyzes the hydrolysis of pectin polymers and have been used in various applications for different industrial preparations such as clarification of fruits and vegetable juices, degumming of plant bast fibers, textile industries for removing non-cellulosic impurities, papermaking industries, wine clarification, coffee and tea fermentation, wastewater treatment as well as isolation of protoplasm for research purpose. The pectinase has been also used for the production of pectin-derived oligosaccharides (POS), which are consider as prebiotic molecules. The low stability of native enzyme under industrial conditions varnishes the industrial application of pectinase. Immobilization has been utilized by different researchers for engineering of pectinase and tried to makes it able to resist the harsh industrial conditions. Immobilization not only enhanced the stability of various pectinases, but also, made these enzymes reusable for continuous industrial processes. Different techniques such as entrapment, binding to a support and enzyme crosslinking have been utilized for the immobilization of pectinases. The supports such as sodium alginate, agar-agar, polyacrylamide, aminated silica gel, nanocomposite microspheres, chitosan, nylon, silica coated porous glass etc. have been tested for the

immobilization of pectinase through different methods. Further research will be required for the engineering of this enzyme to make an efficient pectinase bioreactor, which can be utilize for various industrial applications.

Ethical Statement

This study do not involve human subjects and/or animals study.

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Competing Interests

There is no conflict of interests.

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Authors' Contribution

All authors equally contribute in writing this paper.

References

Alkorta, I., Garbisu, C., Llama, M. J. and J. L. Serra, (1995). "Viscosity decrease of pectin and fruit juices catalyzed by pectin lyase from *Penicillium italicum* in batch and 708 continuous-flow membrane reactors." *Biotechnological Technology,* **9**:95-100.

Amin, F., Bhatti, H.N. and M. Bilal, (2019). "Recent advances in the production strategies of microbial pectinases—A review." *International Journal of Biological Macromolecules*, **122:** 1017-1026.

Bahraman, F. and I. Alemzadeh, (2017). "Optimization of l-asparaginase immobilization onto calcium alginate beads." *Chemical Engineering Communications*, **204**: 216-220.

Bahrami, A., and P. Hejazi, (2013). "Electrostatic immobilization of pectinase on negatively charged AOT-Fe_3O_4 nanoparticles." *Journal of Molecular of Catalysis B: Enzyme*, **93**:1– 7.

Bílková, Z., Mazurvá, J., Churácek, J., Horák, D. and J. Turková, (1999). "Oriented immobilization of chymotrypsin by use of suitable antibodies coupled to a nonporous solid support." *Journal of Chromatograpy A*, **852**: 141-149.

Blamey, J. M., Fischer, F., Meyer, H. P., Sarmiento, F. and M. Zinn, (2017). "Enzymatic biocatalysis in chemical transformations: a promising and emerging field in green chemistry practice." In: Brahmachari, G., Demain, A.L. and J.L. Adrio, (Eds), *Biotechnology of Microbial Enzymes,* Academic Press, Elsevier, London, pp. 347-403.

Blanco, R.M., Terreros, P., Fernandez, M-P., Otero, C. and G-G. Diaz, (2004). "Functionalization of mesoporous silica for lipase immobilisation characterization of the support and the catalysts." *Journal of Molecular Catalysis B. Enzyme,* **30**:83–93.

Brena, B.M. and Batista-Viera, F. (2008). "Immobilization of enzymes." In: Bickerstaff G. (Ed), *Methods in biotechnology: immobilization of enzymes and cells,* Totowa, New 728 Jersey: Humana Press.

Buga, M.L., Ibrahim, S. and A.J. Nok, (2010). "Physico-chemical characteristics of immobilized polygalacturonase from *Aspergillus niger* (SA6)." *African Journal Biotechnology*, **9**: 737 8934-8943.

Cao, L., Van, L.L.M, Van R.F. and R.A. Sheldon, (2001). "Cross-linked aggregates of penicillin G acylase: Robust catalysts for the synthesis of h-lactam antibiotics." *Journal of Molecular Catalysis B. Enzymes*, **11**:665–670.

Carrin, M.E., Ceci, L. and J.E. Lozano, (2000). "Effects of pectinase immobilization during 750 hollow fiber ultrafiltration of apple juice." *Journal of Food Process Engineering*, **23**: 281-298.

Celestino, S.M.C., Freitas, S.M., Medrano, F.J., Sousa, M.V. and E.X.F. Filho, (2006). "Purification and characterization of a novel pectinase from *Acrophialophora nainiana* with emphasis on its physicochemical properties." *Journal of Biotechnology*, **123**: 33-42.

Chan, S.Y., Choo, W.S., Young, D.J. and X.J. Loh, (2017). "Pectin as a rheology modifier: Origin, structure, commercial production and rheology." *Carbohydrate Polymers*, **161**: 118-139.

Chapman, J., Ismail, A.E. and C.Z. Dinu, (2018). "Industrial applications of enzymes: Recent advances, techniques, and outlooks." *Catalysts*, **8**(6): 238.

Chauhan, S., Hiteshi, K. and R. Gupta, (2013). "Immobilization of microbial pectinases: A review." *Academia Journal of Food Research,* **1**:19-32.

Chen, J., Liu, W., Liu, C., Li, T., Liang, R. and S. Luo, (2015). "Pectin modifications: a review." *Critical Review in Food Science and Nutrition*, **55**:1684–1698.

Chung, W.S.F., Meijerink, M., Zeuner, B., Holck, J., Louis, P., Meyer, A.S., Wells, J.M., Flint, H.J. and S.H. Duncan, (2017). "Prebiotic potential of pectin and pectic oligosaccharides to promote anti-inflammatory commensal bacteria in the human colon." *FEMS Microbiology Ecology,* **93**: fix127.

Connell, L.S., Gabrielli, L., Mahony, O., Russo, L., Cipolla, L. and J.R. Jones, (2017). "Functionalizing natural polymers with alkoxysilane coupling agents: reacting 3-glycidoxypropyl trimethoxysilane with poly (γ-glutamic acid) and gelatin." *Polymer Chemistry*, **8**: 1095-1103.

Costa, S.A., Tzanov, T., Carneiro, A.F., Paar, A., Gubitz, G.M. and A. Cavaco-Paulo, (2002). "Studies of stabilization of native catalase using additives." *Enzyme Microbial Technology*, 30: 764 387-391.

Dai, X.Y., Kong, L.M., Wang, X.L., Zhu, Q., Chen, K. and T. Zhou, (2018). "Preparation, characterization and catalytic behavior of pectinase covalently immobilized onto sodium alginate/graphene oxide composite beads." *Food Chemistry*, **253**: 185-193.

Dal Magro, L., de Moura, K.S., Backes, B.E., de Menezes, E.W., Benvenutti, E.V., Nicolodi, S., Klein, M.P., Fernandez-Lafuente, R. and R.C. Rodrigues, (2019). "Immobilization of pectinase on chitosan magnetic particles: Influence of particle preparation protocol on enzyme properties for fruit juice clarification." *Biotechnology Reports*, **24**: e00373.

Dalal, S., Sharma, A. and M.N.A. Gupta, (2007). ″Multipurpose immobilized biocatalyst with pectinase, xylanase and cellulase activities.″ *Chemistry Central Journal,* **1**:16-22.

Das R.D., Maji S., Das S. and C.R. Chaudhuri (2010). ″Optimization of covalent antibody immobilization on macroporous silicon solid supports.″ *Applied Surface Science*. **256**:5867–5875.

Demir, N., Acar, J., Sarioğlu, K. and M. Mutlu, (2001). ″The use of commercial pectinase in fruit juice industry. Part 3: immobilized pectinase for mash treatment.″ *Journal of Food Engineering*, **47**: 275-280.

Demirel, D. and M. Mutlu, (2005). ″Performance of immobilized pectinex ultra SP-L on magnetic duolite-polystyrene composite particles. Part II: a magnetic fluidized bed reactor study.″ *Journal of Food Engineering*, **70**: 1-6.

Diano, N., Grimaldi, T., Bianco, M., Rossi, S., Gabrovska, K., Yordanova, G., Godjevargova, T., Grano, V., Nicolucci, C., Mita, L., Bencivenga, U., Canciglia, P. and D.G. Mita, (2008). ″Apple juice clarification by immobilized pectolytic enzymes in packed or fluidized bed reactors.″ *Journal of Agricultural Food Chemistry*, **56**:11471-11477.

Echavarría, A. P., García-Valls, R., Torras, C., Pagan, J. and A. Ibarz, (2012). ″Effect of pectinase immobilization in a polymeric membrane on ultrafiltration of fluid foods.″ *Separation Science and Technology*, **47**: 796-801.

Esawy, M.A., Gamal, A.A., Kamel, Z., Ismail, A.S. and A.F. Abdel-Fattah, (2013). ″Evaluation of free and immobilized *Aspergillus niger* NRC1ami pectinase applicable in industrial processes.″ *Carbohydrate Polymers*, **92**: 1463-1469.

Esquivel, J.C.C. and C.E. Voget, (2004). ″Purification and partial characterization of an acidic 798 pectinase polygalacturonase from *Aspergillus kawachii*.″ *Journal of Biotechnology*, **110**: 21-28.

Farooqui, M., Saleemuddin, M., Ulber, R., Sosnitza, P. and T. Scheper, (1997). Bioaffinity layering: a novel strategy for the immobilization of large quantities of glycoenzymes. *Journal of Biotechnology*, **55**: 85-98.

Fernandez, M.L. (2001). ″Pectin: Composition, chemistry, physicochemical properties, food applications and physiological effects.″ In: Cho, S. and M.L. Dreher, (Eds), *Handbook of dietary fiber.* New York: Marcel Dekker, Inc. pp. 583-601.

Fritea, L., Tertis, M., Sandulescu, R. and C. Cristea, (2018). ″Enzyme–Graphene Platforms for Electrochemical Biosensor Design with Biomedical Applications.″ in: Kumar, C.V. (Ed), *Methods in Enzymology*, vol. 609**,** Academic Press, pp. 293-333.

Gadre, R.V., Driessche, G.V., Beeumen, J.V. and M.K. Bhat, (2003). ″Purification, characterization and mode of action of an endo-polygalacturonase from the psychrophilic fungus *Mucor flavus*.″ *Enzyme Microbial Technology*, **32**: 321-330.

Garcia-Galan, C., Berenguer-Murcia, Á., Fernandez-Lafuente, R. and R.C. Rodrigues, (2011). ″Potential of different enzyme immobilization strategies to improve enzyme performance.″ *Advanced Synthesis & Catalysis*, **353**: 2885-2904.

Goto, M., Hatanakaa, C. and M. Goto, (2005). ″Immobilization of surfactant-lipase complexes and their high heat resistance in organic media.″ *Biochemical Engineering Journal*, **24**: 91-94.

Gullón, B., Gómez, B., Martínez-Sabajanes, M., Yáñez, R., Parajó, J.C. and J.L. Alonso, (2013). ″Pectic oligosaccharides: Manufacture and functional properties.″ *Trends in Food Science and Technology*, **30**: 153-161.

Gummadi, S.N. and T. Panda, (2003). ″Purification and biochemical properties of microbial pectinases-a review.″ *Process Biochemistry*, **38**: 987-996.

Haas, K. T., Wightman, R., Meyerowitz, E. M. and A. Peaucelle, (2020). ″Pectin homogalacturonan nanofilament expansion drives morphogenesis in plant epidermal cells.″ *Science*, **367**: 1003-1007.

Hector, L.R., Ana, I.B., Juan, Ú. and A. María, (2013). ″Immobilization of pectinase by adsorption on an alginate-coated chitin support.″ *Biotecnología Aplicada*, **30**: 101-104.

Henriksson, G., Akin, D.E., Slomczynski, D. and K.E.L. Eriksson, (1999). ″Production of highly efficient enzymes for flax retting by *Rhizomucor pusillus*.″ *Journal of Biotechnology*, **68**: 115-123.

Hodgson, A. and L. Kerr, (1991). ″Tropical fruit products.″ In: Walter, R.H. and C.A. San Diego, (Eds), *The chemistry and technology of pectin*. Academic Press, Inc. pp. 67-86.

Hoondal, G.S., Tiwari, R.P., Tiwari, R., Dahiya, N. and Q.K. Beg, (2002). ″Microbial alkaline pectinase and their industrial application.″ *Applied Microbiology and Biotechnology*, **59**: 409-418.

Hosseini, S.S., Khodaiyan, F., Mousavi, S.M.E., Azimi, S.Z. and M. Gharaghani, (2020). ″Immobilization of pectinase on the glass bead using polyaldehyde kefiran as a new safe cross-linker and its effect on the activity and kinetic parameters.″ *Food Chemistry*, **309**: 125777.

Jayani, R.S., Saxena, S. and R. Gupta, (2005). ″Microbial pectinolytic enzymes: a review.″ *Process Biochemistry*, **40**: 2931-2944.

Kapoor, M., Beg, Q.K., Bhushan, B., Dadhich, K.S. and G.S. Hoondal, (2000). ″Production 835 and partial purification and characterization of a thermo-alkali stable polygalacturonase from *Bacillus* sp. MG-cp-2.″ *Process Biochemistry*, **36**: 467-473.

Karr, A.L. (1976). ″Cell wall bigenesis.″ In: Bonner J. and J.E. Varner, (Eds) *Plant biochemistry,* New York: Academic Press. pp. 405-426.

Kashyap, D.R., Vohra, P.K., Chopra, S. and R. Tewari, (2001). ″Applications of pectinases in the commercial sector: a review.″ *Bioresource Technology*, **77**: 215-227.

Kaur, G., Kumar, S. and T. Satyanarayana, (2004). ″Production, characterization and application of a thermostable polygalacturonase of a thermophilic mould *Sporotrichum thermophile apinis*.″ *Bioresource Technology*, **94**: 239-243.

Keeling T.T. and J.D. Brennan, (2001). ″Fluorescent probes as reporters on the local structure and dynamics in sol-gel-derived nanocomposite materials.″ *Chemistry Materials*, **13**:3331– 3350.

Khajeh, K., Ranjbar, B., Naderi-Manesh, H., Habibi, A. E. and M. Nemat-Gorgani, (2001). ″Chemical modification of bacterial α-amylases: changes in tertiary structures and the effect of additional calcium.″ *Biochimica et Biophysica Acta*, **1548**: 229-237.

Kharazmi, S., Taheri-Kafrani, A. and A. Soozanipour, (2020). ″Efficient immobilization of pectinase on trichlorotriazine-functionalized polyethylene glycol-grafted magnetic nanoparticles: A stable and robust nanobiocatalyst for fruit juice clarification.″ *Food Chemistry*, **325**: 126890.

Kobayashi, T., Higaki, N., Suzumatsu, A., Sawada, K., Hagihara, H., Kawai, S. and S. Ito, (2001). ″Purification and properties of a high molecular-weight, alkaline exopolygalacturonase from a strain of a strain of *Bacillus*.″ *Enzyme Microbial Technology*, **29**: 864 70-75.

Li Y., Gao F., Wei W.W., Qu J.B., Ma G.H., and W.Q. Zhou (2010). ″Pore size of macroporous polystyrene microspheres affects lipase immobilization.″ *Journal of Molecular Catalysis B. Enzyme,* **66**:182–189.

Li, T., Li, S., Wang, N. and L. Tain, (2008). ″Immobilization and stabilization of pectinase by 875 multipoint attachment onto an activated agar-gel support.″ *Food Chemistry*, **109**: 703-708.

Li, T., Wang, N., Li, S., Zhao, Q., Guo, M. and C. Zhang, (2007). ″Optimization of covalent immobilization of pectinase on sodium alginate support.″ *Biotechnology Letters*, **29**: 1413- 878 1416.

Liang, L.I. and Y. Luo, (2020). ″Casein and pectin: Structures, interactions, and applications.″ *Trends in Food Science and Technology*, **97**: 391-403.

Liu P., and J. Guo, (2006). ″Polyacrylamide grafted attapulgite (PAM-ATP) via surface initiated atom transfer radical polymerization (SI-ATRP) for removal of Hg(II) ion and dyes.″ *Colloids Surface A*., **282**: 498–503.

Liu, K., Li, X., Li, X., He, B. and G. Zhao, (2010). ″Lowering the cationic demand caused by PGA in papermaking by solute adsorption and immobilized pectinase on chitosan beads.″ *Carbohydrate Polymers*, **82**: 648-652.

Liu, K., Zhao, G., He, B., Chena, L. and L. Huanga, (2012). ″Immobilization of pectinase and lipase on macroporous resin coated with chitosan for treatment of whitewater from papermaking.″ *Bioresource Technology*, **123**: 616-619.

Maldonado, M.C., Cáceres, S., Galli, E. and A.R. Navarro, (2002). ″Regulation of the production of polygalacturonase by *Aspergillus niger*.″ *Folia Microbiologica (Praha),* **890**(47): 409-412.

Matsunaga, T., Ishii, T., Matsumoto, S., Higuchi, M., Darvill, A., Albersheim, P. and M.A. O'Neill, (2004). ″Occurrence of the primary cell wall polysaccharide rhamnogalacturonan II in pteridophytes, lycophytes and bryophytes. Implications for the evolution of vascular plants.″ *Plant Physiology*, **134**: 339-351.

Minshull, J., Govindarajan, S., Cox, T., Ness, J.E. and C. Gustafsson, (2004). ″Engineered protein function by selective amino acid diversification.″ *Methods*, **32**: 416-427.

Mohamed, S.A., Christensen, T.M.I.E. and J.D. Mikkelsen, (2003). ″New polygalacturonases from Trichoderma reesei: characterization and their specificities to partially methylated and acetylated pectins.″ *Carbohydrate Research,* **338**: 515-524.

Mohamed, S.A., Farid, N.M., Hossiny, E.N., and R.I. Bassuiny, (2006). ″Biochemical characterization of an extracellular polygalacturonase from *Trichoderma harzianum*.″ *Journal of Biotechnology*, **127**: 54-64.

Mohammadi, M., Mokarram, R.R., Shahvalizadeh, R., Sarabandi, K., Lim, L.T. and H. Hamishehkar, (2020). ″Immobilization and stabilization of pectinase on an activated montmorillonite support and its application in pineapple juice clarification.″ *Food Bioscience*, **36**: 100625.

Mohnen, D. (2008). ″Pectin structure and biosynthesis.″ *Current Opinion Plant Biology*, **11**: 266- 277.

Nguyen, H.H. and M. Kim, (2017). ″An overview of techniques in enzyme immobilization.″ *Applied Science and Convergence Technology*, **26**: 157-163.

O'Neill, M.A., Albersheim, P. and A.G. Dravill, (1990). ″The pectic polysaccharides of primary cell walls.″ In: Dey, P.M. (Ed), *Methods in plant biochemistry*, London: Academic Press, vol. 2. pp. 415-441.

O'Neill, M.A., Ishii, T., Albersheim, P. and A.G. Darvill, (2004). ″Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide.″ *Annals Review of Plant Biology*, **55**:109-139.

Oumer, O. J. (2017). ″Pectinase: substrate, production and their biotechnological applications.″ *International Journal of Environment, Agriculture and Biotechnology*, **2:** 238761.

Oumer, O.J. and D. Abate, (2017). ″Characterization of pectinase from *Bacillus subtilis* strain Btk 27 and its potential application in removal of mucilage from coffee beans.″ *Enzyme Research*, **2017**: 7686904.

Pagan, J. (2014). ″Utilization of enzymes in fruit juice production.″ In: Falguera, V. and A. Ibarz, (Eds.) Juice processing: quality, safety and value-added opportunities. CRC Press, pp. 151-170.

Power, J.B. and E.C. Cocking, (1969). ″A simple method for the isolation of very large number of leaf protoplasts by using mixture of cellulase and pectinase.″ *Biochemical Journal*, **111**: 33.

Poznansky, M.J. (1983). ″Enzyme-protein conjugates new possibilities for enzyme therapy.″ *Pharmacology Therapeutics*, **21**: 53-76.

Prakash, O. and N. Jaiswal, (2011). ″Immobilization of a thermostable α-amylase on agarose and agar matrices and its application in starch stain removal.″ *World Applied Science Journal*, **13**: 922 572-577.

Qamar, S. A., Asgher, M. and M. Bilal, (2020). ″Immobilization of alkaline protease from Bacillus brevis using Ca-alginate entrapment strategy for improved catalytic stability, silver recovery, and dehairing potentialities.″ *Catalysis Letters*, **150**: 3572-3583.

Quirk, R.A., Chan, W.C., Davies, M.C., Tendler, S.J.B. and K.M. Shakeshe, (2001). ″Poly (L926 lysine)-GRGDS as a biomimetic surface modifier for poly (lactic acid).″ *Biomaterials*, **927**(22): 865-872.

Rebello, S., Anju, M., Aneesh, E.M., Sindhu, R., Binod, P. and A. Pandey, (2017). ″Recent advancements in the production and application of microbial pectinases: an overview.″ *Reviews in Environmental Science and Bio/Technology*, **16**: 381-394.

Rehman, H.U., Qader, S.A.U. and A. Aman, (2012). ″Polygalacturonase: Production of pectin depolymersing enzyme from *Bacillus licheniformis* KIBGE IB-21.″ *Carbohydrate Polymers*, **90**: 387-391.

Rehman, H.U., Aman, A. Silipo, A., Qader, S.A.U., Molinaro A., and A. Ansari, (2013). ″Degradation of complex carbohydrate polymer: Immobilization of pectinase from *Bacillus licheniformis* KIBGE-IB21 using calcium alginate as a support.″ *Food Chemistry*, **934**(139): 1081–1086.

Rehman, H.U., Aman, A., Zohra, R.R., and S.A.U. Qader, (2014). ″Immobilization of pectin degrading enzyme from *Bacillus licheniformis* KIBGE IB-21 using agar-agar as a support.″ *Carbohydrate Polymers*, **102**: 622-626.

Renard, C.M.G.C. and J.F. Thibault, (1993). ″Structure and properties of apple and sugar beet pectins extracted by chelating agents.″ *Carbohydrate Research*, **244**: 99-114.

Rombouts, F.M. and W.L. Pilnik, (1980). ″Pectic enzymes.″ In: Rose, A.H. (Ed), *Economic microbiology: Microbial enzymes and bioconversions*. Academic Press, London, England, Vol. 5, pp. 227-282.

Roy, I., Sardar, M. and M.N. Gupta, (2003). ″Evaluation of a smart bioconjugate of pectinase for chitin hydrolysis.″ *Biochemical Engineering Journal*, **16**: 329-335.

Sai, H.C. and R.A. Doong, (2007). ″Preparation and characterization of urease-encapsulated biosensors in poly (vinyl alcohol)-modified silica sol-gel materials.″ *Biosensors and Bioelectronic*, **23**:66–73.

Sangeeta, Y., Yadav, P.K., Dinesh, Y. and Yadav, K.D.S. (2009). ″Pectin lyase: a review.″ *Process Biochemistry*, **44**(1): 1-10.

Sardar, M. and M.N. Gupta, (2005). ″Immobilization of tomato pectinase on Con A–Seralose 4B by bioaffinity layering.″ *Enzyme Microbial Technology*, **37**: 355–359.

Satapathy, S., Rout, J.R., Kerry, R.G., Thatoi, H. and S.L. Sahoo, (2020). ″Biochemical prospects of various microbial pectinase and pectin: An approachable concept in pharmaceutical bioprocessing.″ *Frontiers in Nutrition*, **7**: 117.

Sheldon, R. A. (2019). ″CLEAs, combi-CLEAs and 'smart' magnetic CLEAs: Biocatalysis in a bio-based economy.″ *Catalysts*, **9**: 261.

Sheldon, R.A. (2007). ″Enzyme immobilization: the quest for optimum performance.″ *Advance Synthesis and Catalysis*, **349**: 1289-1307.

Sheldon, R.A., Schoevaart, R. and L.L.M. Van, (2005). ″Cross-linked enzyme aggregates (CLEAs): a novel and versatile method for enzyme immobilization.″ *Biocatalysis and Biotransmation*, **23**:141-147.

Shet, A.R., Desai, S.V. and S. Achappa, (2018). ″Pectinolytic enzymes: classification, production, purification and applications.″ *Research Journal* of *Life Sciences*, *Bioinformatics*, *Pharmaceutical and Chemical Sciences*, **4**: 337-348.

Singh, A., Varghese, L.M., Battan, B., Patra, A.K., Mandhan, R.P. and R. Mahajan, (2020). ″Eco-friendly scouring of ramie fibers using crude xylano-pectinolytic enzymes for textile purpose.″ *Environmental Science and Pollution Research*, **27**: 6701-6710.

Singh, R. S., Singh, T. and A. Pandey, (2019). ″Microbial enzymes—an overview.″ *Advances in Enzyme Technology*, **2019**: 1-40.

Soozanipour, A., Taheri-Kafrani, A., Barkhori, M. and M. Nasrollahzadeh, (2019). ″Preparation of a stable and robust nanobiocatalyst by efficiently immobilizing of pectinase onto cyanuric chloride-functionalized chitosan grafted magnetic nanoparticles.″ *Journal of Colloid and Interface Science*, **536**: 261-270.

Suziki, T., Yokotani, K., Tsubura, H., Yoshida, S., Kusakabe, I., Yamada, K., Miki, Y. and K. Hasegawa, (2002). ″Plant growth promoting oligosaccharides produced from tomato waster.″ *Bioresource Technology*, **81**: 91–96.

Taheri-Kafrani, A., Kharazmi, S., Nasrollahzadeh, M., Soozanipour, A., Ejeian, F., Etedali, P., Mansouri-Tehrani, H.A., Razmjou, A., Yek, S.M.G. and R.S. Varma, (2020). ″Recent developments in enzyme immobilization technology for high-throughput processing in food industries.″ *Critical Reviews in Food Science and Nutrition*, **2020**: 1-37.

Tang, Z.G., Zhou, R.Q. and Z.T. Duan, (2001). ″Adsorption and desorption behavior of taurine on macroporous adsorption resins.″ *Journal of Chemical Technology and Biotechnology*, **76**:752–756.

Tanriseven, A. and Y. Aslan, (2005). ″Immobilization of Pectinex Ultra SP-L to produce fructooligosaccharides.″ *Enzyme Microbial Technology*, **36**: 550-554.

Thakur, B.R., Singh, R.K., Handa, A.K. and M.A.D. Rao, (1997). ″Chemistry and uses of pectin- a review.″ *Critical Review in Food Science and Nutrition*, **37**: 47-73.

Tsai, H.C. and R.A. Doong, (2007). ″Preparation and characterization of urease-encapsulated biosensors in poly (vinyl alcohol)-modified silica sol–gel materials.″ Biosensors and Bioelectronics, 23(1): 66-73.

Ursini, A., Maragni, P., Bismara, C. and B. Tamburini, (1999). ″Enzymatic method of preparation of opticallly active trans-2-amtno cyclohexanol derivatives.″ *Synthetic Communications*, **29**(8): 1369-1377.

Valdés, A., Burgos, N., Jiménez, A. and M. C. Garrigós, (2015). ″Natural pectin polysaccharides as edible coatings.″ *Coatings*, **5**: 865-886.

Vauquelin, M. (1790). ″Analyse du tamarin.″ In: De Morveau, L.B.G. (Ed), *Annals de Chimie*, vol. 5, Forgotten Books, London, pp. 92-106.

Vincken, J.P., Schols, H.A., Oomen, R.J.F.J., McCann, M.C., Ulvskov, P., Voragen, A.G.J. and R.G.F. Visser, (2003). ″If homogalacturonan were a side chain of 993 rhamnogalacturonan I: Implications for cell wall architecture.″ *Plant Physiology*, **132**(994): 1781-1789.

Voragen, A.G.J., Coenen, G., Verhoef, R.P. and H.A. Schols, (2009). ″Pectin, a versatile polysaccharide present in plant cell walls.″ *Structural Chemistry*, **20**: 263-275.

Wang, D., Yeats, T.H., Uluisik, S., Rose, J.K. and G.B. Seymour, (2018). ″Fruit softening: revisiting the role of pectin.″ *Trends in Plant Science*, **23**: 302-310.

Willats, W.G., McCartney, L., Mackie, W. and J.P. Knox, (2001). ″Pectin: cell biology and prospects for functional analysis.″ *Plant Molecular Biology*, **47**: 9-27.

Xue, L., Long, J., Lu, C., Li, X., Xu, X. and Z. Jin, (2021). ″Immobilization of polygalacturonase for the preparation of pectic oligosaccharides from mango peel wastes and assessment of their antibacterial activities.″ *Food Bioscience*, **39**: 100837.

Yamaguchi, H., Kiyota, Y. and M. Miyazaki, (2018). ″Techniques for preparation of cross-linked enzyme aggregates and their applications in bioconversions.″ *Catalysts*, **8**: 174.

Yapo, B.M. (2011). ″Pectin Rhamnogalacturonan II: On the small stem with four branches 998 in the primary cell walls of plants.″ *International Journal of Carbohydrate Chemistry*, **29**: 964521.

Zdarta, J., Meyer, A.S., Jesionowski, T. and M. Pinelo, (2019). ″Multi-faceted strategy based on enzyme immobilization with reactant adsorption and membrane technology for biocatalytic removal of pollutants: A critical review.″ *Biotechnology Advances*, **37**: 107401.

Zhang, J. (2006). ″Biochemical study and technical applications of fungal pectinase.″ Digital comprehensive summaries of Uppsala dissertations from faculty of science and technology, Acta Universitatis Upsaliensis Uppsala, pp. 14.

