Immobilization of microbial pectinases: A review

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ABSTRACT

Immobilization technology creates exciting new opportunities for commercial development in a wide range of industries. There are several reasons for using an enzyme in an immobilized form. In addition to more convenient handling of the enzyme, it provides for its facile separation from the product, thereby minimizing or eliminating protein contamination of the product. To date, pectinases have been immobilized by various techniques (adsorption, cross linked enzyme aggregates (CLEAs), covalent attachment, etc.). In this review, we have focused on pectinase immobilization on various matrices such as polyacrylonitrile copolymer membrane, bones, aminated silica gel, macroporous polyacrylamide, polyethylene, nanocomposite microspheres, silica coated chitosan, Nylon-6, magnetic particles and porous glass support. Even CLEC and CLEA have been used as matrices. Various applications of immobilized pectinases in hollow fibre ultra filtration of apple juice, apple juice clarification, papermaking industries, mash treatment, production of short chain fructooligosaccharides are thoroughly reviewed. Microbial pectinases have remarkable potential to offer mankind which can be efficiently used by applying immobilization principles on them.

Key words: Chitosan, CLEAs, immobilization, Nylon-6, pectinases.

INTRODUCTION

Pectinolytic enzymes are a group of enzymes which hydrolyze pectin, one of the main polysaccharides in plant cell wall (Jayani et al., 2005). Pectin is structurally and functionally the most complex polysaccharide in plant cell wall (Mohnen, 2008). The primary chain of pectin is composed of α-1, 4-linked residues of D-galacturonic acid (Jayani et al., 2005). The enzymes depolymerising pectin can be divided into hydrolases and lyases (Sakai et al., 1993). Pectinases are distributed in many higher plants and microorganisms. They play a very important role in plants since they help in cell wall extension and softening of some plant tissues (Jayani et al., 2005). Pectinases are produced by a large number of organisms such as bacteria (Magro et al., 1994), fungi (Servili et al., 1992) and yeasts (Fontana and da Silveira, 2012). Pectinases can often be produced at high concentrations by strains of filamentous fungi belonging to the Aspergillus genus. Certain Aspergillus species can be characterized by the types of pectinolytic enzymes they are able to produce (Alimardani-Theuil et al., 2011, Maciel et al., 2011, Fontana and da Silveira, 2012).

The most widely occurring enzymes are polygalacturonase (PGs), pectin methylesterase (PMEs) and pectate lyase (PLs) produced during the infection process and during culturing (Jia et al., 2009). The fixed bed reactor with orange peel support and using Aspergillus niger URMS162 is a promising process for polygalacturonase production at the industrial level (Maciel et al., 2013). Alkaline pectinases find application in degumming and retting of plant material, plant protoplast formation and treatment of fruit-processing waste streams. Acidic pectinases are widely used for extraction and clarification of fruit juice. Alkaline pectinases are predominately produced by alkaloophilic bacteria like Bacillus sp. (Kashyap et al., 2001), whereas acidic pectinases are excreted by fungal sources, mainly Aspergillus sp. (Tuttolabello and Mill, 1961). The physicochemical properties show that the majority of fungal PGs (polygalacturonases) have pH optimum between pH 3.0 and pH 6.0 (Kester and Visser, 1990; De Lourdes et al., 1991; Waksman et al., 1991; Devi and AppuRao, 1996; Rao et al., 1996; Gainvors et al., 2000; Nature and Pant,
Some biochemical properties of PGs produced by different fungi are shown in Table 1.

Pectinases find commercial application in production of fruit juices and wines, mainly for juice clarification and for improving pressing and extraction of the juice from fruits and vegetables (Kashyap et al., 2001). The raw fruit juice obtained after pressing is very turbid, viscous and contains a significant amount of colloidal compounds, mainly pectin which causes cloudiness. The amount of colloids present in fruit juices is in the range of 100-1000 mg L⁻¹. Clarification involves the removal of juice haze by enzyme hydrolysis with pectolytic enzymes. After its degradation pectin-protein complexes flocculate giving a juice with liquid viscosity which is easier to filter (Alvarez et al., 1998, Alvarez et al., 2000). Attractive color is one of the most important sensory characteristics of fruit and berry products, and elderberry juice is widely used as natural colorant. When pectinase preparations were used in the production of elderberry juice for clarification, a concomitant decrease of anthocyanins and thus a color loss were observed this is due to side glycosidase activities contained in commercial pectinase preparations from Aspergillus sp. (Pricelius et al., 2009).

Polymerase chain reaction (PCR) has reduced the amount and need for purity of nucleic acids used for many genetic analyses; however, larger amounts of purer DNA or RNA still potentially are needed for some applications, including restriction fragment length polymorphism analyses (RFLP), minisatellite analyses, library construction, DNA cloning protocols and microchip surveys. Although many plants yield a large amount of nucleic acids that are pure enough for these latter types of studies when previously published protocols (most of which are Cetyl Trimethyl Ammonium Bromide extraction protocol derivatives of earlier publications, (Doyle and Doyle, 1987) are used, other plants may yield contaminating substances that coprecipitate with the DNA in the final steps of these protocols. These contaminating substances usually have been described as general polysaccharides, polyphenolics, or secondary metabolites (Scott and Playford, 1996), and in extreme cases, attempts to rehydrate alcohol-precipitated DNA results in a viscous slurry. The higher the contamination, the greater the possible interference with further manipulations of the DNA, such as treatment with enzymes or subsequent electrophoresis. Pectinase treatment of extracted DNA contaminated with such substances modifies the substances so that they are removed more easily (Rogstad et al., 2001).

The term immobilized enzymes refers to enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously. Immobilization means associating the biocatalysts with an insoluble matrix, so that it can be retained in proper reactor geometry for its economic reuse under stabilized conditions. Immobilization thus allows, by essence, to decouple the enzyme location from the flow of the liquid carrying the reagents and products. It is well known that immobilization of enzymes offers several advantages that include reuse of the enzyme and its easier separation from the product. Since in food industry it is preferable to avoid the presence of extraneous compounds in the final products, the possibility of removing the enzyme is a significant advantage. In literature there are data about immobilization of pectinolytic enzymes on different supports by various methods (Vaillant et al., 2000; Rao et al., 2000; Sarioglu et al., 2001; Demirel et al., 2004; Sardar and Gupta, 2005; Brenna and Batista-Viero, 2006). Immobilization can be performed by several methods, namely, entrapment/microencapsulation, binding to a solid carrier, and cross-linking of enzyme aggregates, resulting in carrier-free macromolecules. The latter presents an alternative to carrier-bound enzymes, since these introduce large portion of noncatalytic material. This can account for about 90% to more than 99% of the total mass of the biocatalysts, resulting in low space yields and productivities, and often leads to the loss of more than 50% native activity, which is particularly noticeable at high enzyme loadings (Sheldon, 2007). A broad, generalized overview of the advantages and drawbacks of the different immobilization approaches is given in Table 2. A typical example of the patterns suggested by data in Table 2 was observed by Abdel-Naby et al. (1999) when evaluating the immobilization of α-amylase through different methods.

Despite excellent catalytic properties of pectinase, the native enzymes as biocatalysts always present some drawbacks, such as poor stability under operational conditions, difficulty of product recovery, and impossibility of multiple reuses in an industrial process (Sheldon et al., 2007). To overcome these problems, enzyme immobilizations were involved to improve the catalytic features of enzymes against several forms of denaturation as well as to make the use of expensive enzymes economically viable, strengthening repeat use (Bukowski et al., 2005; Jin and Brennan, 2005; Mateo et al., 2007). There has been an increased interest in the preparation of immobilized pectinase for the clarifications and depectinization of fruit juices using a wide variety of carriers and methods. Satisfactory results without any limitations have been rarely achieved (Alkorta et al., 1996). Several methods have been developed for the preparation of immobilized pectinase. Each has its own advantages and disadvantages (Fiedurek et al., 1992; Alkorta et al., 1996; Dinella et al., 1996). Until now, pectinase has been immobilized on various supports including nylon (Lozano et al., 1987), ion exchange resin (Kminkova and Kucero, 1983), silk (Zhu et al., 1998), and chitin (Iwasaki et al., 1998). Recently, pectinase was immobilized in alginate by simple inclusion (Ipsita et al., 2003, Busto et al., 2006), however, its residual activity was slightly lower or had a lower stability making it difficult to use on an industrial scale, pectinase was covalently immobilized onto the
Table 1. Some biochemical properties of fungal polygalacturonases.

<table>
<thead>
<tr>
<th>Fungal source</th>
<th>PG form</th>
<th>pH optimum</th>
<th>Mw (kDa)</th>
<th>pI</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus niger</td>
<td>II</td>
<td>3.8 – 4.3</td>
<td>61</td>
<td>-</td>
<td>Singh and AppuRao, 2002</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>3 – 4.6</td>
<td>38</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>Exo I</td>
<td></td>
<td></td>
<td></td>
<td>Sakamoto et al., 2002</td>
</tr>
<tr>
<td></td>
<td>Exo II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspergillus awamori</td>
<td>I</td>
<td></td>
<td>41</td>
<td>6.1</td>
<td>Nagri et al., 2002</td>
</tr>
<tr>
<td>Aspergillus carbonarius</td>
<td>I</td>
<td>4.0</td>
<td>61</td>
<td>-</td>
<td>Devi and AppuRao, 1996</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>4.1</td>
<td>42</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>4.3</td>
<td>47</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Aspergillus japonicus</td>
<td>I</td>
<td>4.0</td>
<td>38</td>
<td>5.6</td>
<td>Semenova et al., 2003</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>5</td>
<td>65</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>Aspergillus tubingensis</td>
<td>Exo PG</td>
<td></td>
<td>78</td>
<td>3.7-4.7</td>
<td>Kester et al., 1996</td>
</tr>
<tr>
<td>Aspergillus kawachii</td>
<td>I</td>
<td>2-3</td>
<td>60</td>
<td>3.55</td>
<td>Contreras and Vogt, 2004</td>
</tr>
<tr>
<td>Aspergillus ustus</td>
<td>I</td>
<td>5.0</td>
<td>36</td>
<td>8.2</td>
<td>Rao et al., 1996</td>
</tr>
<tr>
<td>Botrytis cinerea</td>
<td>Exo I</td>
<td></td>
<td>65</td>
<td>8.0</td>
<td>Cabanne and Doneche, 2002</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td></td>
<td>52</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td>Fusarium oxysporum f. sp. Lycopersici</td>
<td>I &amp; II</td>
<td></td>
<td>37</td>
<td>-</td>
<td>Strand et al., 1976</td>
</tr>
<tr>
<td>Fusarium oxysporum</td>
<td>I</td>
<td></td>
<td>35</td>
<td>8.3</td>
<td>Garcia-Marceira et al., 2001</td>
</tr>
<tr>
<td>Fusarium oxysporum f. sp. lycopersici</td>
<td>Exo PG2</td>
<td>5</td>
<td>74</td>
<td>4.5</td>
<td>Di Pietro and Roncero, 1996</td>
</tr>
<tr>
<td>Kluyveromyces marxianus</td>
<td>I</td>
<td>4</td>
<td>41.7</td>
<td>-</td>
<td>Niture et al., 2001</td>
</tr>
<tr>
<td>Mucor cirrinhelloloides</td>
<td></td>
<td>-</td>
<td>5.5</td>
<td>65</td>
<td>Pahwa et al., 2010</td>
</tr>
<tr>
<td>Neurospora crassa</td>
<td>I</td>
<td>4.6</td>
<td>37</td>
<td>-</td>
<td>Polizeli et al., 1991</td>
</tr>
<tr>
<td>Penicillium frequentans</td>
<td>I</td>
<td>3.9</td>
<td>74</td>
<td>4.2</td>
<td>dos Santos et al., 2002</td>
</tr>
<tr>
<td>Penicillium frequentans</td>
<td>I</td>
<td>4.0-4.7</td>
<td>20</td>
<td>5.6</td>
<td>De Fatima et al., 1996</td>
</tr>
<tr>
<td>Postia placenta</td>
<td>I</td>
<td>3.2-3.9</td>
<td>34</td>
<td>3.3</td>
<td>Clausen and Green, 1996</td>
</tr>
<tr>
<td>Phytophthora parasitica</td>
<td>I</td>
<td>-</td>
<td>39.2</td>
<td>5.2</td>
<td>Yan and Liou</td>
</tr>
<tr>
<td>Rhizoctonia fragariae</td>
<td>I</td>
<td></td>
<td>36</td>
<td>6.76</td>
<td>Gervone et al., 1977</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td></td>
<td>36</td>
<td>7.08</td>
<td></td>
</tr>
<tr>
<td>Rhizopus oryzae</td>
<td>I</td>
<td>4.5</td>
<td>31</td>
<td></td>
<td>Saito et al., 2004</td>
</tr>
<tr>
<td>Sclerotinia borealis</td>
<td>I</td>
<td>4.5</td>
<td>40</td>
<td>7.5</td>
<td>Takasawa et al., 1997</td>
</tr>
<tr>
<td>Sclerotinia sclerotiorum</td>
<td>I</td>
<td></td>
<td>42</td>
<td>4.8</td>
<td>Martel et al., 1998</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td></td>
<td>41.5</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>Sclerotinia sclerotiorum</td>
<td>Exo I</td>
<td>5</td>
<td>60</td>
<td>-</td>
<td>Riou et al., 1992</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>I</td>
<td>3-4.5</td>
<td>42</td>
<td>-</td>
<td>De Lourdes et al., 1990</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>I</td>
<td>5.5</td>
<td>65</td>
<td>-</td>
<td>Blanco et al., 1994</td>
</tr>
<tr>
<td>Thermomyces lanuginosus</td>
<td>I</td>
<td>5.5</td>
<td>59</td>
<td>-</td>
<td>Kumar and Palanivelu, 1999</td>
</tr>
<tr>
<td>Thermoasus aurantiacus</td>
<td>I</td>
<td>5.5</td>
<td>30</td>
<td>-</td>
<td>Martins et al., 2007</td>
</tr>
<tr>
<td>Trichoderma harzianum</td>
<td>II</td>
<td>5.0</td>
<td>31.0</td>
<td>4.5</td>
<td>Mohamed et al., 2006</td>
</tr>
</tbody>
</table>

Macroporous polyacrylamide microspheres (Lie and Jiang, 2006) and immobilized on an activated agar-gel support by multipoint attachment (Li et al., 2008). Pectinases are used in the food processing industry (Rombouts and Pilnik, 1990) and especially in the clarification of fruit juices. Pectinases have been immobilized on various matrices. The endo-polygalacturonase from A. niger has been immobilized by adsorption on porous polyethylene terephthalate (Rexova-Bankova et al., 1982). Omelkova et al. (1985) and Omelkova et al. (1985) immobilized 1978; Uhlig, 1990) and especially in the clarification of fruit endopolygalacturonase on to porous poly (6-caprolactam) activated by glutaraldehyde with a relative activity of 24%
(The relative activity is the ratio of the activities of the bound and free enzyme expressed in percentage.) Other matrices which have been used for immobilization of polygalacturonases are poly 2, 6- dimethyl-p-phenylene oxide (Rexova-Benkova et al., 1983), granular poultry bones (Findlay et al., 1986), porous glass (Romero et al., 1987) and nylon (Lozano et al., 1987). Pectinase was immobilized on Fe₃O₄/SiO₂-g-poly (PStNa) nanocomposite microspheres by covalent attachment (Lie et al., 2009). Szaniawski and Spencer (1997) examined the effect of immobilized pectinase on the microfiltration of dilute pectin solutions by macroporous titanium membranes and immobilized enzyme was found to be very effective for the degradation of the pectin solution. Endopeptidylase immobilization onto tailor-made core-shell microspheres is another research on the immobilization of the pectolytic enzymes (Dinella et al., 1996).

**PECTINASE IMMOBILIZATION ON POLYACRYLONITRILE COPOLYMER MEMBRANE**

In the present study, polyacrylonitrile copolymer membrane was used to immobilize commercial pectinase from *A. niger* via adsorption method. This method was simple and effective. The chosen support has suitable pore size that allows easy penetration of the enzyme; it has high mechanical, temperature and chemical stability and can be separated from the reaction mixture without contaminating the final product (Delcheva et al., 2007).

**IMMOBILIZATION OF PECTINEX ULTRA SP-L PECTINASE**

Pectinex Ultra SP-L, a commercial pectinase with fructosyl-transferase (FTF) activity, is able to catalyze the production of short chain fructooligosaccharides (FOS). It was immobilized onto an anion exchange resin by a combined method. Pectinex Ultra SP-L, a commercial enzyme, was used. Combining the adsorption of a commercial pectinase, Pectinex Ultra SP-L having significant fructosyl transferase activity, onto Amberlite IRA900 Cl anion exchange resin and the formation of cross-links between the adsorbed protein molecules, an immobilized enzyme product was prepared. Optimal pH value of the immobilized biocatalyst was found to be similar to that of the free enzyme but the immobilization has increased the optimal temperature a little (Csanadi and Sisak, 2006).

**PECTINASE IMMOBILIZATION BY ELECTROSTATIC ADSORPTION**

Immobilization of an enzyme on a support material by adsorption is generally regarded as the simplest and most economical procedure (Whitaker, 1991). Binding mechanism involves a combination of van der waals' ionic and hydrogen bonds. Despite the fact that binding is weak and displacement can occur in the presence of molecules with higher binding energy, the capability of regenerating the immobilized enzyme activity in place is another advantage of adsorption technique (Bernarth and Venkatasubramaniam, 1986; Shuler and Kargi, 1992). As stated by Bayindirli (1996), support material and chemicals for immobilization are not allowed to contaminate the final products, especially foods. Thus ion exchange resin, Dowex Marathon WBA was used to obtain strong electrostatic interaction for the immobilization of commercial pectinase without using any other chemicals like glutaraldehyde, carbodiimide or cyanogens bromide. Pectolytic enzyme preparation was immobilized on to anion exchange St-DVB macroporous base resins, and the kinetics of immobilized commercial pectinase was studied (Demir et al., 2001).

### Table 2. A generalized characterization of immobilization methods.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Covalent</th>
<th>Ionic</th>
<th>Adsorption</th>
<th>CLEAs, CLECs</th>
<th>Entrapment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity</td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td>Intermediate/High</td>
<td>High</td>
</tr>
<tr>
<td>Range of application</td>
<td>Low</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Low</td>
<td>Intermediate/High</td>
</tr>
<tr>
<td>Immobilization efficiency</td>
<td>Low</td>
<td>Intermediate</td>
<td>High</td>
<td>Intermediate</td>
<td>Intermediate</td>
</tr>
<tr>
<td>Cost</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
<td>Intermediate</td>
<td>Intermediate/ Low</td>
</tr>
<tr>
<td>Preparation</td>
<td>Easy</td>
<td>Easy</td>
<td>Difficult</td>
<td>Intermediate</td>
<td>Intermediate/ Difficult</td>
</tr>
<tr>
<td>Substrate specificity</td>
<td>Cannot be changed</td>
<td>Cannot be changed</td>
<td>Can be changed</td>
<td>Cannot be changed</td>
<td>Can be changed</td>
</tr>
<tr>
<td>Regeneration</td>
<td>Possible</td>
<td>Possible</td>
<td>Impossible</td>
<td>Impossible</td>
<td>Impossible</td>
</tr>
</tbody>
</table>

**Pectinex Ultra SP-L**

L's ionic mobilized biocatalyst. As Simon, 1987)., 2001)., granular poultry. As Simon, 1986) was used to immobilize commercial pectinase, Pectinex Ultra SP-L, a commercial enzyme, was combined with the adsorption of a commercial pectinase, Pectinex Ultra SP-L having significant fructosyl transferase activity, onto Amberlite IRA900 Cl anion exchange resin and the formation of cross-links between the adsorbed protein molecules, an immobilized enzyme product was prepared. Optimal pH value of the immobilized biocatalyst was found to be similar to that of the free enzyme but the immobilization has increased the optimal temperature a little (Csanadi and Sisak, 2006).
BONE AS SOLID SUPPORT FOR IMMOBILIZATION OF PECTINASE

Bone has great potential for enzyme immobilization since it is inexpensive, abundant, chemically functional, porous, non-toxic and mechanically strong. The results of enzyme coupling to dry bone using the various techniques are presented in Table 3 (Findlay et al., 1986).

PECTINASE IMMOBILIZATION ON AMINATED SILICA GEL

Aminated silica gel was used as a support for the covalent immobilization of the enzyme. Endo-polygalacturonase from *Aspergillus ustus* when immobilized on to modified silica gel retained 28% of its original activity. The immobilized enzyme could be re-used through 10 cycles of reaction with almost 90% retention of its original activity. It had increased thermostability over its soluble form: the half-life of the soluble enzyme at 40°C was less than 10 h whereas the immobilized enzyme retained 82% of its activity after 10 h at 40°C. Similarly, at 50°C the half-life of the soluble enzyme was 30 min whereas that of the immobilized enzyme was 5 h. The free aldehyde obtained by activating ethanolamine treated silica gel with glutaraldehyde was linked to the side-chain amino groups of the enzyme, through Schiff's base formation. When the enzyme was treated with trinitrobenzene sulfonate, a reagent which binds specifically to lysine residues in proteins, the enzyme failed to bind to the matrix, indicating that binding takes place through the \(-\)amino groups of lysine residues on the surface of the enzyme. Previous studies on chemical modification of the active site residues of the endo-polygalacturonase had shown that lysine is not essential for catalytic activity (Narsimha et al., 1996).

Cross linked enzyme crystals and cross linked enzyme aggregates

There are numerous methods for immobilization of biocatalysts on rigid supports, such as covalent immobilization (Guin, 1998), ionic immobilization (Mateo et al., 2000, Torres et al., 2002), and hydrophobic adsorption (Palomo et al., 2002). Methods to immobilize enzymes without the use of supports are gaining in importance, however, because they offer the advantages of high volumetric productivity and lower production costs and also because they are composed only of protein and a small amount of cross-linking agent. Examples of such carrier-free preparations include crosslinked enzyme crystals (CLECs) (Quirocho and Richards, 1964; Alter et al., 1967; Clair and Navia, 1992) cross-linked enzymes (Habeeb, 1997; Jansen and Olson, 1969), and the methodology of cross-linked enzyme aggregates (CLEAs) (Cao et al., 2000). In this procedure, the enzyme is precipitated from an aqueous solution by adding a salt or a water-miscible organic solvent or polymer, such as poly(ethylene glycol). In a subsequent step, the physical aggregates of enzyme molecules are cross-linked with a bifunctional agent (Cao et al., 2000; Cao et al., 2001; Lopez et al., 2002). Polyfunctional polymers with a high molecular weight (e.g., 100 to 200 kDa), containing numerous reactive aldehyde groups, are known to be effective cross-linkers of proteins or subunits (Kazan et al., 1997; Fernandez et al., 1999a; Fernandez et al., 1999b). An interesting feature of the CLEAs is that these preparations do not require extensive purification of the enzyme activities. In this respect, CLEAs differ from the CLEC™, another form of enzyme aggregates prepared by chemical cross-linking of enzyme crystals (Presichelli et al., 1995). It has been shown that it is possible to form a CLEA that can catalyze more than one reaction. Thus, a CLEA may catalyze a sequence of reactions. Such CLEAs have been called Combi-CLEAs (Sheldon et al., 2005; Dalal et al., 2006). The general protocol for the preparation of CLEAs consists of precipitating the enzyme activity by adding salt or an organic solvent (Schoevaart et al., 2004; Shah et al., 2006). This is followed by addition of cross-linking reagent, which is generally glutaraldehyde. A multipurpose CLEA with substantial activities of pectinase, xylanase and cellulase was prepared and characterized. The other two activities, xylanase and cellulase, also have well known and extensively documented applications in biotechnology (White and Brown, 1981; Subramaniam and Prema, 2002). Table 4 shows the remarkable thermostabilization of the enzymes present in the preparation. In all three of the cases, half-lives have increased upon CLEA formation. Cellulase activity was most thermostable and its thermostabilization was measured at 70°C. Pectinase was least stable and its thermostabilization was measured at

### Table 3. Immobilization of enzyme to bone by various methods.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Immobilization method</th>
<th>Initial enzyme activity in immobilizing solution (U)</th>
<th>Activity immobilized on bone (U/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pectinase</td>
<td>Acyl-Azide</td>
<td>28</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>Glutaraldehyde</td>
<td>32</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>Carbodiimide</td>
<td>30</td>
<td>0.10</td>
</tr>
</tbody>
</table>
50°C. The largest increase in stability was in the case of pectinase in which the half-life increased from 17 to 180 minutes (Dalal et al., 2007).

Another example is the immobilization of pectinase in egg shell for the preparation of low-methoxyl pectin. The immobilized biocatalyst could be reused for 32 times at 30°C, and it was used in a fluidized-bed reactor, operated at an optimum flow rate of 5 mLh⁻¹ and 35°C. Pectin is widely used in the preparation of jams, jellies and other food gels (Nighojkar et al., 1995). The gels obtained from low methoxyl pectin (LM-pektin) are softer, more spreadable and relatively independent of pH compared to high methoxyl pectin (HM-pektin) gels. Pectin is widely used as a texturizer, stabilizer, and emulsifier in a wide variety of fields, among them the food and feed industry. Depending on the degree of methoxylation, pectins are classified into (1) LM- (low methoxyl, with a 25–50% degree of methoxylation) and (2) HM- (high methoxyl, with a 50–80% degree of methoxylation) pectins. These pectins can form gels of two types with occasional intermediates, which are called acid or calcium gels, depending on whether they are formed from HM or LM-pectins, respectively (Thakur et al., 1997). Briggs (1997) suggested that pectins with both low methoxyl content and low molecular weight (<10000 kDa) could be effectively used to prevent cancer metastasis, whereas pectins with both high methoxyl content and high molecular weight are efficient cholesterol-lowering agents (Yamaguchi et al., 1995; Liu et al., 2001). The enzyme, pectinmethylsterase (PME: EC 3.1.1.11), has been found in plants as well as in pathogenic fungi and bacteria (Giovane et al., 1994) and catalyzes the hydrolysis of the methyl ester groups from pectin. After this the pectin can be hydrolyzed by polygalacturonase action. Plant PME is used in the preparation of LM-pectin and in the destabilization of cloud in fruit juices (Nighojkar et al., 1995). PME has been covalently immobilized onto porous glass beads (Wiebel et al., 1975), nylon polyisonitrile derivatives (Viyalakshami et al., 1979), activated Sepharose 4B and polyethylene terephthalate (Markovic and Machova, 1987). The total PME and partially purified PME showed that PME specific activity increases with temperature. Both samples of PME (total or partially purified) showed highest thermal stability. The total PME needed 110 and 2.17 min for its inactivation at 98 and 106°C, respectively (Assis et al., 2002). Then, because the PME is very thermostable, its immobilization is potentially interesting. The total and partially purified enzyme pectinmethylsterase from acerola fruit was covalently immobilized on porous silica particles (Assis et al., 2006).

### PECTINASE IMMOBILIZATION ON MACROPOROUS POLYACRYLAMIDE

The essential requirement for any carrier is the need to have a large surface area. In this respect, porous polymeric materials, which have obvious advantage of high internal surface areas, have been increasingly employed as the solid supports (Blanco et al., 2004; Li et al., 2010). It has been found that the pore sizes and specific surface area play an important role in the enzyme loading and activity expression (Keeling and Brennan, 2001; Tsai and Doong, 2007; Das et al., 2010). However, a very high loading may produce diffusion constraint, which is not favorable for enzyme immobilization. It is convenient to use supports with a very large specific surface, such as macroporous polyacrylamide (PAM), which provide substrate and product transport with the least diffusional restriction. Macroporous PAM microspheres, a kind of macroporous amino resin (Liu and Guo, 2006) were chosen as immobilization supports because of their prominent advantages, such as availability of plentiful surface amino groups, perfect mechanical strength, large surface area (Tang et al., 2001) amenable to chemical modifications, adjustable particle size, easy regeneration, low operational cost, high performance of antipollution, good selectivity, and favorable chemical stability. The advantages above may provide the pectinase immobilization: (i) a certain number of available binding sites and a very simple, mild, and time-saving process, (ii) the reuse support (Pessela et al., 2003) (iii) the reduction of immobilization costs. Pectinase was immobilized onto the macroporous PAM. The immobilized pectinase exhibited higher relative activity and stability than the free enzyme in the solution.

The SEM (scanning electron microscope) images of the resulting macroporous PAM microspheres are shown in Figure 1. It can be seen in Figure 1 that the macroporous PAM microspheres, after being washed with methanol, are perfect microspheres, with a diameter of less than 50 μm (Figure 1a), and that their surfaces are smooth. The surface morphologies of the macroporous PAM microspheres exhibit porous structures (Figure 1b). Their porous structures did not change much after being washed by methanol, and the diameter of their porous is about 25 nm.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Enzyme</th>
<th>Half life(t½)(min)Free</th>
<th>CLEAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>Pectinase</td>
<td>17</td>
<td>180</td>
</tr>
<tr>
<td>60</td>
<td>Xylanase</td>
<td>22</td>
<td>82</td>
</tr>
<tr>
<td>70</td>
<td>Cellulase</td>
<td>32</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 4. Half-life of pectinase, xylanase and cellulase in CLEAs
Figure 1c showed that the internal morphologies of the macroporous PAM microspheres exhibit porous structures (Lie and Jiang, 2006).

IMMOBILIZATION OF POLYGALACTURONASE ON ACTIVATED POLYETHYLENE

Polyethylene is a convenient matrix for enzyme immobilization. It is easily removed from fruit juice, is inexpensive, inert, non-toxic and readily available. The use of synthetic polymers for enzyme immobilization has several advantages viz. inertness to microorganisms, higher chemical resistance and option to use complex buffer system mostly required in biosensor systems (Lei and Bi, 2007). \textit{A. niger} Van Tieghem (MTCC 3323) produced polygalacturonase when grown in modified Riviere's medium containing pectin as single carbon source by fed-batch culture. The enzyme was precipitated with ethanol and purified by gel filtration chromatography (Sephacryl S-100) and immobilized onto glutaraldehyde-activated polyethylene. The method is very simple and time saving for enzyme immobilization. Various characteristics of immobilized enzyme such as optimum reaction temperature and pH, temperature and pH stability, binding kinetics, efficiency of binding, reusability and metal ion effect on immobilized enzymes were evaluated in comparison to the free enzyme. Both the free and immobilized enzyme showed maximum activity at a temperature of 45°C and pH 4.8. Maximum binding efficiency
was 38%. The immobilized enzyme was reusable for 3 cycles with 50% loss of activity after the third cycle (Saxena et al., 2008).

**IMMobilization of Pectinase on Polymer Nanocomposite Microspheres**

Polymer nanocomposite microspheres (PNCMs) represent an attractive family of composite materials in which the nanometer sized reinforcing fillers are uniformly dispersed in the polymer on a nanometer scale compared to conventional phase-separated macrocomposites (Dyal et al., 2003; Weng and Wei, 2003; Kahraman et al., 2007). Polymer nanocomposite microspheres (PNCMs) as solid supports can improve the efficiency of immobilized enzymes by reducing diffusional limitation as well as by increasing the surface area per mass unit. The PSStNa support presents a very simple, mild, and time-saving process for enzyme immobilization, and this strategy of immobilizing pectinase also makes use of expensive enzymes economically viable, strengthening repeated use of them as catalysts following their rapid and easy separation with a magnet. In this work, to build more stable assembly, the polyelectrolyte brush PSStNa was grafted onto the surface of Fe₃O₄/SiO₂ composite particles by surface-initiated atom transfer radical polymerization (SI-ATRP) using modified magnetic silica as initiator. Subsequently, introducing a layer-by-layer (LbL) method, deposition occurs by electrostatic interactions between the adsorbed PSStNa and chitosan layer with opposite charges. It was further found that Fe₃O₄/SiO₂-g-PSStNa nanocomposite microspheres with modified multishells enhanced the stability of both nanoparticles (compared to adsorption) in solution and the immobilized pectinase. This strategy of pectinase immobilization opens new avenues for the application of bioparticles and represents a promising route for the creation of complex catalytic particles (Lie et al., 2009).

**Pectinase Immobilization on Silica Coated Chitosan**

Chitosan can be easily cross-linked by reagents such as glutaraldehyde to form rigid aqua-gels (Yang and Shao, 2000). As a matter of fact, chitosan has been shown to be a superior supporter for the enzyme immobilization, compared to polysaccharides such as alginate (Centinus and Oztop, 2000; Ibrahim et al., 2002). Furthermore, chitosan has been found to exhibit a considerable protein-binding capacity and a high recovery of enzyme activity, allowing that the enzyme immobilized thereon remains considerably active (Galli Fuoco et al., 1998). However, severe shrinkage and deformation could not be easily avoided upon drying the chitosan carriers into the conres-ponding gels (Michael and Arlon, 2001). This can be improved in conjunction with other solid powders to increase its density and strengthen its physical properties, and thus to expand its applications (Bai et al., 2002). The layer-by-layer (LbL) technique provides an easy, low cost, and versatile method for the fabrication of the silica coated chitosan support. Briefly; this technique is based on the alternating adsorption of oppositely charged polyelectrolytes onto a charged substrate. The major advantage of the layer-by-layer technique is its multifunctionality, allowing the incorporation of a broad variety of functional groups. By virtue of the attraction of oppositely charged molecules, chitosan, owing to its cationic polyelectrolyte nature, spontaneously forms water insoluble complexes with anionic polyelectrolyte (Dumitriu and Chornet, 1998; Kubota and Kikuchi, 1998; Singla et al., 2001). Pectinase was immobilized onto a new type of silica-coated chitosan support from layer by layer approach and the properties of immobilized enzyme were compared with those of free pectinase. The immobilized pectinase revealed acceptable pH stability over a broad experimental range. This simple strategy seems to permit very good results in terms of immobilization rate and stability, offering some advantages when compared to the immobilization on glutaraldehyde pre-activated supports (Lie and Bi, 2007).

**Pectinase Immobilization on Nylon-6**

A particularly convenient, cheap and relatively inert matrix for immobilization of enzyme is Nylon-6. Unlike most other nylons, Nylon-6 is not a condensation polymer, but instead it is formed by ring-opening polymerization. During polymerization, the peptide bond within each caprolactam molecule is broken; with the active groups on each side reforming two new bonds as the monomer becomes part of the polymer backbone. Nylon-6 resembles natural polypeptides more closely; in fact, caprolactam would become an amino acid if it were hydrolyzed. Also, it is non-toxic and readily available and can be obtained in a number of forms. The activation of nylon involved partial acid hydrolysis of the Nylon-6 surface to generate amino groups (and carboxyl groups), which could be coupled to proteins with glutaraldehyde (Sundaram and Hornby, 1970). Pectin lyase [PNL, poly (methoxygalacturonide) lyase; E.C. 4.2.2.10] from Penicillium italicum was immobilized by covalent binding to Nylon 6 in order to compare physicochemical and kinetic properties of the soluble and immobilized counterpart. The immobilization caused a marked increase in the thermal stability of the enzyme. The immobilized PNL was extraordinarily stable during storage at 4°C. No loss of activity was observed when the immobilized enzyme was used for 12 consecutive cycles of operation (Alkorta et al., 1996). Polygalacturonase from A. niger Van Tieghem was immobilized by covalent binding method on glutaraldehyde activated Nylon-6 and used for
apple juice clarification (Shukla et al., 2010).

**IMMOLIZATION OF ASPERGILLUS NIGER PECTINASE ON MAGNETIC PARTICLES**

A commercial preparation of pectinase, pectinex was purified with the help of alginate-magnetite beads. The purified pectinase was immobilized on magnetic latex beads via carbodiimide coupling. The pH optimum (pH 4.5 for both free as well as immobilized enzyme) and $K_m$ (0.7 mg/ml for free enzyme; 1 mg/ml for immobilized enzyme) did not vary significantly upon immobilization. While the half life of free enzyme was calculated as 9 min, the immobilized preparation remained stable upto 3 h at 60°C (Tyagi and Gupta, 1995). In a previous study pectinase from Leucoagaricus gongylophorus immobilized on magnetic particles (Adalberto et al., 2012).

**IMMOLIZATION OF ENDO-POLYGALACTURONASE ON MACROMOLECULAR SUPPORTS**

Endo-polygalacturonase (endo-PG) was immobilized on a wide range of natural and synthetic macromolecular supports and their modified derivatives representing many chemical classes, including esters, amides, phenols, alkyl- and aryl-amines and carboxyl derivatives. The immobilization entailed methods of adsorption alone as well as covalent bond formation using glutaraldehyde or carbodiimide or via the diazo-coupling reaction. The most promising system proved to be immobilization on trimalehylchitosan (TMC) via adsorption followed by treatment with glutaraldehyde (GA). Various properties of immobilized endo-PG were evaluated (Pifferi et al., 1989). Pectinase can also be immobilized on macroporous resin coated with chitosan which can further be used for treatment of whitewater for papermaking. The highest hydrolysis activities of the immobilized enzymes were achieved by using chitosan with 10×10 (5) DaMW for coating and 0.0025% gluteraldehyde cross linking (Liu et al., 2012).

**CO-IMMOMLIZATION OF PECTINESTERASE AND ENDO-D-POLYGALACTURONASE IN A POROUS GLASS SUPPORT**

Derivatives of pectinesterase and polygalacturonase, both individually immobilized and co-immobilized, were obtained and characterized. Immobilization or co-immobilization did not change the optima pH or temperature for the enzymes. However, optimum ionic strength was displaced toward higher values for immobilized pectinesterase; while for polygalacturonase immobilization resulted in a wider range of activity. $K_m$ value remained nearly unchanged for pectinesterase, and decreased for polygalacturonase. The $V_{max}$ value decreased with the immobilization process for the two enzymes, except for polygalacturonase immobilization in presence of pectinesterase. Thermal stability of pectinesterase was not improved after immobilization. On the contrary, the thermal stability of endo-D-polygalacturonase was improved slightly by presence of pectinesterase, and to a greater extent by immobilization. Individually immobilized and co-immobilized pectinesterase activities kept at 90 and 60%, respectively, of their initial values after more than one year stored at 3-5°C (Manjon et al., 1992).

**IMMOLIZATION OF SCLEROTIUM ROLFSII PECTINASE**

Immobilization of pectinase from S. rolfsii was studied on different matrices of which Amberlite XAD-7 showed maximum adsorption and expression of the enzyme. The most active preparation was obtained when XAD-7 was activated with 2% glutaraldehyde and 1.7 μkat of enzyme per g resin was used for immobilization at pH 5.5 and temperature 28°C. Optimum pH and temperature of the S. rolfsii pectinase remained unaltered, 3.5 and 55°C, respectively, after immobilization. Both soluble and immobilized enzyme preparations were most stable at pH 4.0. The immobilized enzyme preparation was more stable than the soluble enzyme (Channe and Shewale, 1995).

**APPLICATIONS**

**Immovlized pectinase in hollow fibre ultrafiltration (HFUF) of apple juice**

The objectives of clarified apple juice concentration are mainly to reduce costs and to increase shelf-life. Before concentration, pressed juice must be clarified. A conventional clarification process includes hydrolysis of pectin and starch with specific enzymes, flocculation of turbidity with clarifying agents (bentonite, gelatin and/or silica-sol) and filtration through plate and frame or vacuum Oliver-type filters, in order to eliminate insoluble solids and destroy pectic substances. This process involves several separate operations, which are hard work and time consuming. The application of ultrafiltration (UF) as an alternative to conventional processes for clarification of apple juice was demonstrated by Heatherbell et al. (1977). However, the acceptance of UF in the fruit processing industry is not yet completed, because there are problems with the operation and fouling of membranes. During UF two fluid streams generation, the ultrafiltered solids free juice (permeate), and the retentate with variable content of insoluble solids which, in the case of apple juice, are mainly remains of cellular walls and pectin. Permeate flux results from the difference between a convective flux from the bulk
of the juice to the membrane and a counter diffusive flux or outflow by which solute is transferred back into the bulk of the fluid (Cheryan, 1986). As a result of this mechanism, pectin and other large solutes are brought to the membrane surface by convective transport during the HFUF of apple juice. Consolidation of this gel layer on the HFUF membrane has a drastic effect on the performance of the operation. Commercial pectic enzymes or pectinases are used in apple juice manufacturing to depectinize pressed juices in order to remove turbidity and prevent cloud-forming (Grampp, 1976). The available commercial pectinase preparations used in apple processing generally contain a mixture of pectinesterase (PE), polygalacturonase (PG) and pectinlyase (PL) enzymes (Dietrich et al., 1991). Endopolygalacturonase and pectinlyase among others, have been immobilized on different organic and inorganic supports, with uneven results (Pifferi and Prezioso, 1987; Spagna et al., 1995). Enzyme immobilization by physical adsorption is a simple and well established technique (Gekas, 1986; Szaniawski, 1996). However, immobilized pectinase enzymes are not currently available commercially. In view of the high molecular weight and viscosity of pectin, the use of immobilized pectinase in most fruit processing applications may be rather limited (Kulp, 1975). Despite the different types of supports and reactor configurations proposed for a continuous performance of enzymatic reaction, immobilization of enzymes on micro, or ultrafiltration membranes, appear as interesting alternatives for treating cloudy fruit juices (Alkorta et al., 1995). The use of pectinase immobilized on ultrafiltration membranes is expected to hydrolyze the pectin to lower molecular weight species (mainly anhydrogalacturonic acid, AGA) at the membrane-permeate interface, resulting in an increase of the permeate flux or at least an extension of the membrane operation without cleaning. Glutaraldehyde has been used as enzyme molecules crosslinking and spacing agent (Atallah and Hultin, 1977; Synowiccki et al., 1982).

**Apple juice clarification**

The catalytic behavior of a mixture of pectic enzymes, covalently immobilized on different supports (glass microspheres, Nylon 6/6 pellets, and PAN beads), was analyzed with a pectin aqueous solution that simulates apple juice. The following parameters were investigated: the rate constant at which pectin hydrolysis is conducted, the time in which the reduction of 50% of the initial viscosity is reached, and the time required to obtain complete depectinization. The best catalytic system was proven to be PAN beads, and their pH and temperature behavior were determined. The yields of two bed reactors, packed or fluidized, using the catalytic PAN beads, were compared to the circulation flow rate of real apple juice. The initial pectin concentration was the one that was present in apple juice sample. No differences were observed at low circulation rates, while at higher recirculation rates, the time required to obtain complete pectin hydrolysis into the fluidized reactor was found to be 0.25 times smaller than in the packed bed reactor: 131 min for the packed reactors and 41 min for the fluidized reactors (Diano et al., 2008). A commercial pectinase, immobilized on appropriately functionalized γ-alumina spheres, was loaded in a packed bed reactor and employed to depolymerise the pectin contained in a model solution and in the apple juice. The activity of the immobilized enzyme was tested in several batch reactions and compared with the one of the free enzyme. A successful apple juice depectinisation was obtained using the pectinase immobilized system (Dinella et al., 2008).

**Paper making industries**

Pectins or polygalacturonic acids (PGA) were originated from alkaline peroxide bleaching of mechanical pulps and considered as the dominant troublesome substance, which seriously decreased the runability of the paper machine in process water closure. To find wide application of enzymes in lowering PGA concentration in papermaking industries, cross-linked chitosan beads were prepared. Results showed that the PGA-adsorption capability of chitosan beads was greatly affected by its cross-linking degree. The activity of immobilized pectinase on cross-linked chitosan beads were also investigated and the highest activity of binary immobilized pectinase on cross-linked chitosan was achieved using 1.00% of activating reagent or 0.005% of glutaraldehyde. Cationic demand of PGA solutions was obviously lowered by increasing the temperature of enzymatic treatment. GPC (Gel Permeation Chromatography) analysis showed a sharp decrease in the molecular weight of PGA after enzymatic treatment (Liu et al., 2010).

**Mash treatment**

Enzymatic mash treatment is a well-known modern process for gaining more juice from fruits and vegetables. According to the technique, cell wall and middle-lamina pectin of the fruit are degraded by pectinase activities. Besides increasing press capacity and the yield of juice up to 20%, it has also a positive effect to achieve high carotene and dry matter content of the product. The aim of the research was to investigate the activity and reusability of immobilized commercial pectinase named as Pectinex Ultra SP-L on carrot puree. Immobilization process was carried out by using ion exchange resin particles. An average yield increment was 30.23% with respect to the yield obtained from non-enzymic processed carrot juice (Demir et al., 2001).
Production of short chain fructooligosaccharides (FOS)

Functional foods as prebiotic fructooligosaccharides have become important and their significance has risen recently because of their favourable properties shown in human and animal nutrition as health foods and special feed additives (Yun, 1996). They have advantageous effects on the intestinal bacterial population and the general health conditions in the body (Borinet et al., 2002; Tuonay et al., 2003). FOS are non-digestible oligosaccharides (NDO) and they are not decomposed in the small intestine by the digestive enzymes, so they reach the colon where they are fermented by the microbial flora (e.g. Bifidobacteria sp., Lactobacillus sp.) to lactic acid and short chain fatty acids. Consequently, FOS stimulate the growth and fermentation of these microbes and decrease pH in the colon, inhibiting the growth of harmful pathogens (Losada and Olleras, 2002). In addition, they have low sweetness intensity, their caloric value is low, approximately 8–9 kJ g⁻¹ (Durieux et al., 2001) and because they avoid the digestion in the upper intestine, they cause no caries. These properties make them applicable as raw materials of diabetic products (Kaplan and Hutkins, 2000). FOS are natural components of many vegetables, for example onion, asparagus, rice, sugar beet, wheat, etc. The industrial scale recovery from these plants is not economical since their concentration is low. For this reason, FOS is produced commercially via biosynthetic as well as hydrolytic methods. The raw material of the biosynthetic way is sucrose; the process is catalyzed by fructosyl-transferase (Yun, 1996). The partial hydrolysis of inulin is also used practically to produce fructooligosaccharides (Kaur and Gupta, 2002). Pectinex Ultra SP-L, a commercial pectinase with fructosyl-transferase (FTF) activity, is able to catalyze the production of short chain fructooligosaccharides (FOS). It was immobilized onto an anion exchange resin by a combined method (Csanadi and Sisak, 2006).

CONCLUSION

It is clear from this review that the subject of pectinase immobilization continues to attract considerable attention from researchers in both industry and academia. Materials such as polyacrylonitrile copolymer membrane, bones, aminated silica gel, macroporous polyacrylamide, polyethylene, nanocomposite microspheres, silica coated chitosan, Nylon-6, magnetic particles and porous glass support have been used for immobilization of microbial and commercial pectinases. Most of the studies performed so far on immobilization of pectinases resulted in applications of these immobilized enzymes in apple juice clarification, mash treatment and in paper making industries. These examples are just a few of the many ways enzymes touch our lives, more work is needed on this topic which is going on in many institutes and industries.

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REFERENCES


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