

An Overview of Enzyme Immobilization

*Ajayi, A. A., Onibokun, E. A.

Department of Biological Sciences, Covenant University, Ota

*Corresponding author: adesola.ajayi@covenantuniversity.edu.ng

Orcid ID: 0000-0001-7771-4130

Abstract: The use of enzymes as biological catalysts has gained increasing importance in industries. Although enzymes can be obtained from plant and animal origin, microbial enzymes have several advantages over enzymes derived from other sources. Due to the high cost of separation of enzymes from product, the instability of enzymes and reduced enzyme activity, several strategies are now being explored to develop immobilized enzymes. Immobilized enzymes have been produced by cell immobilization techniques. Immobilized enzymes have found several industrial applications where they provide the advantages of easy separation of the enzyme from the product, reuse of the enzyme, convenient handling, high stability under extreme physical and chemical conditions, being applicable for all types of reactors with varied interior design, and provides easier process control. However, despite these advantages, enzyme immobilization techniques continue to pose some challenges. These challenges notwithstanding, the development of industrial by-products based on immobilization techniques is very promising.

[Ajayi, A. A., Onibokun, E. A. **An Overview of Enzyme Immobilization**. *Researcher* 2017;9(12):1-7. ISSN 1553-9865 (print); ISSN 2163-8950 (online). <http://www.sciencepub.net/researcher>. 1. doi: [10.7537/marsrsj091217.01](https://doi.org/10.7537/marsrsj091217.01).

Keywords: Enzymes; Immobilization; Adsorption; Entrapment, Covalent binding, Crosslinking

Introduction

Enzymes are biological macromolecules that are produced by living organisms which act as catalysts to bring about specific biochemical reactions (Gurung *et al.*, 2013). With the exception of a small group of catalytic RNA molecules, all enzymes are proteins (Agarwal, 2006). Enzymes are classified as either simple or complex. Simple enzymes are composed only of proteins while complex enzymes are composed of proteins and a relatively small group of organic molecule known as the prosthetic group (Ajayi *et al.*, 2014). Enzymes are central to every biochemical process where they act in organized sequences to catalyze a hundred of stepwise reactions that degrade nutrient molecules, and make biological macromolecules from simple precursors (Ajayi *et al.*, 2014).

Microbial enzymes, due to the numerous advantages they offer are more readily applied in several industries such as food, for the clarification of fruit juices, extraction of lycopene (Ajayi *et al.*, 2015; Ajayi *et al.*, 2013) beverage, textile, pulp and paper, pharmaceutical, agriculture, petroleum, medical industries and in waste management. The current demands of sustainable green methodologies have increased the use of enzymatic technology in industrial processes (Mohamad, 2015).

Due to the high cost of separating enzymes from product, the instability of enzymes and reduced enzyme activity (Fernandez-Lafuente, 2017), immobilization have now become an acceptable technique in the production of many useful metabolites including industrial enzymes.

Immobilization is a process by which an enzyme is fixed to or within solid supports, creating a heterogeneous immobilized enzyme system (Homaei *et al.*, 2013). It also 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 (Hassan *et al.*, 2016).

History of Immobilized Enzymes

The technique of enzyme immobilization arose after the discovery of biofilms by Anthony van Leeuwenhoek which he initially described as aggregated microorganisms in the 'scurf of his teeth' and 'particles scraped off his tongue' (Hoiby, 2014). Scientists soon observed that microorganisms were capable of normal physiological activities when growing attached to surfaces as with their free-living forms. However, it was not until 1978 that the term 'Biofilms' was coined by Bill Costerton (Chandki, 2011). Biofilms are surface attached microbial communities consisting of multiple layers of cells embedded in hydrated matrices (Kierk-Pearson and Karatan, 2005). They are microbially derived sessile communities characterized by the cells that are irreversibly attached to a substratum or to each other (Hassan *et al.*, 2011).

Biofilms have been found growing on various naturally occurring supports like rock immersed in a stream, an implant in the human body, a tooth, a water pipe or conduit etc. (Mahmoud and Helmy, 2009). However, it was not until 1916 that the first immobilized enzyme was reported by Nelson and Griffin. They reported that invertase extracted from

yeast and adsorbed on charcoal showed the same activity as the native enzyme (Nelson and Griffin, 1916). Again in 1948 it was found that urease from jack bean became water insoluble on standing in 30% alcohol and sodium chloride for 1-2 days at room temperature, and the water-insoluble urease was active (Mahmoud and Helmy, 2009). During 1950s and 1960s, different methods besides adsorption were employed in enzyme immobilization e.g immobilization of aminoacylase from *Aspergillus oryzae* in 1967 (Chibata *et al.*, 1967). From 1960s, to date more than 5,000 publications and patents have been published on enzyme immobilization techniques (Brena and Batista-Viera 2006). Today several hundred enzymes have been immobilized using different techniques and a good number of them are being employed in industrial processes.

Enzyme Immobilization Methods

Enzyme immobilization techniques may be carried out based on two broad approaches; reversible and irreversible methods. In the reversible method, the cell can easily be detached from the matrix/support while in the irreversible method the cells cannot be detached from the matrix/support without either destroying the cell or the support (Elakkiya *et al.*, 2016).

Reversible Methods

- Adsorption

This is a non-specific physical interaction between the enzyme protein and the surface of the matrix brought about by mixing a concentrated solution of enzyme with the solid (Abdelmajeed *et al.*, 2012). The method is most suitable for the immobilization of viable cells. It is considered one of the easiest techniques of cell immobilization (Hassan *et al.*, 2016). The enzyme is attached directly to the matrix (based on the affinity of the enzyme for the matrix) without washing. Advantages of this method include ease at which it is carried out, low cost and the fact that it is less disruptive to the enzyme since it does not involve chemical interactions between the functional groups which make up the active site of the enzyme and the matrix/support.

- Entrapment

Entrapment is defined as the caging of enzymes by covalent or non-covalent bonds within gels or fibers (Datta *et al.*, 2013). The gels for support should be chosen such that it has the largest possible pore volume to maintain its mechanical strength and pore size suitable for fitting the enzyme to retain its optimal structure to catalyze the transformation of the substrate (Trevisan *et al.*, 2000). Advantages of this method include speed, relatively low cost and cause less conformational change of the enzyme.

Irreversible Methods

- Covalent binding

Enzymes are joined through intermolecular covalent bonds between chemical groups present on the matrix and common chemical groups on the enzyme (Heck *et al.*, 2013). Matrices commonly used are either natural (e.g. Sephadex, Agarose) or synthetic (e.g. acrylamide, methacrylic acid, styrene) (Dwevedi, 2016). The success of this method of immobilization depends on the covalent bonds being formed between reactive groups that do not make up the active site of the enzyme in order to prevent a conformational change in the enzyme structure thus resulting in loss of enzyme activity. Advantages of the method includes less desorption problem (i.e enzymes leaking out of the support) and higher stability of the immobilized enzymes.

- Cross-linking

This may also be referred to as co-polymerization. The method carried out by cross linking enzymes to each other using a cross linking agent to produce a large, three-dimensional complex structure (Ribeiro and Rabaça, 2011). The method does not require the use of a matrix or support. This method usually requires the chemical modification of the surface of the support. Glutaraldehyde is the most widely used cross-linking agent (Peng *et al.*, 2017) and has been successfully used to immobilize several industrial enzymes e.g glucose isomerase and penicillin amidase.

Choosing a Matrix

A matrix is a support that holds the enzyme or cells for immobilization. A suitable matrix for immobilization should be affordable, inert (should neither react with the product or the support), have physical strength, have the ability to increase enzyme activity and reduce product inhibition (Singh, 2009). A suitable support should also have a pore size small enough to retain the enzyme and prevent desorption but large enough to ensure all substrate to flow in and products to flow out. Mohamad *et al* (2015) grouped into two major categories as organic (natural polymer, synthetic polymers) and inorganic polymers (Table 1)

Table 1: Matrix/support for Enzyme Immobilization

| Inorganic Polymers | Organic Polymers |
|--------------------|---------------------|
| Ceramics | Chitosan and chitin |
| Glass | Gelatin |
| Silica | Starch |
| Activated carbon | Cellulose |
| Charcoal | Alginate |
| Zeolite | Collagen |

Applications of Immobilized Enzymes

- Glucose Biosensors

A biosensor can be defined as a “compact analytical device or unit incorporating a biological or

biologically derived sensitive recognition element integrated or associated with a physio-chemical transducer (Turner, 2000). Enzymes being highly selective towards their substrates make them good candidates as sensing elements in biosensors added to the fact that their catalytic action remains unaltered till the end of the reaction hence the sensors can be used continuously (Patel *et al.*, 2016). Glucose biosensors have evolved to be more reliable, rapid, and accurate and are also more compact and easy to use method of monitoring patients' blood sugar levels (Niraj *et al.*, 2012). Generally, glucose measurements are based on interactions with one of three enzymes: hexokinase, glucose oxidase (GOx) or glucose-1-dehydrogenase (GDH) (Price, 2003) GOx is easy to obtain, cheap, and can withstand greater extremes of pH, ionic strength, and temperature than many other enzymes (Bankar *et al.*, 2009). The basic concept of the glucose biosensor is based on the fact that the immobilized GOx catalyzes the oxidation of β -D-glucose using dissolved oxygen to produce hydrogen peroxide (Putzbach and Ronkainen, 2013). The concentration of glucose can be determined by measuring the amount of hydrogen peroxide produced by the enzyme reaction (Niraj *et al.*, 2012; Yoo and Lee, 2010).

- Antibiotic Production

Benzylpenicillins and Phenoxyethylpenicillins (Penicillins G and V, respectively) are produced by fermentation and are the basic precursors of a wide range of semi-synthetic antibiotics, e.g. ampicillin (Bagherinejad *et al.*, 2012). Enzymes have been generally employed in industry for the hydrolysis of penicillins G and V. Penicillin amidases (also called penicillin acylases) have been particularly employed for their ability to hydrolyse penicillins G and V *without* causing the hydrolysis of the essential β -lactam ring. A number of supports have been utilized for immobilization of Penicillin amidase have been obtained from *E. coli* including cyanogen bromide-activated Sephadex G200. It is one of the earliest successful processes involving immobilized enzymes and is generally used in batch or semi-continuous processes where it may be reused over 100 times (Bagherinejad *et al.*, 2012).

- Chill Proofing of Beer

During the manufacture of beer, organic components such as proteins, polyphenols and carbohydrates (α -glucans, β -glucans) may be present which results in haze formation (Steiner *et al.*, 2010). Immobilized papain has been successfully used to chill-proof beer (Venkatasuermanian *et al.*, 1975). The enzyme is capable of digesting dissolved proteins—with amino acids as a by-product—and thus increasing the clarity of beer. This immobilized-enzyme treatment is carried out simultaneously during process of fermentation (Homaei, 2015).

- Production of High Fructose Corn Syrup (HFCS)

Immobilized glucose isomerase is employed on a commercial scale in the hydrolysis of glucose to fructose and is used in the corn syrup industry for producing HFCS (Converti and Borghi, 1998). The isomerization of glucose to fructose enhances the sweetening power of the syrup as fructose is general sweeter than glucose. Immobilization of Glucose isomerase enhanced the stability of the immobilized enzyme (Saha *et al.*, 2009).

- Hydrolysis of Lactose

Lactose is the principal carbohydrate of milk. However due to its poor solubility, low degree of sweetness and inability to certain individuals to digest it, the consumption of lactose is limited (Rosolen *et al.*, 2015). Undigested lactose in chyme retains fluid, bacterial fermentation of lactose results in production of gases, diarrhea, and bloating, abdominal cramps after consumption of milk and other dairy product (Mahmoud and Helmy, 2009). This situation may be avoided if lactose is hydrolyzed by lactase to the readily utilizable sugars, glucose and galactose to produce lactase-treated milk and lactose-reduced milk. Lactases are rather expensive. The high price makes it essential that the enzyme be immobilized if they are to be used in large-scale manufacturing processes. Several methods and immobilizing agents are now being employed; adsorption, crosslinking with glutaraldehyde, and covalent coupling with a solid carrier e.g alginate beads for the production of "high-purity immobilized lactase for use in hydrolysis of milk sugar" (Twaddell, 2014).

- Cleaning up Pesticide

Pesticides are regarded as the most effective methods of controlling plant pests in conventional farming practices. When applied, they are capable of lowering production cost, improving the yield of crops and reducing soil erosion (.). However, the toxic nature of some pesticides and their eventual adverse effect on human health had led to the demand for green methodologies that have increased the use of enzyme technology in several industrial processes (Mohamad *et al.*, 2015). Currently, immobilized enzymes are capable of breaking down a range of organophosphate insecticides (Mahmoud and Helmy, 2009) making them a more eco-friendly option for the control of plant insect pest.

- Biodiesel Production

Biodiesel has continued to gain advantage over fossil fuels in recent times due to its eco-friendly nature as against fossil fuels with the several environmental concerns associated with fossil fuels (Khan and Alzohairy, 2010). Again biodiesel does not produce sulfur oxide, carbon monoxide and soot (Iso *et al.*, 2001). Biodiesel produced by transesterification

has attracted considerable attention for use as renewable energy source (Tiwari *et al.*, 2007). Lipase-catalyzed transesterification is often preferred since the glycerol is easily removed and the purification process simple (Dizge and Keskinler, 2008). Immobilized lipase could be employed in biodiesel production with the aim of reducing production cost by reusing the enzyme (Jegannathan *et al.*, 2008). Different immobilization supports like ceramics, silica and zeolites has been used for lipase immobilization (Yagiz *et al.*, 2007). Immobilized enzyme treatment not only reduces cost but also reduces environmental pollution (Khan and Alzohairy, 2010).

Advantages of Immobilized Enzymes

- An Immobilized enzyme provides easier product separation. Tischer and wedekind (1999) reported that ease of separation of enzyme from product simplifies several enzyme applications and provides a more efficient reaction. This also minimizes or eliminates the chances of protein contamination of the product (Sheldon and van Pelt, 2013).

- An immobilized enzyme has reusability (particularly for costly enzymes). Reuse of enzymes provides cost advantages which are often an essential prerequisite for establishing an enzyme-catalyzed process in the first place (Tischer and Wedekind, 1999).

- Higher stability under extreme physical and chemical conditions (Guisan, 2006). The stability of the immobilized enzymes is based on the temperature and time of reaction. For most immobilized enzymes, the activity of the enzyme is retained throughout series of cycles (Nisha *et al.*, 2012). Immobilized enzymes are generally more stable than the free ones, allowing the repeated reuse of the biocatalyst (Flores-Maltos, 2011).

- An immobilized enzyme is applicable for all types of reactors with varied interior design (Sheldon, 2007) and provides easier process control (Dwevedi, 2016). In all instances, immobilization of enzymes allows for its reuse. Immobilization greatly simplifies the design of the reactor and the control of the reaction (Katchalski-Katzir, 1993). By simply filtering the enzymes, it is easy to stop the reaction making it possible to be used in all kinds of bioreactor designs (Mateo *et al.*, 2007).

Disadvantages of Immobilized Enzymes

Despite the many advantages associated with the use of immobilized enzymes, immobilized enzymes technology has raised various issues such as

- Lowering the activity of the enzyme due to changes that may occur in the enzyme structure.
- The possibility of enzyme denaturation
- Additional cost of carriers

- Lower efficacy in the presence of insoluble substrates (Dwevedi, 2016; Secundo, 2013).

However, the issues mentioned are not applicable to all types of immobilization systems. Protein engineering methodologies have been recommended which may now be followed by immobilization for establishing robust processes in various immobilization systems (Singh *et al.*, 2013).

Protein Engineering

Protein engineering is the process by which novel proteins with desired properties are developed (Chica, 2015). Protein engineering has helped to develop commercially available enzymes into better industrial catalysts. The technique aims at modifying the sequence of a protein, and hence its structure, to create enzymes with improved functional properties such as stability, specific activity, and selectivity towards non-natural substrates (Singh *et al.*, 2013). One of such protein engineering approaches is site-directed mutagenesis.

Site-Directed Mutagenesis

Site-directed mutagenesis is an in vitro method of choice for altering a gene or vector sequence at a selected location (Hutchison *et al.*, 1978). It is a general method for systematically replacing amino acids in an enzyme (Kohrer and RajBhandary, 2013). Most common method of site-directed mutagenesis is polymerase chain reaction (Trehan *et al.*, 2016). In site-directed mutagenesis, the primers are designed to include the desired mutation which could be base substitution, addition, or deletion. The mutation is then incorporated into the amplicon, replacing the original sequence during the PCR cycle resulting in the synthesis of an entirely new protein.

Conclusion

The technique of immobilized enzymes is still at an experimental. Several researches on enzyme immobilization have been reported making it one of the most promising techniques for highly efficient and economic biotechnological processes. The increasing cost associated with industrial processes will continue to give rise for newer production methods for minimizing cost making it even eminent that immobilized enzymes will provide better advantages for industrial applications.

Acknowledgement

The authors hereby acknowledge Covenant University, Ota, Ogun State Nigeria for providing a suitable platform for research.

References

1. Abdelmajeed, N. A., Khelil, O. A. and Daniel, E. N. (2012). Immobilization techniques for

- enhancing bio-products industry. *African Journal of Biotechnology* 11(71): 13528-13529.
2. Ahmad, R. and Sardar, M. (2015). Enzyme Immobilization: An Overview on Nanoparticles as Immobilization Matrix. *Biochemistry and Analytical Biochemistry* 4(2): 1-8.
 3. Ajayi, A. A., Adedeji, O. M., Olasehinde, G. I., Ayanda, O. O. and Adejuwon A. O. (2013). Extraction of lycopene with cell wall degrading enzymes from tomato (*Lycopersicon esculentum* Mill) fruits deteriorated by *Aspergillus niger*. *Nature and Science* 11(4): 110-113.
 4. Ajayi, A. A., Osunlalu, E. O., Peter-Albert, C. F. and Adejuwon, A. O. (2014). Studies on the pectinolytic and proteolytic enzymes from deteriorated grapes. *Covenant Journal of Physical and Life Sciences (CJPL)* 1(2): 1-15.
 5. Ajayi, A. A., Peter-Albert, C. F., Akeredolu, M. and Shokunbi, A. A. (2015). Clarification of tomato juice with polygalacturonase obtained from tomato infected with *Aspergillus niger*. *Pakistan Journal of Biological Sciences* 18(2): 74-80.
 6. Argawal, P. K. (2006). Enzymes: an integrated view of structure, dynamics and function. *Microbial cell factories* 5: 1-12.
 7. Bagherinejad, M. R., Korbekandi, H., Tavakoli, N. and Abedi, D. (2012). Immobilization of penicillin G acylase using permeabilized *Escherichia coli* whole cells within chitosan beads. *Research in Pharmaceutical Sciences* 7(2): 79-85.
 8. Bankar, S. B., Bule, M. V., Singhal, R. S. and Ananthanarayan, L. (2009). Glucose oxidase--an overview. *Biotechnology Advances* 27: 489-501.
 9. Brena, B. M. and Batista-Viera, F. (2006). Immobilization of Enzymes - A Literature Survey. *Methods in Biotechnology: Immobilization of Enzymes and Cells*, Second Edition Edited by: J. M. Guisan © Humana Press Inc., Totowa, NJ. <http://www.springer.com/978-1-58829-290-2>.
 10. Chandki, R., Banthia, P. and Banthia R. (2011). Biofilms: A microbial home. *Journal of Indian Society of Periodontal* 15(2): 111-114.
 11. Chibata, I., Tosa, T., Sato, T. and Mori, T. (1967). Production of L-amino acids by aminoacylase adsorbed on DEAE- Sephadex. *Methods in Enzymology* 44: 746-759.
 12. Chica, R. A. (2015). Protein Engineering in the 21st Century. *Protein Science* 24(4): 431- 433.
 13. Converti, A. and M. I (1998). Kinetics of glucose isomerization to fructose by immobilized glucose isomerase in the presence of substrate protection. *Bioprocess Engineering* 18: 27-33.
 14. Datta, S., Christena, L. R. and Rajaram, Y. R. S. (2013). Enzyme immobilization: an overview on techniques and support materials. *3 Biotech* 3(1): 1-9.
 15. Datta, S., Christena, L. R. and Rajaram, Y. R. S. (2013). Enzyme immobilization: an overview on techniques and support materials. *3 Biotech* 3(1): 1-9.
 16. Dizge, N. and Keskinler, B. (2008). Enzymatic production of biodiesel from canola oil using immobilized lipase. *Biomass Bioenergy* 32: 1274-278.
 17. Dwevedi, A. (2016). Enzyme Immobilization-Advances in Industry, Agriculture, Medicine and the Environment. Available at <http://www.springer.com/978-3-319-41416-4> accessed on Tuesday 18th of July at 12:10pm.
 18. Elakkiya, M., Prabhakaran, D. and Thirumarimurugan, M. (2016). Methods of Cell Immobilization and Its Applications. *International Journal of Innovative Research in Science, Engineering and Technology* 5(4): 5429-5433.
 19. Fernandez-Lafuente, R. (2017). Special Issue: Enzyme Immobilization 2016. *Molecules* 22:601.
 20. Flores-Maltos, A., Rodríguez-Durán, L. V., Renovato, J., Contreras, J. C., Rodríguez, R. and Aguilar, C. N. (2011). Catalytic Properties of Free and Immobilized *Aspergillus niger* Tannase Enzyme Research Volume 2011, Article ID 768183, 6 pages doi:10.4061/2011/768183.
 21. Górecka, E., Jastrzębska, M. (2011). Immobilization techniques and biopolymer carriers. *Biotechnology and Food Science* 75:65-86.
 22. Guisan, J. M. Immobilization of enzymes as the 21st century begins. In: Guisan JM, editor. *Immobilization of enzymes and cells*. 2nd ed. NJ: Humana Press Inc; 2006. pp. 1-13.
 23. Gurung, N., Ray, S., Bose, S. and Rai, V. (2013). A broader review: microbial enzymes and their application in industries, medicine and beyond. *Biomed Research International* 2013: 1-8.
 24. Hassan, A., Usman, J., Kaleem, F., Omar M., Khalid, A. and Iqbal, M. (2011). Evaluation of different methods of biofilm formation in the clinical isolates. *Brazilian Journal of Infectious disease* 15(4): 305-311.
 25. Hassan, M. E., Tamer, T. M. and Ahmed, O. M. (2016). Methods of Enzyme Immobilization. *International Journal of Current Pharmaceutical Review and Research* 7(6): 385-392.
 26. Heck, T., Faccio, G., Richer, M. and Thony-Meyer L. (2013). Enzymes catalyzed protein crosslinking. *Applied Microbiology and Biotechnology* 97(2): 461-175.

27. Hoiby, N. (2014). A personal history of research on microbial biofilms and biofilm infections *Pathogens and Disease* 70: 205–211.
28. Homaei, A. (2015). Enzyme Immobilization and its application in the food industry. *Advances in Food Biotechnology* 9: 145-164.
29. Homaei, A. A., Sariri, R., Vianello, F. and Stevanato, R. (2013). Enzyme immobilization: an update. *Journal of Chemical Biology* 6(4): 185-205.
30. Hutchison, C. A., Phillips, S., Edgell, M. H., Gillam, S., Jahnke, P. and Smith, M. (1978). Mutagenesis at a specific position in a DNA sequence. *Journal of Biological Chemistry* 253: 6551-6560.
31. Iso, M., Chen, B., Eguchi, M., Kudo, T and Shrestha, S. (2001). Production of Biodiesel from triglycerides and alcohol using immobilized lipase. *Journal of Molecular Catalysis B: Enzyme* 16: 53-58.
32. Jeganathan, K. R., Abang S., Poncelot, D., Chan, E. S. and Ravindra P. (2008). Production of biodiesel using immobilized lipase. A critical review. *Critical Review Biotechnology* 28: 253-264.
33. Katchalski-Katzir, E. (1993). Immobilized enzymes: learning from past successes and failures.
34. Khan, A. A and Alzohairy, M. A. (2010). Recent Advances and Applications of Immobilized Enzymes- A Review. *Research Journal of Biological Sciences* 5(8): 565-575.
35. Kierek-Pearson, K. and Karatan, E. (2005). Biofilm development in Bacteria. *Advances in Applied Microbiology* 57: 79.
36. Kohrer, C. and RajBhandary, U. L. Proteins Carrying One or More Unnatural Amino Acids. In: Madame Curie Bioscience Database [Internet]. Austin (TX): Landes Bioscience; 2000-2013. Available at <https://www.ncbi.nlm.nih.gov/books/NBK6136/> accessed Friday 14th of July 2017 at 11:50am.
37. Lee, K. Y. and Mooney, D. J. (2012). Alginate: properties and biomedical applications. *Progress in Polymer Science* 37(1): 106–126.
38. Mahmoud, D. A. R. and Helmy, W. A. (2009). Potential Application of Immobilization Technology in Enzyme and Biomass Production (Review Article). *Journal of Applied Sciences Research* 5(12): 2466-2476.
39. Martins, S. C., Martins, C. M., Fiúza, G. C. and Santaella, S. T. (2013). Immobilization of microbial cells: A promising tool for treatment of toxic pollutants in industrial wastewater. *African Journal of Biotechnology* 12(28): 4412-4418.
40. Mateo, C. M., Palomo, J. M., Fernandez-Lorente, G. Guisan, J. M. and Fernandez-Lafuente, R (2007). Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme and Microbial Technology* 40(6), 1451-1463.
41. Mohamad, N. R., Marzuki, N. H., Buang, N. A., Huyop, F. and Wahab, R. A. (2015). An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. *Biotechnology, Biotechnological Equipment* 29(2): 205–220.
42. Nelson, J. M. and Griffin, E. G. (1916). Adsorption of invertase. *Journal of the American Chemical Society* 38(5): 1109–1115.
43. Niraj, Gupta, M. M., Varshney, H. M., Pandey, S. and Singh, S. (2012). Sensors for diabetics: glucose biosensors by using different newer techniques: A review. *International Journal of Therapeutic Applications* 6: 28-37.
44. Nisha, S., Arun Karthick, S and Gobi, N. (2012). A Review on Methods, Application and Properties of Immobilized Enzyme. *Chemical Science Review and Letters* 2012, 1(3), 148.
45. Patel, S., Nanda, R., Sahoo, S., and Mohapatra, E. (2016). Biosensors in Health care: The milestones achieved in the Development towards Lab-on-chip analysis. *Biochemistry Research International* 2016. Article ID 3130469. 12 pages.
46. Peng, Y. Y., Glattauer, V. and Ramshaw, J. A. (2017). Stabilisation of Collagen Sponges by Glutaraldehyde Vapour Crosslinking. *International Journal of Biomaterials* 2017: 1-6.
47. Price, C. P. (2003). Point-of-care testing in diabetes mellitus. *Clinical Chemistry and Laboratory Medicine* 41: 1213–1219.
48. Putzbach, W. and Ronkainen, N. J. (2013). Immobilization techniques in the Fabrication of Nanomaterial-based electrochemical biosensors- A review. *Sensors (Basel)* 13(4): 4811–4840.
49. Ribeiro, M. H. L. and Rabaça, M. (2011). Cross-Linked Enzyme Aggregates of Naringinase: Novel Biocatalysts for Naringin Hydrolysis. *Enzyme Research* 2011: 1-8.
50. Roger A. Sheldon and Sander van Pelt (2013). Enzyme immobilisation in biocatalysis: why, what and how. *Chemical Society Reviews* 42: 6223-6235.
51. Rosolen, M. D., Gennari, A., Volpato, G. and Volken de Souza, C. F. (2015). Lactose Hydrolysis in Milk and Dairy Whey Using Microbial β -Galactosidases. *Enzyme Research* 2015: 1-7.
52. Sabel and Brookman (2012). Methods for site-directed mutagenesis. Available at

- www.idtdna.com/pages/decoded/decoded-articles/coreconcepts/decoded/2012/01/10/methods-for-site-directed-mutagenesis accessed on Thursday 20th of July 2017 at 10:05am.
53. Saha, B.C., Bothast, R.J., Jordan, D. B. 2009. Enzymes, Industrial (Overview). In: Schaechter, M., editor. *Encyclopedia of Microbiology*. Oxford: Elsevier. p. 281-294.
 54. Secundo, F. (2013). Conformational changes of enzymes upon immobilization. *Chemical Society Reviews* 42: 6250-6261.
 55. Sheldon, R. A. (2007). Cross-linked enzyme aggregates (CLEAs): stable and recyclable biocatalysts. *Biochemical Society* 35(6):1583–1587.
 56. Singh B. D. *Biotechnology expanding horizons*. India: Kalyani; 2009.
 57. Singh, R. K., Tiwari, M. K., Singh, R. and Lee, J. (2013). From Protein Engineering to Immobilization: Promising Strategies for the Upgrade of Industrial Enzymes. *International Journal of Molecular Sciences* 14(1): 1232–1277.
 58. Steiner, E., Becker, T. and Gast, M. (2010). Turbidity and Haze formation in beer-insights and overview. *Journal of Institute of Brewing* 116(4): 360-368.
 59. Tischer, W. and Wedekind, F. (1999). Immobilized Enzymes: Methods and Applications. *Topics in Current Chemistry* 200: 95-126.
 60. Tiwari, A. K., Kumar A. and Raheman, H. (2007). Biodiesel production from jatropha oil (*jatropha cureas*) with high free fatty acids. An optimized process. *Biomass Bioenergy* 31: 569-575.
 61. Traving, S. J., Thygesen, U. H., Riemann, L. and Stedmon, C. A. (2015). A Model of Extracellular Enzymes in Free-Living Microbes: Which Strategy Pays Off? *Applied and Environmental Microbiology* 81(21): 7385–7393.
 62. Trehan, A., Kiebus, M., Czapinski, J., Stepulak, A., Huhtaniemi, I. and Rivero-Müller, A. (2016). REPLACR-mutagenesis, a one-step method for site-directed mutagenesis by recombineering. *Scientific Reports* 6: 10121.
 63. *Trends in Biotechnology* 11: 471-478.
 64. Trevisan, H. C., Mei, L. H. I. and Zanin, G. M. (2000). Preparation of silica with controlled pore sizes for enzyme immobilization. *Brazilian Journal of Chemical Engineering* 17(1): 71-77.
 65. Turner, A.P. (2000). Biosensors--sense and sensitivity. *Science* 290: 1315–1317.
 66. Twaddell I. (2014). Investigating Immobilized Enzymes. Available at <https://bigpictureeducation.com/investigating-immobilised-enzymes> accessed on Monday 17th of July 2017 at 1:45pm.
 67. Venkatasueramanian, K., Saini, R., and Vieth, W. R. (1975). Immobilization of Papain on Collagen and the use of Collagen-papain membranes in beer chill proofing. *Journal of Food Science* 40 (1): 109-113.
 68. Yagiz, F. Kazan, D. and Akim, A. N. (2007). Biodiesel production from waste oils by using lipase immobilized on hydrotalcite and zeolites. *Chemical Engineering Journal* 1: 262-267.
 69. Yoo, E. and Lee, S. (2010). Glucose biosensors: An overview of use in clinical practice. *Sensors (Basel)* 10(5): 4558-4567.

12/12/2017