

Chitosan and its Significant Properties: A Review

Ezenwelu, Chijioke, O.¹, Okeke, Chisom M.¹, Udemezue, Onyeka, I.², Ngwu, Ogochukwu, R.¹, Ogana, Joy¹, Oparaji, Emeka, H.^{3*}

¹Department of Applied Biochemistry, Nnamdi Azikiwe University, Awka, Anambra State

²Department of Applied Microbiology and Brewery, Nnamdi Azikiwe University, Awka, Anambra State.

³Department of Biochemistry, State University of Medical and Applied Sciences, Igbo-Eno, Enugu State.

*Corresponding Author

Oparaji, Emeka, H.

Department of Biochemistry, State University of Medical and Applied Sciences, Igbo-Eno, Enugu State.

Article History

Received: 01.03.2024

Accepted: 21.03.2024

Published: 06.04.2024

Abstract: Chitin a precursor to chitosan is the penultimate versatile polymer thereafter the β -linked sugar polymer; they are reflective sugars, reactive and with non-brittle properties present in the cellular walls of fungi and in the shells of crustaceans and other insects. Chitosan and their formulations are non toxic to human and biotic surroundings (GRAS) with good characteristics such adaptability and blendable, non-toxicity and financially effective. The properties of this amine linked biocomposite expose their numerous biotechnological applications as enlisted. Search for renewability of their supply is a biotechnological topnotch to meet with their over-rising demand in the today global market.

Keywords: chitin, chitosan, amine, biocomposite, properties.

Background of the Study

The amino sugar linked polymer is a naturally existing unbranched sugar compounds of composites of β -(1,4)-linked glucosamine units (2-amino-2-deoxy- β -d-glucopyranose) together with some repeating proportions of *N*-acetylglucosamine units (2-acetamino-2-deoxy- β -d-glucopyranose) (Milosavic *et al.*, 2005). Chitosan is the most abundant cationic ($-NH_2$) biopolymer in existence. Because the amino groups in chitosan are protonated (H^+), it dissolves in diluted solutions of organic and inorganic acids ($pH < 6$). Because of its polycationic nature, it can combine with polyanions to form polyelectrolyte complexes (Bobu *et al.*, 2011). It has excellent film forming ability and can also be prepared in films, fibers, hydrogels and micro/nanoparticles (Chang *et al.*, 2011). The polar hydroxyl and amino reactive groups of the compounds permit the vast applicability of the different

formulations of the based compounds. Chitin a precursor to chitosan is the penultimate versatile polymer thereafter the β -linked sugar polymer; they are reflective sugars, reactive and with non-brittle properties present in the cellular walls of fungi and in the shells of crustaceans and other insects (Korma *et al.*, 2018). Chitosan and their formulations are non toxic to human and biotic surroundings (GRAS) with good characteristics such adaptability and blendable, non-toxicity and financially effective (Thanou *et al.*, 2001). Chitosan are ecological friendly with high molecular weight and similarity to cellulose (Korma *et al.*, 2018). Increasingly demand on chitosan commercially as suitable resource material due to their unique features including biocompatibility, biodegradability, adsorption, and ability to form films, and to chelate metal ions in waste water composites (Trombotto *et al.*, 2008).

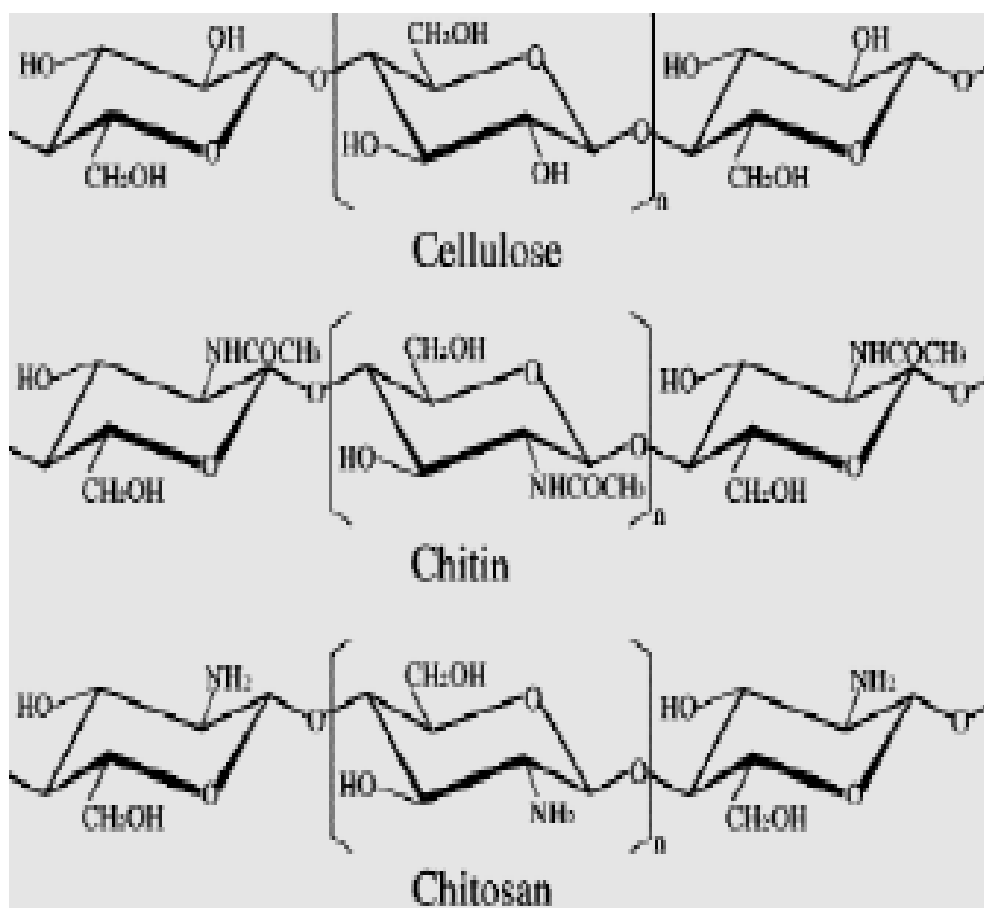


Fig 1. Structure of cellulose, chitin and chitosan (Korma *et al.*, 2018).

Sources, production and characterization of chitosan

Chitosan which is a derivative of chitin is widely seen in certain organisms and sea animals; their abundance in these animals is made manifest by the percentage composition of chitin in these animals (Davis, 2011). Various insects such as wing termites, soldier ants while filamentous fungi of *Zygomycete* and *Deutromycete* family are said to have these biopolymers in abundance. Sea animal of crustacea family (crab, snails, periwinkle, prawns etc) are implicated sources of chitosans (Korma *et al.*, 2018).

Production and purification of chitosan from biopolymers composites comprises of cascade of experimental steps generally divided into two major groups:

- a. Chemical steps
- b. Enzymatic steps

The chemical steps

The chemical procedures can be further alkali and acid based treatment methods. These treatments are basically grouped under the following: Demineralization with 1M HCl, Deproteinization with one molar (1M) caustic soda, Depigmentation with 1% depigmenting agents such as hypochlorites, alcohols, acetones, acidified permanganates etc and Deacetylation of the produced chitin to form chitosan using 40% caustic soda (Krishnaveni and Ragunathan, 2009). Chitin is extracted from fungi in a manner akin to that of crustaceans and other ants. The primary distinction is that the latter has a larger percentage of minerals like limestone, which is a significant part of crustacean waste (Zamani, 2010). Proteins and lipids are typically removed from chitin using 5% NaOH in the first step, and the dissolved mineral—which dissolves in acid—is removed using 30% HCl (Zamani, 2010). Chitin is the insoluble fraction that is left over following these processes. In order to extract chitosan from shells, carbonate is first dissolved or removed using 5% HCl or EDTA. Next, the proteins and lipid components are removed using 40% caustic soda at 110 °C while chitin is concurrently deacetylated to produce chitosan. Typically, chitin makes up 14–27% of the dry weight of shells; of that, 60–80% can be transformed into chitosan (Senel and McClure, 2004).

Enzymatic steps

Enzymatic hydrolysis with chitosanolytic enzymes particularly chitosanase have been another promising path and bio-technical input in chitosan production and processing from chitin (Yeul and Rayalu, 2013). Enzymes are known important bio-catalysts with specific affinity to their substrate(s); their presence in a reaction mixture speeds up a chemical reaction with less energy (enthalpy) inputs by their ability to reduce the barrier activation energy of every reaction path (Korma *et al.*, 2018). Better resolute chitosan from lignocellulosic material are produced with the presence of lignolytic enzymes such as chitosanase, arrays of peroxidases (laccase, Manganese peroxidases), pectinases, xylanase (Korma *et al.*, 2018).

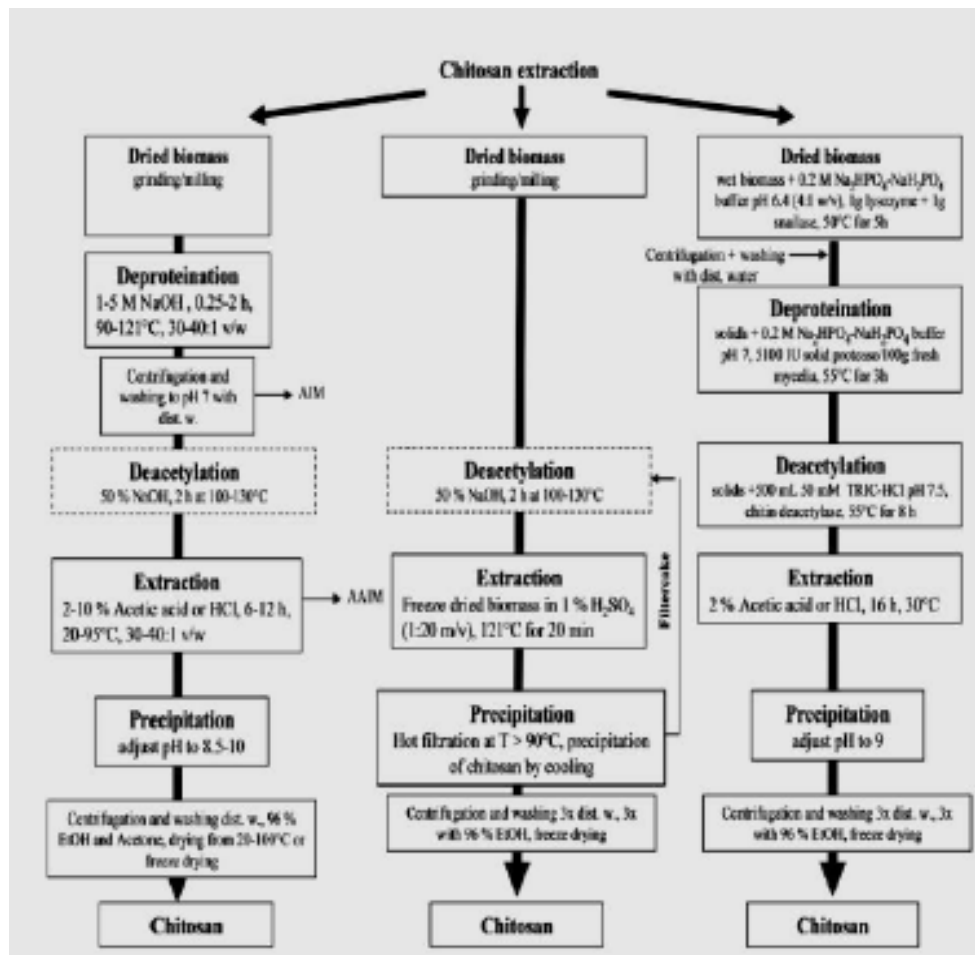


Fig. 2 Different extraction pathways of chitosan from different sources (Korma *et al.*, 2018).

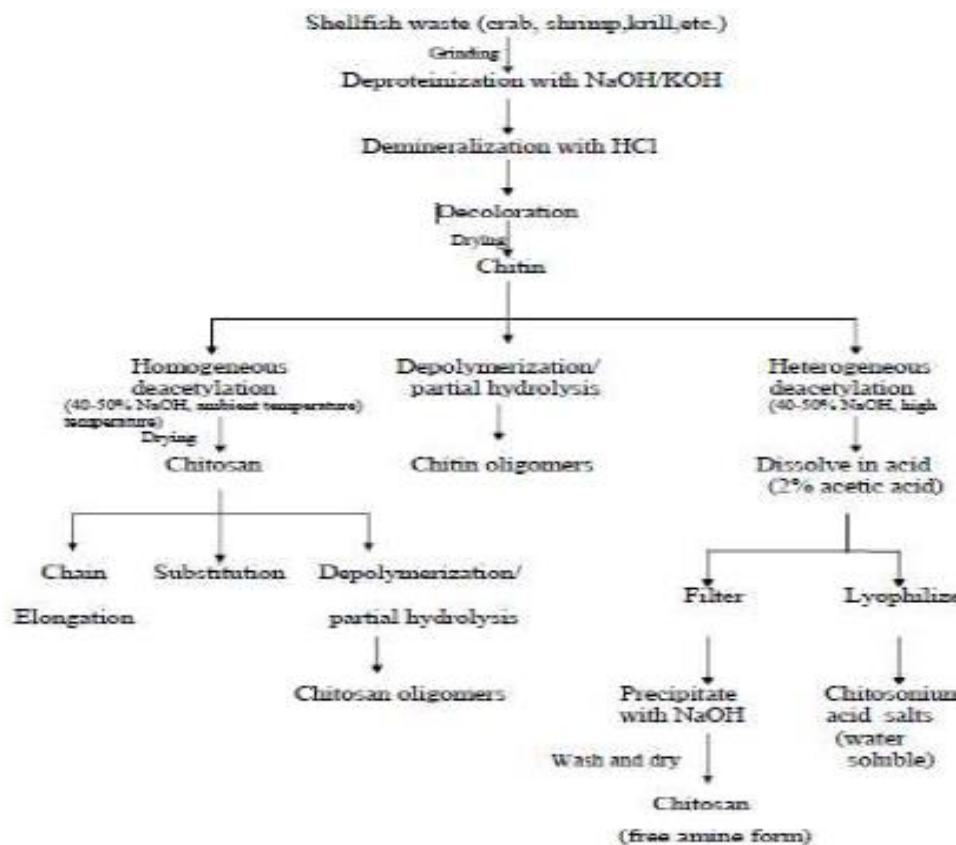


Fig. 3 industrial method of chitosan production from shells of sea animals using chemical means (Korma *et al.*, 2018).

General properties of Chitosan

Chitosan is a non-toxic biodegradable biopolymer with high molecular weight and similarity to cellulose (Korma *et al.*, 2018). Increasingly demand on chitosan commercially as suitable resource material due to their excellent properties including biocompatibility, biodegradability, adsorption, and ability to form films, and to chelate metal ions in waste water composites (Trombotto *et al.*, 2008). The following properties are of peculiarity to chitosans, they include:

Degree of Deacetylation

Deacetylation which is the last bioprocessing step in chitosan production from chitinous materials includes cutting the acetyl groups (COCH_3) off the chitin molecular chain, leaving behind chitosan, which is chemically active and has a highly reactive amino group (NH_2) (Akila, 2014). Because it influences the physicochemical properties of chitosan and, consequently, defines its acceptable uses, the degree of deacetylation (DD) is a crucial attribute of chitosan (Korma *et al.*, 2018).

Deacetylation largely affects the persistent (biodegradability) and immunological potential of chitosans. Chitosan's degree of deacetylation varies from 56% to 99%, with an average of 80%, contingent upon the type of crustacean and the techniques used during preparation. Chitosan is the term used to refer to chitin that has been deacetylated to a degree of 75% or above (Kasaai, 2009). The degree of chitosan deacetylation can be determined using a variety of techniques, according to reports. The ninhydrin test, hydrogen bromide titrimetry, nuclear magnetic resonance spectroscopy, near-infrared spectroscopy, linear potentiometric titration, and first derivative UV spectrophotometry were among them (Stephenie, 2008)

Molecular weight

Chitosan are generally characterized with larger atomic mass and thus reflective in weight. Depending on its chemical constituents, the molar mass of the compound is a reflection of the sourced based materials contents and the modalities of production from the source samples (Korma *et al.*, 2018). Molecular weight of native chitin is usually larger than one million Daltons while commercial chitosan molecular weight range of 100,000 – 1,200,000 Daltons depending on the process and grades (levels) of the product (Kasaai, 2009).

Certain physical processes have been reported to affect the molecular weight of chitosans; at high temperature, dissolved oxygen and shear stress can cause degradation of chitosan. Also, maximal depolymerization of chitosan caused by utilization of high temperature or concentrated acids such as hydrochloric acid, acetic acid and sulphuric acid results in degradation of chitosan molecular structure with overall effects on the molecular weight (Akila, 2014). The molecular weight of chitosan can be determined using various analytical tools such as chromatography, light scattering, and viscometry (Wu *et al.*, 2005).

Viscosity

In order to determine the molecular weight of chitosan and determine its potential commercial applications in intricate biological systems, viscosity is a crucial physical element (Kim *et al.*, 2011). Comparing higher molecular weight chitosans to lower viscosity chitosans, which provide easier application, the former

frequently produce highly viscous solutions, which may not be ideal for industrial handling. The degree of deacetylation, molecular weight, solution concentration, ionic strength, pH, and temperature are some of the processing variables that have an impact on the synthesis of chitosan and its characteristics. As the demineralization period increases, chitosan's viscosity reduces (Korma *et al.*, 2018). It also rises when the degree of deacetylation and chitosan concentration rise, but falls when pH and temperature fall. (Kim *et al.*, 2011). It also rises when the degree of deacetylation and chitosan concentration rise, but falls when pH and temperature fall. There is a reputation for chitosan's ability to improve complexation. The biopolymer with a high degree of deacetylation exhibits a higher formulated chitosan (Kim *et al.*, 2011).

Solubility

Chitin, an acetylated While chitosan is easily soluble in diluted acidic solutions below pH6.0, it is insoluble in the majority of non-polar solvents (organic solvents). Chitosan is dissolved using organic acids including lactic, formic, and acetic acids as a solvent (Korma *et al.*, 2018). The standard solution that is most frequently used is 1% acetic acid solution. Chitosan is insoluble in phosphoric and sulfuric acids but soluble in 1% hydrochloric acid. Chitosan's solubility in inorganic acids is somewhat restricted (Akila, 2014). Concentrated acetic acid solutions at high temperature can cause depolymerization of chitosan. Chitosan's solubilization stability declines at neutral pH. Higher pH values tend to cause precipitation or gelation, with the chitosan solution forming poly-hydrocolloid and gelling as a result. To impart the appropriate functionality, the concentration ratio of acid to chitosan is crucial.

Chitosan continues to act as a viscosifier, keeping the solution smooth even at concentrations of up to 50% organic solvent. Physical parameters that are known to affect the solubility of chitosan include temperature and duration of deacetylation, alkali concentration (NaOH), ratio of chitin to alkali solution, particle size, and previous treatments used to isolate chitin (Abdelaziz, 2012). However, the degree of deacetylation controls solubility; to obtain the necessary solubilization, deacetylation is expected to need to be at least 85% complete. The acid-soluble chitosans with >95% solubility in 1% acetic acid at a 0.5% concentration may be prepared by treatment of the original chitin with 45% NaOH for 10-30 min (Akila, 2014).

Antimicrobial Properties

Antimicrobial activity of chitosan have revealed in the ability of the biopolymer in inhibiting the growth of bacteria (bacterostatic) and other adjourning pathogens (Kim *et al.*, 2011). The type of invasive pathogen and the molecular weight of chitosan determine its antibacterial capabilities. With the exception of *Lactobacillus* sp. strains, chitosan with 470 KDa is the most efficient against gram-positive bacteria; chitosan with 1,106 KDa, on the other hand, is effective against gram-negative bacteria (Knorr, 1982). In the presence of 0.1% chitosan, gram-positive bacteria (*Bacillus megaterium*, *B. cereus*, *Staphylococcus aureus*, *Lactobacillus plantarum*, *L. brevis*, and *L. bulgaris*) generally exhibited stronger bactericidal effects than gram-negative bacteria (*fluorescens*, *Salmonella typhymu parahaemolyticus*) (Almenar *et al.*, 2009).

N-carboxybutyl chitosan a derivative of chitosan during chitosan modification showed bacteriostatic and bactericidal activities on 298 different pathogenic microorganisms upon invivo

antimicrobial sensitivity tests carried out and they were marked morphological alterations in treated microorganisms when examined by electron microscopy (Korma *et al.*, 2018). Conversely, growth inhibition and attenuation of moulds and yeasts depend on chitosan concentration, pH, and temperature (Korma *et al.*, 2018). Both internal and external factors can affect chitosan's antimicrobial activity. These include the kind of chitosan (plain or derivative, for example), the degree of polymerization, the host's nutrient composition, the chemical and/or nutrient composition of the substrate, and environmental factors like substrate water activity (Akila, 2014). It has been demonstrated that chitosan coating greatly slows down fruit deterioration or vegetable and fruit decay at various temperatures (Rout, 2001).

Conclusion

Exceptional properties of the amine charged polymer has enlisted it as a promising biological polymer for present and future biotechnological applications. However there limited information as regards to the benefit of the compound in the agricultural sector. Owing to its physicochemical attributes it can pave way(s) for next generational biofertilizer.

Funding Information

This work was solely funded by **DR. Ezenwelu, C. and his research laboratory**

Ethical Delarations

The authors have disclosed no ethical concerns that might surface following the release of this work.

REFERENCES

1. Kasaai, M. R. (2009). Various methods for determination of the degree of N-acetylation of chitin and chitosan: a review. *Journal of agricultural and food chemistry*, 57(5), 1667-1676.
2. Kaushal, J., Singh, G., & Arya, S. K. (2018). Immobilization of catalase onto chitosan and chitosan–bentonite complex: a comparative study. *Biotechnology Reports*, 18, e00258.
3. Khan, A. A., Akhtar, S., & Husain, Q. (2006). Direct immobilization of polyphenol oxidases on Celite 545 from ammonium sulphate fractionated proteins of potato (*Solanum tuberosum*). *Journal of Molecular Catalysis B: Enzymatic*, 40(1-2), 58-63.
4. Kim, C. K., Chung, H. S., Lee, M. K., Choi, L. N., & Kim, M. H. (1999). Development of dried liposomes containing β -galactosidase for the digestion of lactose in milk. *International Journal of Pharmaceutics*, 183(2), 185-193.
5. Kim, K. W., Min, B. J., Kim, Y. T., Kimmel, R. M., Cooksey, K., & Park, S. I. (2011). Antimicrobial activity against foodborne pathogens of chitosan biopolymer films of different molecular weights. *LWT-Food Science and Technology*, 44(2), 565-569.
6. Kitagawa, Y., Kanayama, Y., & Yamaki, S. (1995). Isolation of β -galactosidase fractions from Japanese pear: activity against native cell wall polysaccharides. *Physiologia Plantarum*, 93(3), 545-550.
7. Klewicki, R. (2007). Formation of gal-sorbitol during lactose hydrolysis with β -galactosidase. *Food Chemistry*, 100(3), 1196-1201.
8. Knorr, D. (1982). Functional properties of chitin and chitosan. *Journal of Food Science*, 47(2), 593-595.
9. Korma, S., Niazi, S., Alahamad, I., Ammar, A. and Alyousef, H. (2018). Production, classification, properties and application of different chitosan. *International Journal of Research in Agricultural Science*.
10. Krajewska, B. (2004). Application of chitin-and chitosan-based materials for enzyme immobilizations: a review. *Enzyme and microbial technology*, 35(2-3), 126-139.
11. Krishnaveni, B., & Ragunathan, R. (2015). Extraction and characterization of chitin and chitosan from *f. Solani* cbnr bkrr, synthesis of their bionanocomposites and study of their productive application. *Journal of Pharmaceutical Sciences and Research*, 7(4), 197.
12. Krivtsov, A. V., & Armstrong, S. A. (2007). MLL translocations, histone modifications and leukaemia stem-cell development. *Nature reviews cancer*, 7(11), 823-833.
13. Kumar, M. N. R. (2000). A review of chitin and chitosan applications. *Reactive and functional polymers*, 46(1), 1-27.
14. Ladero, M., Ruiz, G., Pessela, B. C. C., Vian, A., Santos, A., & Garcia-Ochoa, F. (2006). Thermal and pH inactivation of an immobilized thermostable β -galactosidase from *Thermus* sp. strain T2: Comparison to the free enzyme. *Biochemical Engineering Journal*, 31(1), 14-24.
15. Lamb, S. B., & Stuckey, D. C. (2000). Enzyme immobilization on colloidal liquid aphrons (CLAs): the influence of system parameters on activity. *Enzyme and microbial technology*, 26(8), 574-581.
16. Laridi, R., Kheadr, E. E., Bénéch, R. O., Vuillemand, J. C., Lacroix, C., & Fliss, I. (2003). Liposome encapsulated nisin Z: optimization, stability and release during milk fermentation. *International dairy journal*, 13(4), 325-336.
17. Liu, X., Guan, Y., Shen, R., & Liu, H. (2005). Immobilization of lipase onto micron-size magnetic beads. *Journal of Chromatography B*, 822(1-2), 91-97.
18. Lo, S., Dugdale, M. L., Jeerh, N., Ku, T., Roth, N. J., & Huber, R. E. (2010). Studies of Glu-416 variants of β -galactosidase (*E. coli*) show that the active site Mg²⁺ is not important for structure and indicate that the main role of Mg²⁺ is to mediate optimization of active site chemistry. *The protein journal*, 29, 26-31.
19. Loeffler, R. T., Sinnott, M. L., Sykes, B. D., & Withers, S. G. (1979). Interaction of the lacZ β -galactosidase of *Escherichia coli* with some β -D-galactopyranoside competitive inhibitors. *Biochemical Journal*, 177(1), 145-152.

20. Carrillo-López, A., Cruz-Hernández, A., Cárabaz-Trejo, A., Guevara-Lara, F., & Paredes-López, O. (2002). Hydrolytic activity and ultrastructural changes in fruit skins from two prickly pear (*Opuntia* sp.) varieties during storage. *Journal of Agricultural and Food Chemistry*, 50(6), 1681-1685.
21. Luo, Y., & Wang, Q. (2013). Recent advances of chitosan and its derivatives for novel applications in food science. *Journal of Food Processing & Beverages*, 1(1), 1-13.
22. Mabel, M., Parthasarathy, N., Rajkumar, J., Gururaj, P. and Priyadharshini, M. (2016). A study on the immobilization of acid xylanase on chitosan from chitin of shrimps shells. *Journal of chemical and Pharmaceuticual Science*, 9(1): 268-271.
23. Mammarella, E. J., & Rubiolo, A. C. (2006). Predicting the packed-bed reactor performance with immobilized microbial lactase. *Process Biochemistry*, 41(7), 1627-1636.
24. Mandal, M., Kundu, S., Ghosh, S. K., Panigrahi, S., Sau, T. K., Yusuf, S. M., & Pal, T. (2005). Magnetite nanoparticles with tunable gold or silver shell. *Journal of colloid and interface science*, 286(1), 187-194.
25. Mateo, C., Grazu, V., Palomo, J. M., Lopez-Gallego, F., Fernandez-Lafuente, R., & Guisan, J. M. (2007). Immobilization of enzymes on heterofunctional epoxy supports. *Nature Protocols*, 2(5), 1022-1033.
26. Mateo, C., Palomo, J. M., Fernandez-Lorente, G., Guisan, J. M., & Fernandez-Lafuente, R. (2007). Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme and microbial technology*, 40(6), 1451-1463.
27. Matthews, B. W. (2005). The structure of *E. coli* β -galactosidase. *Comptes rendus. Biologies*, 328(6), 549-556.
28. Matto, M., & Husain, Q. (2006). Entrapment of porous and stable concanavalin A–peroxidase complex into hybrid calcium alginate–pectin gel. *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology*, 81(7), 1316-1323.
29. Mbachu, A. E., Chukwura, E. I., & Mbachu, N. A. (2016). Isolation and characterization of hydrocarbon degrading fungi from used (spent) engine oil polluted soil and their use for polycyclic aromatic hydrocarbons (PAHs) degradation. *Universal Journal of Microbiology Research*, 4(1), 31-37.
30. Migneault, I., Dartiguenave, C., Bertrand, M. J., & Waldron, K. C. (2004). Glutaraldehyde: behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. *Biotechniques*, 37(5), 790-802.
31. Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical chemistry*, 31(3), 426-428.
32. Monnard, P. A. (2003). Liposome-entrapped polymerases as models for microscale/nanoscale bioreactors. *Journal of Membrane Biology*, 191(2), 87.
33. Montalto, M., Curigliano, V., Santoro, L., Vastola, M., Cammarota, G., Manna, R., ... & Gasbarrini, G. (2006). Management and treatment of lactose malabsorption. *World journal of gastroenterology: WJG*, 12(2), 187.
34. Mozhaev, V. V., Melik-Nubarov, N. S., Sergeeva, M. V., Šikšnis, V., & Martinek, K. (1990). Strategy for stabilizing enzymes part one: increasing stability of enzymes via their multi-point interaction with a support. *Biocatalysis*, 3(3), 179-187.
35. Muzzarelli, R. A., & Rocchetti, R. (1985). Determination of the degree of acetylation of chitosans by first derivative ultraviolet spectrophotometry. *Carbohydrate Polymers*, 5(6), 461-472.
36. Nilsson, T. K., & Johansson, C. A. (2004). A novel method for diagnosis of adult hypolactasia by genotyping of the–13910 C/T polymorphism with Pyrosequencing™ technology. *Scandinavian journal of gastroenterology*, 39(3), 287-290.
37. Rodríguez-Nogales, J. M., & López, A. D. (2006). A novel approach to develop β -galactosidase entrapped in liposomes in order to prevent an immediate hydrolysis of lactose in milk. *International Dairy Journal*, 16(4), 354-360.
38. Özbek, B., & Ülgen, K. Ö. (2000). The stability of enzymes after sonication. *Process Biochemistry*, 35(9), 1037-1043.
39. Pakula, T. M., Salonen, K., Uusitalo, J., & Penttilä, M. (2005). The effect of specific growth rate on protein synthesis and secretion in the filamentous fungus *Trichoderma reesei*. *Microbiology*, 151(1), 135-143.
40. Palomo, J. M., Segura, R. L., Fernandez-Lorente, G., Fernandez-Lafuente, R., & Guisán, J. M. (2007). Glutaraldehyde modification of lipases adsorbed on aminated supports: A simple way to improve their behaviour as enantioselective biocatalyst. *Enzyme and Microbial Technology*, 40(4), 704-707.
41. Panesar, P. S., Panesar, R., Singh, R. S., Kennedy, J. F., & Kumar, H. (2006). Microbial production, immobilization and applications of β -D-galactosidase. *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology*, 81(4), 530-543.
42. Parascandola, P., Branduardi, P., & de Alteriis, E. (2006). PVA-gel (Lentikats®) as an effective matrix for yeast strain immobilization aimed at heterologous protein production. *Enzyme and Microbial Technology*, 38(1-2), 184-189.
43. Pedroche, J., del Mar Yust, M., Mateo, C., Fernández-Lafuente, R., Girón-Calle, J., Alaiz, M., ... & Millán, F. (2007). Effect of the support and experimental conditions in

- the intensity of the multipoint covalent attachment of proteins on glyoxyl-agarose supports: correlation between enzyme-support linkages and thermal stability. *Enzyme and Microbial Technology*, 40(5), 1160-1166.
44. Penner, R. M., Roth, N. J., Rob, B., Lay, H., & Huber, R. E. (1999). Tyr-503 of β -galactosidase (*Escherichia coli*) plays an important role in degalactosylation. *Biochemistry and cell biology*, 77(3), 229-236.
 45. Pessela, B. C., Mateo, C., Carrascosa, A., Fernández-Lafuente, R., & Guisan, J. M. (2007). Selective adsorption of large proteins on highly activated IMAC supports in the presence of high imidazole concentrations: purification, reversible immobilization and stabilization of thermophilic α - and β -galactosidases. *Enzyme and microbial technology*, 40(2), 242-248.
 46. Picard, C., Fioramonti, J., Francois, A., Robinson, T., Neant, F., & Matuchansky, C. (2005). bifidobacteria as probiotic agents—physiological effects and clinical benefits. *Alimentary pharmacology & therapeutics*, 22(6), 495-512.
 47. Pierre, A. C. (2004). The sol-gel encapsulation of enzymes. *Biocatalysis and Biotransformation*, 22(3), 145-170.
 48. Pillai, C. K., Paul, W., & Sharma, C. P. (2009). Chitin and chitosan polymers: Chemistry, solubility and fiber formation. *Progress in polymer science*, 34(7), 641-678.
 49. Pivarnik, L. F., Senecal, A. G., & Rand, A. G. (1995). Hydrolytic and transgalactosyl activities of commercial β -galactosidase (lactase) in food processing. *Advances in food and nutrition research*, 38, 1-102.
 50. Plantz, B. A., Nickerson, K., Kachman, S. D., & Schlegel, V. L. (2007). Evaluation of Metals in a Defined Medium for *Pichia pastoris* Expressing Recombinant β -Galactosidase. *Biotechnology progress*, 23(3), 687-692.
 51. Price, N. and Stevens, L. (2000). Fundamentals of Enzymology. 3rd Edn. *Oxford Press, London*. pp 155-161.
 52. Ranwala, A. P., Suematsu, C., & Masuda, H. (1992). The role of β -galactosidases in the modification of cell wall components during muskmelon fruit ripening. *Plant physiology*, 100(3), 1318-1325.
 53. Rebroš, M., Rosenberg, M., Mlichová, Z., Křištofiková, L. U., & Paluch, M. (2006). A simple entrapment of glucoamylase into LentiKats® as an efficient catalyst for maltodextrin hydrolysis. *Enzyme and Microbial Technology*, 39(4), 800-804.
 54. Rech, R., & Ayub, M. A. Z. (2007). Simplified feeding strategies for fed-batch cultivation of *Kluyveromyces marxianus* in cheese whey. *Process Biochemistry*, 42(5), 873-877.
 55. Rios, G. M., Belleville, M. P., Paolucci, D., & Sanchez, J. (2004). Progress in enzymatic membrane reactors—a review. *Journal of Membrane Science*, 242(1-2), 189-196.
 56. Rodriguez-Nogales, J. M., & Delgadillo, A. (2005). Stability and catalytic kinetics of microencapsulated β -galactosidase in liposomes prepared by the dehydration–rehydration method. *Journal of Molecular Catalysis B: Enzymatic*, 33(1-2), 15-21.
 57. Roth, N. J., Penner, R. M., & Huber, R. E. (2003). β -Galactosidases (*Escherichia coli*) with Double Substitutions Show That Tyr-503 Acts Independently of Glu-461 but Cooperatively with Glu-537. *Journal of protein chemistry*, 22, 663-668.
 58. Rout, S. K. (2001). *Physicochemical, functional and spectroscopic analysis of crawfish chitin and chitosan as affected by process modification*. Louisiana State University and Agricultural & Mechanical College.
 59. Schramm, V. L. (2003). Enzymatic transition state poise and transition state analogues. *Accounts of chemical research*, 36(8), 588-596.
 60. Selwood, T., & Sinnott, M. L. (1990). A solvent-isotope-effect study of proton transfer during catalysis by *Escherichia coli* (*lacZ*) β -galactosidase. *Biochemical Journal*, 268(2), 317-323.
 61. Şenel, S., & McClure, S. J. (2004). Potential applications of chitosan in veterinary medicine. *Advanced drug delivery reviews*, 56(10), 1467-1480.
 62. Şener, N., Apar, D. K., & Özbek, B. (2006). A modelling study on milk lactose hydrolysis and β -galactosidase stability under sonication. *Process Biochemistry*, 41(7), 1493-1500.
 63. Sinha, R., Radha, C., Prakash, J., & Kaul, P. (2007). Whey protein hydrolysate: Functional properties, nutritional quality and utilization in beverage formulation. *Food chemistry*, 101(4), 1484-1491.