Fermentative production of fungal Chitosan, a versatile biopolymer (perspectives and its applications)

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ABSTRACT

Worldwide every year approximately 140 million tonnes of synthetic polymers are produced. These polymers are stable and their degradation cycle in the biosphere is limited. This necessitates the need for natural biodegradable polymers which fit into the ecological cycle. Among these biopolymers, chitosan, a partially deacetylated form of biopolymer chitin has been receiving more attention due to its versatile nature. Commercial chitosan is obtained from crustacean shells such as crabs, lobsters and shrimps are loaded with many demerits and limited the potential industrial acceptance of chitosan. Mycelial wastes from fermentation processes as a source of fungal chitin and chitosan would offer a stable non-seasonable source of raw material and would be more consistent in character and of high quality. In this context, the present review discusses biopolymers, the merits and demerits of various sources of chitosan and its various biological activities.

Keywords: Biopolymer, chitin, chitosan, crustacean , fungi, fermentation.
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INTRODUCTION

Plastics and polymers are an integral part of our daily existence [1]. Worldwide polymer production was estimated to be 260 million metric tonnes per year [2]. Plastic debris poses considerable threat by choking and starving wildlife, distributing non-native and potentially harmful organisms, absorbing toxic chemicals and degrading to micro-plastics that may subsequently be ingested   Accumulation and fragmentation of plastic debris in global environments[3]. Environmental problem associated with plastics have stimulated the formulations and legislations regulating polymer use with increasing public and political awareness and to satisfy the environmental imperatives, research is directed towards finding suitable substitutes that are biodegradable besides retaining the properties of the conventional plastics. Of particular interest are biodegradable polymers which fit into the ecological cycle[4].

1. Existence of biopolymers
Living matter is able to synthesize a wide range of different polymers, and in most organisms these biopolymers contribute the major fraction of cellular dry matter. The functions of biopolymers are in most cases, essential for the cells and as manifold as their structures[5].Biopolymers may be naturally occurring materials: most materials formed in nature during the life cycles of green plants, animals, bacteria and fungi are polymers or polymer matrix composites. Biopolymers include the polymers such as cellulose, starch, the carbohydrate polymers produced by bacteria and fungi and animal protein based biopolymers such as wool, silk, gelatin and collagen: biopolymers, especially the carbohydrate origin, have been found very promising industrial application in various forms [6].

2. Chemical classification of biopolymers
Organisms are able to synthesize an overwhelming variety of polymers which can be distinguished into eight major classes according to their chemical structure.
1. Polynucleotides such as nucleic acids
2. Polyamides such as proteins and poly(α- or β-) aminoacids

3. Polymers such as starch, cellulose, glucans, mannans, chitin and chitosan [7].
4. Organic polyoxoesters such as poly hydroxy alkanoic acids, polymalic acid and cutin
5. Polythioesters such as Poly (3HB-*co*-3MP) which were reported recently.
6. Inorganic polyesters such as polyphosphate.
7. Polyisoprenoids such as natural rubber or Gutta percha
8. Polyphenols such as lignin or humic acids.

3. Functions of Biopolymers

These biopolymers fulfill a range of quite different essential functions for the organisms such as conservation and expression of genetic information, catalysis of reactions, storage of carbon, energy (or) other nutrients, defending and protecting against the attack of other cells, hazardous environmental factors, sensing of biotic and abiotic factors, communication with the environment and other organisms, mediation of the adhesion to surfaces of other organisms (or) of non-living matter and many more. Alternatively, they may be structural components of cells like chitin, chitosan, tissues and entire organisms. Microbial polymers are used by industry for technical applications in medicine, pharmacy, agriculture, as packaging materials and in many other areas. Biotechnological production of these polymers is mostly achieved by the fermentation of microorganisms in stirred tank bioreactors and the biopolymers can be obtained as extracellular (or) intracellular compounds.

4. Principles of Biopolymer Synthesis

Location of Biosynthesis

All biopolymers are synthesized by enzymatic processes in the cytoplasm, in the various compartments (or) organelles of cells, at the cytoplasmic membrane (or) at cell wall components, at the surface of cells (or) even extracellularly. Polymers can be also excreted from cells into the environment. This occurs for example in enzyme proteins which hydrolyze polymeric nutrients or lipids[8].

5. Biopolymers and Renewable Resources

Biopolymers can be obtained from agriculture or biotechnological processes overview on the major products which are available from the plantation of crops and trees which are used for non-food applications. With the exception of triacyl glycerols and sucrose, these products are mainly polymers such as starch, cellulose, lignin and natural rubber. Many biopolymers possess rarer chemical structures and compositions and are therefore not available from the chemical synthesis. Biopolymers like almost all products of living matter are generally biodegradable but this is not a general feature of synthetic polymers[9]. These few aspects indicate reasons for the growing interest of industry to produce and use biopolymers for a steadily increasing number of applications [10].Only, few biopolymers have been commercialized so far, this is mainly due to production costs, which are often much higher than synthetic polymers that have been established in the market during the last decades. However this situation may change for some biopolymers in the future, when biotechnological production processes have been further optimized and if our knowledge of the material properties and processing of biopolymers has increased.

6. Isolation and Synthesis of Biopolymers

There are different ways to produce biopoymers inorder to make them available for interesting technical applications: (i) Many biopolymers occur abundantly in nature and are isolated from plants and algae which grow in natural environments or are cultivated on plantations. Cellulose and starch are isolated from several agricultural crops and trees such as *Zea maize* or *Pinus silvesteris*, respectively. Natural rubber is isolated from plantations of rubber tree *Hevea brasiliensis* and agar and alginates are isolated from red algae belonging to the genus *Gelidium* [11] or from various brown algae also referred to as seaweeds [12]. Hyaluronic acid is extracted from the umbilical cords of newborn children. DNA polymerases in the polymerase chain reaction (PCR) are used to produce mono disperse defined DNA molecules[13]. Dextran can be produced on a technical scale with isolate *Leuconostoc mesenteroides* [14] Polymers such as xanthan from *Xanthomonas campestris* [15] and dextran from *Leuconostoc mesenteroide* [16] by fermentation technology. Microbial cellulose obtained by ferementation of *Acetobacter xylinum* has more medical applications such as wound dressings, scaffolds for tissue engineering and artificial blood vessels over plant cellulose. Polyhydroxy butyrate was available under the trade name "Biopol" from ZENECA and Monsanto, have been also produced on an industrial scale by employing *Ralstonia eutropha*. However, genetically engineered bacteria can also be employed for the production of biopolymers.

Intracellular and Extracellular Production of Biopolymers

The biotechnological production of biopolymers occurs intracellularly or extracellularly. This causes several severe consequences regarding the limitations of the production and downstream processes to obtain the biopolymers in a purified state.

PHAs [17], Cyanophycin[18] , Glycogen [19], Starch [20] and polyphosphate[21] are examples of biopolymers which are accumulated in the cytoplasm of cells. The availability of space in the cytoplasm therefore limits the amount of polymer that can be produced by a cell. This is particularly relevant for fermentation production processes mostly employing microorganisms. Intracellular chitin, chitosan synthesis occurs also in the process of wall formation during spore development within the sporangia of zygomycetes [22]. Therefore, the yield per volume is limited or determined by the cell density and the fraction of biopolymer in the biomass. The tedious processes must follow the production of the biomass containing the biopolymer to disintegrate the cells or tissues and to release the biopolymer from the cells. Furthermore, it should get separated from other cell constituents which are released concomitantly along with the biopolymer. Poly-(γ-D-Glutamate) [23] and many polymers such as alginates [24], dextran, xanthan and microbial cellulose[25] are examples of biopolymers which occur outside the cells, either as a result of extracellular synthesis or of excretion by the cells. Polylactide acid is produced by the combination of biotechnological and chemical approach [26]. The third strategy is to convert an intracellular process into an extracellular process by metabolic and genetic engineering of the cells or organisms.

7. Biopolymers from fungi and yeast

Pullulan from *Aureobasidium* (*Pullularia) pullulans*[27,28],Extracellular polysaccharide composed of N-acetyl-D-glucosamine and N-acetyl-D-glucuronic acid residues from *Rhinocladiella mansonii*[29], Extracellular polysaccharide composed of 2-acetamido-2-deoxy-D-glucuronic acid residues from *Rhinocladiella elatior*[30],Water soluble polysaccharide from *Armillaria mellaria*[31],Capsular polysaccharide from *Leptosphaeria albopunctata*[32],Extracellular branched D-glucan from *Monilinia fructigena*[33],Phosphomannans from *Hansenula holstii* and *Hansenulacapsulata*[34-37]

Polysaccharide Y-1401 from *Cryptococcus laurentii*[38],Tremella heteropolymers from *Tremellames enteric*[39],Sclero glucan from *Claviceps purpurea*[40],Chitin from *Streptomyces cerevisiae, Pythium ultimum, Fusarium oxysporum, Rhizoctoniasolani, Cryptococcus neoformans, Rhizopus oryzae*[41-43],Chitosan from *Mucor, Absidia, Rhizopus species* and *Lentinus edodes*[44]

8. Factors affecting the Synthesis of Polysaccharides

a. Nutritional factors and mode of cultivation

Production of microbial polymers is generally favoured by a high carbon or nitrogen ratio in the medium. The nitrogen source is growth limiting nutrient and its concentration is set to produce the required biomass concentration. Since cations can affect the rheological properties of polymers solutions, care must be taken in optimizing the concentration of salts provided as nutrients in the medium.

Continuous culture is not used for the production of microbial polymers. At higher growth rates, which are desirable for high productivity, an increasing proportion of the carbon source is used to produce biomass rather than polymers. Furthermore, some polymers producing microorganisms are not stable in continuous culture whereas strain stability is less of a problem in batch cultures which are of shorter duration [45].

For some microorganism the carbon source determines both the quantity and quality of formation and the quantity of the product synthesized. Ordinarily the carbon source concentration affects the efficiency of carbon source conversion into polymers e.g. conversion efficiency of glucose to polymer by *Xanthomonas campestris* by increased concentration of glucose [46].

Although a nitrogen source is necessary for both cell growth and enzyme synthesis for polymers formation, an excess of nitrogen, in general reduces conversion of carbohydrate substrate to extracellular polymers.

b. Environmental Factors

Temperature often is critical in polymer synthesis. In general, the optimal temperature for cell growth also is optimum for product formation. Most of the polymer producers are mesophilic.

Large amount of hot air and high degrees of agitation are applied during fermentation to combat the inefficient mass transfer due to high viscosity. Another important factor is pH. The bacterial polymers of possible commercial significance appear to have an optimal pH for synthesis between 6.0 and 7.5[47]. For fungal gum production, the optimum pH lies between 4.0 and 5.5[48] .

Many microorganisms have strict requirements for certain mineral elements. Among these elements are K, P, Mg, Ca, Mo, Fe, Cu and Zn are required. However certain elements can inhibit the product formation. As a result, the mineral requirements for polymer synthesis vary from species to species .
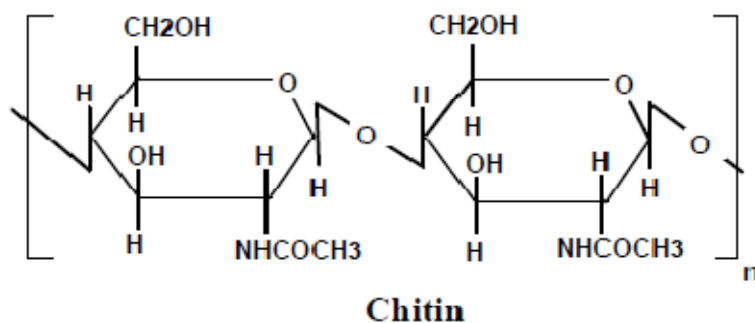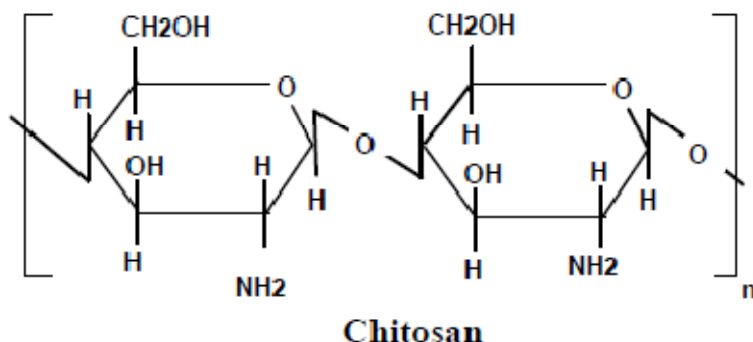
9.   **Chitin and chitosan**
a. *Background*
Professor Henri Braconnot, Director of the Botanical Garden in Nancy, France first isolated a fraction called fungine in 1811 from the cell walls of mushooms. In 1823 Odier renamed fungine as chitin almost 3 decades before the isolation of cellulose [49].

b.*Definitions and Chemical structures*
Biopolymers originate from natural sources and are biologically renewable, biodegradable and biocompatible. Chitin and Chitosan are the biopolymers that have received much research interests due to their numerous potential applications in agriculture, food industry, biomedicine, paper making and textile industry.The amino glucan chitin (poly-GlcNAc) is the second most abundant organic compound in nature after cellulose (Ruiz Herrera, 1978). Chitin is a polysaccharide made of N- acetyl –D- glucosamine units connected by β(1→4) linkage.

Chitin

Chitosan was purportedly first discovered in 1859 by Rouget while he was experimenting with thermal and chemical manipulation of the natural fiber chitin by loosing acetyl group[50].

Chitosan

However, commonly available chitin and chitosan are not strict homopolymers but they exist as copolymers.

b. *Occurrence and Composition of Chitin and Chitosan*
Chitin is widely distributed in marine invertebrates, insects, fungi and yeast [51]. However, chitin is not present in higher plants and higher animals.

c. *Crustacean chitin*
The crustacean shells of crabs, shrimps and crawfish (Allan and Hadwiger, 1979) consist of 30-40% protein, 30-50% calcium carbonate and calcium phosphate and 20-30% chitin[52]. Studies of Ashford and co-workers (1977) demonstrated that chitin represents 14-27% and 13-15% of dry weight of shrimp and crab processing wastes respectively[53].

e. *Fungal chitin*
The chitin of fungi possesses principally the same structure as that of the crustacean chitin. However a major difference results from the fact that fungal chitin is associated with other polysaccharides such as glucans and mannans which do not occur in the exoskeleton of arthropods[54].

Chitin is widely distributed in fungi, occurring in Basidiomycetes, Ascomycetes and Phycomycetes where it is a component of the cell walls and structural membranes of mycelia, stalks and spores . However, not all fungi contain chitin and the polymer may be absent in one species that is closely related to another. It is the major component in

primary septa between mother and daughter cells of *Sacchromyces cerevisiae* and also one of the main components of the hyaline outer wall of spores of four arbuscular mycorrhizal glomus species [55]. The chitin of the white-rot fungus *Rigidoporus lignosus* is degraded by enzymes excreted as a defense response by the host cell and therefore not detectable during the process of infection [56]. Another white rot fungus, *Phellius noxius* do not contain chitin [57]. The mycelia, the caps and stalks of fruiting bodies of four edible mushooms, *Lentinus edodes, Lycophyllum shimeji, Caju* and *Volvariella volvacea* contain chitin as a minor component [58].

f. Composition of fungal chitin
The molecular mass of chitin in fungi is not known. However it was estimated that bakers yeast synthesizes rather uniform chains containing 120-170 GlcNAc monomer units which corresponds to 24,000 – 34,500 daltons [59]. In *Sacchromyces cerevisiae* terminal reducing ends of chitin chains are attached though β (1, 4) or β (1, 2) linkages to the non-reducing end of β (1, 6) glucan. Attachment of chitin to glucan is catalyzed by chitin synthase [60]. A mannoprotein is attached to β (1, 6) glucan though a glycosyl phosphatidyl inositol anchor containing five α - linked mannosyl residues[61]. A mutant of *Sacchromyces cerevisiae* with a reduced β (1,3) glucan content shows increased cross linking of mannoproteins to chitin trough β (1,6) glucan[62].

g. Crustacean chitosan
Commercial chitosan mostly available in the market is derived from crustacean chitin by chemical deacetylation.

h. Fungal chitosan
Chitosan occurs naturally in the mucorales such as *Mucor, Absidia and Rhizopus* species. There is apparently only one report on the presence of chitosan in a basidiomycete, *Lentinus edodes* (Shiitake mushoom). Slime molds (Myxomycetes) and bacteria (Schzomycetes) are devoid of chitin

Physiological Function
Chitin serves as a fibrous strengthening element responsible for cell wall rigidity. However, there are other functions of chitin and chitosan as revealed by mutants bearing a defect in the complex machinery of chitin biosynthesis, intracellular trafficking of chitin synthases or deposition of the polysaccharide in cell walls, although the morphology of a mutant may be indistinguishable from that of wild-type [63].

j. Chemical Analysis and Detection
Frequently, GlcN is quantified by colorimetric methods in hydrolyzates of alkali-resistant fractions to determine the amounts of chitin and chitosan [64]. GlcN was also determined in acid hydrolyzates by high- performance liquid chromatography (HPLC) of 9- fluroenyl methoxy carbonyl (FMOC) or phenyl isothio cyanate (PITC)-GlcN [68] or by gas chromatography-mass spectrometry (GC-MS)[65]. Localization of chitin in cell walls or spores of fungi is achieved by using dyes that intercalate with polysaccharides. Calcofluor white shows enhanced fluorescence when binding to β (1,4) glucans such as chitin, chitosan and cellulose, whereas β (1,3) glucans are selectively stained with aniline blue (Nicholas *et al.,*1994). Various wheat germ agglutinin (WGA) labeling techniques in combination with fluorochromes or gold labeling are also described for the detection of chitin in fungi.[66].Fourier transform (FT) Raman spectroscopy of cell walls of fungi was applied to discriminate between different mixed species in culture media[68].

k. Biosynthesis of chitin and chitosan
Chitin is biosynthesized in all chitinous fungi, including the relatively few investigated examples of mucorales which contain chitosan. Biosynthetic pathway of chitin and chitosan[69]. (Figure 3)
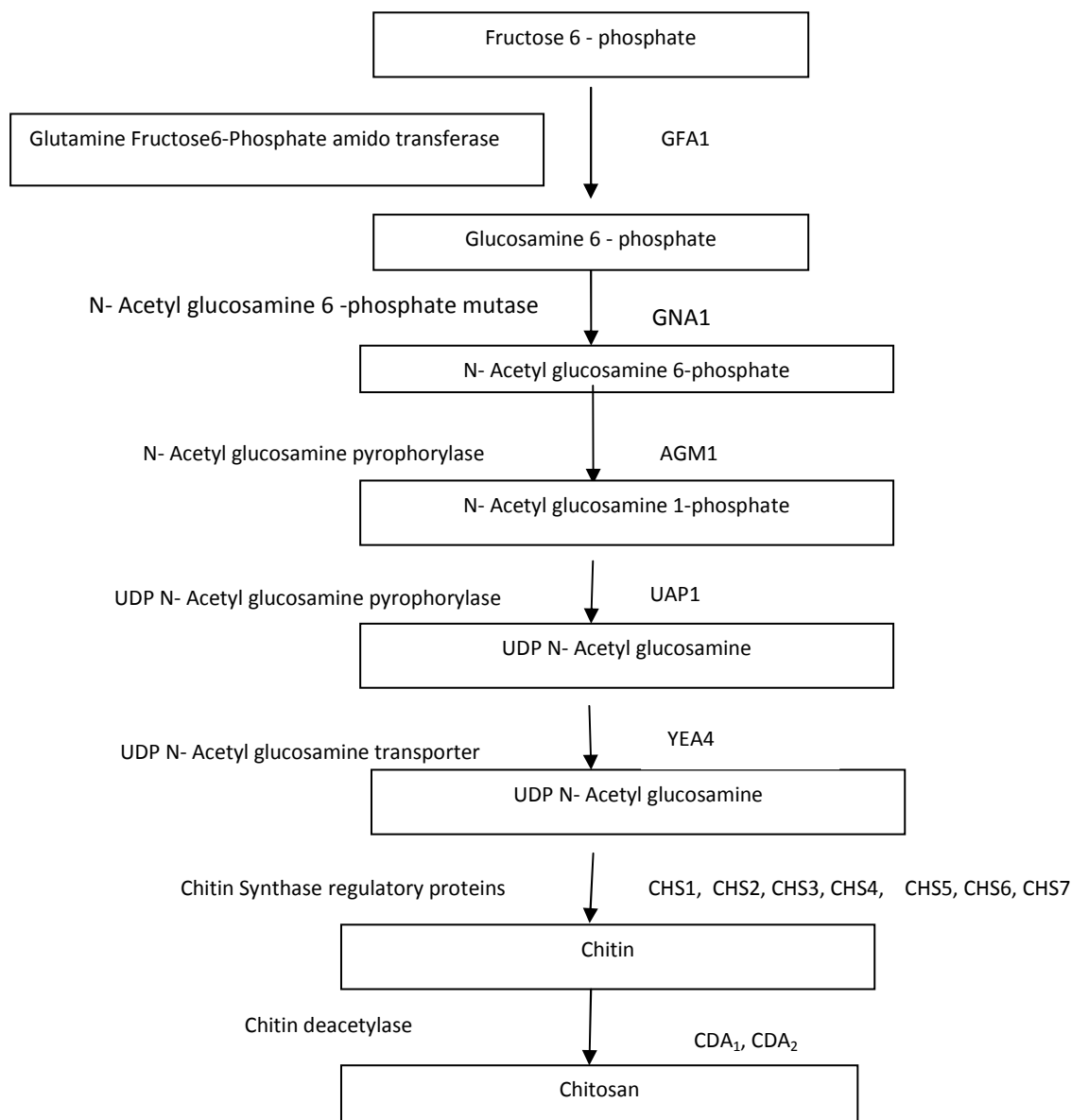
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                        ┌─────────────────────────────────┐
                        │       Fructose 6 - phosphate     │
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│ Glutamine Fructose6-Phosphate amido transferase│  GFA1   │
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                        │      Glucosamine 6 - phosphate    │
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   N- Acetyl glucosamine 6 -phosphate mutase        GNA1   │
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       N- Acetyl glucosamine pyrophorylase           AGM1  │
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      UDP N- Acetyl glucosamine transporter          YEA4  │
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  Chitin Synthase regulatory proteins    CHS1, CHS2, CHS3, CHS4,  CHS5, CHS6, CHS7
                        ┌─────────────────────────────────┐
                        │              Chitin               │
                        └─────────────────────────────────┘
       Chitin deacetylase              CDA₁, CDA₂         │
                        ┌─────────────────────────────────┐
                        │             Chitosan              │
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Figure 3 Biosynthesis of chitin and chitosan

l. Chitin synthases
In contrast to the situation in arthopods many details of chitin biosynthesis are known in fungi. Most of the current knowledge is based on studies on baker's yeast *Sacchromyces cerevisiae*. The various aspects of chitin synthesis in fungi have been reviewed [70-75].

m.Chitin Deacetylase (CDA)
Enzymatic Deacetylation of chitin by CDA is apparently restricted to fungi and bacteria [76, 77]. It was first identified and partially purified from extracts of the fungus *Mucor rouxii* [78]. Since then, the presence of this enzyme activity has been reported in several other fungi [79]. Eventhough chitin biosynthesis, enzymology and cytology in fungi have been extensively studied; there is limited information on chitosan biosynthesis. CDA from *Mucor rouxii* is a secreted enzyme and that its function is localized in the perisplasmic space[80].

10. Synthesis of Chitin and Chitosan Production
a. Industrial and Traditional Synthesis of Chitin and Chitosan
The annual biosynthesis of chitin is estimated as 100 billion tons [81]. The best available source of chitin is the seafood waste, primarily by the crabs and shrimp shells. The annual world wide production of crustacean shells has been estimated as 1.2×10^6 tons [82].

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Crustacean chitin is naturally closely associated with proteins, minerals, lipids and pigments. The industrial process consists of 3 basic steps: Demineralization to remove $CaCO_3$, Deproteinization to remove protein and Decoloration to remove pigments[83].Based on the basic steps, many industrial procedures have been developed for the demineralization and deproteinization parameters. Thus Percot *et al.,* 2003 found that demineralization and deproteinization were completely achieved within 15 min at ambient temperature in 0.25M HCl and within 24 h in 1M NaOH at a temperature around 70°C without damage to the molecular weight or the degree of deacetylation respectively[84].Chitosan is generally produced from chitin by treatment with 40 - 50% of sodium hydroxide or potassium hydroxide solution at 80 - 150°C.

b. Synthesis of fungal Chitosan
Synthesis of chitin and chitosan from fungal mycelium has recently received increased attention due to significant advantages. The crustacean waste supplies are limited by seasons and sites of fishing industry, while the fungal mycelium can be obtained by convenient fermentation process that does not have geographic or seasonal limitations[85].

Fungal mycelia have lower level of inorganic materials compared to crustacean wastes and thus no demineralization treatment is required during the processing [86].Crustacean chitin and chitosan may vary in the physico-chemical properties, while fungal chitin and chitosan have relatively consistent properties because of the controlled fermentation conditions[87].Fungal chitin and chitosan are apparently more effective in inducing the plant immune response and are potentially more suitable for agricultural applications [88].Many fungal species including *Absidia glauca, Absidia coerulea, Aspergillus niger, Mucor rouxii, Gongronella butleri, Phycomyces blakesleeanus, Absidia blakesleeanus, Rhizopus oryzae, Trichoderma reesei and Lentinus edodes* have been investigated for the production of chitin and chitosan [89-93]. Among all investigated species, the most commonly researched one is *Mucor rouxii* and quantities of chitin and chitosan in its mycelia can reach 35% of cell wall dry weight..Fungi are usually harvested at their late exponential growth phase to obtain the maximum yield for chitin and chitosan. Although fungi can be grown on solid media, cultivation for chitin and chitosan isolation is usually carried out in the Yeast Peptone Glucose Broth (YPG), Potato Dextrose Broth (PDB) or Molasses Salt Medium (MSM).Extraction process from fungal sources is similar to industrially utilized except that no demineralization treatment is required due to low mineral content in fungal mycelia.

It involves 3 steps
a. Alkaline treatment to remove protein and alkali soluble polysaccharides.
b. Acid reflux to separate chitin and chitosan
c. Precipitation of chitosan under alkaline conditions.

Removal of proteins by alkaline treatment is commonly performed with 1M NaOH at 95°C from 1 to 6 h and at 121°C from 0.24 h to 1 h. Separation of chitosan by acid treatment is usually carried out by 2 to 10% acetic acid or HCl at 95°C for 3 to 14 h. 2%NaOH was used at 90°C during 2 h for alkali treatment and 10% acetic acid at 60°C during 6 h for acid reflux during the extraction of chitin and chitosan from *Mucor rouxii*. Hu *et al.,* 1999 adopted autoclaving at 121°C in both alkaline and acid treatment of *Absidia glauca* mycelia. However, the temperature and time of treatment had to be reduced to 25°C and 1h to avoid polymerization of chitosan during extraction from zygomycetes.Most of the studies in this field, concentrate on the fermentation process to produce fungal mycelia for chitin and chitosan extraction.Relatively few studies have focused on the fungal waste from industrial fermentations or mushoom industry. However, considering the amount of waste that accumulates during processing, citric acid industry and mushoom industry specifically from *Agaricus bisporus* growing practices can provide plenty of raw materials for fungal chitin and chitosan production. Citric acid is the most widely used organic acid in food, beverage and pharmaceutical industries. The industrial production is based on *Aspergillus niger* submerged fermentation. The current world requirements for citric acid production are estimated to be 400,000 tons/year [94]. Managing this waste presents an extra expense for the producers and alternative solution for mycelial disposal has been evaluated. One of the potent outputs for the spent mycelia is in food supplements. However, this type of feed seems to be difficult to compete with the other low price feeds.

11. Pharmacokinetics
Experimental evidence suggests that chitosan is partially digested and absorbed owing to the acidic environment of the stomach, enzymes present in saliva and gastric juice and bacterial enzymes in the large intestine. Oral intake of 1 g/day of chitosan increases the serum concentration of N-acetyl D-glucosamine[95].

12. Adverse effects and Toxicity
Chitosan is generally considered to be non-toxic (Muzzarelli, 1999). In clinical studies, very few adverse effects which are mild and transient like nausea, flatulence, thoat irritation, itching [96, 97].

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13. Interactions

Theoretically, there has been concern that chitosan could bind fat-soluble vitamins and deprive the body of these essential nutrients. It has been suggested that vitamin supplements should be taken at a separate time from chitosan inorder to avoid any potential interaction . Absorption of vitamin A and β-carotene is not altered by chitosan intake[98]. Studies have shown that absorption of trace metal ions such as Fe and Zn is not inhibited by chitosan administration.

14. Products

Chitosan is available in dietary supplements sold under several properiatary names. Products may be combined with a starch excipient. Additionally, chitosan may be found in combination with other substances, such as appetite suppressants, stimulants, chromium picolinate, carnitine or amino acids in products marketed for weight loss [99]. Chitosan has been sold in Europe and Japan for the past 20 years as a nonprescription product to inhibit fat absorption[100].

15. Physico-chemical Properties
a. Physical properties
It is off white amorphous translucent flake or powder with pearly colour.

b. Solubility
Chitin is insoluble in most organic solvents; Chitosan is readily soluble in dilute acidic solutions below pH 6.0. Organic acids such as acetic, formic and lactic acids are used for dissolving chitosan. The most commonly used is 1% acetic acid solution at about pH 4.0 [101].

c. Degree of deacetylation
This term is used to characterize chitosan and gives the proportion of monomeric units (glucopyranose) of which the acetylic groups that have been removed, indicating the proportion of free amino groups on the polymer. DDA is a key property that influences the physical and chemical characteristics of chitin and chitosan including solubility, chemical reactivity and biological activity[102]. DDA could vary from 70 to 100% depending on the manufacturing method used. This parameter is important since it indicates the cationic charge of the molecule after dissolution in a weak acid [103].Various techniques have been proposed to determine the degree of deacetylation for chitin and chitosan. The methods include infrared spectroscopy (IR) ,^{13}C solid NMR, Ultraviolet spectrometry. Potentiometric titration and High pressure Liquid chromatography (HPLC)[104-109].Among these techniques IR is the most utilized one because of its convenience in minimal sample preparation, but IR requires the precise calibration using a wide spectrum of chitin and chitosan standards with known DDA. The solid ^{13}C solid state NMR appears to be the most reliable technique and is often used as the reference method [110-112]. But, it is not available in many laboratories due to the high cost of the instrument. Ultraviolet spectrometry and potentiometric titration are techniques that require dissolved samples and thus are not applicable for chitins and chitosans with DDA < 50%. The degree of deacetylation by infrared spectroscopy for microbial chitin and chitosan can be determined utilizing the following baselines.

i.   DDA = 100-[(A1655/ A3450) x 100/1.33] [113]
ii. DDA = 97.67-[26.486X (A1655 / A3450)][114]
iii. DDA = 100 – [(A1655 / A3450) X 115] [115]
iv. DDA = 118.883-[40.1647 X (A1655/A3450)] [116]

d. Molecular weight
Chitosan is a biopolymer of high molecular weight. Like its composition, the molecular weight of chitosan varies with the raw material sources and the method of preparation [117]. The molecular weight of chitin has been reported to be as high as many Daltons. However, the harsh chemical treatment tends to bring down the molecular weight of chitosan ranging from 100 kDa to 1500 kDa.The low molecular weight of chitosan could be produced by either enzymatic or chemical methods [118].

The molecular weight of chitosan can be determined by methods such as chromatography , Light scattering, Viscometry [119-120].

Mark-Houwkinsequation $\eta = K[M_v]^a$ [121].

Where $K = 1.81 \times 10^{-3}$ and a= 0.93.

e. Purity

The purity of the product is vital particularly for high-value product (biomedical or cosmetic area) purity is quantified as the remaining ashes, proteins, insolubles and also in the bio-burden (microbes, yeasts, moulds and endotoxins). Even in the lower value chitosan such as that used for waste water treatment, the purity is a factor because the remaining ashes or proteins tend to block active sites, the amine grouping. Application of chitosan can be classified mainly in three categories according to the requirement on the purity of chitosan such as technical grade for agriculture and water treatment,pure grade for the food and cosmetic industries and ultra-pure grade for biopharmaceutical uses.

16. Applications of chitosan

The poor solubility of chitin is the major limiting factor in its utilization.Chitosan is considered as a potential polysaccharide because of its free amino groups that contribute polycationic, chelating and dispersion forming properties along with ready solubility in dilute acetic acid. Chitosan possess exceptional biological and chemical qualities that can be used in a wide variety of industrial, medical and pharmaceutical applications.

Table 3 General Applications of chitosan

Waste water treatment	Removal of metal ions, flocculant/coagulation of protein, dye, aminoacids
Food industry	Removal of dye, suspended solids, as a preservative, colour stabilization, food stabilizer, thickener and gelling agent, animal feed additive etc.
Agriculture	Seed coating, fertilizer, controlled agro chemical release, in prevention of decay of fruits and vegeTable s.
Biotechnology	Enzyme immobilization, protein separation, cell recovery, chromatography.
Medical	Wound and bone healing, blood cholesterol control, skin burn, contact lens, surgical sutures, dental plaque inhibition, clotting agent, anti-tumor, etc.
Cosmetics	Moisturizer, face, hand and body creams, bath lotion, etc.

Pharmaceutical Applications of chitosan [122-157]

- Diluent in direct compression of Tablets
- Binder in wet granulation
- Slow-release of drugs from Tablets and granules
- Drug carrier in microparticle systems
- Films controlling drug release
- Preparation of hydrogels, agents for increasing viscosity in solutions
- Wetting agent and improvement of dissolution of poorly soluble drug substances
- Disintegrant
- Bioadhesive polymer
- Site-specific drug delivery(stomach or colon)
- Absorption enhancer (nasal or oral drug delivery)
- Biodegradable polymer (implants, microparticles)   Carrier in relation to vaccine delivery or gene therapy

17.Biological Activities of chitosan

a. Antioxidant activity of chitosan

Reactive oxygen species and free radicals are generated naturally in the body during aerobic metabolism and cause oxidation of lipids, proteins, sugars, sterols and nucleic acids. During ageing process, antioxidant defense systems weaken, resulting in the accumulation of (ROS) and free radicals. Uncontrolled formation of these free radicals is toxic as they cause cellular damage leading to many pathological conditions such as arthitis, cancer, stroke, arthosclerosis, retinal damage, diabetes and heart attack. The body has developed natural antioxidants to fight against these free radicals. However the capacity of such systems gradually decreases with ageing, resulting in imbalances in the redox status. Therefore, the body must be nourished with a diet that includes adequate antioxidants. Scavengers of free radicals are preventive antioxidants, and their presence breaks the oxidative sequence at different levels. Antioxidative nutraceuticals, such as tocopherols, ascorbic acid, caroteinoids, polyphenols and chitosan can help to minimize oxidative damage and reduce the risk for age-related disorders by preventing the accumulation of ROS and free radicals. In addition antioxidant property of chitin and chitosan depend on degree of deacetylation and molecular weight. Low MW chitosan was recently reported to be more effective antioxidant than high MW chitosan. Chitin and chitosan are known to adsorb metal ions, with chitosan exhibiting an increased affinity as a result of its free amino groups. The ability to chelate metal ions involved in oxidative reactions can be highly useful in food preparation and storage, health care, water treatment, and pharmaceutical products [158, 159].

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b. Antitumor activity of chitosan

 Cancer is currently the cause of 12% of all deaths worldwide. Cancer prevalence in India is estimated to be around 2.5 million, with over 8, 00,000 new cases and 5, 50,000 deaths occurring each year due to this in the country [160]. Incorporation of anticarcinogenic and antioxidative nutraceuticals such as phytochemicals (i.e., flavanoids, polyphenols, retinoids etc.) into the diet can help to reduce the occurrence of some cancers by a variety of mechanisms, including prevention of DNA mutagenenesis and induction of apoptosis. In addition to the ability to decrease oxidative stress and subsequent DNA damage, some nutraceuticals can adsorb mutagens and thereby inhibit their carcinogenic activity. Most of the anticarcinogenic studies showed that chitin and chitosan and its oligomers, inhibit heavy metal induced genotoxicity and possess growth inhibitory and antimetastatic effects against a variety of cancer tumors. The protective effect of chitosan against environmental mutagens could be quite useful in the field of nutraceuticals. For example, dietary supplement of chitin or chitosan could potentially adsorb carcinogens and transport them out of the digestive tract, thereby providing protection of variety of cancers.

c. Antidiabetic activity of chitosan

Diabetes Mellitus is classified into two types, type1 (Insulin dependent) and type 2 (Non-insulin dependent). Type 2 Diabetes Mellitus is divided into 2 categories, obese type with hyperinsulinemia and non-obese type with hypoinsulinemia. It is established that obesity causes peripheral insulin resistance which leads to hyperinsulinemia. Obesity related type 2 Diabetes mellitus is also characterized by hypertriglyceridemia as well as hyperinsulinemia. Improving the abnormality of lipid metabolism as well as glucose metabolism may be useful in preventing the development or progression of obesity –related type 2 Diabetes Mellitus. Miura *etal* (1995) first showed that chitosan given as a 5% food mixture produces consistent blood glucose and lipid lowering effects in normal mice and neonatal streptozotocin induced diabetic mice. Hayashi *etal* (2002) examined the effect of long-term administration of LMW chitosan, given as drinking water, on hyperglycemia, hyperinsulinemia and hypertriglyceridemia of diabetes mellitus in male genetically obese type NIDDM mice. Therefore, it is unlikely that antidiabetic action of LMW chitosan is due to D-glucosamine, a monosaccharide contained in chitosan. It is believed that chitosan may be primarily absorbed after it has been transformed into oligosaccharide, a high fat diet by inhibiting the intestinal absorption of dietary fat. [161,162]

d.. Antiulcer activity of chitosan

Ulcers are open, non-healing sores that can occur in a number of locations through out the body. Peptic ulcers generally occur as sore or holes in the lining of stomach or duodenum and are characterized by symptoms such as abdominal pain, nausea, bloating and loss of apetite. These ulcers are a result of imbalances in pepsin, an aggressive stomach acid and protective mucosal coating, which is weakened by the bacteria *Helicobacter pylori*. Chitin and chitosan are widely used as wound dressings to promote rapid healing of external cuts or burns. Since similar agents used for healing skin ulcers have also been reported to be effective at preventive gastric mucosal injury and gastric ulcers, chitin and chitosan were also expected to exhibit some protective effects.

Low molecular weight chitosan may have been more effective because of its ability to dissolve more easily in acid. It was proposed that solubilised chitosan may have exerted its protective effect by coating the ulcerated area and neutralizing H^+ and pepsin in the gastric juices as a result of their antioxidant capacity[163].

CONCLUSION

In this context, the present review discusses the potential of fungal biomass resulting from various biotechnological industries or grown on negative/low cost agricultural and industrial wastes and their by-products as an inexpensive source of chitosan. Biologically derived fungal chitosan offers promising advantages over the chitosan obtained from crustacean shells with respect to different physico-chemical attributes. The different aspects of fungal chitosan extraction methods and various parameters having an effect on the yield of chitosan are too discussed. This review also deals with essential attributes of chitosan for high value-added applications in different fields. This review conveys that fungal chitosan could be an alternative source for commercial crustacean chitosan and stresses that further research works should be continued and intensified.

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