Chapter 14
ChiBio: An Integrated Bio-refinery for Processing Chitin-Rich Bio-waste to Specialty Chemicals

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1 Marine Chitin: Sources, Structures, and Properties

Chitin is the second most abundant biopolymer in the world next to cellulose, with 10^{10}–10^{11} tons available [1, 2]. Chitin is a main constituent of fungal, yeast, and algal cell walls [3–5] as well as of cuticles of arthropods and such of crustacean shells. Worldwide more than 13,000,000 tons of crustaceans are caught from marine habitats each year giving rise to a substantial source of chitin of marine origin. Up to 50% of this catch is shell waste [6] and consists of 30–40% protein, 30–50% calcium carbonate, and 20–30% chitin, depending on the species and with seasonal variations [7]. Chitin itself is a linear polysaccharide and is composed of two subunits:

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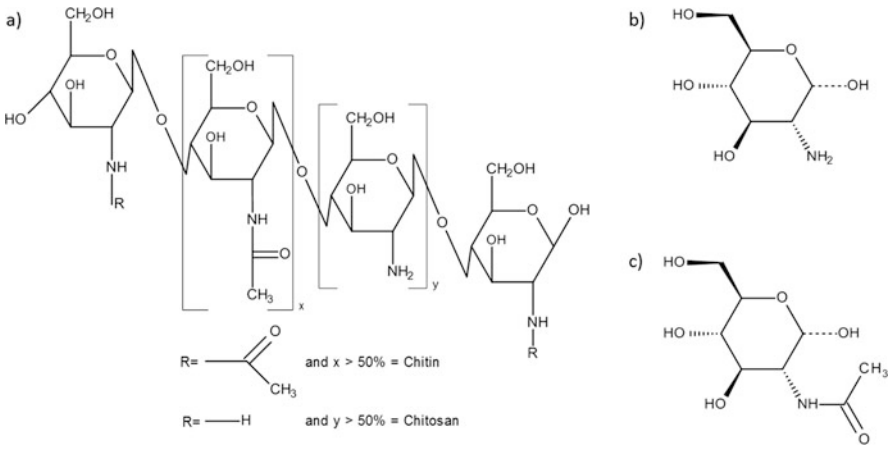

Fig. 14.1 Chemical structures of (**a**) chitin/chitosan, (**b**) glucosamine, and (**c**) N-acetylglucosamine

D-glucosamine and *N*-acetyl-D-glucosamine (Fig. 14.1). In general chitin has a ratio of 90:10 of *N*-acetyl-D-glucosamine to D-glucosamine, which varies depending on the chitin source [8–10]. Due to its high acetylation degree, chitin is hydrophobic and therefore insoluble in water and most organic solvents [11, 12]. The acetylation degree is responsible for the physicochemical properties. By deacetylation these properties can be changed. In general acetylation degrees above 50% are responsible for the insolubility of chitin, while acetylation degrees below 50% make the material generally soluble in acidic aqueous solvents and are used to differentiate chitosan from chitin. Besides its solubility the deacetylation degree is also influencing flexibility, polymer conformation, chemical reactivity, viscosity, and bioactivity [13–15]. Structurally chitin is similar to cellulose but closely associated with proteins, calcium carbonate, lipids, and pigments in crustacean shells [16]. As cellulose in plants, chitin is responsible for the structural integrity in crustaceans. Based on the crystal structure, chitin occurs in three distinct polymorphic forms: alpha-, beta-, and gamma-chitin. The difference is in the arrangement of the molecular chains. Alpha-chitin, the by far most abundant form, has an antiparallel chain arrangement, while in beta-chitin the chains are arranged in parallel. Gamma-chitin is a mixture of the alpha and beta form [17–19].

Chitin and chitosan have attracted huge interest for a wide range of different applications due to their excellent properties as biocompatible material. Both are vulnerable to enzymatic hydrolysis by lysozyme, and it was also shown that lipases in human fluids are able to hydrolyze chitosan [20]. These activities lead to nontoxic degradation products, showing the biodegradability of chitin and chitosan, which is an important factor for applications in medicine and pharmacy.

2 Chitin and Chitosan Applications

Chitin and chitosan attracted huge interest for a number of applications due to their unique properties such as biodegradability, biocompatibility, and nontoxicity especially in the field of medicine and pharmacy. Mainly chitosan- and chitin-derived products, such as oligosaccharides or glucosamine and *N*-acetylglucosamine, are used. In 2015 the chitosan market had a volume of USD 63 billion, and the amount of chitosan on the market was 13,700 tons [21], which is expected to increase to 124,000 tons and USD 4.2 billion [22] in 2020.

2.1 Applications in Medicine and Pharmacy

Chitin but mainly chitosan has attracted huge attention in this field. As investigations have shown, chitosan is able to interact with plasma proteins and blood cells through its free amino groups, leading to the formation of clots [23] and activating the complement and blood coagulation system [24–26] as polymeric contact material. Water-soluble chitosan and chitosan oligomers show this activity only after their sulfatation. In combination with the biocompatibility, biodegradability, and low toxicity, chitin and chitosan are therefore very attractive materials for the treatment of wounds and burns [27]. The adhesive properties of chitosan in combination with its antimicrobial effect and oxygen permeability led to numerous patents and products on the market [28].

In pharmacy, chitosan is widely used for the preparation of drug delivery systems. Although alone chitosan films showed only a limited use due to the low release control of these systems, in combination with hydrophilic polymers, membranes with excellent properties for drug release can be prepared [29]. Therefore, chitosan was mixed with pectin, alginate, or polyacrylate to form polyelectrolyte complexes for the development of controlled release systems [30]. Chitosan-xanthan microspheres have been described for the delivery of drugs to the gastrointestinal tract, according to their biodegradability and pH sensitivity [31]. Nanoparticles of chitosan have been developed as drug delivery system for the nasal mucosa, increasing drug penetration into the human body [32]. Plenty of other reports describe the usage of chitosan as well as chitin in film, gel, or powder form for drug delivery systems, showing the huge potential of these molecules [33, 34].

2.2 Applications in Agriculture

Chitin and chitosan are already widely used in agriculture for protecting plants from infections by pathogenic fungi, bacteria, and viruses but also for the improvement of soil. Especially, chitin and chitosan oligomers have been shown to have a direct anti-

pathogenic effect on all types of microbes and are potent inducers of the plant protection system [35]. In the soil chitin supports the growth of symbiotic plant partners and chitin-degrading microorganisms [36]. Through their chitin-degrading activity, these microorganisms reduce the growth of pathogenic fungi and nematodes [37]. In addition, chitin and chitosan have a beneficial effect on plant growth by improving plant metabolism and resulting in higher yields.

2.3 Application in Wastewater Treatment

Chitosan is widely used as water treatment agent. Due to its polycationic nature, it has excellent flocculation properties and can also be used as chelating agent. It binds a variety of metal ions like Hg^{2+}, Cu^{2+}, or Zn^{2+} [38], dyes [39], hydrocarbons [40], and organic residues in wastewater and effluent sludge [41]. Organic- as well as mineral-contaminated water can be cleared, and in combination with its biodegradability, chitosan is often the method of choice.

2.4 Application in Food and Cosmetics

The *N*-acetylglucosamine monomer from chitin is present in human milk and improves the growth of *Bifidobacterium* sp. in the human gut. Disturbance of the bifidobacterial flora leads to lactose intolerance, because they are responsible for the lactase production. Investigations showed that the addition of chitinous material to food increases the tolerance toward whey-containing products [29]. Chitin and chitosan are also used for the preservation of food [42] or as antioxidants [43]. Chitosan blocks the absorption of dietary fat and cholesterols, reducing the overall cholesterol value [44]. Additionally, it has been shown that chitosan contributes weight and body fat loss in human [45]. Therefore, chitosan has the potential to increase the nutritional value of food. Chitosan is also employed as additive in creams and lotions due to its antimicrobial activity and as thickening agent [46]. It was also tested as additive for nail lacquers.

3 Chitin and Chitosan Production

Up to 50% of the total weight of crustacean, such as shrimps, crabs, or lobsters, consists of shell material. This waste product of the fishery industry is currently the most important source for chitin and chitosan production. For the extraction of the valuable chitin from the shell materials, harsh conditions are necessary to break up the complex protein-chitin-calcium carbonate structure, which guarantees the exoskeleton stability. Over the last decades, several chemical as well as biological

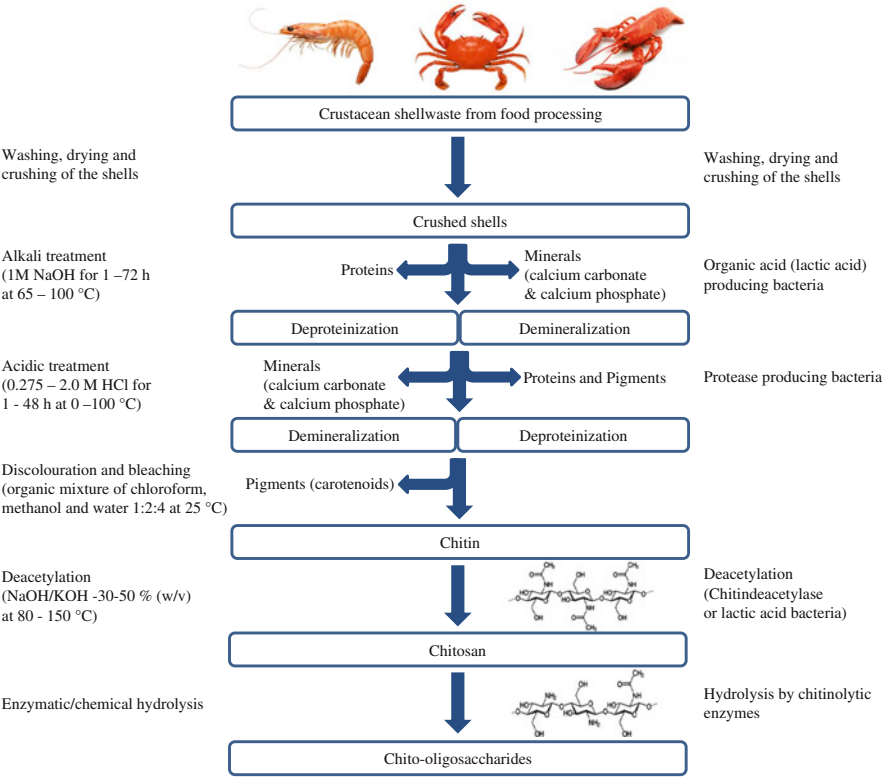

Fig. 14.2 Extraction of chitin from crustacean shell waste by chemical and biological methods

processes were developed to prepare pure chitin and chitosan from shell waste material and are commercially used (Fig. 14.2). The processing steps determine the properties of the final product, such as purity, acetylation degree, and molecular weight. Depending on the process parameters, the resulting products have different properties which have a strong impact on their applicability. All processes follow in general the same route from deproteinization (DP) and demineralization (DM), discoloration (DC), and in the case of chitosan production to deacetylation (DA).

3.1 Classical Chemical Processing of Crustacean Shells for Chitin and Chitosan Production

DP is performed by alkaline treatment using high amounts of NaOH and increased temperatures. DM is performed by acid treatment using HCl, HNO_3, H_2SO_4, CH_3COOH, and HCOOH, with HCl as preferred agent [8, 47]. Although NaOH and HCl treatment results in nearly complete removal of proteins and minerals, these

harsh conditions lead to undesired side reactions such as polymer hydrolysis and deacetylation, impairing the final product quality [48–51]. Different chitin qualities can therefore be explained by the used chemicals in their corresponding processing steps. Pigments, mainly melanin and carotenoids, are finally removed by treatment with potassium permanganate, hydrogen peroxide, sodium hypochlorite, or organic solvents resulting in a white colorless product. The final step in chitosan preparation is performed either by a harsh alkaline hydrolysis treatment or by enzymes. Chitosan with different deacetylation degrees can be obtained by the variation of process temperature and incubation time. The most important parameters in chitin and chitosan synthesis are the final product quality in terms of molecular weight, acetylation/deacetylation degree, and polydispersity. As already mentioned, the usage of harsh chemicals such as HCl and NaOH in the processing of chitin can have a negative impact on the product quality, limiting its use in applications. Quality improvements can be achieved by using stirred reactors to increase the contact between the material and the chemicals and therefore reducing reaction times and needed reaction temperature [13]. Besides the product quality impacts of the used chemicals, they also create a waste problem, since neutralization and decontamination of the wastewater are necessary. Therefore, the chemical processing of crustacean shell waste is neither sustainable nor environmentally friendly. In view of these disadvantages, eco-friendly, efficient, and economically viable technologies are needed.

3.2 Biotechnological Processing of Crustacean Shells for Chitin and Chitosan Production

An alternative to the chemical extraction route for chitin is to use biological approaches. The biological methods for chitin extraction follow the same route as the chemical ones (Fig. 14.2). By using enzymes and microorganisms instead of harsh chemicals, the biological processing of crustacean shell waste has the advantages of being more eco-friendly, safe, technologically flexible, and economically feasible [52, 53]. Ensiling of these waste materials is the oldest method using lactic acid-producing microorganisms [54]. By using low-cost substrates, such as lignocellulose, an economically feasible processing is possible. The lactic acid produced by the microorganisms during ensiling is responsible for the demineralization by reacting with calcium carbonate forming calcium lactate, which can be precipitated and removed. Through the lowering of the pH, proteases are activated, which then catalyze the deproteinization, leading to a liquefaction of the nonsolid compounds of crustacean shell waste. The liquor is rich in proteins, amino acids, fats, and carotenoids [55]. Additionally, the low pH suppresses the growth of spoilage microorganisms. Besides these pure microbial processes, several studies examined the usage of commercial enzymes for deproteinization [56, 57] or demineralization [58] alone

or in combination with microbial agents [59, 60]. Also the combination of lactic acid fermentation with chemical steps has been investigated [61–63]. In general, three different strategies for the biological deproteinization and demineralization of crustacean shell waste were developed: (1) lactic acid fermentation, (2) non-lactic acid fermentation, and (3) co-fermentation. Table 14.1 gives a comprehensive overview of studies in these fields. In the following section, only selected examples are presented.

3.2.1 Lactic Acid Fermentation

Starting from shrimp waste, Rao et al. [66] used *L. plantarum 541* and controlled the pH during fermentation. By using acetic acid at a pH of 6 and a surplus of glucose, approximately 75% DP and 86% DM could be achieved. In the absence of pH control, only 68% DP and 64% DM were reached. Fermentation of minced scampi waste (*Nephrops norvegicus*) using *Lactobacillus paracasei A3* and added glucose resulted in 77.5% DP and 61% DM after 5 days at 30 °C, while the solid fraction contained 17.5% chitin (dry mass) [88]. Crayfish waste was also used in fermentation with *L. paracasei* and dextrose yielding 94% DP and 97% DM [89]. A mixture of proteolytic enzyme-producing bacteria (*L. plantarum, L. salivarius, S. faecium*, *and P. acidilactici*) was used for the anaerobic fermentation of prawn [90]. This showed to be an effective method to break down shell waste. 91% DM efficiency was reached on average in combination with nearly unmodified chitin which was identified through elemental analysis measuring the nitrogen content of the resulting material.

3.2.2 Non-lactic Acid Fermentation

The amount of acid and proteases produced by *Bacillus subtilis* during fermentation allowed shell demineralization as well as deproteinization. Using shrimp shell waste (*Metapenaeopsis dobsoni*), the fermentation process yielded 84% DP and 72% DM [91]. *Pseudomonas aeruginosa* strain K-187 produces proteases as well as chitinases and lysozyme during cultivation with shrimp and crab shells. After 5 days DP reached 82% in a solid-state process [92]. Using *P. aeruginosa F722* with crab shell wastes at 30 °C, 92% DM and 63% DP after 7 days were reported [93]. The deproteinization and demineralization of crab shell waste using *Serratia marcescens FS-3* reached 47% and 84% after 7 days. In combination with 1% Delvolase®, the deproteinization could be increased from 47 to 90%. For the degradation of shrimp shell waste, *Bacillus cereus* and *Exiguobacterium acetylicum* were used in a fermentation at 37 °C [94]. Deproteinization yielded 97 and 93% and demineralization 95 and 92%, showing the huge potential of these strains for chitin-rich waste processing.

Table 14.1 Fermentation-mediated extraction of chitin from crustacean shell wastes

Waste source	Strains and/or proteolytic enzymes	DP	DM	Refs.
Lactic acid fermentation				
Penaeus sp.	*Lactobacillus* spp. B2	85	87.6	[64]
Demineralized *Nephrops norvegicus*	Stabisil: *Streptococcus faecium* M74, *L. plantarum, Pediococcus acidilactici*	40	n.d.	[61]
Nephrops norvegicus	Sil-All$^{4 \times 4}$: *L. plantarum, L. salivarius, S. faecium, P. acidilactici*	n.d.	90.99	[65]
Nephrops norvegicus	*L. paracasei* A3	77.5	61	[63]
One-step shrimp fermentation	*L. plantarum* 541	75	86	[66]
Pretreated *Procambarus clarkii* (crayfish)	*L. paracasei* A3	94	97.2	[67]
Procambarus clarkii	Immobilized *Lactobacillus pentosus* 4023	81.5	90.1	[68]
Chionoecetes japonicus	*L. paracasei* ssp. *tolerans* KCTC-3074	54.7	55.2	[69]
Parapenaeus longirostris	*L. helveticus*	91	44	[70]
Shrimp shell	*Lactobacillus plantarum* PTCC 1058	–	82% (date syrup) 75% (glucose) 71% (sucrose)	[71]
Shrimp bio-waste	Non-amylolytic strain *L. plantarum* 541	59.8	81.4	[72]
	Amylolytic strain *L. plantarum* A6	52.2	65.5	[72]
Crab shell (CS) waste	*L. paracasei* subsp. *tolerans* KCTC-3074	–	89–92	[73]
The teguments of white shrimp, *Parapenaeus longirostris*	*L. helveticus* strain Milano	76	60	[70]
Non-lactic acid fermentation				
Metapenaeus dobsoni	*Bacillus subtilis*	84	72	[74]
Shrimp and crab shell	*Pseudomonas aeruginosa* K-187	82	–	[75]
Shrimp and crab shell powder	Proteases of *P. aeruginosa* K-187	72	–	[76]
Natural shrimp shells	Immobilized proteases of *P. aeruginosa*	78	–	[76]
Acid-treated natural shrimp shell	Immobilized proteases of *P. aeruginosa*	45	–	[76]
Shrimp and shell crab powder	Immobilized proteases of *P. aeruginosa*	67	–	[76]
Crab shell powder	*P. aeruginosa* F722	63	92	[74]

(continued)

Table 14.1 (continued)

Waste source	Strains and/or proteolytic enzymes	DP	DM	Refs.
Chionoecetes opilio (natural crab shell waste)	*Serratia marcescens* FS-3	47	84	[77]
	Delvolase®	90	–	[77]
	Combination of Delvolase® and *Serratia marcescens* FS-3	85	–	[77]
	S. marcescens FS-3 supernatant culture	81		[77]
Shrimp shell waste	*Bacillus cereus*	97.1	95	[78]
	Exiguobacterium acetylicum	92.8	92	[78]
Squid pen	*Bacillus sp*. TKU 004	73	n.d	[79]
Penaeus monodon	*Pediococcus acidilactici* CFR2182	97.9 ± 0.3	72.5 ± 1.5	[52]
Shrimp shells	*Pediococcus sp*. L1/2	n.d.	83	[80]
Fresh shrimp waste (FSW) or shrimp waste powder (SWP)	*Pseudomonas aeruginosa* A2	56% and 85% for SWP and FSW	–	[81]
Shrimp waste	Crude alkaline proteases extract from the viscera of the striped seabream (*Lithognathus mormyrus*)	79	–	[82]
Shrimp waste	*Bacillus cereus* SV1	88	–	[83]
Co-fermentation				
Two-step fermentation of *Penaeus monodon* and *Crangon crangon*	First step: anaerobic deproteinization by autochthonous flora of Indonesian shrimp shells and/or proteolytic bacteria	97.4	99.6	[84]
	Second step: *L. casei* MRS1	90.8	99.7	[84]
Prawn waste	*Lactobacillus lactis*	66.5	78.8	[85]
	Teredinibacter turnerae	77.8	23.3	[85]
	Co-fermentation of both species	95	95	[85]
Red crab shell waste	One-step fermentation: *L. paracasei* ssp. *tolerans* KCTC-3074 and *S. marcescens* FS-3	52.6	97.2	[86]
	Successive two-step fermentation	94.3	68.9	[86]
	Two *Bacillus licheniformis* strains with treatment of the final fermentation product with 0.9% lactic acid	99	98.8	[87]

3.2.3 Co-fermentation

For the extraction of chitin from prawn waste, lactic acid-producing bacterium *L. lactis* and a protease-producing bacterium *Teredinibacter turnerae* were combined. Both bacteria were cultivated separately and used in combination for fermentation. *L. lactis* alone reached a DP of 66% and DM 79%, while *T. turnerae* alone reached 78% DP and 23% DM [95]. The co-fermentation of *L. paracasei* ssp.

tolerans KCTC-3074 with *S. marcescens FS-3* yielded a DM level of 97% but only a DP of 53% after 7 days with red crab shell waste [71]. By using both strains in a successive two-step fermentation process, the DP efficiency was nearly constant at 94% and the DM efficiency was increased to 69% [96]. Two from shrimp shell waste isolated *B. licheniformis* strains were used for the fermentation of shrimp shells [87]. After optimization of fermentation parameters and a subsequent demineralization step with 0.9% lactic acid after fermentation, 99% DP and 99% DM could be reached.

4 ChiBio: A Chitin Bio-refinery

In general a bio-refinery is the sustainable processing of biomass into a spectrum of marketable products. At the beginning of the development of bio-refineries, all kind of biomass was used, especially for the production of biofuels, raising the food or fuel discussion [97]. Nowadays, novel-developed bio-refinery concepts only use biomass waste streams, e.g., straw, as starting material [98]. Within the EU-funded project ChiBio, a bio-refinery on the basis of crustacean shell waste was developed. It aims at the development of a novel bio-refinery process for a sustainable, waste-free, low energy conversion route of negative value crustacean shell waste streams into high-value, high-performance chemical intermediates and products for the polymer industry. As stated before, crustacean shells are a waste product of food production from shrimps, prawns, or lobsters all over the world. Most of this shell waste is dumped into the sea, which is a main pollutant to coastal areas. In Europe, sea dumping is forbidden, and a cost-intensive proper waste management is a burden for the fishery industry [99]. There are more than 300,000 t/a of shell waste material available in Europe alone, showing the potential of this yet under-explored biomass. As processing of crustacean shell wastes is currently mainly chemically based with all the negative environmental and ecological impact, the ChiBio project targeted the development of a bio-refinery concept using state-of-the-art biotechnological methods for the processing of crustacean shell waste in a sustainable and eco-friendly manner (Fig. 14.3). Starting from the raw material, an effective biological treatment of the shell waste was developed yielding chitin, calcium carbonate, and a protein- and lipid-rich liquid. Chitin is further processed into its basic building blocks glucosamine and *N*-acetylglucosamine by enzymatic degradation. Both molecules were used as substrates for the production of novel bio-based monomers, which were finally evaluated in polymer materials to reveal novel applications in the material sector. All by-products of the ChiBio bio-refinery, e.g., calcium carbonate and the protein- and lipid-rich liquid, are valuable side products for their usage in the construction and the biogas industry. In connection with a life cycle analysis, ChiBio showed that a sustainable bio-refinery concept for crustacean shell waste is feasible and sustainable.

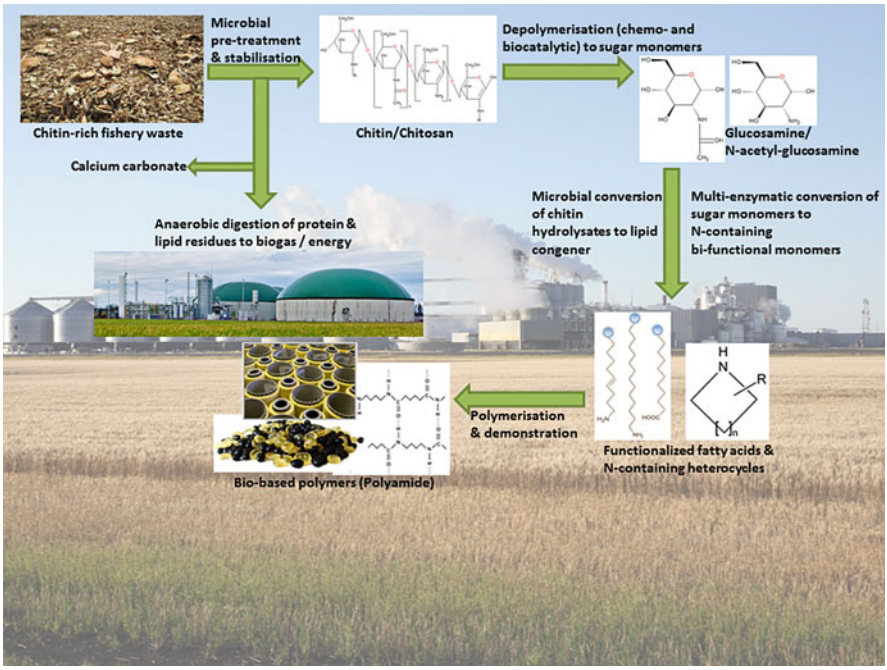

Fig. 14.3 Schematic overview of the ChiBio bio-refinery process. Chitin-rich crustacean shell waste is deproteinized and demineralized by microorganisms to chitin/chitosan. As by-products calcium carbonate and a liquid rich in protein and lipids are obtained, which are raw materials for the construction industry and for the production of biogas. Chitin and chitosan are further depolymerized to their basic building blocks glucosamine and N-acetylglucosamine. Through microbial and enzymatic conversion technologies, these building blocks are transformed into functional fatty acids and N-containing heterocycles, which are suitable building blocks for the production of novel bio-based polymers

4.1 Pretreatment

For the pretreatment step developed within ChiBio, we compared the demineralization and deproteinization of brown crab shell using conventional chemical methods and optimized biological methods using commercial *Lactobacillus* spp., *Pseudomonas aeruginosa,* and *Serratia marcescens* strains in addition to proteolytic and acid-producing bacterial isolates from crab shell waste.

An initial survey of the mineral content of obtained brown crab shell waste showed that calcium in the form of $CaCO_3$ was by far the most abundant mineral.

In 5-day fermentations utilizing the mixed exogenous microbiota present on the shell, ash mineral content and FTIR results showed that self-fermented crab shells were neither fully decalcified nor deproteinized. At the end of the fermentation, a pH

of 5.7 ± 0.4 was observed with a yield of chitinous material of 31.80 (±0.07) %, an ash content of 45.00 (±1.20) %, and a calcium content of 3.4 (±0.5) g per 100 g. Untreated crab, in comparison, contained 66.30 (±0.06) % ash and 4.3 (±0.1) g per 100 g calcium. Chemically produced chitin from brown crab treated using 1 mol/l hydrochloric acid and 1 mol/l sodium hydroxide yielded 15.19 (±0.04) % chitinous material with 4.50 (±0.05) % ash and 0.94 (±0.36) g per 100 g calcium [100]. During a 5-day fermentation using 1×10^6 cfu ml^{-1}, each of a combination of *Bacillus cereus* and *Pseudomonas spp.* isolated from crab shell, resulted in 98.8% demineralization with an overall yield of chitinous material of 15.4 (±1.56)%. The degree of acetylation was 81.9 (±1.0) % as calculated from FTIR analysis [101]. Experiments were performed using either 5 or 10 g of shell waste in 100 ml of a 10% (w/v) glucose solution. All of these initial experiments were performed in a shaking incubator at 175 rpm and 30 °C using a shell particle size ranging from 250 to 750 μm.

One-step fermentations at 30 °C using 1×10^6 cfu per ml of commercial strains *Serratia marcescens* (DSMZ 30121), *Pseudomonas aeruginosa* (DSMZ 8924), or *Pseudomonas aeruginosa* (DSMZ 7232) yielded unsatisfactory results for either deproteinization or demineralization. Subsequently, further trials using sequential fermentations incorporating 1×10^6 cfu ml^{-1} of the lactic acid bacterium *Lactobacillus plantarum* subsp. *plantarum* (DSMZ 20174) to further reduce the overall pH of the reaction were performed. The most effective fermentation was obtained in a sequential two-step fermentation using *S. marcescens* (DSMZ 30121) for 5 days and *L. plantarum* subsp. *plantarum* (DSMZ 20174) for 7 days, both at 30 °C in a shaking incubator at 180 rpm. The use of these organisms successfully decreased the pH rapidly from pH 6.07 ± 0.2 from the first (*S. marcescens*) fermentation to 4.6 ± 0.3 using *Lactobacillus plantarum* subsp. *plantarum*. No difference in acid production was observed between 8% (w/v) and 10% (w/v) glucose. The decrease in pH was rapid enough to inhibit the endogenous microbiota as observed by plate culture. The overall yield of chitinous material from the whole brown crab by the two-step fermentation was 19 (±0.02) %, an ash content of 7.0 (±0.1) %, and a calcium content of 2.1 (±1.0) g per 100 g. The degree of acetylation was 82 (±11) % as calculated from FTIR analysis [100]. This was comparable to the yield obtained with native microorganisms isolated from crab shell and from other studies [102]. Like the traditional chemical treatment using 1 mol/l hydrochloric acid and 1 mol/l sodium hydroxide, the production of acids using the facultative heterofermentative and aero-tolerant *L. plantarum* also allowed for successful removal of $CaCO_3$ by predominantly producing D- and L-lactate by stereospecific lactate dehydrogenase enzymes [103]. In literature, demineralization efficiencies of 94.3% and >99% were commonly achieved by lactic acid-producing bacteria and with ±10% glucose as a carbon source [56, 84]. Demineralization efficiency, as seen by FTIR spectroscopy, of 93% using 8% glucose was achieved using the methods established by ChiBio. *S. marcescens* was used to effectively remove residual proteins from the shells through the production of extracellular proteases without affecting the degree of acetylation from endogenous chitinases [86]. A successive two-step fermentation *using S. marcescens* followed by *L. plantarum* was required to achieve optimal

results for the removal of residual proteins and minerals from brown crab since co-fermentations were ineffective due to differing acid tolerances and generation times under prescribed conditions. The order of the fermentative organisms applied was important as demineralization proved more effective when the shell was already deproteinized. Fermentation times were comparable to other studies demonstrating that the use of proteolytic organisms and lactic acid fermentation could provide a viable alternative to chemical treatments for the extraction and recovery of chitinous materials even though the calcium content is still higher than in commercial samples available today. However, the process costs for microbial chitin recovery as demonstrated here do not support the recovery of chitin from crustacean waste using a mainstream carbon source such as glucose even though cost analysis revealed similar expenditures to chemical production (approx. USD20/kg [100]). Other low-cost carbon sources such as molasses, whey, corn steep liquor, lignocellulose, and other waste streams could be potentially substituted and could in turn significantly reduce production costs.

4.2 Enzymatic Chitin Depolymerization

The microbial processed chitin was used as starting material for the further degradation into its basic building blocks glucosamine and *N*-acetylglucosamine. Two strategies were followed to yield enzyme systems for the targeted degradation of chitin: (1) chitinolytic enzyme cocktails from chitin-degrading microorganisms and (2) cloning and heterologous expression of single chitin-degrading enzymes for their usage in defined enzyme reactions.

4.2.1 Chitinolytic Enzyme Cocktails

Chitin-degrading microorganisms are well-known, and in-depth analysis of their enzymatic machinery has been done for some microorganisms [104, 105]. *Serratia marcescens* is one of the best known organisms, with well-defined chitinolytic enzyme machinery. It includes four enzymes, ChiA and ChiB (EC 3.2.1.14), which are in opposite direction working chitinases; ChiC (EC 3.2.1.14), an endo-acting non-processive chitinase; and CBP21 (EC 1.14.99.B7), a lytic polysaccharide monooxygenase that acts through oxidative cleavage. These enzymes degrade the chitin polymer into short-chain oligomers, which are then processed by the enzyme chitobiase, *N*-acetylhexosaminidase, into monomeric *N*-acetylglucosamine [106]. Besides *S. marcescens*, also *Cellvibrio japonicus*, *Amantichitinum ursilacus* (*A. ursilacus*), and *Andreprevotia ripae* were analyzed for their chitinolytic potential within ChiBio [107, 108]. In a first step methods for the production of chitinolytic enzyme cocktails of the natural strains were developed, which were then used for the development of a chitin degradation process. The strains were cultivated under standard conditions using M9 media with 2% chitin (w/v) at 37 °C under shaking

for 5 days. The broth was then centrifuged to remove cells and chitin, and the supernatant was filtered through 0.22 μM filters and used as chitinolytic enzyme cocktail. The final aim was to generate hydrolysates containing the chitin monomers glucosamine and *N*-acetylglucosamine from the pretreated crustacean shell waste. The best results were obtained with the chitinolytic enzyme cocktail from *S. marcescens*, hydrolyzing up to 77% of the used chitin at 50 °C and pH 6 after 24 hours. To reach this degree of depolymerization, it was necessary to mill the material to a fine powder with an approximate particle size of 0.2 mm, larger particles reduced the efficiency. In addition the used chitin was from a chemical pretreatment step. Using biologically derived chitin reduced the yield to 20% under the same conditions.

4.2.2 Chitin-Degrading Enzymes

All described enzymes from *S. marcescens* were overexpressed in *E. coli* and purified by affinity chromatography [109]. For optimization of the mono-component enzymes, the amount of ChiA, ChiB, ChiC, and CBP21 was varied from 0 to 90% and analyzed in combination with varying process parameters (pH, temperature, and incubation time). Depending on the chitin source and the pretreatment method, different enzyme cocktails yielded optimal conversion rates (Table 14.2). For chemically pretreated brown crab shell chitin, a monomer yield of 57.3% could be achieved (ChiA 30.4%, ChiB 28.1%, ChiC 10.6%, CHB 10.1%, and CBP21 20.8%). On the other hand, biologically pretreated brown crab shell chitin only yielded 45.9% monomer (ChiA 39.6%, ChiB 28.9%, ChiC 21.0%, CHB 10.0%, and CBP21 0.5%). For comparison commercially available chitin yielded 61.7% monomer (ChiA 38.1%, ChiB 29.7%, ChiC 14.0%, CHB 10.2%, and CBP21 8%). The data shows the potential of the enzymatic toolbox for the targeted degradation of chitin. In addition this data clearly reveals that independent on the chitin composition, different enzyme mixtures are necessary to maximize the monomer yields.

Although neither chitinolytic enzyme cocktails nor mono-component enzyme mixtures gave full conversion of chitin into its monomers, these tools open the route for the production of glucosamine and *N*-acetylglucosamine. The combination

Table 14.2 Summary of optimization of chitin active enzymes for maximal degradation of benchmark substrates

Chitin source	Pretreatment	ChiA (%)	ChiB (%)	ChiC (%)	CHB (%)	CBP21 (%)	Yield (%)
Commercial chitin	Chemical	38.1	29.7	14	10.2	8	61.7 ± 3.5
Brown crab shells	Chemical	30.4	28.1	10.6	10.1	20.8	57.3 ± 1.7
Brown crab shells	Biological	39.6	28.9	21.0	10.0	0.5	45.9 ± 1.5

of both systems might be a way to realize the full conversion of chitin into its basic building blocks.

4.3 Monomer Synthesis

The demand for plant- and animal-based lipids for the food and pharmaceutical industry and for the production of biofuels has driven the search for new sources of relevant lipids, such as the very-long-chain omega-3 (ω-3) polyunsaturated fatty acids (VLC-PUFAs), eicosapentaenoic acid (EPA; 20:5 Δ5,8,11,14,17), and docosahexaenoic acid (DHA; 22:6 Δ4,7,10,13,16,19). To date, these PUFAs are derived primarily from fish and crustaceans, which results on a negative impact on the marine food chain and related ecosystems [110, 111]. The need for more environmentally friendly alternatives of VLC-PUFAs has become more economically competitive driving forward the establishment of new technologies focused on providing ω-3 VLC-PUFAs from plant, algae, and yeast biomass. In particular, advances in process and metabolic engineering of oleaginous yeasts such as *Yarrowia lipolytica* take advantage of fast growth rates and high yields of designer lipids in combination with waste biomass feedstocks to offer a sustainable production approach for non-food lipid production [112, 113]. However, most *Y. lipolytica* are ex novo lipid producers and thus require fatty acids in their growth medium.

In the course of the ChiBio project, a new de novo lipid-producing yeast from a crab shell waste disposable site was isolated, which by 18S rDNA and ITS taxonomic identification was classified as *Trichosporon oleaginosus* (phylum *Basidiomycota*, order *Tremellales*). Initial physiological characterization showed that this strain could metabolize *N*-acetylglucosamine as well as a range of other pentose and hexose sugars without any metabolic preferences, making it ideal for fermentative high-value lipid production on cost-efficient waste biomass hydrolysates or alternative biotechnological waste streams [114]. Enzymatically liquefied crude crab shell hydrolysate proved to be a sufficient growth substrate for the yeast and de novo lipid accumulation. *N*-acetylglucosamine, a monomeric product of the enzymatic degradation of chitinous materials, was used as a carbon and energy source without adverse effects on de novo lipid biosynthesis, indicating high tolerance of higher nitrogen concentrations. However, due to the nitrogen content of *N*-acetylglucosamine, only phosphate limitation could be utilized for the induction of lipogenesis. Nonetheless, lipid accumulation was 35% (w/dcw) in *Trichosporon* fermentations after 72 hours and remained constant up to 168 hours. At 24 hours about 50% of the final lipid content was already present. However, with fermentation substrates where *N*-limiting conditions could be applied, *Trichosporon* sp. was able to accumulate up to 60% (w/dcw) triglycerides. The lipid fraction based on crude crab shell hydrolysate as a feedstock consisted of 47% (w/w) oleic acid as principal fatty acid component which is a suitable building block in the polymer and lubricant industry. For the production of VLC-PUFAs such as alpha-linoleic and eicosadienoic acid, genetic engineering approaches of fatty acid biosynthesis

pathways were surveyed. With no detailed genome information and molecular biology tools at hand for *Trichosporon*, a random genome integration protocol was developed [115]. Initial gene transfer methods using conventional techniques, such as electroporation, were unsuccessful. However, in the course of the ChiBio project, an *Agrobacterium tumefaciens*-mediated DNA transfer protocol used in plant engineering was adapted for *Trichosporon* applications. Existing plasmid systems were modified and utilized reported promoters from related *Basidiomycetes*. These strategies allowed random genomic integration of recombinant genes into *Trichosporon* sp. However, the functional expression of the genetic elements was inefficient. Whole genome sequencing of *Trichosporon oleaginous* recovered the full-length sequence of native, constituent promoter systems [116]. The native *Trichosporon* promoter regulating the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase finally provided for strong, constitutive expression of recombinant genes [117]. Subsequently, various *Trichosporon* mutants that produce enzymatically modified fatty acid profiles could be generated. A detailed GC-MS-based fatty acid profiling of the *Trichosporon* mutants showed that in vivo desaturation of linoleic acid to yield alpha-linoleic acid and its congener eicosatrienoic (ETE) and eicosadienoic acid (EDA) was particularly successful.

4.4 Novel Bio-based Polymers from Chitin-derived Monomers

ChiBio aims to develop novel value chains by producing bio-based monomers for the polymer industry on the basis of chitin-derived building blocks. Therefore, ChiBio-derived building blocks were used for initial technical trials to produce polyamide test bars as demonstrators and to characterize their mechanical properties. From the two monomers, being 1.19-nonadecane dicarboxylic acid and an aromatic dicarboxylic acid (ADCA), respectively, the latter was chosen for such demonstrator activities. The co-polyamide 6.12 doped with 3% of the ChiBio ADCA had been polymerized at pilot-plant scale. The granulate polymer as obtained was then used to manufacture testing bars by injection molding as the demonstrator production series. It could indeed be molded under such common industrial conditions; however most machine parameters had to be adjusted to quite uncommon values. The demonstrator production series yielded a good number of test bars. With this batch of demonstrator parts, the physical mechanical properties of this ChiBio-influenced polyamide had then been scrutinized. These demonstrator specimens exhibited a sharp drop of ductility features compared to neat homo-PA6.12 (Table 14.3). Nevertheless, the co-polyamide could be processed under standard technical conditions, in the present case on common injection-molding equipment, albeit with processing parameters unusual for thermoplastic polymers. Toward future applications and developments, using ADCA as the comonomer in bulk machined parts appears unlikely, considering the lack of ductility performance. However, it might likely be fruitful when looking at

Table 14.3 Characteristic physical mechanical data of the bulk-produced polyamide (PA) 6.12, containing 3% of one of the ChiBio key monomers, ADCA

Properties	Measurement values	
E-modulus [MPa]	2900 ± 100	
Tension at break [MPa]	6.5 ± 0.1	
Elongation at break [%]	0.2 ± 0.1	
Impact strength (+23 °C and −30 °C, resp.; unnotched)	1.7 ± 0.1	1.3 ± 0.1
Impact strength (+23 °C and −30 °C, resp.; notched)	0.8 ± 0.1	0.5 ± 0.1

surface applications, given that this monomer would render a co-polyamide equipped with a high degree of heteroaromatic and NH functional chemical moieties.

4.5 Life Cycle Analysis

The utilization of crustacean waste for the production of value-added products is a viable option for the European fishery industry as about 60 w/w-% of the crustacean catch is accumulating as waste. Besides potentially profiting from selling value-added products, the saving of disposal costs which range from about 60 €/t for landfilling to 160 €/t for incineration could create an additional boost for the concept, and illegal ocean dumping could be avoided.

The main cost factors identified in the economic process analysis are the stirred tank reactors for the pretreatment, the *Lactobacillus* seed, the enzymatic depolymerization, and especially the monomer synthesis. Summarized, at this early state the process is not cost-efficient enough. The presented estimations are not integrating effects of experience and learning curves. As approximation learning rates decrease the unit costs of technologies by constant percentage for each doubling of experience. This process runs over time and leads to a decrease of unit costs [118]. A progress ratio of about 10–15% seems to be appropriate for a couple of technologies. A massive process development and further intense research on the process time and particularly enzyme efficiency could eventually result in a marketable price.

International reported prices range between USD6/kg for plain chitin and USD20/kg for pharma grade chitosan as a benchmark for the pretreatment section and raw material supply. Consequently the pretreatment of the raw material to yield the chitin is a key step in the process starting from a material with negative to low input price resulting to a significant price of pure chitin/chitosan. This cost structure and the various competing application of chitin/chitosan derivatives require an integrative bio-refinery approach including cost-effective biotechnological pretreatment as substitute for the harsh conditions and high chemical load in the chemical processing route. Despite positive results presented in the literature [68, 119, 120], an effective biotechnological pretreatment procedure could not be established within the project. Future research effort has to address this process step within the bio-refinery process chain.

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