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REVIEW

Progress in bioextraction processes of chitin from crustacean biowastes

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Abstract The most common methods for the recovery of chitin from crustacean shells are chemical procedures using strong acids and bases. The chemical demineralization and deproteinization processes have several drawbacks such as depolymerization, anomerization, and deacetylation. Moreover, chemical treatments are hazardous, energy consuming, and environmentally unfriendly. As an alternative to chemical processes for extracting chitin, fermentation processes using microorganisms or proteolytic enzymes have been studied for decades for various crustacean shells. Bioextraction of chitin is emerging as a green and eco-friendly process, thus overcoming some of the shortcomings of chemical extraction. Microorganism-mediated fermentation processes are desirable due to easy handling, simplicity, controllability through optimization of process parameters, ambient temperature, and less environmental impact. The main drawbacks of the biotechnological process are the lower yield and quality of the products and thus a higher cost than the chemical processes. We briefly discuss the critical issues encountered in biotechnological processes for chitin recovery from crustacean shells, together with compiling the most advanced findings during the last decades.

Keywords Bioextraction · Chitin · Crustacean waste · Demineralization · Deproteinization

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Introduction

Crustacean shells are the most important source of chitin for commercial use due to their high chitin content and availability. Chitin is tightly associated with calcium carbonate, proteins, lipids, and pigments in crustacean cuticles. Chitin is isolated from crustacean shells in three steps: demineralization (DM), deproteinization (DP), and elimination of lipids and pigments. The chemical methods for the preparation of chitin from crustacean shell waste consist of mechanical grinding, DM with strong inorganic acids, and DP with alkali at elevated temperature (Aye and Stevens 2004; No et al. 1989; Percot et al. 2003). The chemical DM and DP processes have several drawbacks such as poorly controlled depolymerization, resulting in the reduction of molecular weight and viscosity and hydrolytic deacetylation. In addition, chemical treatments engender hazardous environmental problems such as disposal of wastewater, making this process ecologically aggressive and a source of pollution to the environment because of the high concentration of mineral acids and caustic chemicals employed (Gortari and Hours 2014). Chemical processes also render the protein component useless (Cheong et al. 2014; Manni et al. 2010; Pacheco et al. Sini et al. 2007; Xu et al. 2008).

As an alternative to chemical processes for extracting chitin, fermentation processes using proteolytic microorganisms or proteolytic enzymes have been studied for decades for various crustacean shells such as crab shells (Jo et al. 2008; Jung et al. 2006; Oh et al. 2007), shrimp waste (Cira et al. 2002; Xu et al. 2008; Younes et al. 2012), crayfish exoskeleton (Bautista et al. 2001; Cremades et al. 2003), scampi waste (Zakaria et al. 1998), and prawn waste (Fagbenro 1996). Upon fermentation of crustacean wastes, two additive portions of protein and organic acid salts

could be recovered for feed, fertilizer, and chemical reagent purposes (Manni et al. 2010; Oh et al. 2007; Sini et al. 2007; Sorokulova et al. 2009; Xu et al. 2008). Along with increased demands for an environmentally friendly society, more eco-friendly processes using enzymatic and microbiological methods for producing chitin have attracted great interest in the field of green biotechnologies. Procedures for chitin and chitosan production by chemical and biotechnological treatments are briefly compared in Fig. 1 (Jo et al. 2010).

Many critical reviews are available on the production, characteristics, and applications of chitin and its derivatives (Domard 2011; Gortari and Hours 2014; Jung and Park 2014; Kurita 2006; Pillai et al. 2009; Surinder and Singh 2015; Synowiecki and Al-Khateeb 2003; Tharanathan and Kittur 2003). Chitin production from crustacean shell waste through biotechnological means is still at its early stage of development but it is undergoing rapid progress in the recent years and showing a promising prospect (Daum et al. 2007; Healy et al. 2003). Recently, Gortari and Hours (2014) typically reviewed a potential of biotechnological processes using microbial fermentation and enzymatic extraction for chitin recovery out of crustacean waste. In this review, we briefly review and discuss the critical issues encountered in biotechnological processes for chitin recovery from crustacean shells, together with compiling the most advanced findings during the last decades as in Tables 1 and 2.

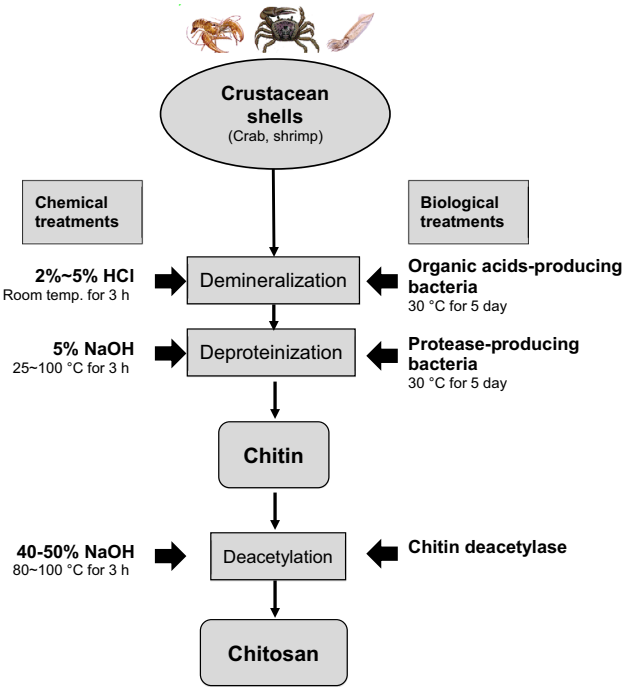

Fig. 1 Chitin and chitosan production by chemical and biological treatments

Rapid acidification with addition of organic acid

A major problem with crustacean shell fermentation is the high perishability of the material (Ploydee and Chaiyanan 2014; Prameela et al. 2010; Rao et al. 2000, 2001; Wang and Chio 1998). Appropriate technology should be applied to prevent decay and to convert the biomaterial into valuable products. Rapid acidification of the medium is very important, especially on an industrial scale, to preserve the shell materials, thus minimizing a possible reduction in chitin polymers. Rao and Stevens (2005) treated shrimp biowaste with *Lactobacillus* and acetic acid to reduce the initial waste pH and the spoilage of biowaste, which resulted in a higher DM and lower DP than individual treatments. Through the inoculation with *Lactobacillus,* a high-quality protein liquor was produced. This result showed that the combined treatment of *Lactobacillus* and acetic acid was a choice for the suppression of growth of spoilage microorganisms and thus putrefaction in waste shells, and therefore being an environmentally friendly procedure for the fermentation of shrimp waste (SW). Suppression of the putrefaction was observed at pH values below 5.5. Rapid acidification of the medium using organic acids could be an efficient method of preservation, allowing the recovery of proteins, pigments, and enzymes from crab or shrimp shell wastes (Rao and Stevens 2005; Wang and Chio 1998). The addition of glucose also facilitates medium acidification through organic acid production, which suppresses the growth of spoilage microorganisms. That is why lactic acid-producing bacilli were mostly adopted for the acidification and decalcification processes (Adour et al. 2008; Gortari and Hours 2014; Shirai et al. 2001). Over the first 48 h of scampi waste fermentation in 10 % glucose and 10 % inoculum (*Lactobacillus paracasei* strain A3), the pH of the liquor reached a minimum value of 5.0 (Zakaria et al. 1998).

Large-scale extraction of chitin

The biotechnological processing of crustacean wastes is one of the economic and environmental advantages, because it is relatively simple, less polluting, and less expensive, thus overcoming some of the shortcomings of chemical treatment. However, it suffers from its low efficiency of DM and DP, as being the major disadvantage. Thus, improvement of the efficiency and quality is the most important challenge to overcome. Less-expensive and more eco-friendly technology for large-scale extraction of chitin remains to be developed in the future, as environmental regulations become stricter. At present, most studies are performed in shaking flasks under laboratory conditions and thus may not be applicable for large-scale operations.

Table 1 Summary of microbial treatment of crustacean shell wastes for chitin production

Shell sources	Microorganisms	Products	DM (%)	DP (%)	Reference
Red crab (*Chionoecetes japonicus*)	*Lactobacillus paracasei* KCTC-3074	OA[a]	97.2	52.6	Jung et al. (2006)
	Serratia marcescens FS-3	Protease	94.3	68.9	Jung et al. (2007)
	Pseudomonas aeruginosa F722	OA, protease	92.0	63.0	Oh et al. (2007)
Crab (*Callinectes bellicosus*)	*Lactobacillus* sp. B2	Lactic acid	88.0	56.0	Flores-Albino et al. (2012)
Squid pen (–)	*Bacillus* sp. TKU004	Protease	–[a]	73.0	Wang et al. (2006)
Shrimp (*Penaeus* spp.)	*Lactobacillus* sp. strain B2	OA	87.6	85.0	Cira et al. (2002)
	Lactobacillus plantarum 541	OA	86.0	75.0	Rao et al. (2000)
Shrimp (*Penaeus* spp.)	*Aspergillus niger*	Protease	–	62.2	Teng et al. (2001)
	Bacillus licheniformis	Protease	98.8	>99.0	Waldeck et al. (2006)
Shrimp (*Penaeus japonicas*)	*Pseudomonas maltophilia*	Protease	–	82.0	Wang and Chio (1998)
Shrimp (*P. monodon*)	*L. casei* MRS1	OA, protease	99.6	94.7	Xu et al. (2008)
Shrimp (*Crangon crangon*)			99.7	90.8	Xu et al. (2008)
Shrimp (*P. semisulcatus*)	*Lactobacillus* sp.	OA	–	–	Khanafari et al. (2008)
Shrimp (*Acetes chinensis*)	*Bacillus* sp. SM98011	Protease	–	–	He et al. (2006)
Shrimp (*Metapenaeopsis dobsoni*)	*B. subtilis* ACC No. 121	OA, protease	72.0	84.0	Sini et al. (2007)
Shrimp (–)	*Bacillus cereus*	–	95.0	97.1	Sorokulova et al. (2009)
Shrimp (–)	*Exiguobacterium acetylicum*	–	92.0	92.8	Sorokulova et al. (2009)
Shrimp (*Penaeus vannamei*)	*Serratia marcescens* B742	Protease	93.0	94.5	Zhang et al. (2012)
	Lactobacillus plantarum ATCC 8014	Chitinase			
Shrimp (*Metapeneaus monoceros*)	*Bacillus subtilis* A26	Protease	79.9	91.3	Ghorbel-Bellaaj et al. (2012)
	Bacillus mojavencis A21	Protease	78.7	88.0	
	Bacillus licheniformis RP1	Protease	55.6	90.8	
Shrimp (*Metapeneaus monoceros*)	*Bacillus pumilus* A1	Protease	88.0	91.0	Ghorbel-Bellaaj et al. (2013)
Shrimp (*Metapeneaus monoceros*)	*Bacillus cereus* SV1	Lactic acid	–	88.8	Mani et al. (2010)
Shrimp shells (*Litopenaus vannamei*)	*Pediococcus* sp. L1/2	–	83.5	–	Choorit et al. (2008)
Shrimp shells (*Litopenaus vannamei*)	*Lactobacillus* L7+ *Bacillus thuringiensis* SA	–	94.7	85.4	Ploydee and Chaiyanan (2014)
Shrimp waste	*Lactobacillus* sp. probiotic	–	95.6	81.9	Lopez-Cervantes et al. (2010)
Shrimp waste (*Penaeus monodon*)	Natural probiotic (milk curd)	Lactic acid	69.0	89.0	Prameela et al. (2010)
		Protease			
Shrimp waste (*Litopenaeus vannamei*)	*Lactobacillus plantarum*	Lactic acid	92.0	94.0	Pacheco et al. (2011)
		Protease			
Shrimp shells (*Penaeus vannamei*)	*Bacillus licheniformis* F11-1 + *Lactobacillus acidophilus*	–	50.2–88.7	47.4–79.6	Wahyuntari et al. (2011)
Shrimp shells (*Penaeus vannamei*)	*Lactobacillus acidophilus* SW1	Lactic acid	99.3	92.2	Duan et al. (2012)
		Protease	99.0	91.6	
Shrimp shells	*Lactobacillus plantarum* 1058	–	54.0	45.0	Khorrami et al. (2012)
Crayfish (*Procambarus clarkii*)	*Lactobacillus pentosus*-4023	OA	90.1	81.5	Bautista et al. (2001)
	L. paracasei A3	OA	97.2	94.0	Cremades et al. (2003)
Scampi (*Nephrops norvegicus*)	*L. paracasei* A3	OA	61.0	77.5	Zakaria et al. (1998)
Prawn (*Macrobrachium vollenhovenii*)	*L. plantarum*	OA	–	–	Fagbenro (1996)
Prawn shell (*Nephrops norvegicus*)	*Lactobacillus salivarius* + *Enterococcus faecium* + *Pediococcus acidilactici*	Lactic acid	68.3	49.4	Beaney et al. (2005)

Table 1 continued

Shell sources	Microorganisms	Products	DM (%)	DP (%)	Reference
Prawn shell waste	*Lactococcus lactis* spp. *Lactis* NRRL-B-1821	Lactic acid Protease	47.2–78.8	66.5–69.4	Aytekin and Elibol (2010)
Prawn shell waste	*Teredinobacter turnirae*	Lactic acid Protease	18.5–37.3	63.2–77.8	Aytekin and Elibol (2010)

OA organic acid, *DM* demineralization, *DP* deproteinization

[a] Not mentioned

Table 2 Summary of enzymatic treatment of crustacean shell wastes for chitin production

Waste source	Enzyme source	DM (%)	DP (%)	Reference
Snow crab (*C. opilio*)	*S.marcescens* FS-3 culture supernatant + Delvolase	47.0	84.0	Jo et al. (2008)
King crab (*Paralithodescamtschaticus*)	Protease cocktail (hepatopancreas of king crab)	–[a]	–	Sila et al. (2014)
Crab species (*Hyasaraneus; Lithodesmaja*)				
Shrimp (*C. crangon*)	Alcalase	94.4	89.0	Valdez-Pena et al. (2010)
Shrimp (*Xiphopenaeuskroyeri*)	Alcalase 2.4L	97.5	93.41	Duarte de Holanda and Netto (2006)
	Swine pancreatin	97.9	92.23	
Shrimp head (*Litopenaeusvannamei*)	Alcalase 2.4L FG	74.42	ND	Mukhin and Novikov (2001)
	Alcalase 2.4L FG+	82.15		
Shrimp shell (*Metapenaeusmonoceros*	Crude protease (*Bacillus cereus* SV1)	99.56	88.8	Manni et al. (2010)
Shrimp waste (*Metapenaeusmonoceros*)	Crude protease (*B. mojavensis*A21)	98.1	88	Younes et al. (2012)
Shrimp waste (*Penaeuslongirostris*)	Fish protease (*Barbuscallensis*)	–	80	Younes et al. (2012)
Shrimp waste (*Metapenaeusmonoceros*)	Fish crude protease (*Balistescapriscus*)	–	78	Younes et al. (2012)
Shrimp waste (–)	Papain + GBW protease	–	–	Rao et al. (2001)
Prawn shells	Crude enzyme (*P. Maltophilia*)	82	64	Bustos and Michael (1994)

DM demineralization, *DP* deproteinization

[a] Not mentioned

Hence, modifications of the specific conditions and processing procedures might be necessary for the commercial scale (Zhang et al. 2012). The bioextraction technology should be applicable to bulky amount of the biowaste from the industrial processing of sea food. It should be fast, simple, and more eco-friendly with less waste water efflux and full recovery of additive ingredients such as protein, organic acids, carotenoids, flavors, and other value-added compounds (Cheong et al. 2014).

Biotechnological process vs chemical process

The efficiency of lactic acid fermentation for DM of crab waste shells with *L. paracasei* KCTC-3074 was compared with that of chemical treatments such as 2 N HCl, 0.1 M EDTA, and 0–10 % lactic acid (Jung et al., 2005). The DM rates were 75–82 %, depending on the amount of inoculum, which were lower than those of the chemical processes. Although such DM and DP percentages are lower than those of chemical treatment, enzymatic DP helps avoid drawbacks of chemical treatment such as heavy metal contaminations and degradation of chitin. The physicochemical properties and quality of chitin extracted by a lactic acid bacterial fermentation were compared with those of chitin produced by a chemical technique by Beaney et al. (2005). DP was not sufficient and DM was 68.29 % in the bio-extracted chitin. Generally, the chemical process seems to be the most effective way of obtaining chitin of the highest purity. Only one report has argued that biotechnological processes are more effective in chitin extraction. The efficiency of lactic acid bacterial fermentation of *Penaeus semisulcatus* biowaste for chitin recovery was compared with a chemical method (2 % NaOH for DP

and 10 % acetic acid for DM) (Khanafari et al., 2008). The microbial method using *Lactobacillus* spp. was more effective, demonstrating the effectiveness of the lactic acid fermentation as an alternative to chemical treatment.

Biotechnological process combined with chemical process

As mentioned, minerals and proteins are not completely removed by biological treatments. In order to produce chitin of standard quality, the residual protein and mineral could be further removed from the fermented materials by mild chemical treatment. Sini et al. (2007) treated the materials after fermentation (84 % DP and 72 % DM of shrimp shells) with mild chemicals (0.8 N HCl and then 0.6 N NaOH) to obtain characteristic chitin (*N*-acetylation 84.4 %, protein 0.81 %, and ash 0.85 %). Pacheco et al. (2011) produced chitin by lactic acid fermentation and mild treatment with HCl and NaOH to eliminate the remaining minerals and proteins. Manni et al. (2010) treated SW with a crude enzyme preparation from *B. cereus* SV1 and removed the residual minerals by mild acid treatment for isolation of pure chitin. Cira et al. (2002) also conducted lactic fermentation of shrimp for chitin recovery and obtained a DM of 85 % and a DP of 87.6 % for 6-day culture. The crude chitin was treated with HCl (0.5 M) and NaOH (0.4 M) to completely remove mineral and protein. The combination of a biotechnological process with a chemical process is reasonable and practical for standard chitin quality and environmental advantages. If a greater purity of chitin is required for medical applications, the fermented materials can be further treated with mild chemicals to remove the spoilage bacteria, residual proteins, and minerals. The optimal concentrations of acid and alkali were found to depend on the contents of residual protein and ash, the ratio of solid to liquid, the type of raw materials, and treatment time (Sini et al. 2007).

Mono- versus co-cultivation and one- versus two-step fermentation

Various fermentation procedures were evaluated, which included auto-fermentation, a single-step and two-step fermentation, mono- and co-fermentation, enzymatic treatment, in combination or not with chemical treatment, and other different processes. For one-step extraction of crude chitin from red crab shell waste, co-fermentation using a lactic acid bacterium *L. paracasei* subsp. *tolerans* KCTC 3074 and a protease-producing bacterium *S. marcescens* FS-3 was conducted (Jung et al. 2006). The DM level of KCTC 3074 + FS-3 (1:1) co-fermentation increased to 97.2 % after 7 days of fermentation, suggesting that co-fermentation was highly effective for removal of ash, but DP was 52.6 %. In this process, FS-3 contributed less to DP. The proliferation of FS-3 would be affected by the severe variation in pH, resulting in less secretion of proteases and thus less DP. In the co-fermentation with at least two different strains for DM and DP together in a one-batch culture, this kind of problem would be always encountered. Thus, the discovery and characterization of novel microorganisms and other protocols which employ proliferating organisms that secrete organic acids and proteases will be necessary in order to improve the co-removal of proteins and minerals from crustacean shell wastes. They also conducted a successive two-step fermentation using the same organisms and the same biomaterial, combining the 1st step with *L. paracasei* subsp. *tolerans* KCTC 3074 and the second step with *S. marcescens* FS-3, and vice versa as discussed later (Jung et al. 2007). Similarly, using the conditions optimized by the Taguchi experimental design with orthogonal array, a chitin yield of 18.9 % with final DP and DM rates of 94.5 and 93.0 %, respectively, was obtained from shrimp shell powders by successive two-step fermentation of *S. marcescens* B742 and *L. plantarum* ATCC 8014 (Zhang et al. 2012). When *L. lactis*, a protease producer, and a marine bacterium *Teredinobacter turnerae* were evaluated in bioextraction of chitin from prawn waste, individual application of *L. lactis* and *T. turnerae* was not a good choice for chitin extraction (Aytekin and Elibol 2010). However, in all the co-culture processes, DM rates were higher than those of the mono-cultivation. The highest process yield (95.5 %) was recorded in the co-cultivation of *T. turnerae* and *L. lactis*in a medium containing 5 % glucose. All these results indicate that co-fermentations and two-step fermentations are recommendable for efficiency and quality of chitin.

Order of microbiological DP and DM

One can use one microorganism that produces both organic acids and proteases or two different ones, that is, one is an organic acid producer and the other is a protease producer. Often the acidification and DM process come first for a stable waste ensilation. A successive two-step fermentation firstly with lactic acid-producing *L. paracasei* KCTC-3074 and secondly with protease-producing *S. marcescens* FS-3 resulted in 94.3 % DM and 68.9 % DP from red crab shells, indicating that DP still remained unsatisfactory and needed additional treatments (Jung et al. 2007). These results suggest that the sequential order of processing could become important. Wahyuntari et al. (2011) compared the effectiveness of the order in microbiological DP and DM for chitin extraction from shrimp shell waste. In the first experiment, a DP (*B. licheniformis*) was followed by DM

(*L. acidophilus* FNCC116) process (DP–DM), and in the second one, DM process was followed by DP (DM–DP). They found out that DM–DP produced higher chitin extraction efficiency than DP–DM. Very recently, Liu et al. (2014) applied a successive co-fermentation to extract chitin from shrimp head waste in combination with a protease-producing bacterium, *B. licheniformis* 21886 (B. 21886), and an acid-producing bacterium, *Gluconobacter oxydans* DSM-2003 (G. 2003). Three different co-cultivation strategies, namely B+G (simultaneous inoculation), B-G (first B. 21886 then G. 2003), and G-B (first G. 2003 then B. 21886) were employed, and the resultant changes in the proteolytic activity, pH, and total titratable acidity (TTA) contents were followed. For DM, single cultivation with G. 2003 achieved a high removal efficiency (93.7 %), and a similar result (93.5 %) was observed in the successive inoculation of B. 21886 and G. 2003. For DP, single cultivation with B. 21886 achieved a high removal efficiency (83.1 %), and in the co-cultivation system reached a slightly higher efficiency (87 %). Overall, the successive fermentation with a combination of B. 21886 and G. 2003 (as in B-G) yielded the best co-removal of $CaCO_3$ and proteins from shrimp head waste. In the case of G-B, DP and DM were 71 % and 51.7 %, respectively. The removal rates were lower than those of B-G, indicating the importance of the order of DM and DP processing. This result is contradictory to that of Wahyuntari et al. (2011), in which DM–DP resulted in the best co-removal of $CaCO_3$ and proteins from shrimp shell. This may be mainly due to the difference in raw material (shrimp shell waste *vs* shrimp head waste), microorganism (*B. licheniformis–L. acidophilus* FNCC116 pair *vs B. licheniformis* 21886–*G. oxydans* DSM-2003 pair), and cultivation conditions. In the co-fermentation broth, eight organic acids with a total amount of 16 g/L were found. These organic acids must suppress the growth of spoilage microorganisms by rapid acidification of the medium along with DM. In industrial-scale extraction of chitin, suppression of putrefaction in waste shells is the most important step in preserving the biomaterials fresh as mentioned above. All these results indicate that a combination of the 1st step with a lactic acid bacterium and the second step with a protease-producing bacterium is more efficient and practical than other combinations in co-removal of CaCO3 and proteins from crustacean shell waste. This combination also creates merits of rapid acidification of medium to prevent decay of biomaterials and reduce hydrolysis of chitin polymers.

Optimization of culture conditions

Optimization of the biological processing of crustacean wastes is critical for improvement of DP and DM to produce high-quality chitin and to reduce the efflux of waste water. The efficiency of fermentation depends on factors such as inoculum levels, shell content in medium, shell size, carbon sources such as glucose, sucrose, malt, cassava, molasses, and date juice, initial pH and pH change during fermentation, the fermentation type including liquid-phase and solid-phase cultures, aerobic and anaerobic conditions, and the order in which these factors are implemented in the case of successive fermentation consisting of DM and DP processes (Jung et al. 2007; Oh et al. 2007; Prameela et al. 2010; Rao and Stevens 2005). In recent years, the statistical approaches including the Plackett and Burman design (Ghorbel-Bellaaj et al. 2013), Box–Behnken design (Younes et al. 2014), Taguchi experimental design (Zhang et al. 2012), and response surface methodology (Arbia et al. 2013; Bhaskar et al. 2007; Choorit et al. 2008) have been applied in process and medium optimization. These methods are useful and powerful tools for understanding interactions among various parameters using a minimal number of experiments. Response surface methodology is a collection of statistical techniques that is useful for designing experiments, evaluating the effects of different factors, and searching for optimal conditions for desirable responses (Arbia et al. 2013; Choorit et al. 2008). Through a central composite design and response surface methodology, Arbia et al. (2013) achieved 98 % DM and 78 % DP by fermentation of 4.84 g of shell of 1.53 mm size per 100 mL medium. Oh et al. (2007) studied the efficiency of DM and DP using a high protease producer *P. aeruginosa* F722 with various concentrations of glucose as carbon source and two particle sizes of crab shell waste. DM and DP efficiencies decreased with the increase of the solid to liquid ratio, and the best efficiency in DM and DP was found for 5 % crab shell waste. At the optimal incubation temperature (30 °C), DM was 92 % and DP was 63 % after 7 day culture. The efficiency of DM was largely dependent on the glucose concentration, the solid to liquid ratio, and the decrease in pH of the medium. Regression analysis determined the correlation of DM and DP with the measured variables: glucose concentration, pH, TTA, and protein in the medium. Positive relationships were found between DM and glucose concentration ($R^2 = 0.821$) and between DP and glucose concentration ($R^2 = 0.787$), whereas a negative relationship was between DM and pH ($R^2 = 0.793$). Shell particle size had a relatively small effect on DM and DP.

Enzymatic treatment for deproteinization

Proteolytic enzymes have been used for the DP of crustacean wastes. Tuna proteinase, papain, and a bacterial proteinase have been used for DP (Indra Jasmine et al.

2006). Gagne and Simpson (1993) showed that the residual protein levels in SW after the DP were 1.3 and 2.8 % for chymotrypsin- and papain-treated samples, respectively. A high enzyme to waste ratio (E/W) was needed for maximum DP; typical E/W values were 0.7 and 1.0 % (w/w) for chymotrypsin and papain, respectively. Rao et al. (2001) applied the combination of papain and GBW protease on the DP of SW and found that the protein removal rates from the waste with these enzymes were low. A crude enzyme preparation from *B. cereus* SV1was applied for the isolation and characterization of chitin from SW (Manni et al. 2010). After extraction of chitin, residual minerals were removed by mild acid treatment. These treatments resulted in the chitin recovery of 16.5 % of its initial mass, even though removal of the residual protein associated with the chitin was not complete. Younes et al. (2012) assayed six crude microbial proteases from *B. mojavensis* A21, *B. subtilis* A26, *B. licheniformis* NH1, *B. licheniformis* MP1, *Vibrio metschnikovii* J1 and *Aspergillus clavatus* ES1 for chitin extraction from shrimp biowaste. The DP rates obtained with the crude enzymes were 76 % for A21, A26, J1, and MP1, and 65 and 59 % for NH1 and ES1, respectively. After enzymatic extraction of chitin, residual minerals were also removed by mild acid treatment. The commercial enzymes such as Delvolase, Cytolase PCL5, Econase CEPi, Econase MP 1000, Maxazme NNP, and Ccllupulin MG were applied for DP of crab shell waste by Jo et al. (2008). They also compared four treatments on the crab shells: FS-3 inoculum, Delvolase, FS-3 culture supernatant, and FS-3 supernatant plus Delvolase. After biological treatment, the DP was in the 81–90 % range and DM was in the 0.01–47 % range, showing that little DM occurred in the treatment with enzymes alone. The commercial enzyme Delvolase was most rapid and effective in DP of the crab shells, but did not completely remove residual protein associated with the chitin. A commercial enzyme, Alcalase 2.4L, a serine endopeptidase from *B. licheniformis* was used for an enzymatic DP of previously demineralized (10 % HCl treated) shrimp shells (Synowiecki and Al-Khateeb 2000). The enzymatic DP allowed the isolation of chitin containing only about 4 % of protein impurities and 0.31–1.56 % of ash. Interestingly, Bustos and Michael (1994) have compared the DP effects between microbes and enzymes. A maximum value of 82 % DP was achieved with *P. maltophilia* after 6 days of incubation, but a maximum of 64 % DP was achieved using a purified microbial protease under the same condition, suggesting the effectiveness of the microbial enzyme complex secreted during fermentation when compared with a single purified enzyme. The application of proteolytic enzymes for the treatment of crustacean biowastes is summarized in Table 2. Results obtained for DP varied between 27 and 95 % and for DM between 47 and 99.5 %.

Genetic engineering and other emerging biotechnologies

Genetic engineering is both applicable and promising for chitin bioextraction using recombinant enzymes. Cell immobilization technology (Lopez-Cervantes et al. 2010) and chitin recovery using an enzymatic DP combined with microwave-assisted DM (Valdez-Pena et al. 2010) and sonication-assisted DP (Zhang et al. 2012) could be promising applications in the field of biotechnology. To obtain long-chain chitin, two parameters are of utmost importance: high proteolytic activity and no chitinolytic activity during the process. In this line, *B. licheniformis* F11 (wild type) was genetically modified to completely abolish chitinase activity, and the modified strains F11.1, F11.2, F11.3, and F11.4 were applied for efficient DP of shrimp shells (Hoffmann et al. 2010). The application of the variants resulted in the production of long-chain chitin, showing that genetic engineering is therefore a promising tool for the production of chitin. Immobilized cells of a commercial probiotic strain (*Lactobacillus* sp.) were applied to the lactic acid fermentation of SW to facilitate the separation of products such as raw chitin and protein-rich liquid hydrolysates (Lopez-Cervantes et al. 2010). This fermentation technology facilitates the separation and partial purification of the main components of SW, even though the raw chitin obtained showed a lower DM and DP with an average purity of 59.1 %. Lactic acid was produced from whey lactose in a fed-batch fermentation process, using immobilized cells of *Lactobacillus pentosus* 4023 (Bautista et al. 2001). Protein and mineral contents were significantly reduced (81.5 and 90.1 %, respectively) in chitin obtained from crayfish exoskeleton. These results show that complete DM was not achieved by in situ production of lactic acid and a final treatment with 0.5 M HCl was necessary. Nevertheless, the process proposed for crayfish chitin purification was less polluting than the traditional HCl–NaOH procedure. Valdez-Peña et al. (2010) tried chitin extraction using commercial proteolytic enzymes combined with microwave-assisted DM to shorten the reaction time. The use of microwave-assisted technology for DM also promoted the elimination of the residual protein from the shrimp heads. Zhang et al. (2012) adopted the sonication technology for DP and improved DP and DM rates to 94.5 and 93.0 %, respectively. These emerging technologies help improve the efficiency and quality of chitin obtained by fermentation technology and recovery of the by-products.

Recovery of by-products

It is worth mentioning that the recovery of by-product protein has received much attention from researchers for

use in animal feed supplements and for the preparation of essential amino acids complex for human nutrition (Cheong et al. 2014). This would reduce the environmental problems associated with the high biological oxygen demand of the effluent. Cremades et al. (2003) obtained carotenoproteins and chitin from crawfish by a combined process based on flotation–sedimentation and in situ semisolid lactic acid fermentation. During fermentation of the shrimp, oligopeptides were produced as an additive by-product using *Bacillus* sp. SM98011 (He et al. 2006). Aye and Stevens (2004) reported that physical treatments, such as drying, grinding, and sieving, were sufficient for the recovery of 50 % shrimp protein as a dry powder, and pretreatment of shells with shearing in acidified water resulted in 60 % removal of the protein and a reduction of the mineral content. As expected, recovery of calcium salts of organic acids, mainly calcium lactate, from the culture medium requires attention, but there has been limited reports on this aspect so far. Calcium lactate is formed during fermentation and precipitates, which can be recovered by washing (Gortari and Hours 2014).

New enzyme sources

There is increasing interest in applying protease-, chitinase-, and lactic acid-producing bacteria to extract chitin. The high cost of enzymes and the low extraction efficacy are some of the pitfalls of the biotechnological method. Screening of potential strains that are robust secretors of organic acids or proteases is important, and their combined cultures are necessary for efficiency and quality of chitin. Crude microbial and fish protease preparations must be attractive for chitin extraction as a means of cost reduction and bulky treatment of crustacean waste. Recombinant enzymes and genetically modified strains could promote the efficacy and quality, and lower production costs. The digestive system of marine vertebrates and invertebrates is promising as a potential source of proteolytic enzyme preparation. A proteinase cocktail isolated from the hepatopancreas of king crab was tested for crustacean processing waste to recover chitin and protein hydrolysates (Mukhin and Novikov 2001). Sila et al. (2014) also extracted chitin from SW material using fish protease-aided process. A high DP rate (80 %) was recorded with enzyme/substrate ratio of 10 U/mg. After DP, the DM was completely achieved within 6 h at room temperature in 1.25 M HCl, and the residual content of calcium in chitin was below 0.01 %. Along these lines, chitin was recovered in another study through enzymatic DP of shrimp processing by-products (Younes et al. 2014). Nine different microbial and fish viscera protease preparations were tested for their DP efficiency. High levels of protein removal of about 77 and 78 % were recorded using *B. mojavensis* A21 and *Balistes capriscus* proteases, respectively, after 3 h of hydrolysis at 45 °C using an enzyme/substrate ratio of 5. All these results suggest a potential applicability of the digestive system of marine vertebrates and invertebrates as a source of enzyme preparations for chitin extraction (Mukhin and Novikov 2001; Sila et al. 2014). Ploydee and Chaiyanan (2014) prepared chitin from shrimp heads and abdominal shells using a two-step biotreatment process and shrimp head extract solution (SHES). The calcium carbonate of the shells was almost completely solubilized in 48 h with glucose fermentation by *L. pentosus* L7 to lactic acid (pH 3.9). The amount of residual calcium and crude protein was further eliminated by the proteolytic activity of *Bacillus thuringiensis* SA. After DM and DP of the shells, the amounts of residual calcium and crude protein of chitin flakes were 1.7 and 3.8 %, respectively. Together with this, they showed a possibility to overcome the high cost of media for cultivating lactic acid bacteria and *B. thuringiensis* using SHES. The price of synthetic culture medium (broth) for lactobacilli was about 400 baht/L, while the cost for preparation of culture medium made from SHES plus 2 % glucose was about 9 baht/L. The solid residue after SHES preparation still contains some nutritional value and can be used as an animal feed supplement and a raw material for chitin extraction.

Conclusions

The bioextraction of chitin from crustacean shell wastes has been studied mostly at the laboratory scale. A cost-effective, fast, and easily controllable industrial bioprocess remains to be developed for producing chitin of high molecular mass on the commercial level. Its maximal potential will be exploited through screening new enzyme sources, optimization of fermentation conditions, preservation of the biomaterials, and recovery of the by-products for yield efficiency and chitin quality.

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