

Chitosan obtained from black soldier fly larval cuticles expands the value chain and is effective as a biocontrol agent to combat plant pathogens

Carolina Escobar Rodríguez^{a,b,1}, Valeriia Zaremska^{a,1}, Thomas Klammersteiner^{b,c}, Ioannis Kampatsikas^a, Nils Münstermann^d, Oliver Weichold^d, Sabine Gruber^{a,b,*}

^a FH Campus Wien University of Applied Sciences, Department of Bioengineering, Vienna, Austria

^b Universität Innsbruck, Department of Microbiology, Innsbruck, Austria

^c Universität Innsbruck, Department of Ecology, Innsbruck, Austria

^d RWTH Aachen University, Institute of Building Materials Research, Aachen, Germany

ARTICLE INFO

Keywords:

Chitosan
Black soldier fly
Insect upcycling
Biocontrol

ABSTRACT

The industrial use of certain insects, such as the black soldier fly (BSF, *Hermetia illucens*), has become a burgeoning way of converting residual biomass into a valuable source of biomolecules for the feed and food industry. Here, the integration of a valuable step as an upcycling technology using biological waste from the 5th instar BSF larvae processing as a source of bioactive chitosan was explored. The goal is to pave the way for sustainable chitosan production from insect-based resources in addition to proteins, lipids, and fertilizers. The macronutrient profile of the larval waste was evaluated for chitosan extraction. After homogenization and mechanical separation of the larval pulp, enzyme-assisted extraction of chitosan from the chitin-rich matrix was performed using different combinations of commercially available proteases and lipases, followed by chemical deacetylation. The antifungal efficacy of the recovered chitosan varied depending on the extracted product and tested fungal plant pathogens. Furthermore, the plant priming effect of chitosan on *Beta vulgaris* promoted plant vigor and disease resistance. Seed coating resulted in improved germination and primary root growth. Thus, the extraction of chitosan from BSF larval cuticles offers an optimistic outlook for expanding the technological competence of the insect upcycling industries.

1. Introduction

As the global population expands, agricultural land resources that form the foundation for producing high-quality proteins have become increasingly constrained. Along these lines, the imperative to address climate change has gained urgency, as highlighted in the recent Intergovernmental Panel on Climate Change (IPCC) report (Calvin et al., 2023). Our dependence on livestock proteins still comes with a high environmental cost in terms of land and water usage and, more importantly, greenhouse gas emissions. A catalyst behind the increasing popularity of insect mass rearing is the potential of insect-derived proteins to diminish global reliance on animal feed made from soy and fishmeal, and to strengthen regional protein self-sufficiency.

Insect farming provides a more sustainable alternative to conventional feed and food production, by converting organic biomass from agro-industrial waste streams into valuable fertilizers and biomolecules.

In addition to fats and proteins suitable for food and feed, insects are a promising and sustainable source of chitin with 5–15 % total chitin on a dry weight basis, a range comparable to extractable fungal sources with 1 and 15 % of the fungal cell wall mass (Hahn, Tafi, et al., 2020). Moreover, highly seasonal and unsustainable farming strategies for crustaceans have been greatly debated, and a switch or partial replacement with insect farms for food, feed, and chitin production is now favored (Röthig et al., 2023; Van Huis & Oonincx, 2017).

In contrast, insect farming offers several major advantages, including significantly lower water and land requirements, lower greenhouse gas emissions, the use of low-value waste streams as substrates, and high reproduction rates and feed conversion efficiencies (Van Huis & Oonincx, 2017). In addition, rearing occurs indoors in a controlled environment, rendering insect farming season-independent and enabling better control of GHG emissions. With the variety of substrates on which it can feed on, the larvae of the black soldier fly (BSF; *Hermetia illucens*), it is an ideal organism for the bioconversion of organic side

* Corresponding author at: FH Campus Wien University of Applied Sciences, Department of Bioengineering, Vienna, Austria.

E-mail address: sabine.gruber@fh-campuswien.ac.at (S. Gruber).

¹ Equal contribution.

Abbreviations

BSF	black soldier fly
BSFL	black soldier fly larvae
BSF CHSN	Chitosan extracted from BSFL cuticles

streams into valuable products. To this end, this biowaste-based circular economy contributes to several Sustainable Development Goals (SDGs) and the central goals of the European Green Deal.

BSF larvae (BSFL) consist of 38–56 % protein, 5–28 % fat, and 4–11 % ash, depending on the developmental stage (day 1–14) (Eggink & Dalsgaard, 2023), whereas chitin amounts can reach up to 9 % of the total larval biomass (Eggink & Dalsgaard, 2023; Khayrova et al., 2019). In contrast to the chitinous cuticle of crustaceans, insect chitin has lower levels of inorganic material (Rumpold & Schlüter, 2013), allowing for more gentle demineralization steps during extraction (Jardine & Sayed, 2018). Moreover, BSFL are generally considered a resource with low or no pigmentation until the 5th instar (Bußler et al., 2016; Ushakova et al., 2017), which could be advantageous for the extraction of chitin from larval biomass. In addition, the nutritional composition of the larval diet affects the mineral and protein content of the harvested biomass, whereas the ratio between chitin and proteins is less affected (Mannucci et al., 2024). Therefore, adding extra value to insect farming by opening and improving new resource recovery pathways, such as the extraction of chitin and its conversion to chitosan, could contribute to the growth of an insect-based bioeconomy.

Chitosan is a cationic copolymer consisting of glucosamine and *N*-acetylglucosamine (GlcNAc, 2-(acetylamino)-2-deoxy-D-glucose) units, which are obtained by the chemical or enzymatic deacetylation of chitin from crustaceans, insects, and fungi (Dash et al., 2011). Fully or partially (>50 %) *N*-deacetylated amine groups are characteristic traits of chitosan, accounting for its solubility in diluted acids and bioactivity, making it suitable for use in a wide array of industrial applications (Dutta et al., 2004; Ravi Kumar, 2000), including its potential as a biocontrol agent in agriculture. Several studies have reported that seed soaking and application to the roots or foliage induces defense responses in numerous plant species, enhances germination and vigor, and promotes resistance against pathogens (Badawy & Rabea, 2011; Kappel et al., 2022; Orzali et al., 2017). Most recently, it was demonstrated that the application of commercial crustacean-derived chitosans on sugar beet (*Beta vulgaris*) foliage induced the expression of a pathogenesis-related gene (PR-3), but only supplementation of the substrate resulted in a significant upregulation of phytoalexin synthesis (PAL) and oxidative stress-related genes (GST) as defensive responses in plants. Moreover, repeated foliar applications of commercial chitosan promoted growth, elicited defense responses, and reduced the incidence of Cercospora leaf spot (CLS) in *B. vulgaris*. A strong and sturdy fungicidal effect on severe fungal plant pathogens such as *Cercospora beticola*, *Botrytis cinerea*, and *Fusarium* spp. was demonstrated (Kappel et al., 2022). The antimicrobial mechanisms proposed so far include inhibition of growth by affecting the permeability of the plasma membrane through interactions with negatively charged components in the phospholipid layer (Liu et al., 2004), scavenging essential nutrients (Roller & Covill, 1999), or interfering with microbial mRNA synthesis (Kong et al., 2010). Therefore, chitosan is a natural, non-toxic, biodegradable, and biocompatible polymer that offers an environmentally safe alternative to chemical pesticides as a plant biostimulant and biocide.

Chitosan, however, is not a unique compound but rather a heterogeneous blend of molecules whose compositions vary depending on the source (Achinivu et al., 2022; van den Broek et al., 2020) and the process used during production (Kurita, 2006). Consequently, different chitosans may display varying antimicrobial activities, as they may differ in molecular weight (M_w), deacetylation degree (DDA), and consequently,

solubility. For instance, chitosans of insect origin surpass crustacean products in terms of physicochemical properties, such as molecular weight and solubility. The water-binding capacity of insect chitosans is generally higher than that of crustaceans (Mohan et al., 2020). Furthermore, state-of-the-art insect-derived chitosans are currently produced using harsh thermochemical multi-stage processes with acidic (mostly 1 M–2 M HCl, acetic, or oxalic acid) and alkaline (1–22 M NaOH) treatments at relatively high temperatures (95–120 °C). Such chemical extraction not only raises environmental concerns and leads to high processing costs for waste disposal (Vázquez et al., 2013) but also often yields products with highly variable DDA and M_w distributions, resulting in unreliable properties (Batista & Roberts, 1990). To date, few studies have been published on the partially sustainable extraction of chitin and chitosan from insect matrices using green alternatives to conventional chemical extractions (Pedrazzani et al., 2024), while a large amount of data is available on crustaceans (Gortari & Hours, 2013).

This study aimed to evaluate the use of residual BSF biomass to produce chitosan with antifungal and plant-defense priming properties. The BSFL cuticles were obtained from the waste stream of a commercial insect producer, where BSFL were reared under controlled environmental conditions using a feed of constant quality and composition. These parameters were further correlated with the macronutrient profile of the depulped cuticles. Chitosans were obtained using 14 extraction strategies with unique enzyme-assisted pre-treatments of the cuticles, followed by chemical chitin deacetylation using NaOH. To remove melanin from the matrix, an additional bleaching step with moderate concentrations of hydrogen peroxide was added. Here, a process using commercially available enzymes that yielded BSF-derived chitosan (BSF CHSN) with distinctive properties and promising antifungal and plant growth-promoting properties, as well as comparable efficacy to commercial crustacean-derived chitosan products, is presented. Altogether, these findings provide an optimistic outlook for further development of chitin valorization processes from insect streams, thus enhancing the economic feasibility of industrial insect farming while contributing to developing a circular economy in this sector.

2. Materials and methods

2.1. Chemicals

Chemicals were purchased from Carl Roth (Karlsruhe, Germany) and Sigma-Aldrich (Merck, Darmstadt, Germany). Chitosans of crustacean origin were purchased from Sigma-Aldrich, including a low-molecular-weight (LMW, CAS Number: 448869; DP: 50–190 kDa (based on viscosity), DDA: 75–85 %) and a high-molecular-weight (HMW, CAS Number: 419419; M_w : 310–375 kDa, DDA: >75 %) variety. Enzymatic deproteination and defatting of the larval cuticles were performed using a protease from *Bacillus licheniformis* P3111 (Pedrazzani et al., 2024) and a lipase from *Aspergillus oryzae* from Sigma Aldrich, respectively (Merck, Darmstadt, Germany). Synthetic Melanin was purchased from Thermo Fisher Scientific (Darmstadt, Germany).

2.2. Rearing and harvesting of black soldier fly larvae

Raw larval processing wastes were obtained from a commercial BSF breeder (LIVIN Farms, Vienna, Austria). Adult BSF were reared under a 12:12 light:dark cycle in customized cages illuminated with 50 W LED lights with a spectral range of 350–440 nm. They were not fed and were only provided a water source. Females deposited eggs into customized egg collection devices two days after mating. Eggs were collected daily and allowed to hatch under controlled environmental conditions at 60 % relative humidity and 27 °C. The fresh hatchlings were then transferred to a starter feed, where they were kept for five days (until the 3rd instar) before being transferred to the rearing feed, where they remained for seven additional days (until the 5th instar). At this stage, the BSFL were subsequently harvested by separation from the remaining rearing

residues (frass). Harvested larvae were subjected to a purely mechanical separation process to obtain a protein- and oil-rich fraction and a chitin-rich fraction (larval cuticles). Mechanical separation consisted of hurling, followed by pressing to obtain depulped larval cuticles. The cuticles were then washed with cold water (4 °C), pressed again to remove non-chitin residues, and finally dried at 80 °C for 45 min. To prevent the oxidation of the non-chitin-rich fraction, pre-processing was performed at 18 °C in a cooling chamber. Samples of the depulped cuticles were sent to the Austrian Agency for Health and Food Safety (AGES, Vienna, Austria) for the determination of macronutrient composition, namely the relative amounts [% of dry mass] of crude fiber, protein, fat, and ash. Briefly, the crude fat content was determined by simmering the sample in 3 M HCl and gravimetry of the dried residue. Crude protein content was measured based on total nitrogen content using the Kjeldahl method

(Sáez-Plaza et al., 2013). The crude fiber included all organic compounds in a previously defatted sample that was insoluble in acidic or alkaline solvents and was determined using the Weender method (Van Soest & McQueen, 1973). Finally, the total ash content was determined gravimetrically after incineration at 550 °C.

2.3. Chitosan extraction from cuticles of black soldier fly larvae

All steps for chitosan extraction from the depulped BSFL cuticles were performed in 2-L round-bottomed flasks with continuous stirring (Fig. 1). Ten grams of freeze-dried BSFL were ground with a coffee mill to a fine powder and washed through a Miracloth filter (Calbiochem®) with 300 mL reverse osmosis water per g biomass. The washing was repeated after each step of the extraction process. The washed powder

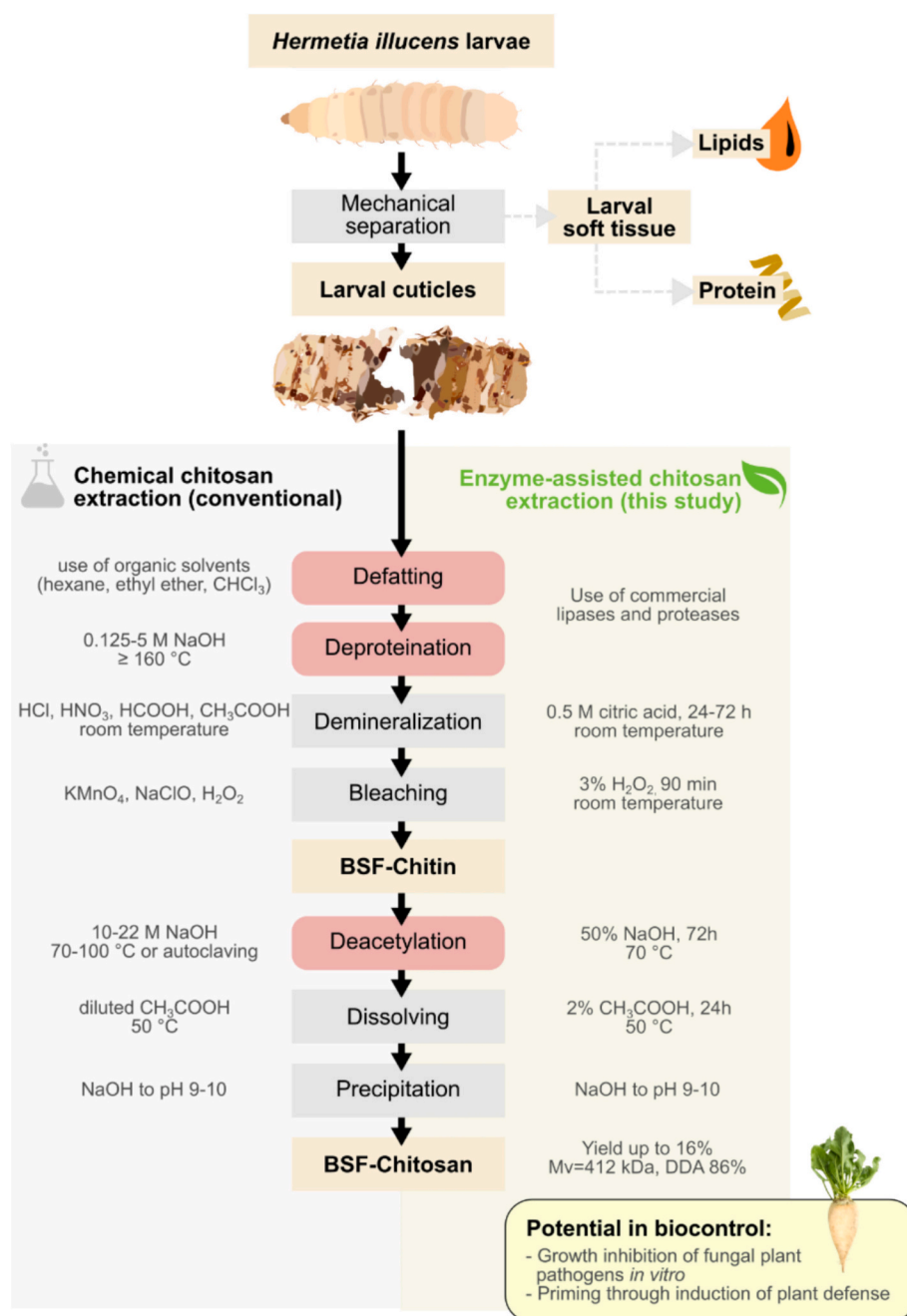


Fig. 1. Conversion of black soldier fly (BSF; *Hermetia illucens*) biomass into valuable products with a focus on chitosan. Conventional chemical approach (left) with relevant process parameters. The sustainable approach (right) is an enzyme-assisted process established in this study. Products derived from BSF are shown in beige.

was first defatted and subsequently treated for the final deproteinization using commercial enzymes as described previously (Pedrazzani et al., 2024). The defatted and deproteinized biomass was demineralized with 0.5 M citric acid for 72 h at room temperature (RT). The obtained chitinous matrix was deacetylated using 50 % NaOH for 72 h at 70 °C. After deacetylation, the alkaline supernatant was separated from the solid fraction by filtration through Miracloth. Chitosan (BSF CHSN) was extracted from this fraction by stirring in 2 % acetic acid (pH 3) for 24 h at 50 °C. The undissolved particles were removed by centrifugation at 20,000 ×g for 20 min. BSF CHSN was precipitated by gradually adding concentrated NaOH to the supernatant under stirring until pH 9–10 and stored for further flocculation overnight at 4 °C. The final BSF CHSN product was recovered by centrifugation, washed with deionized water to neutral pH, and lyophilized. The yield of BSF CHSN was calculated relative to the total amount of larval cuticles used.

2.4. Characterization of black soldier fly chitosan

The degree of acetylation (DA) was determined by conductometric titration according to Dos Santos (Dos Santos et al., 2009) using a GMH 3430 conductometer (Senseca, Regenstauf, Germany). For this, 50 mg of chitosan was dissolved in 20 mL of 0.05 M hydrochloric acid and constantly stirred at room temperature. 0.1 M sodium hydroxide solution was used for titration. All titrations were carried out in duplicate.

Infrared spectra (FTIR) were recorded on a Perkin Elmer Spectrum Two with an ATR attachment. Twenty-four scans were acquired in a measurement range of 400–4000 cm⁻¹.

The intrinsic viscosity was measured with a Xylem 501 13 / Ic Ubbelohde viscometer ($K = 0.03059 \text{ mm}^2/\text{s}^2$) in 0.25 M acetic acid and sodium acetate buffer solution with a pH value of 4.34 at 20 ± 0.05 °C. A series of seven samples in the range of 0.0–3.0 mg/mL in 0.5 mg/mL increments were measured. Before the measurements, the solutions were passed through a 1 µm syringe filter. All measurements were carried out in quadruplicate. The k and α values of the samples as well as the viscosity average molecular mass M_v were calculated using the Mark-Houwink equation following the procedure described by Wang (Wang et al., 1991).

2.5. Antifungal efficacy of black soldier fly chitosan

The ability of BSF CHSN to inhibit the proliferation of fungal plant pathogens, *Fusarium culmorum*, *F. equiseti*, *F. oxysporum*, *F. sporotrichoides*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Rhizoctonia solani*, *Penicillium digitatum*, *P. citrinum*, and *Cercospora beticola* was assessed, by monitoring the growth of all fungi on potato dextrose agar (PDA, BD DIFCO, NJ, USA) plates supplemented with 0.1 % (v/v) chitosan, as described in a previous study (Kappel et al., 2022). Commercial crustacean-derived LMW and HMW chitosans served as controls. Briefly, 1 % (w/v) chitosan solutions were prepared by resuspending them in distilled water and gradually adjusting the pH of the suspension with diluted acetic acid until the chitosan was fully dissolved (pH 5.5). These chitosan solutions were used to supplement liquid PDA to a final chitosan concentration of 0.1 % (v/v) before pouring and solidification of the medium. As a control, PDA was supplemented with the same amount of acidified water (pH 5.5). For monitoring the inhibition of hyphal growth by all chitosan products, circular agar plugs (ca. 5 mm) carrying mycelia of different plant pathogens were placed in the center of each plate and incubated for up to seven days at an optimal growth temperature of 25 °C. The radial growth [cm] on each plate was measured daily. The inhibition of fungal growth [%] was calculated as follows: Inhibition [%] = (Ra [cm] – Rc [cm]) × 100 / Ra [cm], where Ra is the radial growth on the acidic control PDA plate and Rc is the growth on PDA supplemented with chitosan. To assess the ability of chitosan to inhibit the germination of *C. beticola* spores, a suspension bearing 10⁶ spores was given on each plate and spread using a Drigalski spatula. Spore germination on each plate was visually monitored daily for up to

12 days. Each experiment was carried out with two technical replicates per fungal strain.

2.6. Plant priming with black soldier fly chitosan against fungal disease

Treatments of sugar beet (*B. vulgaris* v. Hannibal DE068–001723319) seeds and plants with BSF CHSN were prepared as described by Kappel et al. (Kappel et al., 2022) with some modifications. Native seeds were soaked for 6 h, at room temperature with constant shaking (800 rpm) in 10 mg/mL of LMW, HMW, or BSF CHSN solution. An additional treatment with acidified distilled water at pH 4.9 served as a control. The treated seeds were dried for 48 h on filter paper. Two replicates of 40 seeds per treatment were distributed into two 20 cm square Petri dishes (40 seeds per dish) and placed on a layer of moistened filter paper. The seeds were stored in the dark at RT, and germination was monitored after 48 h and 72 h and compared to the control condition to obtain the total germination rates [%]. Root development was determined by measuring the radicle length of the seedlings five days after coating.

Six seedlings per treatment were selected based on their synchronized developmental stages (two unfolded cotyledons) and transferred to pots containing a conventional peat-based pot mix in a small greenhouse test setup. Plants were grown over three months (June to August 2023) without additional heating or light and an irrigation regime of 100 mL tap water every second day. Eight weeks after repotting, the plants were sprayed with 1 mg/mL solutions of HMW- or BSF CHSN using a hand-held sprayer. This experiment also included a control treatment with deionized water (pH 4.5). Five applications were performed and repeated every two weeks. The average perennial (1981–2010) monthly temperature and hours of sunshine for the region around Wiener Neustadt (local experiment) were as follows: June - 18 °C and 228 h; July - 20.2 °C and 250 h; August - 19.8 °C and 239 h. During the test period, the monthly average temperature was 16–31 °C from June to August 2023.

After the fifth chitosan application, all plants were sprayed with a freshly prepared spore suspension of the fungal pathogen *C. beticola*. Spores were collected by adding 10 mL of sterile distilled water to a Petri dish containing a 10-day-old culture of *C. beticola* and dispersed with a Drigalski spatula. The spore concentration was adjusted to 10⁴ spores/mL. After infection, the plants were covered with transparent polyethylene bags (50 × 100 cm) to maintain the optimal humidity necessary for infestation (Kappel et al., 2022). The severity of the CLS disease was monitored and documented two- and three weeks post-infection using photography and visual assessment of the developed leaf spots.

3. Results

3.1. Rearing and processing of black soldier fly larvae

For this study, 5th instar BSFL were grown on a defined rearing

Table 1

Macronutrient profiles of the substrate used for black soldier fly larva (BSFL) rearing, harvested 5th instar BSFL, and BSFL cuticles. The analyses were performed by the Austrian Agency for Health and Food Safety (AGES) following EU Regulation No. 152/2009.

Component	Rearing substrate	5th instar BSFL	Larval cuticles
Total carbohydrates [% DM]	46.9	–	–
Crude fiber [% DM]	38.5	2.1	21.4 ± 3
Crude protein [% DM]	14.04	15.2	67.6 ± 2
Crude fat [% DM]	5.5	6.2	8.1 ± 3
Crude ash [% DM]	3.7	1.9	2.9 ± 2
Calories [kcal]	425.5	–	–
Moisture [%]	69.5	69	–
Dry matter [%]	30.6	31	100

substrate (Table 1). They were harvested approx. 12 days after hatching. The 5th instar typically yields the highest BSFL biomass, along with desirable nutritional values (Giannetto et al., 2020) but low melanin content compared to later developmental stages.

The mechanical separation of whole larvae generated two product streams: I) a lipid- and protein-rich fraction suitable for animal feed and/or biofuel production, which accounted for approximately 70 % of the fresh biomass, and II) a waste fraction consisting of brown-colored larval cuticles (30 % of the fresh BSFL, Fig. 2A). The macronutrient content of the rearing substrate, whole BSFL, and separated cuticle fractions was investigated to gain insights into the correlation between the rearing substrate and achievable amounts of chitin (and ultimately chitosan) in each of these streams. Compared to the whole BSFL, the cuticle fraction showed significant enrichment of both crude fiber and protein, while fat and ash content remained roughly unchanged (Table 1). This suggested the enrichment of insoluble chitin and N-containing compounds. However, an accurate assessment of chitin content in these samples cannot be assumed because the presence of other recalcitrant compounds (e.g., brown melanins) may cause a falsified overestimation. The chitin content in the larval cuticles of the harvested instar has been previously estimated at 9 % of the total dry biomass (Soetemans et al., 2020), which is within the range of our results.

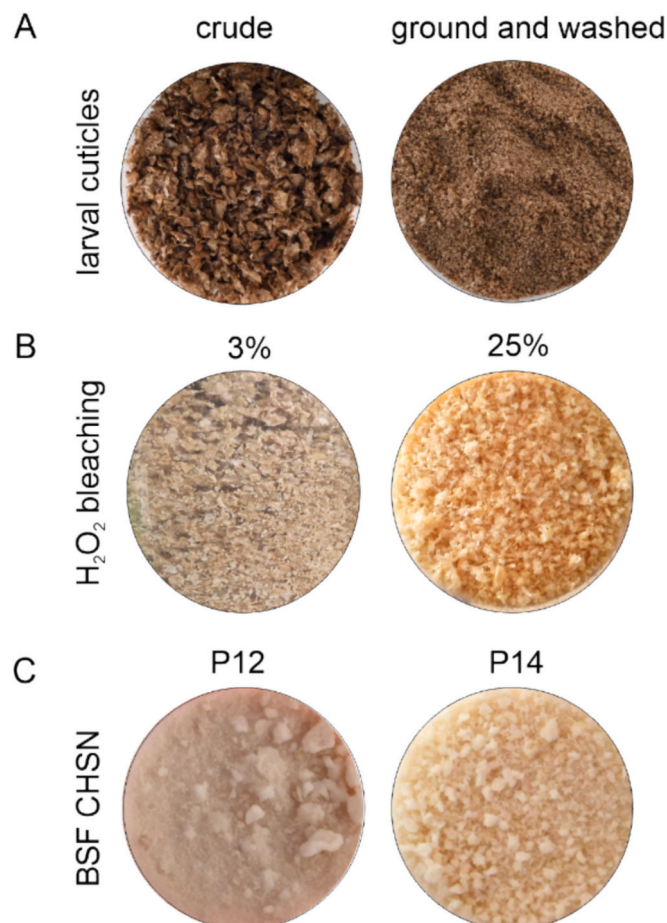


Fig. 2. Preparation of chitin and chitosan from cuticles of black soldier fly (BSF; *Hermetia illucens*) larvae from a waste stream of insect rearing. A) Appearance of the larval cuticles at the 5th instar after mechanical separation (crude) and after grinding and washing. B) Homogenized larval cuticles treated with 3 to 25 % hydrogen peroxide at room temperature resulted in reduced pigmentation. C) BSF-chitosan was obtained by employing two different enzyme-assisted extraction strategies without (P12) and with a bleaching step with 3 % hydrogen peroxide before chemical deacetylation (P14).

3.2. Enzyme-assisted chitosan extraction

The overall aim was to employ enzyme-assisted and thus more sustainable chitosan extraction from BSFL cuticles by circumventing the environmental disadvantages of the standard chemical processes using large amounts of NaOH (Fig. 1). Based on the methodology employed to determine the macronutrient content, it was inferred that the chitin in the obtained BSFL cuticles was represented within the crude fiber (around 21 %, Table 1) as a defatted matrix that was insoluble in both acidic and alkaline solvents (Rinaudo, 2006). Fourteen approaches for defatting and final deproteinization of BSFL cuticles were evaluated, each using a unique combination of commercial enzymes. Based on their efficiency, two enzymatic approaches were selected for the final processes.

For this purpose, the defatted and deproteinized chitin matrix was prepared for chemical deacetylation using NaOH at low incubation temperatures. The prepared BSF CHSN was expected to exhibit antifungal properties against plant pathogens, comparable to those of commercially available crustacean-derived chitosan products. Briefly, BSFL cuticles were homogenized into a fine powder and washed thoroughly with water through a Miracloth filter to remove water-soluble contaminants. Separating solids from the suspension using a Miracloth filter is a time-efficient alternative to centrifugation. Next, 9 % of the original biomass was enzymatically defatted and subsequently treated for deproteinization in suitable buffer systems for 24 h under continuous stirring, as described elsewhere (Pedrazzani et al., 2024).

For demineralization, the deproteinized and defatted biomass was homogenized in 0.5 M citric acid with a brief Ultraturrax pulse and stirred at room temperature for 72 h to replace the harsh HCl reaction used in traditional protocols. Citric acid was chosen as a mild alternative as well as for prolonged demineralization at room temperature to prevent both the degradation of the BSF chitin and unnecessary energy consumption by elevated temperatures. The pigmentation of the BSF chitin remained unaffected by the demineralization reaction and was visually comparable to that of untreated BSFL cuticles (Fig. 2A).

Therefore, a bleaching step to remove covalently bound melanins from the BSF chitin was included by testing two different hydrogen peroxide concentrations, namely 3 % and 25 % (v/v). A reduction in the pigmentation of BSF chitin was observed with increasing concentrations of hydrogen peroxide (Fig. 2B). However, BSF CHSNs obtained from both unbleached and bleached BSF chitin (Fig. 2C; P12 and P14, respectively) did not differ much in color and texture and were similar in appearance to commercial crustacean products (not shown). Furthermore, the reduced amounts of residual melanin were comparable in both P12 and P14 BSF CHSNs (Fig. S1). Since the initial goal was to establish a process with the lowest possible environmental impact, the bleaching step at a lower concentration of 3 % hydrogen peroxide and RT was further pursued to reduce the pigmentation of BSF chitin. The bleached BSF chitin was then filtered through Miracloth and washed with water before deacetylation.

Chemical deacetylation of BSF chitin was performed with 50 % (w/v) sodium hydroxide. A reaction time of 72 h at 70 °C was sufficient to achieve a satisfactory yield of BSF-CSHN. The pellet was washed with deionized water, resuspended in 2 % (v/v) acetic acid, and stirred at 50 °C for 24 h. The solution containing BSF CHSN was then separated by centrifugation and Miracloth filtration, and BSF CHSN was precipitated from the solution by adding NaOH at a pH of 9–10. To improve flocculation, the sample was incubated overnight at 4 °C.

The pellet was washed with deionized water until a neutral pH was achieved. Finally, the BSF CHSN pellets were lyophilized, ground into a fine powder, and weighed to determine the yield in relation to the initial weight of the biomass from the BSFL cuticles. The BSF CHSN yields from all fourteen different extraction strategies ranged from 2 to 16 %. The lowest yield (2.5 %) was obtained from protocols where lipase and protease were used simultaneously. In comparison, extraction with defatting and subsequent deproteinization, as well as an additional

bleaching step with 3 % hydrogen peroxide, gave the highest yield of 16 %, suggesting improved deacetylation and extraction by hydrogen peroxide. In contrast, the process without bleaching yielded only 9 % chitosan, based on the initial amounts of larval cuticles.

3.3. Degree of deacetylation and molecular weight of black soldier fly chitosan

BSF CHSNs from the two selected extraction protocols (bleached P14 and unbleached P12) were characterized in terms of their degree of deacetylation (DDA) and molecular weight (M_v) by conductometric titration and measurement of intrinsic viscosity, respectively. The DDAs of the obtained chitosans were 72.6 % for unbleached and 86.5 % for bleached BSF CHSN. Regarding the viscosity average molecular weight (M_v), the BSF CHSN from the unbleached sample had a higher molecular weight of 730 kDa, whereas the one from the bleached approach was found to have only 412 kDa.

3.4. Biocontrol: inhibition of fungal plant pathogens and plant priming against disease

The BSF CHSNs showed promising chemical properties such as molecular weight and degree of deacetylation for biocontrol (Kappel et al.,

2022). Therefore, their bioactivity in terms of their antifungal properties and their ability to inhibit the growth of 11 plant pathogens in vitro was tested. These pathogens included four *Fusarium* spp., three *Penicillium* spp., *B. cinerea*, *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, and *C. beticola*. All fungi were cultivated on solid PDA plates with 0.1 % commercial crustacean chitosan (LMW and HMW) and both BSF CHSNs (bleached P14 and unbleached P12). Chitosan P12 successfully inhibited the growth of most tested fungal pathogens (Fig. 3A). Moreover, it significantly outperformed commercial products on *Fusarium oxysporum* with an inhibition of 51 %, compared to HMW and LMW chitosans, with 43 % and 46 %, respectively ($p < 0.01$). Similarly, *B. cinerea* was significantly more inhibited by P12 (55 %) than by LMW chitosan (46 %, $p < 0.05$). Interestingly, P14 was exceptionally effective against *R. solani*, inhibiting its growth by 70 %. Furthermore, *S. sclerotiorum* was moderately (around 25 %) or not inhibited at all by any of the BSF chitosans (Fig. 3A). Of particular interest was the effect of BSF chitosan on *C. beticola*, a causative agent of CLS disease in sugar beet (*B. vulgaris*). In the in vitro plate assay, the P12 inhibited the growth of *C. beticola* by 36 %, comparable to both LMW and HMW chitosans. Moreover, this pathogen was inhibited twice as much (60 %) on plates supplemented with P14 (Fig. 3B).

The effects of BSF CHSN on seed germination, seedling development, growth, and priming against disease were investigated using *B. vulgaris*

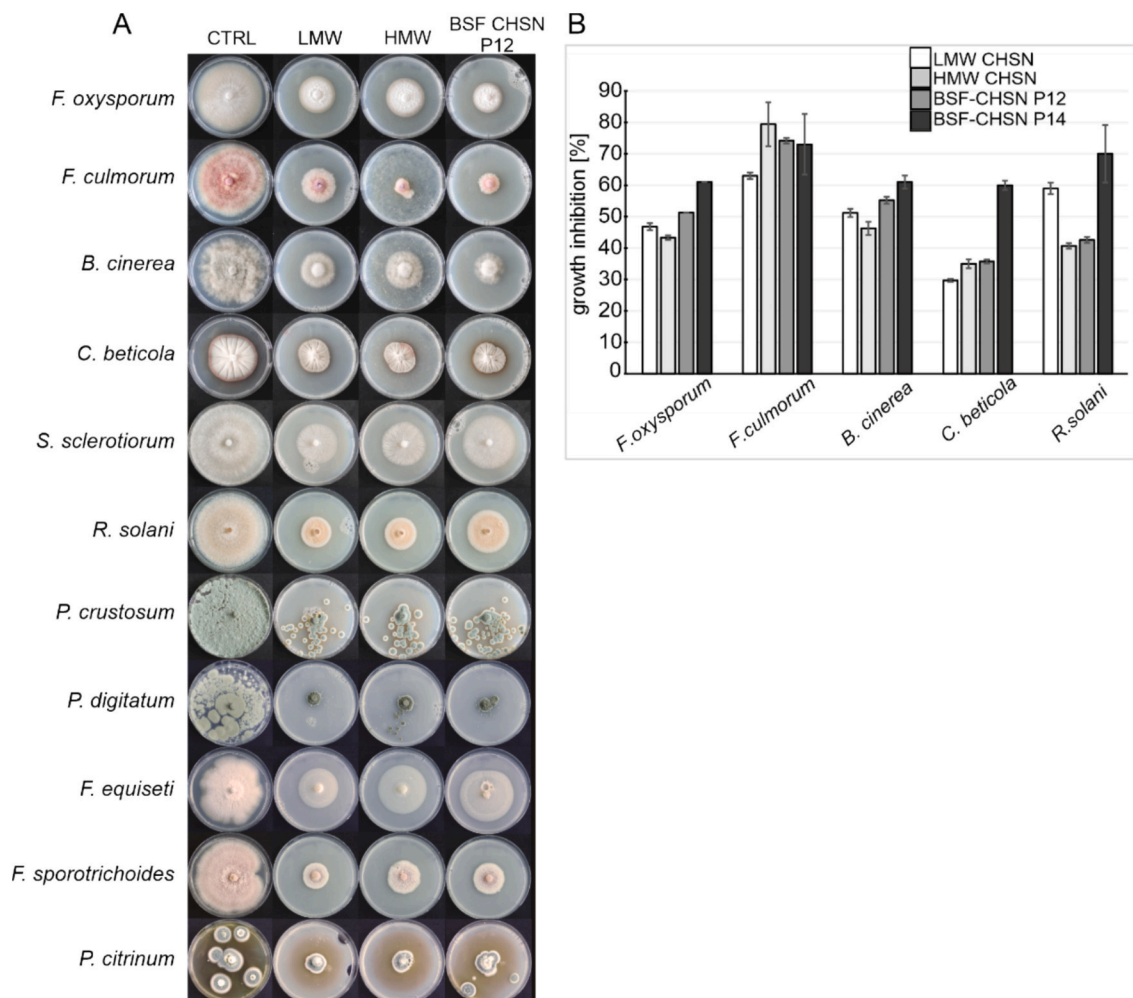


Fig. 3. The biocidal efficacy of black soldier fly (BSF) chitosan (BSF CHSN) against fungal plant pathogens is comparable to commercial products of crustacean origin. A) Inhibition of selected fungal plant pathogens grown on potato dextrose agar plates supplemented with 0.1 % (w/v) chitosans of low molecular weight (LMW), high molecular weight (HMW), and BSF-derived P12 product (BSF CHSN). Control plates were supplemented with acidified water (pH 5.5). Cultures are shown at the time point when the control group reached the edge of the plate. B) The growth inhibition [%] of the selected fungal plant pathogens is shown relative to the control group. Measurements were performed with 2 technical replicates of each pathogen.

as a model plant. The seeds were coated with 0.1 % and 0.5 % P12 BSF chitosan and commercial LMW and HMW chitosans, respectively. The control seeds were soaked in acidified water (see Materials and Methods). Coatings with 0.1 % solutions of all three chitosans significantly increased seed germination rates, while coatings with 0.5 % chitosans delayed germination (Fig. 4A). The root length of the seedlings was measured after five days to assess the effect of BSF CHSN on primary root development. Seeds coated with chitosans developed significantly longer roots than the acidified water (pH 5.5) control treatment (Fig. 4B). Importantly, seeds coated with 0.1 % solutions had longer radicles than those coated with 0.5 % chitosan. Furthermore, in both treatment groups, seeds coated with BSF CHSN P12 showed an earlier onset and longer roots than those coated with HMW and LMW chitosans.

The plant priming effect of BSF chitosan against fungal disease was tested by spraying the foliage of young plants with 0.1 % solutions of both BSF CHSN P12 and HMW chitosan. These treatments were reapplied every second week for a total of five applications. Plant vigor was documented in terms of turgor, shoot height, and appearance. Plants treated with both BSF- and HMW chitosans showed increased turgor and bright green color compared to the control plants sprayed with acidified water (Fig. 5A, B). The mean shoot length of plants treated with both chitosans was comparable to the control condition five weeks after repotting. However, after infection with spores of the pathogen *C. beticola*, the number of plants treated with BSF CHSN P12 was significantly higher than that in the control group (Fig. 5C), as was observed in the plants treated with commercial HMW chitosan. In addition, increased leaf growth and leaf regeneration per plant were observed in plants treated with both chitosans compared with the control group, particularly post-infection (Fig. 5D). Indeed, both P12 and P14 BSF chitosans proved effective in inhibiting the germination of *C. beticola* spores, with comparable efficacy to HMW. In contrast, LMW failed to provide a long-lasting inhibition, delaying germination for less than five days (Fig. 6A).

Consequently, after the manual infection with *C. beticola* spores, plants treated with both BSF- and HMW chitosans had significantly fewer affected leaves than the control group, where severe necrotic leaf tissue was observed (Fig. 6 B, C). In contrast, chitosan-treated plants

developed few scattered CLS symptoms (Fig. 6B). The sturdy protective effects of chitosan applications were evident in the treated plants at two- and three weeks post-infection. Interestingly, 3 weeks after infection, plants primed with BSF CHSN chitosan showed a 20 % reduction in CLS incidence compared to those primed with HMW chitosan, and 44 % less than the control group (Fig. 6C).

4. Discussion

The growing global demand for alternative protein sources has led to increased interest in insect species, such as the black soldier fly (BSF), which is also a sustainable source of chitin, a biopolymer with significant industrial potential. However, traditional chitosan extraction methods from BSF chitin involve harsh chemicals and generate environmentally harmful waste (Tyliaszczak et al., 2020). This study aimed at extracting chitosan from the cuticles of BSF 5th instar larvae, which contain high levels of chitin (Soetemans et al., 2020) and lower levels of melanization compared to later developmental stages (Ushakova et al., 2017) (Fig. 7). For this, an enzyme-assisted approach (Fig. 1) that reduces the need for harsh chemicals was used (Hahn, Tafi, et al., 2020). The extraction process started with enzymatic defatting and deproteinization of BSF larval cuticles using commercially available enzymes, which helped condition the chitin matrix for further treatment (Pedrazzani et al., 2024; Xiong et al., 2023). Following enzymatic treatment, a gentle demineralization step using 0.5 M citric acid was performed to remove residual minerals (Niego et al., 2023; Triunfo et al., 2022), as insect biomass contains lower mineral content compared to crustacean shells (Rumpold & Schlüter, 2013). This method was selected as an environmentally friendly alternative to traditional strong acids, improving the accessibility of the chitin matrix for subsequent deacetylation while minimizing environmental impact (Tyliaszczak et al., 2020).

Since no significant changes in the pigmentation of the BSFL biomass after all three pre-treatment steps were observed (Fig. 2), an additional bleaching treatment on the BSF chitinous matrix was performed. As expected, and based on visual assessment, the reduction in biomass pigmentation was more effective with increasing hydrogen peroxide

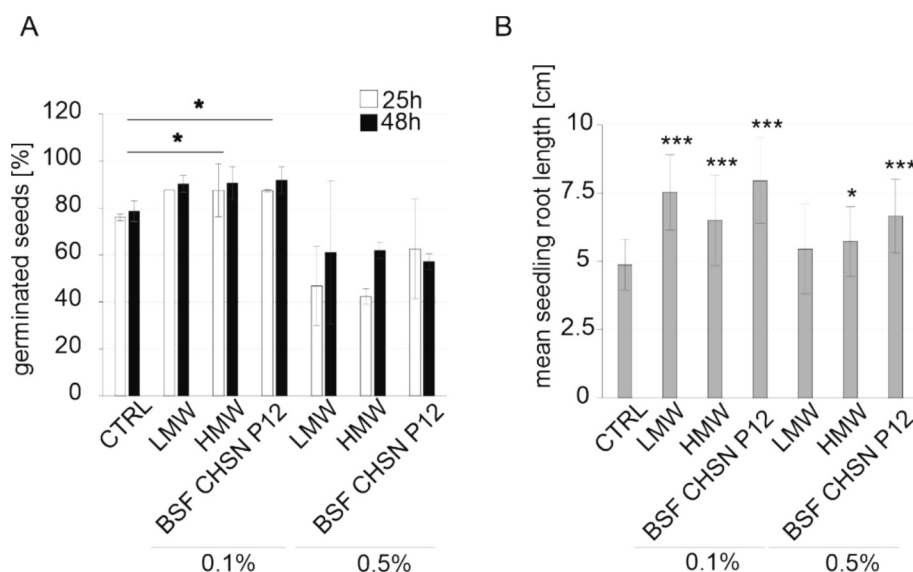


Fig. 4. Black soldier fly (BSF) chitosan (BSF CHSN) coatings enhance seed germination and early root development in sugar beet (*Beta vulgaris*). The seeds were coated with 0.1 % and 0.5 % chitosan (low molecular weight, LMW; high molecular weight, HMW; BSF CHSN) in two replicate experiments ($N = 40$). A) Germination efficiency [%] of seeds was significantly enhanced by coatings with 0.1 % HMW and BSF chitosans ($p > 0.05$). In contrast, all coatings with 0.5 % chitosan delayed seed germination compared to the control group (CTRL). B) Seedling root length was monitored for all germlings five days after coating. All coatings with 0.1 % chitosan successfully enhanced seedling root length compared to the control group ($p < 0.001$). In contrast, only formulations of 0.5 % HMW and BSF chitosans showed a moderate albeit significant effect in root length ($p < 0.05$ and $p < 0.001$, respectively). Asterisks show significance levels for each measurement compared to the no-chitosan control (CTRL).

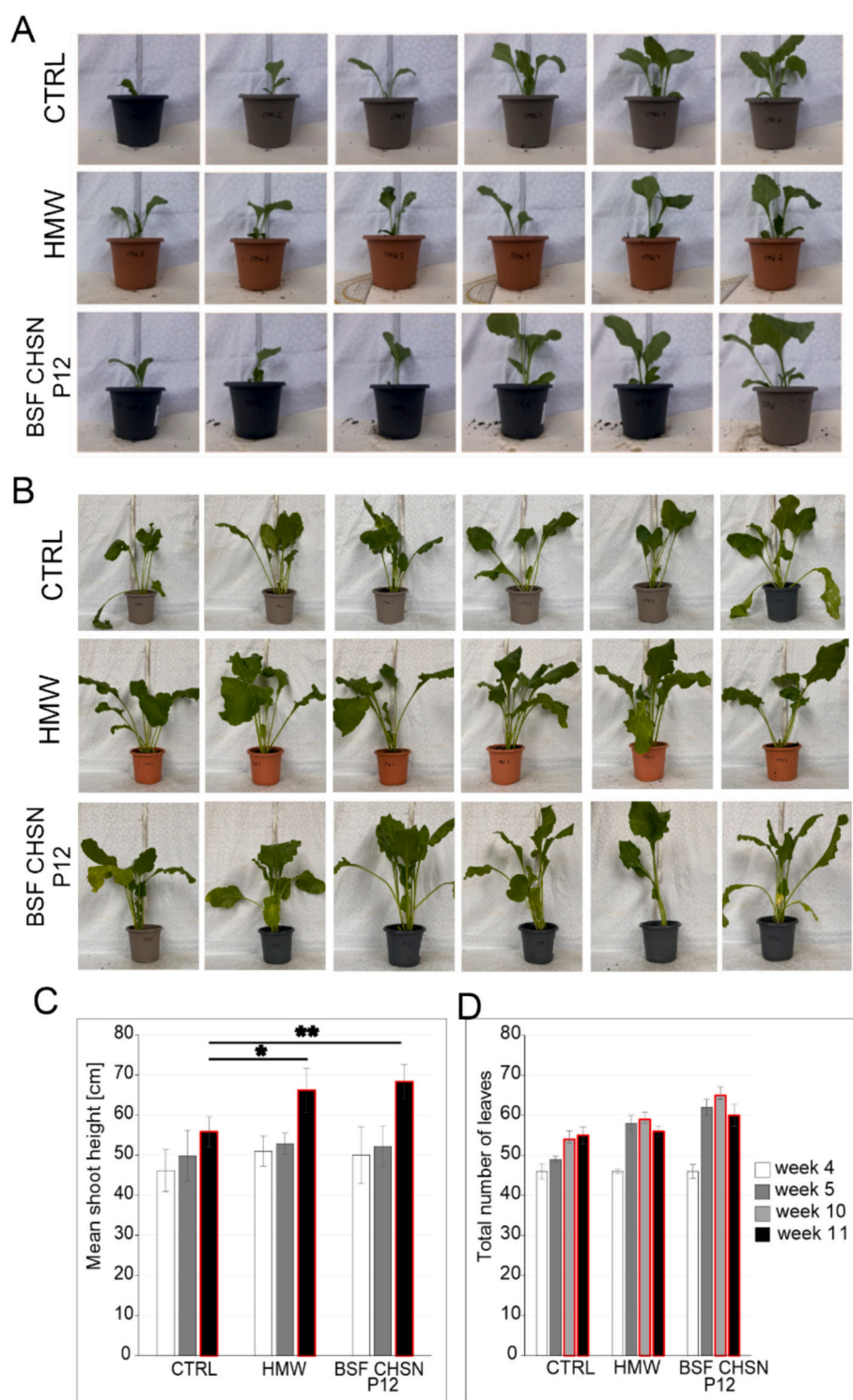


Fig. 5. Plant priming effect of black soldier fly chitosan (BSF CHSN) in young sugar beet (*Beta vulgaris*) plants ($N = 6$) grown in a greenhouse. The foliage was sprayed with 0.1 % high molecular weight (HMW) and BSF CHSN for a total of five applications every two weeks. After the fifth treatment, plants were manually infected with a spore suspension of the fungal pathogen *Cercospora beticola*. Plants at four weeks are shown with increasing size (from left to right for better comparison) before treatment (A) and after the fifth chitosan application (B). Plants treated with chitosan show increased vigor (foliage volume), significant growth promotion, and vitality compared to the control condition (C). The promotion of plant growth is reflected in improved leaf regeneration/development, which was determined by comparing the leaf count of all plants. D) Mean number of leaves per treatment pre- and post-infection remained unaffected. Bars referring to measurements performed after the infection with *C. beticola* are outlined in red. Asterisks show significance levels for each measurement compared to the no-chitosan control (CTRL).

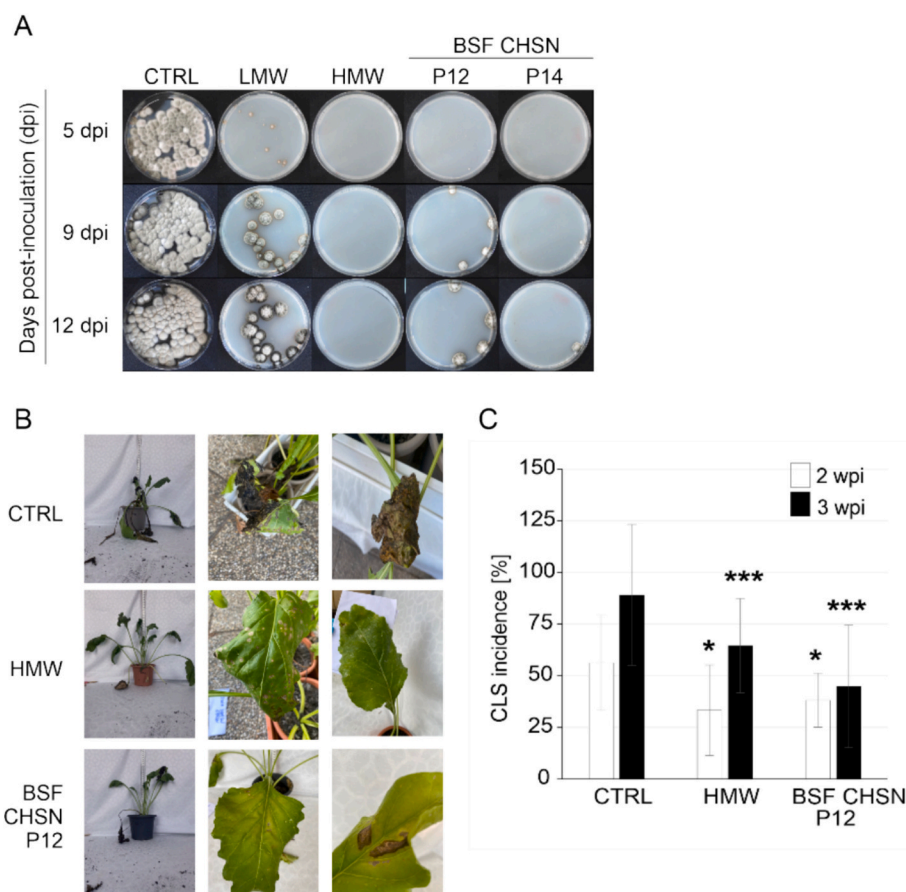


Fig. 6. Black soldier fly (BSF) chitosan induces resistance against *Cercospora* Leaf Spot (CLS) disease A) Inhibition of germination of *Cercospora beticola* spores on potato dextrose agar and plates supplemented with 0.1 % (w/v) chitosans of low molecular weight (LMW), high molecular weight (HMW), and BSF-derived P12 and P14 products (BSF CHSN P12, BSF CHSN P14). B) Appearance of plants ($N = 6$) primed with five applications of 0.1 % chitosan or acidified water (CTRL) after manual infection with a suspension of 10^4 spores/mL of *C. beticola*. Pictures show plants three weeks post-infection (wpi). C) CLS incidence is presented as the percentage of affected leaves per plant.

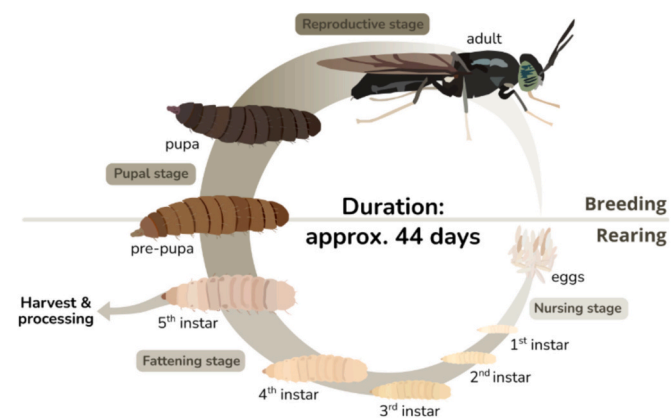


Fig. 7. Black soldier fly (*Hermetia illucens*, L. 1758) life cycle including aspects of industrial rearing.

concentrations. Interestingly, chitosan from unbleached (P12) and bleached (P14) BSF chitin did not show significant levels of melanin as indicated by the absence of the IR bands at 1720 cm^{-1} and 750 cm^{-1} (Fig. S1). Notably, the strategy with the introduced bleaching step yielded more BSF CHSN P14 (16 %) than its unbleached counterpart P12 (9 %), indicating an improvement in the purity of the chitinous matrix prior to deacetylation. An increase in yield may be attributable to the improved accessibility of the bleached chitinous matrix due to the

removal of bound catechols, thus enhancing both the solubility and efficiency of the subsequent deacetylation (Triunfo et al., 2022). Furthermore, both P12 and P14 BSF CHSNs differed in their DDA and molecular weight (M_v). Comparatively, P12 showed a higher M_v than P14 (730 kDa and 412 kDa) and a lower DDA (72.6 % and 86.5 %, respectively). Similarly as shown in two recent studies, chemical extractions from *Tenebrio molitor* (Yellow mealworm) bleached with consecutive treatments of 96 % ethanol, acetone, and diethyl ether yielded chitosans with higher M_v (767–796 kDa) and lower DDA (82–84 %) (Khatami et al., 2024), compared to those from processes employing hydrogen peroxide for decolorization (M_v 153–287 kDa, DDA 98 %) (Yuan et al., 2024). Bleaching with hydrogen peroxide has been reported to reduce the molecular weight of chitin due to the cleavage of glycosidic bonds in the polymer chain, in addition to the liberation of amino groups after the cleavage of bound pigments (Qin et al., 2002; Triunfo et al., 2022), thereby affecting both M_v and DDA. Thus, the introduction of mild bleaching agents can affect the quality of the generated chitosan in terms of the molecular weight and degree of deacetylation.

In general, the obtained results are within the range of published data on the degree of deacetylation of insect-derived chitosan, which can vary greatly depending on the insect species used, developmental stage, and extraction conditions (Hahn, Roth, et al., 2020; Hahn, Tafi, et al., 2020; Khayrova et al., 2019; Triunfo et al., 2022). For instance, recent efforts in exploring the valorization of insect-rearing residues employing conventional chemical extractions report chitosan yields up to 7 % (of total insect dry mass) and DDA between 76 and 83 % from larval cuticles from *Zophobas morio* and *T. molitor*, respectively (Khatami

et al., 2024; Machado et al., 2024). As for the impact of the extraction process, it was recently reported that chemical demineralization, followed by both deproteinization decolorization steps yielded the highest amount of chitosan (yield 4 %, DDA 98 %) from BSF larval cuticles compared to processes where the extraction steps differed in order (yield 3.7 %, DDA 53 %) (Yuan et al., 2024). In the present study, optimization of the yield may rely grossly on the separation method after each process step. For instance, a broad particle size distribution of BSFL cuticles was obtained using a conventional coffee grinder for pulverization. This heterogeneous mixture of variously sized particles made it challenging to separate the biomass from the aqueous components after each reaction or washing step using traditional centrifuges (up to 20,000 rpm) and filter systems. Here, larger particles failed to build a compact pellet after centrifugation, which led to biomass loss and remarkably reduced chitosan yields. Therefore, a filter system using Miracloth was implemented, as it proved to be a more time-efficient and promising alternative to centrifugation. However, the use of Miracloth also resulted in significant losses of the finest particles at each washing/separation step, which in turn led to lower chitin and chitosan yields. Chitinous residues could be extracted from the side streams of the process, suggesting that the reported final yield was underestimated. In short, optimizing the extraction process using more suitable filter systems (e.g., cellulose filters with defined pore sizes) could significantly increase the final yield of chitosan products.

Finally, the bioactivity of the produced chitosan was investigated, with particular interest in the application of chitosan in the biocontrol of fungal plant pests. First, the antifungal properties of the extracted chitosans were investigated in vitro by cultivating a total of eleven plant pathogens of agricultural relevance on solid media supplemented with 0.1 % BSF CHSN. Overall, the fungicidal activity of BSF CHSN P12 was comparable to that of commercial crustacean chitosans of both low and high molecular weight, showing the strongest inhibition of *F. oxysporum* and *B. cinerea* growth. In contrast, pathogens, such as *S. sclerotiorum*, *F. equiseti*, and *R. solani*, were only moderately inhibited by BSF CHSN P12 (Fig. 3). Interestingly, growth inhibition of *R. solani* and *C. beticola* by BSF CHSN P14 (412 kDa) outperformed all P12 and commercial products of crustacean origin. Although the exact molecular mechanisms underlying the inhibition of microbial proliferation have not been clarified yet, it has been suggested that chitosan might prevent nutrient uptake by enveloping the cell, thus influencing cell permeability. This could further cause hyphal agglomeration and impair the normal growth of a fungus (Harris, 2008; Kappel et al., 2022). Moreover, these mechanisms are strongly dependent on the molecular weight and degree of deacetylation of the employed chitosan (Hahn, Tafi, et al., 2020; Mohan et al., 2020; Pellis et al., 2022), which explains the varying but high efficacy of both, rather highly viscous BSF CHSN on different fungal pathogens. Further, the effect of BSF CHSN on germination, seedling development, and priming against disease using sugar beet (*B. vulgaris*) as a model plant was investigated. For this purpose, P12 BSF CHSN was chosen as the product from the extraction with the lowest environmental impact (no bleaching), the highest efficacy towards most fungal pathogens, as well as a strong inhibitor of spore germination in *C. beticola* with comparable efficacy to HMW chitosan. Moreover, P12 with its M_w of 730 kDa was a promising candidate as it overlaps with commercial crustacean products of comparable molecular weight in a related study (Kappel et al., 2022). Seed coatings with 0.1 % (v/w) chitosans improved germination, as opposed to coatings with 0.5 % solutions, which showed the opposite effect (Fig. 4). Nevertheless, regardless of the concentration, seeds coated with BSF CHSN developed longer roots than those treated with crustacean chitosan. The ability of chitosan seed coatings to enhance germination and seedling health has been described for several plant species (Godínez-Garrido et al., 2022; Kappel et al., 2022), especially under biotic and abiotic stresses (Benhamou, 1994; Stanley-Raja et al., 2021). Owing to its film-forming and antimicrobial properties, coatings with chitosan can protect seeds at the early stages of germination when they are most susceptible to pathogen invasion and abiotic

stresses such as the presence of heavy metals in the soil, high salinity in the surroundings, and drought (Riseh et al., 2024). In this process, the semipermeable chitosan film can aid in maintaining moisture around the seed, aiding during imbibition and nutrient uptake, and ultimately, promoting germination. However, our results suggest that at higher chitosan concentrations (0.5 % w/v), coatings may act counterproductively during germination under the conditions tested in this study, as reported previously on wheat (Li et al., 2019). Inhibitory effects on seeds at higher concentrations in coatings presumably occur due to a reduction of optimal permeability around the seed, hindering the uptake of nutrients and/or water and consequently, truncating germination. Furthermore, films with higher chitosan contents may result in diffusion into the seed tissues through cracks during imbibition, where it can interact with embryonic cells, negatively affecting cellular metabolism (Benhamou, 1994; Hadwiger, 2013).

An additional outstanding property of chitosan is its ability to elicit a defense response in plants promoting pathogen resistance and stimulating growth (Orzali et al., 2017; Riseh et al., 2022). The bioactivity of chitosan also relies on its ability to modify plant metabolism. The interaction between chitosan and plant metabolic factors has been shown to be unfavorable for plant pathogens, and when used at proper concentrations, can stimulate plant growth (van den Broek et al., 2020). The results presented in this study show that plants sprayed with both crustacean HMW chitosan and BSF CHSN exhibited improved vigor as reflected in taller shoots, compared to the control group treated with acidified water. These traits became more evident in the later growth stages after repeated chitosan applications (Fig. 5, and supplementary Fig. S2). Next, the primed plants were infected with *C. beticola* to study the protective effect of BSF CHSN on the CLS incidence. Indeed, the number of infected leaves in plants primed with both chitosans two weeks post-infection was significantly lower than that in the control group, and the infestation was limited to isolated clear-cut spots, whereas the control group developed severely necrotic leaf tissue. Furthermore, three weeks post-infection, it was observed that BSF CHSN outperformed crustacean HMW chitosan, as CLS incidence was 20 % lower in plants primed with the latter. Compared with the control group, plants prepared with BSF CHSN were 44 % less infected (Fig. 6).

The observed effects may be attributed to the induction of systemic resistance in plants by chitosan, which has been shown to activate the expression of defense- and pathogenesis-related enzymes, such as phenylalanine ammonia-lyase (PAL), polyphenol oxidases (PPO), and different peroxidases (Orzali et al., 2017; Riseh et al., 2022). For instance, a related study showed that in *B. vulgaris* plants primed with crustacean HMW chitosan, the expressions of PAL and oxidative stress-related enzymes, 6,7-dimethyl-8-ribityl lumazine synthase (DMRL) and glutathione-S-transferase (GST) were upregulated, suggesting the accumulation of antifungal phytoalexins and detoxification of generated reactive oxygen species (ROS), respectively (Kappel et al., 2022). These physiological changes in plants primed with chitosan prevent fungal pathogens such as *C. beticola* from efficient infection through thickening of the plant cell wall (Daniel & Guest, 2005), synthesis of antifungal metabolites, and degradation of ROS, which *C. beticola* produces during successful infection to damage plant cell membranes (Valenzano & Pooler, 1987). In addition, chitosan was shown to weakly bind to the chitin receptor AtCERK1 (Das et al., 2015) mimicking pathogen attack and eliciting a cascade of defense responses.

Overall, this study encompasses the entire process of upcycling a waste stream from industrial BSF rearing, namely BSFL cuticles, into bioactive chitosan circumventing several environmentally tolling steps with gentler and greener alternatives. BSF CHSN obtained in this manner performs well as a plant-priming agent as a replacement for harmful pesticides in agriculture and is a promising alternative to chitosan of crustacean origin.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.carbpol.2024.123023>.

CRediT authorship contribution statement

Carolina Escobar Rodríguez: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Valeriia Zaremska:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Thomas Klammsteiner:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ioannis Kampatsikas:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Nils Münstermann:** Visualization, Investigation, Data curation. **Oliver Weichold:** Writing – review & editing, Methodology, Investigation. **Sabine Gruber:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to thank LIVIN Farms GmbH for their kind support in providing the raw materials and documentation from their rearing process. We express our gratitude to the two anonymous reviewers for their insightful comments and constructive suggestions.

The work was funded by the Austrian Research Promotion Agency (FFG) project number 891131, <https://projekte.ffg.at/projekt/4298910>.

Data availability

Data will be made available on request.

References

- Achinivu, E. C., Shamshina, J. L., & Rogers, R. D. (2022). Chitin extracted from various biomass sources: It's not the same. *Fluid Phase Equilibria*, 552, Article 113286. <https://doi.org/10.1016/j.fluid.2021.113286>
- Badawy, M. E. I., & Rabea, E. I. (2011). A biopolymer chitosan and its derivatives as promising antimicrobial agents against plant pathogens and their applications in crop protection. *International Journal of Carbohydrate Chemistry*, 2011, 1–29. <https://doi.org/10.1155/2011/460381>
- Batista, I., & Roberts, G. A. F. (1990). A novel, facile technique for deacetylating chitin. *Die Makromolekulare Chemie*, 191, 429–434. <https://doi.org/10.1002/macp.1990.021910217>
- Benhamou, N. (1994). Induction of systemic resistance to fusarium crown and root rot in tomato plants by seed treatment with chitosan. *Phytopathology*, 84, 1432. <https://doi.org/10.1094/Phyto-84-1432>
- Buñler, S., Rumpold, B. A., Jander, E., Rawel, H. M., & Schlüter, O. K. (2016). Recovery and techno-functionality of flours and proteins from two edible insect species: Mealworm (*Tenebrio molitor*) and black soldier fly (*Hermetia illucens*) larvae. *Heliyon*, 2, Article e00218.
- Calvin, K., Dasgupta, D., Krinner, G., Mukherji, A., Thorne, P.W., Trisos, C., Romero, J., Aldunce, P., Barrett, K., Blanco, G., Cheung, W.W.L., Connors, S., Denton, F., Diongue-Niang, A., Dodman, D., Garschagen, M., Geden, O., Hayward, B., Jones, C., Jotzo, F., Krug, T., Lasco, R., Lee, Y.-Y., Masson-Delmotte, V., Meinshausen, M., Mintenbeck, K., Mokssit, A., Otto, F.E.L., Pathak, M., Pirani, A., Poloczanska, E., Pörtner, H.-O., Revil, A., Roberts, D.C., Roy, J., Ruane, A.C., Skea, J., Shukla, P.R., Slade, R., Slangen, A., Sokona, Y., Sörensen, A.A., Tignor, M., Van Vuuren, D., Wei, Y.-M., Winkler, H., Zhai, P., Zommers, Z., Hourcade, J.-C., Johnson, F.X., Pachauri, S., Simpson, N.P., Singh, C., Thomas, A., Totin, E., Arias, P., Bustamante, M., Elgizouli, I., Flato, G., Howden, M., Méndez-Vallejo, C., Pereira, J.J., Pichs-Madruga, R., Rose, S.K., Saheb, Y., Sánchez Rodríguez, R., Ürges-Vorsatz, D., Xiao, C., Yassaa, N., Alegría, A., Armour, K., Bednar-Friedl, B., Blok, K., Cissé, G., Dentener, F., Eriksen, S., Fischer, E., Garner, G., Guivarch, C., Haasnoot, M., Hansen, G., Hauser, M., Hawkins, E., Hermans, T., Kopp, R., Leprince-Ringuet, N., Lewis, J., Ley, D., Ludden, C., Niamir, L., Nicholls, Z., Some, S., Szopa, S., Trewin, B., Van Der Wijst, K., Winter, G., Witting, M., Birt, A., Ha, M., Romero, J., Kim, J., Haïtes, E.F., Jung, Y., Stavins, R., Birt, A., Ha, M., Orendain, D.J.A., Ignon, L., Park, S., Park, Y., Reisinger, A., Cammaramo, D., Fischlin, A., Fuglestedt, J.S., Hansen, G., Ludden, C., Masson-Delmotte, V., Matthews, J.B.R., Mintenbeck, K., Pirani, A., Poloczanska, E., Leprince-Ringuet, N., Péan, C., 2023. IPCC, 2023: Climate Change 2023: Synthesis Report. Contribution of Working Groups I, II and III to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, H. Lee and J. Romero (eds.)]. IPCC, Geneva, Switzerland. Intergovernmental Panel on Climate Change (IPCC). Doi:10.59327/IPCC/AR6-9789291691647.
- Daniel, R., & Guest, D. (2005). Defence responses induced by potassium phosphonate in *Phytophthora palmivora*-challenged *Arabidopsis thaliana*. *Physiological and Molecular Plant Pathology*, 67, 194–201. <https://doi.org/10.1016/j.pmp.2006.01.003>
- Das, S. N., Madhuprakash, J., Sarma, P. V. S. R. N., Purushotham, P., Suma, K., Manjeet, K., Rambabu, S., Gueddari, N. E. E., Moerschbacher, B. M., & Podile, A. R. (2015). Biotechnological approaches for field applications of chito oligosaccharides (COS) to induce innate immunity in plants. *Critical Reviews in Biotechnology*, 35, 29–43. <https://doi.org/10.3109/07388551.2013.798255>
- Dash, M., Chiellini, F., Ottenbrite, R. M., & Chiellini, E. (2011). Chitosan—A versatile semi-synthetic polymer in biomedical applications. *Progress in Polymer Science*, 36, 981–1014. <https://doi.org/10.1016/j.progpolymsci.2011.02.001>
- Dos Santos, Z. M., Caroni, A. L. P. F., Pereira, M. R., Da Silva, D. R., & Fonseca, J. L. C. (2009). Determination of deacetylation degree of chitosan: A comparison between conductometric titration and CHN elemental analysis. *Carbohydrate Research*, 344, 2591–2595. <https://doi.org/10.1016/j.carres.2009.08.030>
- Dutta, P. K., Dutta, J., & Tripathi, V. S. (2004). Chitin and chitosan: Chemistry, properties and applications. *Journal of Scientific & Industrial Research*, 63, 20–31.
- Eggink, K. M., & Dalsgaard, J. (2023). Chitin contents in different black soldier fly (*Hermetia illucens*) life stages. *Journal of Insects as Food and Feed*, 9, 855–863. <https://doi.org/10.3920/JIFF2022.0142>
- Giannetto, A., Oliva, S., Ceccon Lanes, C. F., De Araújo Pedron, F., Savastano, D., Baviera, C., Parrino, V., Lo Paro, G., Spanò, N. C., Cappello, T., Maisano, M., Mauceri, A., & Fasulo, S. (2020). *Hermetia illucens* (Diptera: Stratiomyidae) larvae and prepupae: Biomass production, fatty acid profile and expression of key genes involved in lipid metabolism. *Journal of Biotechnology*, 307, 44–54. <https://doi.org/10.1016/j.jbiotec.2019.10.015>
- Godínez-Garrido, N. A., Torres-Castillo, J. A., Ramírez-Pimentel, J. G., Covarrubias-Prieto, J., Cervantes-Ortiz, F., & Aguirre-Mancilla, C. L. (2022). Effects on germination and plantlet development of sesame (*Sesamum indicum* L.) and bean (*Phaseolus vulgaris* L.) seeds with chitosan coatings. *Agronomy*, 12, 666. <https://doi.org/10.3390/agronomy12030666>
- Gortari, M. C., & Hours, R. A. (2013). Biotechnological processes for chitin recovery out of crustacean waste: A mini-review. *Electronic Journal of Biotechnology*, 16, 14.
- Hadwiger, L. A. (2013). Multiple effects of chitosan on plant systems: Solid science or hype. *Plant Science*, 208, 42–49. <https://doi.org/10.1016/j.plantsci.2013.03.007>
- Hahn, T., Roth, A., Ji, R., Schmitt, E., & Zibek, S. (2020). Chitosan production with larval exoskeletons derived from the insect protein production. *Journal of Biotechnology*, 310, 62–67. <https://doi.org/10.1016/j.jbiotec.2019.12.015>
- Hahn, T., Tafi, E., Paul, A., Salvia, R., Falabella, P., & Zibek, S. (2020). Current state of chitin purification and chitosan production from insects. *Journal of Chemical Technology & Biotechnology*, 95, 2775–2795. <https://doi.org/10.1002/jctb.6533>
- Harris, S. D. (2008). Branching of fungal hyphae: Regulation, mechanisms and comparison with other branching systems. *Mycologia*, 100, 823–832. <https://doi.org/10.3852/08-177>
- Jardine, A., & Sayed, S. (2018). Valorisation of chitinous biomass for antimicrobial applications. *Pure and Applied Chemistry*, 90, 293–304. <https://doi.org/10.1515/pac-2017-0707>
- Kappel, L., Kosa, N., & Gruber, S. (2022). The multilateral efficacy of chitosan and Trichoderma on sugar beet. *JoF*, 8, 137. <https://doi.org/10.3390/jof8020137>
- Khatami, N., Guerrero, P., Martín, P., Quintela, E., Ramos, V., Saa, L., Cortajarena, A. L., De La Caba, K., Camarero-Espinosa, S., & Abarrategi, A. (2024). Valorization of biological waste from insect-based food industry: Assessment of chitin and chitosan potential. *Carbohydrate Polymers*, 324, Article 121529. <https://doi.org/10.1016/j.carbpol.2023.121529>
- Khayrova, A., Lopatin, S., & Varlamov, V. (2019). Black soldier fly *Hermetia illucens* as a novel source of chitin and chitosan. *International Journal of Sciences*, 8, 81–86.
- Kong, M., Chen, X. G., Xing, K., & Park, H. J. (2010). Antimicrobial properties of chitosan and mode of action: A state of the art review. *International Journal of Food Microbiology*, 144, 51–63.
- Kurita, K. (2006). Chitin and chitosan: Functional biopolymers from marine crustaceans. *Marine Biotechnology*, 8, 203–226. <https://doi.org/10.1007/s10126-005-0097-5>
- Li, R., He, J., Xie, H., Wang, W., Bose, S. K., Sun, Y., Hu, J., & Yin, H. (2019). Effects of chitosan nanoparticles on seed germination and seedling growth of wheat (*Triticum aestivum* L.). *International Journal of Biological Macromolecules*, 126, 91–100. <https://doi.org/10.1016/j.jbiomac.2018.12.118>
- Liu, H., Du, Y., Wang, X., & Sun, L. (2004). Chitosan kills bacteria through cell membrane damage. *International Journal of Food Microbiology*, 95, 147–155.
- Machado, S. S. N., Silva, J. B. A. D., Nascimento, R. Q., Lemos, P. V. F., Assis, D. D. J., Marcelino, H. R., Ferreira, E. D. S., Cardoso, L. G., Pereira, J. D., Santana, J. S., Silva, M. L. A. D., & Souza, C. O. D. (2024). Insect residues as an alternative and promising source for the extraction of chitin and chitosan. *International Journal of Biological Macromolecules*, 254, Article 127773. <https://doi.org/10.1016/j.jbiomac.2023.127773>
- Mannucci, A., Panariello, L., Abenaim, L., Coltelli, M. B., Ranieri, A., Conti, B., ... Castagna, A. (2024). From food waste to functional biopolymers: Characterization of

- chitin and chitosan produced from prepupae of black soldier fly reared with different food waste-based diets. *Foods*, 13, 278. <https://doi.org/10.3390/foods13020278>
- Mohan, K., Ganesan, A. R., Muralisankar, T., Jayakumar, R., Sathishkumar, P., Uthayakumar, V., Chandrasekar, R., & Revathi, N. (2020). Recent insights into the extraction, characterization, and bioactivities of chitin and chitosan from insects. *Trends in Food Science & Technology*, 105, 17–42. <https://doi.org/10.1016/j.tifs.2020.08.016>
- Niego, A. G. T., Lambert, C., Mortimer, P., Thongklang, N., Rapior, S., Grosse, M., ... Stadler, M. (2023). The contribution of fungi to the global economy. *Fungal Diversity*, 121, 95–137. <https://doi.org/10.1007/s13225-023-00520-9>
- Orzali, L., Corsi, B., Forni, C., & Riccioni, L. (2017). Chitosan in agriculture: A new challenge for managing plant disease. In E. A. Shalaby (Ed.), *Biological Activities and Application of Marine Polysaccharides*. InTech. <https://doi.org/10.5772/66840>
- Pedrazzani, C., Righi, L., Vescovi, F., Maistrello, L., & Caligiani, A. (2024). Black soldier fly as a new chitin source: Extraction, purification and molecular/structural characterization. *LWT*, 191, Article 115618. <https://doi.org/10.1016/j.lwt.2023.115618>
- Pellis, A., Guebitz, G. M., & Nyanhongo, G. S. (2022). Chitosan: Sources, Processing and Modification Techniques. *Gels*, 8, 393. <https://doi.org/10.3390/gels8070393>
- Qin, C. Q., Du, Y. M., & Xiao, L. (2002). Effect of hydrogen peroxide treatment on the molecular weight and structure of chitosan. *Polymer Degradation and Stability*, 76, 211–218. [https://doi.org/10.1016/S0141-3910\(02\)00016-2](https://doi.org/10.1016/S0141-3910(02)00016-2)
- Ravi Kumar, M. N. V. (2000). A review of chitin and chitosan applications. *Reactive and Functional Polymers*, 46, 1–27.
- Rinaudo, M. (2006). Chitin and chitosan: Properties and applications. *Progress in Polymer Science*, 31, 603–632. <https://doi.org/10.1016/j.progpolymsci.2006.06.001>
- Riseh, R. S., Hassanisaadi, M., Vatankhah, M., Babaki, S. A., & Barka, E. A. (2022). Chitosan as a potential natural compound to manage plant diseases. *International Journal of Biological Macromolecules*, 220, 998–1009. <https://doi.org/10.1016/j.ijbiomac.2022.08.109>
- Riseh, R. S., Vazvani, M. G., Vatankhah, M., & Kennedy, J. F. (2024). Chitosan coating of seeds improves the germination and growth performance of plants: A review. *International Journal of Biological Macromolecules*, 278, Article 134750. <https://doi.org/10.1016/j.ijbiomac.2024.134750>
- Roller, S., & Covill, N. (1999). The antifungal properties of chitosan in laboratory media and apple juice. *International Journal of Food Microbiology*, 47, 67–77.
- Röthig, T., Barth, A., Tschirmer, M., Schubert, P., Wenning, M., Billion, A., Wilke, T., & Vilcinskis, A. (2023). Insect feed in sustainable crustacean aquaculture. *Journal of Insects as Food and Feed*, 9, 1115–1138. <https://doi.org/10.3920/JIFF2022.0117>
- Rumpold, B. A., & Schlüter, O. K. (2013). Nutritional composition and safety aspects of edible insects. *Molecular Nutrition Food Research*, 57, 802–823. <https://doi.org/10.1002/mnfr.201200735>
- Sáez-Plaza, P., Navas, M. J., Wybraniec, S., Michałowski, T., & Asuero, A. G. (2013). An overview of the Kjeldahl method of nitrogen determination. Part II. Sample preparation, working scale, instrumental finish, and quality control. *Critical Reviews in Analytical Chemistry*, 43, 224–272. <https://doi.org/10.1080/10408347.2012.751787>
- Soetemans, L., Uytendaele, M., & Bastiaens, L. (2020). Characteristics of chitin extracted from black soldier fly in different life stages. *International Journal of Biological Macromolecules*, 165, 3206–3214. <https://doi.org/10.1016/j.ijbiomac.2020.11.041>
- Stanley-Raja, V., Senthil-Nathan, S., Chanthini, K. M.-P., Sivanesh, H., Ramasubramanian, R., Karthi, S., ... Kalaivani, K. (2021). Biological activity of chitosan inducing resistance efficiency of rice (*Oryza sativa* L.) after treatment with fungal based chitosan. *Scientific Reports*, 11, 20488. <https://doi.org/10.1038/s41598-021-99391-w>
- Triunfo, M., Tafi, E., Guarnieri, A., Salvia, R., Scieuzo, C., Hahn, T., Zibek, S., Gagliardini, A., Panariello, L., Coltelli, M. B., De Bonis, A., & Falabella, P. (2022). Characterization of chitin and chitosan derived from *Hermetia illucens*, a further step in a circular economy process. *Scientific Reports*, 12, 6613. <https://doi.org/10.1038/s41598-022-10423-5>
- Tyliszczak, B., Drabczyk, A., Kudłacik-Kramarczyk, S., & Sobczak-Kupiec, A. (2020). Sustainable production of chitosan. In G. M. Królczuk, M. Wzorek, A. Król, O. Kochan, J. Su, & J. Kacprzyk (Eds.), *Sustainable Production: Novel Trends in Energy, Environment and Material Systems, Studies in Systems, Decision and Control* (pp. 45–60). Cham: Springer International Publishing. https://doi.org/10.1007/978-3-030-11274-5_4
- Ushakova, N. A., Dontsov, A. E., Sakina, N. L., Brodsky, E. S., Ratnikova, I. A., Gavrilova, N. N., Bastrakov, A. I., Kozlova, A. A., & Nekrasov, R. V. (2017). Melanin properties at the different stages towards life cycle of the fly *Hermetia illucens*. *Ukrainian Journal of Ecology*, 7, 424–431. https://doi.org/10.15421/2017_137
- Valenzano, D. P., & Pooler, J. P. (1987). Photodynamic action. *BioScience*, 37, 270–276. <https://doi.org/10.2307/1310590>
- van den Broek, L. A. M., Boeriu, C. G., & Stevens, C. V. (2020). *Chitin and chitosan: Properties and applications, Wiley series in renewable resources*. Hoboken, New Jersey: Wiley.
- Van Huis, A., & Oonincx, D. G. A. B. (2017). The environmental sustainability of insects as food and feed. A review. *Agronomy for Sustainable Development*, 37, 43. <https://doi.org/10.1007/s13593-017-0452-8>
- Van Soest, P. J., & McQueen, R. W. (1973). The chemistry and estimation of fibre. *The Proceedings of the Nutrition Society*, 32, 123–130. <https://doi.org/10.1079/pns19730029>
- Vázquez, J., Rodríguez-Amado, I., Montemayor, M., Fraguas, J., González, M., & Murado, M. (2013). Chondroitin sulfate, hyaluronic acid and chitin/chitosan production using marine waste sources: Characteristics, applications and eco-friendly processes: A review. *Marine Drugs*, 11, 747–774. <https://doi.org/10.3390/md11030747>
- Wang, W., Bo, S., Li, S., & Qin, W. (1991). Determination of the Mark-Houwink equation for chitosans with different degrees of deacetylation. *International Journal of Biological Macromolecules*, 13, 281–285. [https://doi.org/10.1016/0141-8130\(91\)90027-R](https://doi.org/10.1016/0141-8130(91)90027-R)
- Xiong, A., Ruan, L., Ye, K., Huang, Z., & Yu, C. (2023). Extraction of chitin from black soldier fly (*Hermetia illucens*) and its puparium by using biological treatment. *Life*, 13, 1424. <https://doi.org/10.3390/life13071424>
- Yuan, B.-Q., Yu, T.-H., Chen, S.-C., Zhang, Z.-Q., Guo, Z.-K., Huang, G.-X., Xiao, J., & Huang, D.-W. (2024). Physical and chemical characterization of chitin and chitosan extracted under different treatments from black soldier fly. *International Journal of Biological Macromolecules*, 279, Article 135228. <https://doi.org/10.1016/j.ijbiomac.2024.135228>