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Research Letter

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Isolation of proteolytic bacteria from mealworm (*Tenebrio molitor*) exoskeletons to produce chitinous material

Fernanda Kerche Paes da Silva[1], Dieter W. Brück[2] and Wolfram M. Brück[1,2,*]

[1]Institute of Life Technologies, University of Applied Sciences Western Switzerland, Route du Rawyl 47, 1950 Sion, Switzerland and [2]BDW-Consulting, Kattowitzer Str. 34, 51065 Köln, Germany

*Corresponding author: Institute of Life Technologies, University of Applied Sciences Western Switzerland, HES-SO Valais-Wallis, Route du Rawyl 47, 1950 Sion, Switzerland. Tel: +41-0 27-606-86-64; Fax: +41-0-27-606-86-16; E-mail: wolfram.bruck@hevs.ch

One sentence summary: Bacterial isolates from farmed mealworms may aid deproteination and demineralisation of insects through fermentation to produce chitin.

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ABSTRACT

The use of insects as a source of protein is becoming an important factor for feeding an increasing population. After protein extraction for food use, the insect exoskeleton may offer the possibility for the production of added value products. Here, the aim was to isolate bacteria from the surface of farmed mealworms (*Tenebrio molitor* Linnaeus, 1758) for the production of chitinous material from insect exoskeletons using microbial fermentation. Isolates were screened for proteases and acid production that may aid deproteination and demineralisation of insects through fermentation to produce chitin. Selected isolates were used single-step (isolated bacteria only) or two-step fermentations with *Lactobacillus plantarum* (DSM 20174). Two-step fermentations with isolates from mealworm exoskeletons resulted in a demineralisation of 97.9 and 98.5% from deproteinated mealworm fractions. Attenuated total reflectance-Fourier- transform infrared spectroscopy analysis showed that crude chitin was produced. However, further optimisation is needed before the process can be upscaled. This is, to our knowledge, the first report using microbial fermentation for the extraction of chitin from insects.

Keywords: chitin; mealworms; fermentation; bacterial isolation; screening; exoskeleton

INTRODUCTION

By 2050, the world will have an expected population of 9.7 billion (United Nations 2015). In order to provide sufficient nutrition, at least twice as much food needs to be produced as today (van Huis *et al.* 2013). Simultaneously, harsher environmental conditions due to climate change will likely lead to an overburden to current food production techniques. Thus, different and more efficient solutions to feed the planet are essential. Adopting a strictly plant-based nutrition using high-protein plants has been shown to be an environmentally friendly and healthy balanced diet (Neacsu, McBey and Johnstone 2017). In addition, the use of insects as a source of protein is also coming into focus for those wanting to keep some animal protein in their diet (van Huis *et al.* 2013).

Life cycle analysis showed that the production of food insects has several advantages when compared to traditional farmed meats such as pork, chicken or beef: insects emit less greenhouse gases and ammonia and therefore have a lower global warming potential (kg CO_2-eq.) than traditional meats. Furthermore, insects required less land, time and water per 1 kg of protein (Oonincx and de Boer 2012). However, while currently 2 billion people use insects as part of a regular diet, in most western

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cultures, there is a reluctance to consume insects (Fessler and Navarette 2003).

Insects fit a biorefinery concept by offering the possibility of recovering the waste after extraction of the proteins for added value products. The most important by-product right now is the insect exoskeleton of which the main component is chitin, a polysaccharide that resembles cellulose. In its de-acetylated derivative (chitosan), the polymer has many applications, ranging from emergency care, food processing, agriculture, tissue engineering and drug delivery (Knorr 2006; Senthilraja *et al.* 2010; Chang *et al.* 2013; Pant *et al.* 2013; Shang *et al.* 2014). The main sources of chitin currently are crustacean shells and various species of mushroom (Nwe, Furuike and Tamura 2010; Lynch *et al.* 2016). The chitin content of insect exoskeletons (dry weight) is about 10%–20% and is mostly bound to cuticular proteins and lipids (Kaya *et al.* 2012). Currently, chitin from insects is produced similar to chitin from crustacean shells with silkworm pupae, larvae, bees and beetles having previously been used (Haga 1996; Zhang *et al.* 2000; Nemtsev *et al.* 2004; Paulino *et al.* 2006). The process comprises chemical deproteination using a strong alkali such as 0.75–2.5 N NaOH for 2–42 h, and demineralisation with a strong acid such as 1–2 N HCl for 0.3–96 h before a final decoloration step (Haga 1996; Zhang *et al.* 2000; Nemtsev *et al.* 2004; Paulino *et al.* 2006; Nwe, Furuike and Tamura 2010; Liu *et al.* 2012; Lynch *et al.* 2016). HCl treatmeant also deacetylated silkworm chitin (Zhang *et al.* 2000). The overall chitin/chitosan yield was observed to be lower than that achieved with crustacean shell waste which may have been due to acid hydrolysis under the harsh chemical methods involved (Paulino *et al.* 2006). However, the chitin produced with this resource was of high purity. Therefore, process should be developed that would maximise chitin yields.

This study aims to isolate endogenous bacteria on the exoskeleton of mealworm larvae (*Tenebrio molitor* Linnaeus, 1758) with the necessary characteristics for chitin extraction under fermentation conditions. *Tenebrio molitor* larvae have recently been approved as a food and novel/alternative protein source in Switzerland (Anonymous, 2017).

MATERIALS AND METHODS

Agar preparation

Chitin agar was prepared as previously described using (/l) 10 g chitin (Sigma Aldrich, Buchs, Switzerland), 0.5 g $CaCl_2{*}2H_2O$ (Sigma Aldrich), 1 g K_2HPO_4 (Sigma Aldrich), 5 g NaCl (Sigma Aldrich) and 20 g agar (Oxoid, Thermo Fischer Scientific, Reinach, Switzerland) (Harkin, Brück and Lynch 2015). No additional nutrients were added. Tryptic Soy Agar (TSA; Biolife, Milan, Italy) was prepared according to the manufacturer's instructions. All media were autoclaved.

BACTERIAL ISOLATION

Sample collection

Mealworms were purchased from Entomos (Grossdietwil, Switzerland) and stored for 72 h at 4°C before being sieved to remove small particulate matter. Insects were then fully submerged in liquid nitrogen until fully frozen. One hundred grams of aliquots of frozen insects were then homogenised in 200 ml of sterile deionised water containing 2 g ascorbic acid (Sigma Aldrich) for 1 min using an T25 Ultra-Turrax with S25 N 18 G dispersion attachment (IKA, Staufen, Germany). Homogenates were then sieved through a sterile 50 μm sieve to separate liquid and solid fractions. Liquid fractions were stored at –20°C for protein analysis in a separate project.

Screening of bacterial isolates

Ten grams of aliquots of the solid fractions were mixed with 90 ml of diluent containing 1.0 g/l peptone (Biolife) and 8.5 g/l NaCl (Acros Organics, Chemie Brunschwig AG, Basel, Switzerland) and 10-fold serial dilutions were prepared before 100 μl selected dilutions (10^{-2} to 10^{-5}) were pipetted onto TSA (Biolife) and chitin agar and incubated at 25°C for 72 h. Chitinase activity was observed through the formation of a zone of clearance around an isolate that grows on chitin agar.

Protease and acid production

Protease and acid production assays were performed as described previously and adapted from the Casein Digestion Method outlined by Keay and Wildi (1970) and the protease assay by Viswanatha *et al.* (2010) (Jung *et al.* 2007; Harkin, Brück and Lynch 2015). Briefly, overnight cultures were measured at 600 nm and adjusted to equal optical density before pelleting. One hundred microliters of supernatants were added to 200 μl 1% casein solution and 100 μl glycine buffer (pH 10) (Sigma Aldrich). Mixtures were then incubated at 75°C for 10 min before adding 600 μl trichloroacetic acid (Sigma Aldrich). After 10-min incubation at room temperature, the mixture is centrifuged at 12 000 × g for 5 min before 500 μl volumes of supernatant and Na_2CO_3 are added to 50 μl Folin & Ciocalteau's phenol reagent. Samples were mixed and incubated in the dark for 30 min at 37°C. The absorbance of the reaction was measured at 660 nm, and the protease concentration is determined using a tyrosine (Sigma Aldrich) standard curve. According to Keay and Wildi (1970), 'A unit of protease activity was defined as that quantity of enzyme which produced TCA-soluble fragments giving blue colour equivalent to 0.5 μg tyrosine under the conditions of the assay (1 protease unit = 0.5 μg tyrosine liberated in the enzyme reaction)'.

Identification of isolates

Isolates were grown in pure culture on TSA and were identified using standard Gram staining and bacterial mobility followed by Sanger sequencing of 16S rDNA. A GenElute Bacterial Genomic DNA Kit (Sigma Aldrich) was used with added lysozyme incubation at 37°C for 30 min after cell harvesting. 16S rDNA universal primers 27f (5′-AGAGTTTGATCATGCCTCAG-3′) and 1429r (5′-GGTTACCTTGTTACGACTT-3′) (Microsynth Balgach, Switzerland) developed by Frank *et al.* (2008) were used to generate PCR products as outlined by Harkin, Brück and Lynch (2015). PCR products were visualised on a 0.7% agarose gel in 1 X TAE buffer and cleaned using a QIAquick PCR Purification Kit (Qiagen, Hombrechtikon, Switzerland). Purified PCR products were sent to Microsynth for 16S rDNA sequencing. Resulting sequences were edited and aligned using ChromasPro v2.1.5 (Technelysium Pty Ltd, South Brisbane, Australia) and identified using BLAST N (Altschul *et al.* 1990). New sequences from this study were submitted to GenBank and given accession numbers: MF381033–MF381042.

Table 1. Isolates and their protease activity with % similarity to closest relative as identified by NCBI BLAST N, gram stain, motility test and colony morphology.

		Colony morphology									
Isolate	GenBank Accession No.	Form	Elevation	Margin	Colour	Opacity	Gram stain	Mobility	Protease activity (U/ml)	Closest GenBank Match	% Similarity
1 VA	MF381033	Irregular	Flat	Lobate	Red	Translucent	–	Yes	96.78	*Serratia marcescens*	100
1VG	MF381034	Circular	Convex	Entire	Yellow/mustard	Translucent	–	No	79.45	*Chryseobacterium* sp.	99
2VA	MF381035	Circular	Umbonate	Entire	Beige	Translucent	–	Yes	83.20	*Serratia liquefaciens*	99
3VB	MF381036	Irregular	Convex	Entire	Rosé/orange	Translucent	–	Yes	77.12	*Stenotrophomonas rhizophila*	99
10VA	MF381037	Circular	Flat	Entire	White	Translucent	–	Yes	90.18	*Serratia marcescens*	99
16VB	MF381038	Irregular	Convex	Undulate	White/beige	Translucent	–	Yes	97.34	*Serratia liquefaciens*	99
16VC	MF381039	Circular	Convex	Entire	White	Translucent	–	Yes	86.26	*Stenotrophomonas maltophilia*	99
17VA	MF381040	Circular	Convex	Entire	Brown (in)/beige (out)	Translucent	–	Yes	72.46	*Stenotrophomonas maltophilia*	99
17VB	MF381041	Circular	Pulvinate	Entire	White/beige	Translucent	+	Yes	75.81	*Brevibacterium sp.*	99
17VC	MF381042	Circular	Flat	Entire	Beige	Translucent	–	Yes	84.73	*Serratia marcescens*	99

Chitin extraction by fermentation

Fermentation conditions

Single colonies of isolates which exhibited the highest protease and acid production were used to inoculate 50 ml of TSB (Biolife) and incubated at 140 rpm for 24 h at 25°C.

Fermentations (triplicates) used 5 g of dried (100°C, 24 h) solid mealworm fractions with a particle size ≥50 μm as prepared above and 100 ml of sterilised 10% (w/v) glucose solution (Thermo Fischer Scientific) prepared in deionised water. TSB cultures (50 ml) were used were counted using a Neubauer hemocytometer and diluted to 1×10^6 cfu/ml (Xu, Gallert and Winter 2008) using the glucose/mealworm solution. Inoculated flasks were incubated at 175 rpm and 25°C for 5 days. After incubation, the pH of the solution was measured before the cultures were sieved using a sterile 50 μm sieve to recover the solid materials. The obtained solid fraction was sterilised by autoclaving and dried at 100°C for 24 h. The dried samples were weighed before chemical analysis or a second round of fermentation. For the second fermentation, the dried solid residues of the primary fermentation were added to 100 ml of sterilised 10% (w/v) glucose solution prepared in sterile deionised water containing 1×10^6 cfu/ml *Lactobacillus plantarum* (DSM 20 174) and incubated for 7 days at 175 rpm and 30°C. After incubation, the pH of the solution was again measured before the sample was sieved, sterilised and dried for chemical analysis.

Analysis of insect chitin

Residual mass and mineral content

To determine mineral content, the dry weight after fermentation was measured after sample drying at 100°C for 24 h. To mineral content, samples were incinerated in a muffle furnace (Nabertherm, Hägendorf, Switzerland) at 600°C for 6 h (Rao, Muñoz and Stevens 2000; Sorokulova *et al.* 2009). The mass of the residual ash was given as a percentage of the original dried material.

Fourier-transform infrared spectroscopy

Attenuated total reflectance-Fourier- transform infrared spectroscopy (ATR-FTIR) was used to characterise crude chitin from fermented mealworm fraction. Samples were compared with unfermented solid mealworm residue and a chitin previously produced from brown crab (*Cancer pagurus*) shells after chemical treatment (Lynch *et al.* 2016). Spectra were measured with a Nicolet iS50 FT-IR Spectrometer (Thermo Fisher Scientific, Dreieich, Germany) using a diamond ATR unit. The IR spectra were collected across the range 4000–400 cm^{-1}, at 2 cm^{-1} resolution and 100 scans without any further sample preparation. The obtained spectra were submitted to ATR correction to standardise the depth of penetration using OMNIC (v. 9.2; ThermoFisher Scientific, Dreieich, Germany).

RESULTS AND DISCUSSION

Bacterial isolates

Ten acid and protease-producing bacterial strains were identified (Table 1). All protease-producing strains also produced acid. No chitinase activity was observed using the chitin agar method used here. The combination of proteolysis and acidification mimics the traditional chemical extraction process of demineralisation using acid and deproteination using an alkaline step. This enables the liberation of chitin covalently bound to catechol and sclerotin in the insect exoskeleton (Liu *et al.* 2012). Edited and aligned sequences with 96% or above similarity in BLAST N were identified to genus and species level. Two isolates identified as *Serratia marcescens* (1VA) and *Serratia liquefaciens* (16VB) were found to exhibit the highest protease activity at 96.78 and 97.34 U/ml, respectively, under assay conditions. A total of five *Serratia* sp. with varying protease activity were found. *Serratia* have been hitherto identified from whole body samples from various insects such as *Heptophylla picea* Motschulsky and *Lithobius koreanus*, amongst others (Han *et al.* 2014). While *Serratia* have been extensively used for chitin extraction in crab waste and for the deacetylation of chitin to produce chitosan, no previous reports have been found using *Serratia* for the biological extraction of chitin from insects. *Serratia marcescens* has been previously found to exhibit extracellular endochitinases, a chitobiase and a factor (CH1) required for the hydrolysis of 'crystalline' chitin (Monreal and Reese 1969). At least four chitinase enzymes and a chitin-binding protein have been found in *S. marcescens* (van Aalten *et al.* 2000). Jung *et al.* (2006) described *S. marcescens* as a highly proteolytic bacterium that was used to deproteinate the shell waste in fermentations with *Lactobacillus paracasei*. A demineralisation of 97.2% was achieved. Using *S. marcescens* FS-3 as the sole fermenting organism, a demineralisation of 47% and deproteination of 84% were obtained, suggesting that *Serratia* is a useful organism for biological chitin production (Jo *et al.* 2008). Isolate 17VC in this study was identified as *S. marcescens*. However, it did not show any chitinase activity and only presented intermediate protease activity at 84.73 U/ml. Hence, the two *Serratia* with the most proteolytic activities (1VA and 16VB) found here were used to produce chitinous material from solid,

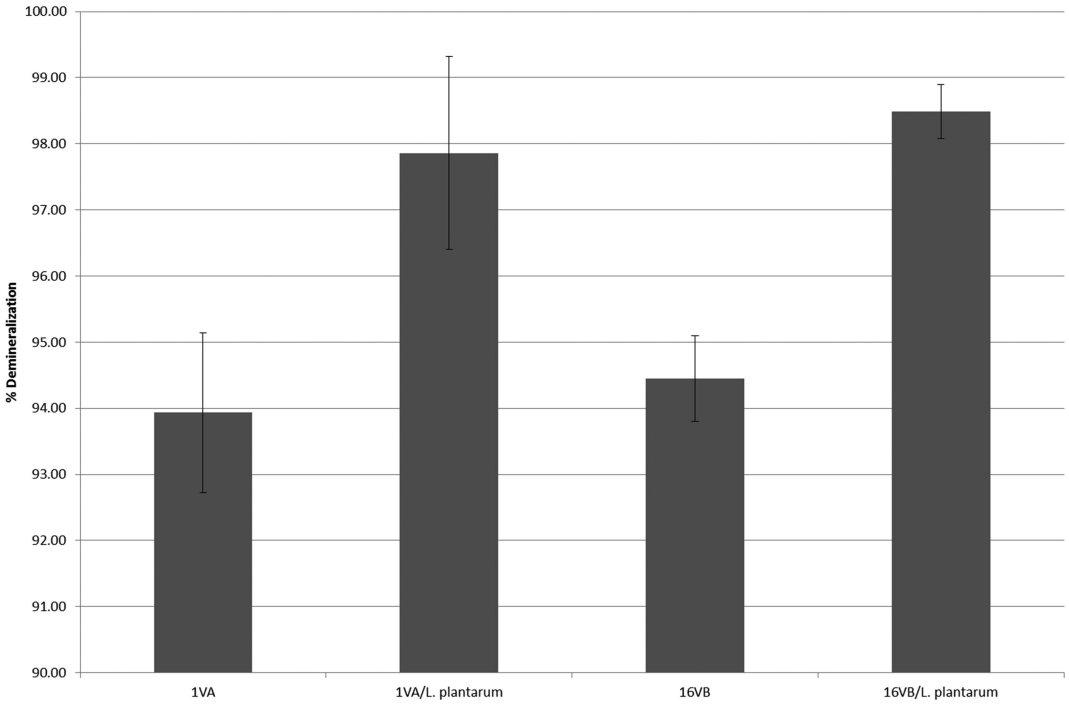

Figure 1. Demineralisation (%) of 5 g solid, deproteinated mealworm fractions achieved by respective isolates in single-step or successive two-step fermentations with *L. plantarum* (DSM 20 174).

deproteinated mealworm fractions through fermentation in this study.

Three further isolates have been identified as *Stenotrophomonas rhizophila* and *St. maltophilia*, an opportunistic human pathogen and common soil organism, part of the rhizosphere (Berg, Roskot and Smalla 1999). *Stenotrophomonas maltophilia* isolate 17VA exhibited the lowest protease activity at 72.46 U/ml under assay conditions in this study. Two further isolates have been identified as *Chryseobacterium* sp., a psychrotrophic and proteolytic bacterium that causes a variety of defects in meat and dairy products, and *Brevibacterium* sp., a Gram-positive soil organism of the order Actinomycetales (Gavrish *et al.* 2004; Bernardet *et al.* 2005).

Yield and mineral content of fermented solid, deproteinated mealworm fractions

Chitin extracted from solid, deproteinated mealworm fractions by fermentation was an odourless, brown, crystalline powder. The darker color indicated that catechol compounds, sclerotin-like proteins and pigment could not completely be removed in the deproteination process using *S. marcescens* (1VA) and *S. liquefaciens* (16VB) isolated from mealworm exoskeletons. The mean yield of chitinous material (dry weight) after treatment was 1.41 g per 5.0 g starting material (±0.15, 28.2%) for 1VA and 1.43 g (±0.11, 28.6%) for 16VB. Analysis of mineral content through ash analysis showed that all chitinous samples produced were about 94% demineralised (Fig. 1). The mean pH after the first fermentation was 3.9 (±0.2) for isolate 1VA and 3.4 (±0.3) for isolate 16VB.

For fermentations with 1VA followed by *Lactobacillus plantarum* (DSM 20 174) and 16VB followed by *L. plantarum*, the mean yield of chitinous material (dry weight) was 0.93 g (±0.09, 18.6%) and 0.86 g (±0.12, 17.2%), respectively. Yields of chitinous material were comparable with those of other studies. Zhang *et al.* (2000) extracted chitin from the larva cuticle and pupa of the domesticated silk moth. Yields of 15%–20% using chemical extraction were achieved. This compared with a chitin yield of 15% from adult Holotrichia *parallela* (Liu *et al.* 2012).

Two-step fermentations using either isolate 1VA or 16VB followed by *L. plantarum* yielded a slightly higher demineralisation (DM) at 97.9 (±1.46) and 98.5 (±0.41) %, respectively (Fig. 1). The pH after the second fermentation was 3.3 (±0.4) and 3.9 (±0.2).

Lactobacillus plantarum is used extensively for biological chitin production and was shown to be more effective than other *Lactobacillus* sp. (Khanafari, Marandi and Sanatei 2008; Zhang *et al.* 2012). Using *L. plantarum*, the chitin yield is identical to that achieved with chemical extraction using *Penaeus semisulcatus* waste (Khanafari, Marandi and Sanatei 2008). When *L. plantarum* was used as a single fermentative organism in combination with low calcium carbonate content waste material such as shrimp shell, a DM of 90% was obtained (Rao, Muñoz and Stevens 2000). *Lactobacillus plantarum* with *S. marcescens* resulted in a slightly higher DM of 94% (Zhang *et al.* 2012). Using Fe $(NO_3)_3$ further optimised chitin extraction conditions for *L. plantarum* and resulted in higher yields (Khanafari, Marandi and Sanatei 2008). The higher demineralisation efficiencies of the fermentation steps in this study may be due to insects including mealworms having lower mineral levels when compared to crustacean shell waste (Tolaimate *et al.* 2003).

FTIR characterisation of fermented solid, deproteinated mealworm fractions

The efficiency of chitin extraction from mealworms was determined by Diamond ATR-FTIR-spectrometry (Fig. 2). In the region of 3700–3000 cm^{-1}, overlapping of the broad ν(O–H) band of bonded OH-groups and of the absorbance of the ν(N–H) stretching vibrations takes place. The spectra further show the absorbance of the amide group of chitin. The ν(C=O) stretching vibration (amide I band) is due to interchain and intrachain hydro-

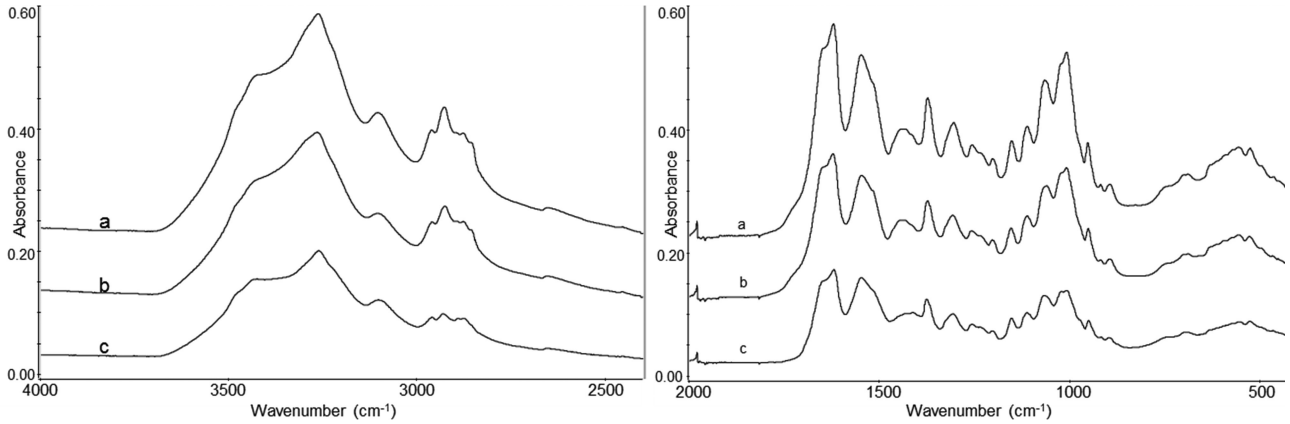

Figure 2. Diamond ATR-FTIR spectra of the solid, deproteinated mealworm fractions after extraction with (**A**) 1VA and *L. plantarum* (DSM 20 174), (**B**) 16VB and *L. plantarum* (DSM 20 174), (**C**) chitinous material from brown crab (*Cancer pagurus*) after chemical treatment (Lynch *et al.* 2016).

gen bonding (–NH···O=C–, and –OH···O=C–, respectively) in the crystalline material split into two bands at 1653 and 1619 cm^{-1}; the δ(N–H) deformation vibration (amide II band) can be found at 1552 cm^{-1}. The spectra of the fermented solid, deproteinated mealworm fractions matched those of chemically treated crab material analysed previously and show the absorbance of crystalline amide groups of chitin (Limam *et al.* 2010; Zaku *et al.* 2011; Hajji *et al.* 2014). This result demonstrates that chitin from brown crab and mealworms are in α form.

CONCLUSIONS

The study showed that isolated bacteria from the solid residues of deproteinated mealworms had the necessary characteristics to extract chitin. Fermentation profiles and results followed closely those of crab shell wastes. Chitin production from food insect wastes through fermentation demonstrates an environmentally friendly way of waste reduction while providing an efficient protein source for a growing population. However, more experience is needed before this technology is ready for pilot scale and commercial production. Variables such as processing time, substrates, substrate quantities and process volumes, amongst others will have to be further analysed in a life cycle analysis in order to estimate production costs and efficiency for a proper comparison between traditional substrates and processes with insect exoskeleton fermentation. Ultimately, a combination of chemical and fermentation protocols to optimise yield and costs while improving product color may be needed.

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