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ORIGINAL ARTICLE

Isolation & identification of bacteria for the treatment of brown crab (*Cancer pagurus*) waste to produce chitinous material

C. Harkin[1], W.M. Brück[1,2,3] and C. Lynch[1,4]

1 Letterkenny Institute of Technology, Letterkenny, Co. Donegal, Ireland
2 BDW-Consulting, Köln, Germany
3 Institute of Life Technologies, University of Applied Sciences Western Switzerland, Sion, Switzerland
4 Earagail Eisc Teoranta (Errigal Seafood), Mín an Aoire, AnCharraig, Co. Donegal, Ireland

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Correspondence
Wolfram Brück, Institute of Life Technologies, University of Applied Sciences Western Switzerland, HES-SO Valais-Wallis, Route du Rawyl 47, 1950 Sion, Switzerland.
E-mail: wolfram.bruck@hevs.ch

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Abstract

Aims: To isolate bacteria from soil for microbial pretreatment of brown crab (*Cancer pagurus*) shell waste and the production of chitin.
Methods and Results: Isolates were screened for protease enzymes and acid production in order to facilitate the removal of protein and calcium carbonate fractions from brown crab shell to yield a chitinous material. Selected isolates were applied in various combinations in successive, two-step fermentations with brown crab shell waste. These isolates were identified as: *Exiguobacterium* spp. (GenBank accession number: KP050496), *Bacillus cereus* (GenBank accession number: KP050499), *B. subtilis* (GenBank accession number: KP050498), *Bacillus licheniformis* (GenBank accession number: KP050497), *Pseudomonas migulae* (GenBank accession number: KP050501), *Pseudomonas* spp. (GenBank accession number: KP050500), *Pseudomonas* spp. (GenBank accession number: KP050502), *Arthrobacter luteolus* (GenBank accession number: KP050503), *Lactobacillus* spp. (GenBank accession number: KP072000) and *Enterococcus* spp. (GenBank accession number: KP071999).
Conclusions: Successive two-step fermentations with isolates in certain combinations resulted in a demineralization of >94% and the extraction of a crude chitin fraction from brown crab processing waste. The highest demineralization, 98·9% was achieved when isolates identified as *B. cereus* and *Pseudomonas* spp. were used in combination. The transfer of fermentations to a larger scale requires further research for optimization.
Significance and Impact of the Study: The successful application of these isolates in successive two-step fermentation of brown crab shell waste to extract chitin means with further research into optimization and scale up, this chitin extraction process may be applied on an industrial scale and provide further commercial value from brown crab shell waste.

Introduction

Chitin is the second most abundant naturally occurring polysaccharide next to cellulose. It is found in the exoskeletons and internal structure of invertebrates such as crabs, shrimp, krill and insect species. It is also found in the cell walls of some fungi. Chitin is a hard, inelastic material which is insoluble in water (Hu *et al.* 2007). Chitin and its de-acetylated derivative, chitosan, are incredibly useful polymers with an ever-expanding variety of applications in areas including wound and burn treatment (Pant *et al.* 2013), food processing (Knorr 2006), agriculture (Senthilraja *et al.* 2010), tissue engineering (Chang *et al.* 2013) and drug delivery (Shang *et al.* 2014).

The largest source of chitin is biowaste generated by crustacean processing. In 2012 alone, the global aquacul-

ture production of crustaceans was estimated to be six and a half million tonnes (Food and Agriculture Organisation of the United Nations 2014). Most of this waste is discarded in landfill sites or dumped in the sea in extremely large amounts which causes major global environmental concern (Gimeno *et al.* 2007; Ghorbel-Bellaaj *et al.* 2012). Brown crab is the third most important marine species landed in Ireland. In 2004, 13 000 tonnes of brown crab was harvested in Ireland (Tully *et al.* 2006) making this species an abundant source of chitin.

Crustacean waste consists primarily of 30–40% protein, 30–50% calcium carbonate and 20–30% chitin, depending on the species and season (Vani and Stanley 2013). In industry, chitin is extracted chemically using strong acids and alkalis to remove unwanted mineral (calcium carbonate) and protein fractions. This proves costly and is a cause of environmental pollution. The application of biological methods has been explored to alleviate these problems and provide a safe, economical alternative. Direct treatment of crustacean waste with organic acids and enzymes (Wang *et al.* 2008; Das and Ganesh 2010) and the fermentation of crustacean waste with microorganisms that produce these reagents has been investigated (Oh *et al.* 2007; Zhang *et al.* 2012).

This study investigates the isolation and identification of bacteria possessing the necessary characteristics to extract chitin specifically from brown crab (*Cancer pagurus*) through processing waste by fermentation methods.

Materials and methods

Bacterial isolation and screening

Sample collection

Drained subpeat mineral soil samples were taken from a previously farmed area in the Carrick region of southwest Donegal, Ireland. Several collection points were selected to allow for variability. Soil samples were collected using a spade and large sterile spoons. The samples were stored in sterile 50 ml falcon tubes at 4°C for short-term storage or −20°C for long-term storage.

Agar preparation

Casein agar was prepared according to a recipe laid out by Jyothis (2010). The stated media contained per litre: 10 g casein (BDH Chemicals, VWR, Radnor, PA, USA), 0·5 g calcium chloride 2-hydrate ($CaCl_2.2H_2O$) (BDH Chemicals), 1 g dibasic potassium phosphate (K_2HPO_4) (Sigma), 5 g sodium chloride (NaCl) (Acros Organics, Fisher Scientific Ireland, Dublin 15, Ireland) and 20 g agar (Oxoid, Oxoid Limited, Basingstoke, UK). Marine agar (BD, Difco Marine Agar 2216) was prepared in 1/5 strength by adjusting the manufacturer's instructions to 1/5 concentration and topping up the agar (Oxoid) concentration to full strength. Chitin agar was prepared using 10 g chitin (Sigma, Sigma-Aldrich Ireland Ltd., Wicklow, Ireland) and 15 g agar (Oxoid). No additional nutrients were added. All other media was prepared according to the manufacturer's instructions in deionised water and sterilised by autoclave at 121°C for 15 min at 1 atm pressure.

Screening of bacterial isolates

Ten-fold serial dilutions of soil samples were prepared in sterile Tryptone Soya Broth (TSB). Selected dilutions (10^{-3} and 10^{-6}) were transferred to agar plates by pipetting 100 μl aliquots of sample dilutions and spreading on several agars: Tryptone Soya Agar, Casein agar (Jyothis 2010), Chitin agar and 1/5 strength Marine agar. The agar plates were incubated at 37°C for 24 h (Binder BF53; Tittlingen, Germany).

Screening of bacterial isolates for acid production

To preliminarily determine acid production, selected colonies from spread plates were transferred into 750 μl sterile Tryptone Soya Broth (TSB) (Oxoid) and incubated on a shaking incubator at 140 rev min^{-1} (Stuart Orbital Incubator Si500; Staffordshire, UK) for 24 h. The optical density (OD) of overnight cultures was measured at 600 nm (SPECTROstar Omega microplate reader; BMG Labtech, Ortenberg, Germany) and diluted accordingly so that all were equal. To a sterile 1·5 ml centrifuge tube 0·025 g of brown crab shell and 500 μl 10% glucose solution (w/v) was added (Jung *et al.* 2007) and 50 μl of the respective isolate in culture was used to inoculate the mixture. Positive and negative controls were set up using acid-producing bacterium *Lactobacillus plantarum* and sterile TSB as inocula respectively. The samples were incubated at 30°C at 140 rev min^{-1} for 72 h and 20 μl of 0·01% methyl red pH indicator (M&B Chemicals, Tokyo, Japan) was added. A positive result was indicated by the production of a red colour (<pH 4·2).

Screening for protease production

Protease production was indicated by the production of a zone of proteolysis surrounding isolated colonies on spread plates prepared on casein agar. The distance from the edge of the colony to the outer edge of the zone of proteolysis was measured to approximate the strength of the isolate's ability to produce proteases. Protease production was further quantified using a colorimetric assay. The method of Vishwanatha *et al.* (2010) was modified and carried out as follows: The optical density (OD) of overnight cultures was measured at 600 nm and adjusted so that all were equal. Cultures were centrifuged to pellet bacterial cells and 100 μl of each culture supernatant was added to a sterile

1·5 ml centrifuge tube containing 200 μl 1% casein solution (BDH Chemicals) and 100 μl of glycine buffer (pH 10) (Merck). The mixture was incubated at 75°C for 10 min. The reaction was terminated by the addition of 600 μl 10% trichloroacetic acid (TCA) (Fisher Chemicals, Fisher Scientific Ireland, Dublin 15, Ireland). The mixture was allowed to stand for 10 min at room temperature and centrifuged at 12 000 ***g*** for 5 min. To a 500 μl aliquot of the supernatant, 500 μl 0·4 mol sodium carbonate (Na_2CO_3) and 50 μl Folin & Ciocalteau's phenol reagent were added. Samples were mixed by inversion and incubated at 37°C in the dark for 30 min. The absorbance was measured at 660 nm. A tyrosine (Fisher Chemicals) standard curve was prepared and used to determine the protease activity of the cultures as follows: one protease unit was defined as the amount of enzyme that released 1 μg of tyrosine ml^{-1} per minute under the above assay conditions.

Identification of isolates

Gram staining of bacterial isolates

A smear was prepared in sterile deionised water using cultures incubated for 18 h and left to air dry. To fix the cells, two drops of 95% (v/v) methanol (Fisher Chemicals) was applied to the smear and left for 2 min to air dry. The gram staining solutions (bioMérieux SA, Marcy l'Etoile, France) were applied to the dry, fixed smear as follows: crystal violet for 1 min, iodine for 1 min, decolouriser, to wash the smear until the solution was clear and safranin for 1 min. Gentle washing using sterile deionised water for 2 s was carried out between applications of solutions. The stained smear was blotted gently with tissue paper and viewed under a light microscope (Olympus BX51, Hamburg, Germany) with a 100× objective lens under oil-immersion.

Motility testing of bacterial isolates

Single colonies (18 h) were picked from nutrient agar plates and transferred to a drop of sterile PBS (1×) (Sigma) on a clean glass slide. A cover slip was placed over the mixture gently and at an angle to avoid air bubbles. The slide was immediately viewed using an inverted microscope (Olympus IX51) with a 40× phase contrast objective (LC Plan fl 40× Ph2).

Genomic DNA isolation

Single colonies were picked from nutrient agar plates and inoculated into 750 μl sterile Luria Broth, Miller (LB) (Fisher Bioreagents, Fisher Scientific Ireland, Dublin 15, Ireland) in sterile 1·5 ml centrifuge tubes and incubated overnight) at 140 rev min^{-1} and 37°C. Un-inoculated media was transferred to a sterile 1·5 ml centrifuge tube and incubated also to ensure its sterility and ensure the cultures were not contaminated. The bacterial cells were harvested by centrifugation at 13 000 ***g*** for 2 min and the supernatant was discarded. A Wizard Genomic DNA Purification Kit (Promega, GmbH, Mannheim, Germany) was used to isolate genomic DNA from bacterial isolates according to the manufacturer's instructions. Genomic DNA was viewed by gel electrophoresis (1% agarose) in tris acetate EDTA buffer (1×) and using a UV transilluminator (Alpha Innotech Corporation, San Leandro, CA, USA) in conjunction with Alpha Digi Doc RT software for visualization of the bands.

PCR and product purification

Two pairs of 16S rRNA Universal primers: 27f (5′-AGA GTTTGATCATGCCTCAG-3′) and 1429r (5′-GGTTACCT TGTTACGACTT-3′) (Eurofins MWG Operon) (Frank *et al.* 2008) and Eco9f (5′-GACTTTGATCCTGGCTCA G-3′) and Lop27rc (5′-GACTACCAGGGTATCTAATC-3′) (Sigma Genosys, Sigma-Aldrich Ireland Ltd., Wicklow, Ireland) (Brück *et al.* 2008) were used to generate PCR products from genomic DNA.

The preparation of PCR reagents was carried out in a PCR workstation (Bigneat, Waterlooville, UK). The PCR mix was prepared in a sterile 1·5 ml centrifuge tube containing the following components in their final concentrations: 10× PCR Rxn buffer, Dinucleotide triphosphate mix (dNTPs) (200 μmol each dNTP), Magnesium chloride ($MgCl_2$) (3 mmol), 16S rRNA forward and reverse primers (0·25 μmol), Go Taq DNA Polymerase (1·25 U) and nuclease free water (NFH_2O). Using this mix, the reaction was set up as follows in 0·5 ml PCR tubes: 45 μl of master mix, 100 ng genomic DNA of respective sample, made up to 50 μl with NFH_2O. The tubes were inserted into a G-Storm Thermal Cycler (Somerset, UK) and the following cycles repeated for each set of primers: 27f & 1429r: 1 cycle 95°C 15 min, 33 cycles of 95°C 45 s, 55°C 30 s and 72°C 1 min 30 s and 1 cycle 72°C 10 min. Eco9f & lop27rc: 1 cycle 95°C 5 min, 30 cycles 94°C 5s, 53°C 1 min 30 s, 72°C 2 min and 1 cycle 72°C 30 min. PCR products were visualized on a 0·7% agarose gel in tris acetate EDTA buffer (1×) and using a UV transilluminator (Alpha Innotech Corporation) in conjunction with Alpha Digi Doc RT software for visualization of the bands.

The PCR product bands were physically cut from the gel using a sterile scalpel and a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) was used to wash and purify the PCR fragments according to the manufacturer's instructions.

16S rRNA sequencing

The purified PCR products were sent to outside companies for 16S rRNA sequencing: Beckman Coulter and

Queen's University Belfast Genomics Core Technology Unit. Resulting sequences were queried using the National Center for Biotechnology Information (NCBI) BLAST software (NCBI, Bethesda, MD, USA) using the 16S ribosomal RNA database.

Chitin extraction by fermentation

Crustacean shell waste preparation

Brown crab (*C. pagurus*) processing waste was supplied by Errigal Seafood (Earagail Eisc Teoranta Ltd). It had been boiled and minced during processing and arrived wet with some crab meat still remaining attached. Initial preparation involved breaking/crushing larger pieces. The shell pieces were then suspended in water and broken down further with a dispenser (Ika Ultra Turrax T25 Basic, Staufen, Germany). The shear force of this technique also separated meat and tissue away from the exoskeleton which floated and was then decanted off. The shell was then dried in the oven at 55°C overnight. Following this, it was ground further using grinders (Krups 75 Offenbach am Main, Germany & de Longhi KG40, Treviso, Italy) and an electric mill (Ika, A 11 Basic Analytical mill, Staufen, Germany). The resulting shell fragments were sifted to ensure a uniform size of approx. 1 mm and stored at 4°C for short-term storage or −20°C for long-term storage.

Fermentation conditions

Single, pure colonies of selected isolates from agar plates were used to inoculate 20 ml of Luria broth, Miller (LB) (Fisher Bioreagents) in sterile falcon tubes to prepare starter cultures. The tubes were incubated at 140 rev min^{-1} for 48 h (Jung *et al.* 2007) at 37°C or 25°C depending on optimum growth conditions of the isolates. To prepare an inoculum for fermentations, 2 ml of the starter cultures was transferred into 20 ml of sterile LB and incubated at 140 rev min^{-1} at 37 or 25°C for 2 days.

Fermentation setup involved the addition of brown crab shell and 10% glucose solution (Fisher Scientific Ireland, Dublin 15, Ireland) in a 1 : 20 (w/v) ratio (Jung *et al.* 2007) to a sterile Erlenmeyer flask with a foil cap. The optical densities (OD) of selected isolates in culture were measured using a Shimadzu Biotech Biospec Mini (Mason Technology, Dublin, Ireland) to determine concentration. The desired concentration of culture (1×10^6 colony forming units (CFU) per millilitre of glucose; Xu *et al.* 2008) was added to the flask. Once inoculated, the flasks were covered, labelled and incubated at 175 rev min^{-1} and 30°C.

After 5 days the pH of the solution was determined by immersion of a pH meter (WTW pH197i, WTW, Welheim, Germany) whose electrode was sterilized using 70% (v/v) ethanol (Lennox), the solutions were filtered and the solid (brown crab shell) material washed with deionised water and sterilised with 70% (v/v) ethanol. To the brown crab shell, fresh 10% glucose solution and fresh inoculum were added and the mixture was incubated for a further 7 days at 175 rev min^{-1} and 30°C. At the end of the 7 day incubation, the pH was determined and the sample filtered, washed and sterilised. In some fermentations the cultures used to inoculate varied for each incubation step. This was based on the respective properties of the isolates and was to test the efficacy of demineralization using a protease-producing isolate first, and an acid-producing isolate second *vs* the use of an acid-producing isolate alone.

Fermentations were initially carried out in a small scale, 5 g brown crab material in 100 ml 10% (w/v) glucose, this was then increased to 10 g in 200 ml 10% (w/v) glucose.

Analysis of chitinous material

Yield and mineral content

Residual dry weight and chitin yield after fermentation was measured after drying the resulting chitinous material at 55°C for 24 h. To determine the mineral content of the samples ash content was determined. A given mass of samples were placed into a muffle furnace (Carbolite AA F 1100, Derbyshire, UK) at 600°C for 6 h (Ra1o *et al.* 2000; Al Sagheer *et al.* 2009; Sorokulova *et al.* 2009). The resulting mass was expressed as a percentage of the mass of the original material.

Characterization by Fourier transform infrared spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) was used to further characterize the crude chitin obtained and compare with unfermented brown crab waste and commercial chitin (Obtained from Biotech Surindo, Jawa Barat, Indonesia). A Perkin-Elmer FT-IR Spectrum BX (Cambridgeshire, UK) system was used in conjunction with SPECTRUM (v5.3.1) software. Samples were dried at 100°C for 24 h and stored in a dessicator until use. Sample discs were prepared by grounding with dried potassium bromide (KBr) (Fisher Chemicals) and applying a pressure of 10 tonnes for 2 min.

Analysis was carried out across the range 4000–400 cm^{-1}; resolution was 2 cm^{-1} with 20 scans per sample. The degree of acetylation (DA %) was measured according to the formula obtained from Hajji *et al.* (2014): DA % = (A1650/A3450)/1·33 × 100.

The 'A' represents the absorbance of the respective wavenumbers 1650 and 3450 cm^{-1}. The amide-I band (1650 cm^{-1}) was used as the analytical band and the hydroxyl band (3450 cm^{-1}) as the internal reference

band. The factor '1·33' denoted the value of the ratio of A1650/A3450 for fully N-acetylated chitin.

High performance liquid chromatography (HPLC)
Organic acids were tested for using a Shimadzu HPLC system (Mason Technology) with CLASS-VP (ver. 6.1) software used in conjunction. A Bio-Rad Organic Acid Analysis Kit was used, containing an Aminex HPX-87H column and a mixed organic acid standard (Oxalic acid, citric acid, malic acid, succinic acid, formic acid and acetic acid) was used. A separate lactic acid (Fluka Analytical, Sigma-Aldrich Ireland Ltd., Wicklow, Ireland) standard was prepared in the mobile phase in concentrations ranging from 5 to 60 mmol. Fermentation supernatant samples were centrifuged and filtered through 0·22 μm syringe filters. The mobile phase was 4 mol sulphuric acid (H_2SO_4) (Barboni *et al.* 2010).

Analysis was carried out with a mobile phase flow rate of 0·6 ml min^{-1} and the column oven temperature at 35°C. Samples and standards were eluted isocratically over 35 min and detected at 210 nm using a photodiode array detector.

Results

Screening of bacterial isolates

A total of 10 isolated bacterial strains that had the highest acidogenic and protease-producing activities were selected for application in fermentations and were identified by 16S rRNA sequencing (Table 2). Two isolates, C5 and C11 were found to exhibit both acidogenic and protease-producing properties, while the remaining isolates exhibited only one.

Protease production screening

Four protease-producing isolates were selected for application in fermentations (Table 1). The measurement of the zone of proteolysis surrounding colonies on casein agar suggested isolates C16 and C14 were equally effective in the production of proteases. However, the colorimetric protease assay identified C16 as the highest protease-producing bacterium with 186·047 U ml^{-1} of protease enzyme produced under assay conditions as opposed to 161·047 U ml^{-1} produced by C14. Isolates C11 and C5 were less effective with 137·21 and 80·814 U ml^{-1} enzyme produced under assay conditions.

Table 1 Protease activity of bacterial isolates used in fermentation with brown crab. The measurement of the zone of hydrolysis surrounding a colony on casein agar is given in mm and the protease activity determined by a protease assay is given in U ml^{-1}. Average values are given ± the standard deviation

Bacterial isolate	Zone of hydrolysis on casein agar (mm)	Protease U ml^{-1}
C16	2 ± 0·61	186·047 ± 0·85
C14	2 ± 0·42	161·047 ± 0·72
C11	1 ± 0·63	137·21 ± 0·91
C5	0·5 ± 0·44	80·814 ± 1·24

Acid production screening

Isolates that tested positive for acid production with a low pH after addition of methyl red indicator were selected to apply in fermentations. There were eight acidogenic isolates: C5, C11, Ch2, Ch9, Ch11, Ch24, Ma22 and A11. Isolates C5 and C11 were also found to produce proteases.

Identification of bacterial isolates

The identification of the final 10 isolates was obtained by querying sequences of purified PCR products of isolates using National Center for Biotechnology Information (NCBI) BLAST software (Table 2) and using the 16S ribosomal RNA sequences (Bacteria and Archaea) database. Partial sequences of isolates C5 and Ma22 were used. Sequence identification with a similarity percentage of 96% or above was identified by genus and species. Sequence identification of 93–96% similarity was identified by genus only.

Efficacy of demineralization

Demineralization (DM) was effective using selected bacterial isolates, with over 94% demineralization in all chitinous samples produced (Fig. 1).

The most successful demineralization of 98·9% was achieved at 10 g with C16 + Ch11 isolates used in combination. Fermentations using C14 + Ma22, Ch9, C16+ Ch11 and C16 + Ch24 all achieved demineralization of >96%, using both 5 and 10 g brown crab shell. Isolate C5 achieved the lowest demineralization at both 5 and 10 g amounts of shell.

The yield of chitinous material was measured from the dry weight of samples and ranged from 12·7–18·1% at 5 g to 13·8–16·3% at 10 g (Table 3). It is estimated in the literature that crustaceans contain 20–30% chitin depending on species and season (Vani and Stanley 2013). Further optimization may serve to increase the yield obtained by this method.

Figure 2 shows the difference in demineralization efficacy between fermentations using acid-producing bacteria alone for both steps, isolates Ma22, Ch11 and Ch24, and when used in combination with a protease-producing iso-

Table 2 Isolates with closest relative as identified by the 16S rRNA database from NCBI BLAST, query cover and per cent similarity, gram stain, motility test and colony morphology

Sequence	Closest relative	GenBank accession number	Cell morphology	Colony morphology	Gram stain	Motility	Similarity (%)	Query cover (%)
C5	*Exiguobacterium* spp.	KP050496	Rods, single	Puctiform-1 mm, cream/orange pigment, slightly translucent, round, entire margin, convex, shiny.	–	+	96	97
C11	*Bacillus licheniformis*	KP050497	Rods, single	Colonies 1 mm, cream pigment, irregular margin, flat elevation, dull surface.	+	+	99	100
C14	*Bacillus subtilis*	KP050498	Rods, clusters	1–2 mm, cream pigment, round, entire margin, crateriform elevation, dull surface.	+	+	99	99
C16	*Bacillus cereus*	KP050499	Rods, pairs	1–2 mm, cream pigment, irregular margin, flat elevation, dull surface.	+	+	99	98
Ch2	*Pseudomonas* spp.	KP050500	Rods, long	Punctiform, cream/yellow pigment, translucent, round, convex, shiny surface.	–	+	98	92
Ch9	*Pseudomonas migulae*	KP050501	Rods, single	Punctiform, round, cream translucent pigment, convex, shiny.	–	+	98	98
Ch11	*Pseudomonas* spp.	KP050502	Rods, single	Punctiform, cream/yellow pigment, round, convex, shiny.	–	+	99	100
Ch24	*Arthrobacter luteolus*	KP050503	Rods, short	Colonies punctiform, yellow pigment, translucent, round, convex, shiny	+	+	99	98
Ma22	*Lactobacillus* spp.	KP072000	Rods, single	Punctiform, white/cream pigment, round, convex, shiny surface	+	–	99	99
A11	*Enterococcus* spp.	KP071999	Cocci, clusters	Punctiform, translucent/white pigment, round, convex, shiny surface	+	–	94	99

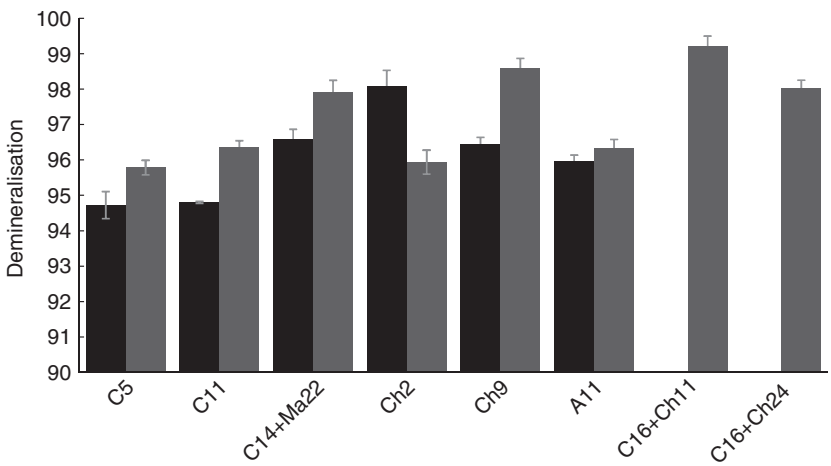

Figure 1 Demineralization (%) of brown crab achieved by respective isolates in successive two-step fermentations using both 5 g (■) and 10 g (■) brown crab. Where, 'isolate + isolate', each was used in one of the fermentation steps. For isolates C16 + Ch11 and C16 + Ch24, there are no data for 5 g brown crab. Error bars represent the standard deviation of duplicate samples.

late, C14 and C16 in the first step. It can be seen from the figure that the demineralization efficacy was increased when the use of a protease-producing isolate was used in the fermentation. For isolates C14 + Ma22, demineralization increased to almost 98% when both acid and protease producers were used compared to a demineralization of below 90% when just the acid-producing Ma22 was used. In the same way, the use of isolate C16 in the first step of the successive fermentation, in combination with isolates Ch11 and Ch24, increased the demineralization from just over 92 and 93% to 99 and 98% respectively.

Chitin quality

It can be seen in Fig. 3 that the FTIR spectra of all isolates are similar to the commercial chitin. Absorption

Table 3 Yield (%) of chitinous material obtained from successive two-step fermentations using selected bacterial isolates for 5 and 10 g brown crab. Where, 'isolate+isolate', each was used in one of the fermentation steps. Average values are given ± the standard deviation

Isolates used	Isolate codes	Yield % (5 g brown crab)	Yield % (10 g brown crab)
Exiguobacterium spp.	C5	12·7 ± 0·56	13·8 ± 0·85
Bacillus licheniformis	C11	13·8 ± 2·23	14·5 ± 0·99
Bacillus subtilis + *Lactobacillus* spp.	C14 + Ma22	18·1 ± 2·40	14·7 ± 1·56
Bacillus cereus + *Pseudomonas* spp.	C16 + Ch11	NA	15·4 ± 1·56
B. cereus + *Arthrobacter luteolus*	C16 + Ch24	NA	14·1 ± 1·41
Pseudomonas spp.	Ch2	20·4 ± 1·97	16·3 ± 0·42
Pseudomonas migulae	Ch9	14·1 ± 0·71	15·5 ± 0·85
Enterococcus sp.	A11	14·1 ± 0·14	14·6 ± 1·13

bands at 1600–1700 cm^{-1} show the C=O stretching vibration. The splitting of this band indicates that this is α-chitin and not β-chitin. The difference is due to the presence of strong hydrogen bonds that are present in α-chitin but not in β-chitin. Other characteristic chitin absorptions include bands at approx. 1550 cm^{-1} which represent a N-H bend, bands at approx. 2900 cm^{-1} represent a C-H stretch from the CH_3 molecule in chitin structure and bands at approx. 1450 cm^{-1} represent a C-N stretch (Limam *et al.* 2010; Zaku *et al.* 2011; Hajji *et al.* 2014).

The degree of acetylation of the chitin samples are listed in Table 4. Dimzon *et al.* (2013) states that if the degree of acetylation is below 60%, the polymer is deacetylated sufficiently to be considered chitosan. In this case, all samples are above 60% acetylation, which means they have not been deacetylated to chitosan during fermentation.

Production of organic acids

Fermentation supernatant from the second step of fermentations using 10 g brown crab were chosen as the most representative samples of the efficacy of the isolates due to the highest demineralization rate achieved using this amount for all isolates except Ch2. Where there are isolates in combination, the data represent the second isolate only.

Lactic acid was produced in the highest concentration by all isolates (mmol concentrations). Isolate Ma22 produced the highest concentration of 124·208 mmol. The lowest concentration of 26·929 mmol was produced by isolate C5 (Fig. 4).

Acetic acid was produced in the second highest concentration by all isolates in concentrations ranging from 46·380 to 42·397 μmol. Isolate C5 produced the highest concentration of acetic acid and isolate Ma22 produced the lowest (Fig. 5).

Most remaining organic acids tested for (oxalic acid, citric acid, malic acid, succinic acid formic acid) are present in μmol concentrations (Fig. 5). All isolates produced oxalic acid in the next highest concentration following acetic acid, except isolate Ma22. Formic acid was produced by isolates Ma22, Ch2 and Ch9. Citric acid was produced in the lowest concentrations for all isolates. All isolates tested have been shown to produce organic acids (Freitas *et al.* 1999; Hoberg *et al.* 2005; Chen *et al.* 2006; Crapart *et al.* 2007; Zhang *et al.* 2012).

Discussion

Isolate C5 was identified as an *Exiguobacterium* spp. (GenBank accession number: KP050496). It tested positive in both acid and protease production assays. *Exiguobacterium*

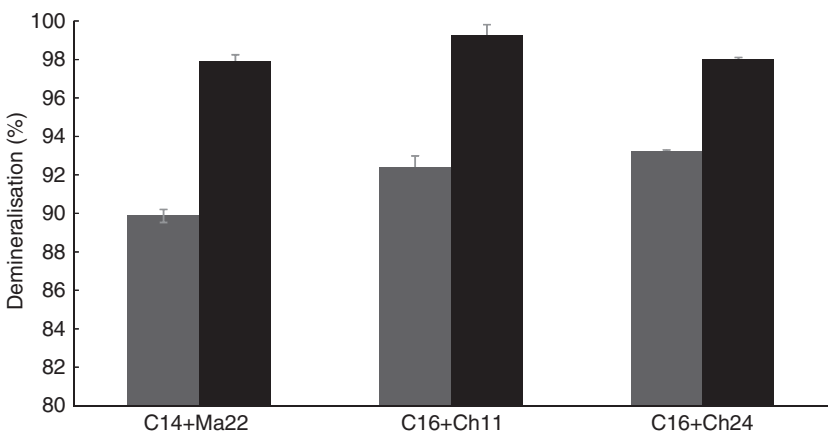

Figure 2 Comparison of demineralization efficacy of brown crab achieved in successive two-step fermentation using; (a) (■) an acid-producing isolate for both steps, Ma22, Ch11 and Ch24 respectively, (b) (■) a protease-producing isolate in step 1, C14 or C16, followed by the acid-producing isolate in step 2. Error bars represent the standard deviation of duplicate samples.

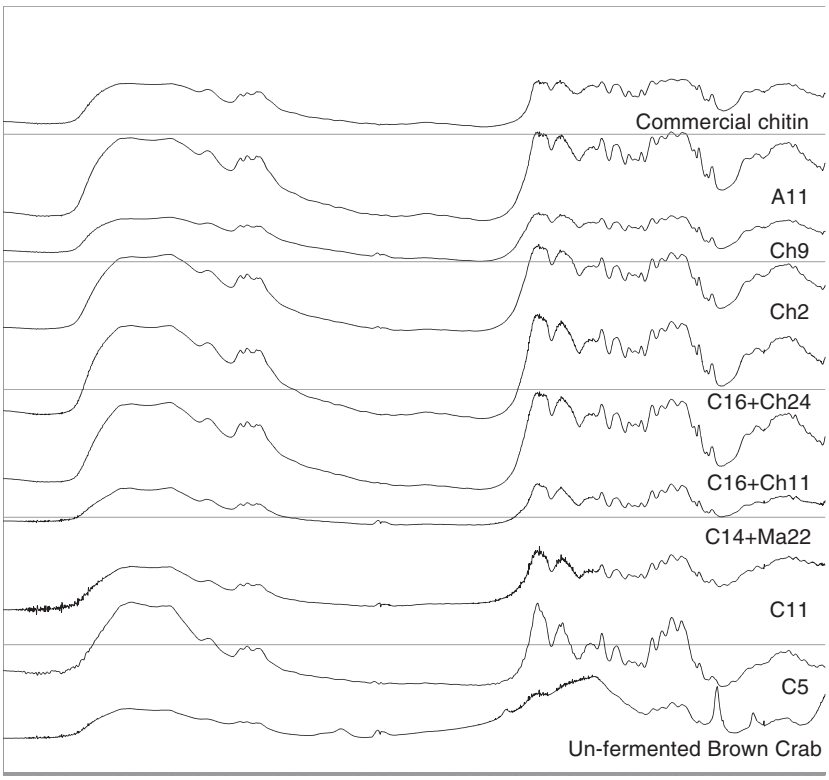

Figure 3 Stacked overlay of FTIR spectra of the crude chitin obtained from successive two-step fermentations using bacterial isolates (middle) in comparison with the spectra of un-fermented brown crab (bottom) and a commercial chitin product (top).

Table 4 Degree of acetylation of chitinous material produced by successive 2-step fermentations using selected bacterial isolates and a commercial chitin calculated using FTIR spectra

Isolate(s) used in generation of chitin sample	Degree of acetylation (DA %)
C5	68·9 ± 1·25
C11	77·3 ± 1·65
C14 + Ma22	82·0 ± 0·78
C16 + Ch11	81·9 ± 0·98
C16 + Ch24	80·6 ± 1·23
Ch2	82·0 ± 1·69
Ch9	78·9 ± 1·56
A11	78·3 ± 0·90
Commercial Chitin	74·7 ± 1·86

species have been shown to produce proteases (Kasana and Yadav 2007; Sorokulova *et al.* 2009). The strain was motile and formed orange colonies on nutrient agar (Table 2), matching descriptions of the species in literature (Crapart *et al.* 2007; Kasana and Yadav 2007). *Exiguobacterium acetylicum* has been used in the extraction of chitin from shrimp in a study by Sorokulova *et al.* (2009).

Isolates C11, C14 and C16 were protease-producing isolates identified as *Bacillus licheniformis* (GenBank accession number: KP050497), *Bacillus subtilis* (GenBank accession number: KP050498) and *Bacillus cereus* (GenBank accession number: KP050499) respectively. Isolate C11 also tested positive for acid production. There are many studies supporting the production of proteases by these *Bacillus* species (Xu *et al.* 2010; Jellouli *et al.* 2011; Qureshi *et al.* 2011). In addition, Sorokulova *et al.* (2009) and Ghorbel-Bellaaj *et al.* (2012) describe the use of *B. cereus* in the fermentation of shrimp to extract chitin.

Isolates Ch2, Ch11 and Ch9 were identified as *Pseudomonas* spp. (GenBank accession number: KP050500), *Pseudomonas* spp. (GenBank accession number: KP050502) and *Pseudomonas migulae* (GenBank accession number: KP050501) respectively. *Pseudomonas aeruginosa* has been used in the extraction of chitin from shrimp in studies by Oh *et al.* (2007) and Ghorbel-Bellaaj *et al.* (2011). The identification of these isolates as Gram-negative, motile rods (Table 2), in addition to the production of organic acids is in agreement with their identification as *Pseudomonas* species (Madigan *et al.* 2012).

Isolate Ma22 was identified as a *Lactobacillus* spp. (GenBank accession number: KP072000). *Lactobacilli* are widely used in the chitin extraction of chitin from crustacean waste due to their ability to produce lactic acid. (Rao *et al.* 2000; Gimeno *et al.* 2007; Zhang *et al.* 2012).

Isolate A11 was identified as an *Enterococcus* spp. (GenBank accession number: KP071999), supported by its cell morphology as Gram-positive cocci. Freitas *et al.* (1999) describes the production of organic acids by *Enterococcus*

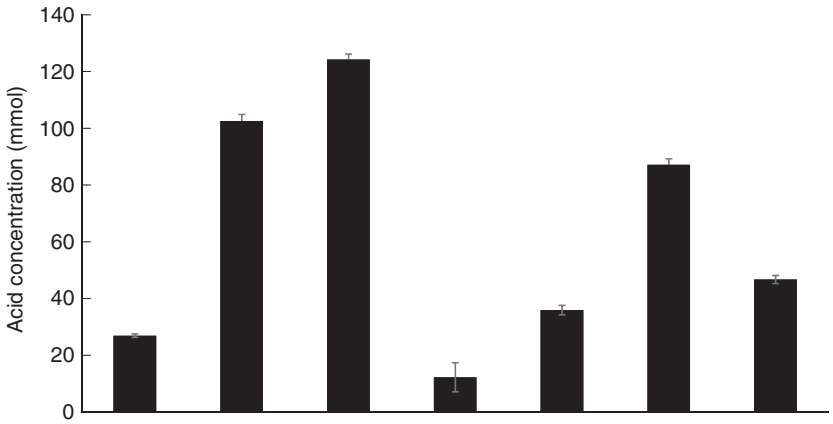

Figure 4 Lactic acid concentration (mmol) in the supernatant of fermentation solutions from the second step of successive two-step fermentations by respective isolates using 10 g of brown crab.

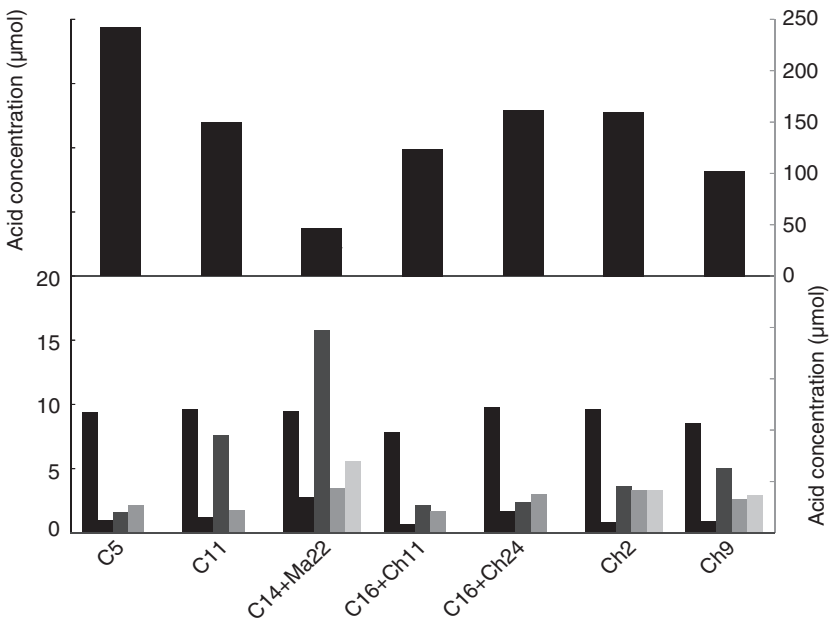

Figure 5 *Top*: Acetic acid concentration (μmol) in the supernatant of fermentation solutions from the second step of successive two-step fermentations by respective isolates using 10 g of brown crab. *Bottom*: Acid concentrations (μmol) in the supernatant of fermentation solutions from the second step of successive 2-step fermentations by respective isolates using 10 g of brown crab. (■) Oxalic acid, (■) Citric acid, (■) Malic acid, (■) Succinic acid and (■) Formic acid.

faecalis and *Enterococcus faecium*. This supports the production of acids observed by isolate A11.

Isolate Ch24 was identified as *Arthrobacter luteolus* (GenBank accession number: KP050503). *Arthrobacter* species have been shown to produce organic acids Chen *et al.* (2006). Wauters *et al.* (2000) described the strain as Gram-positive, motile, obligate aerobes that form yellow colonies on agar as observed in Table 2.

When the isolates were applied in chitin extraction it was found that the use of a protease-producing isolate in step 1 of the fermentation increased the efficacy of demineralization (DM) when compared to fermentations using an acid-producing isolate in both steps (Fig. 2). Jung *et al.* (2007) carried out a similar study investigating the successive fermentation of red crab shell wastes with *Lactobacillus paracasei* KCTC-3074, an acidogenic bacterium and *Serratia marcescens* FS-3, a protease-producing bacterium both separate and in combination. It was found that the use of *Serratia marcescens* followed by *Lactobacillus paracasei* gave the best removal of both proteins and calcium carbonate from the shell waste with 68·9% deproteinization (DP) and 94·3% DM achieved after fermentation.

Exiguobacterium acetylicum has been used in the extraction of chitin from shrimp in a study by Sorokulova *et al.* (2009). Demineralization and deproteinization were 92 and 92·8% respectively. The highest demineralization achieved by isolate C5, identified as *Exiguobacterium* spp. in this study was slightly higher at 95·8%.

Pseudomonas aeruginosa has been used in the extraction of chitin from shrimp in studies by Oh *et al.* (2007) and Ghorbel-Bellaaj *et al.* (2011). In both studies, the

demineralization efficacy exceeded the deproteinization efficacy with DM and DP values of 92 and 63% for Oh *et al.* (2007) and 96 and 89% for Ghorbel-Bellaaj *et al.* (2011). The demineralization of brown crab achieved in this study was greater by isolates Ch2, Ch9 and Ch11, identified as *Pseudomonas* species also, with values of 98·1, 98·6 and 99·2% respectively.

Lactobacillus plantarum is widely used in chitin extraction (Rao *et al.* 2000; Gimeno *et al.* 2007; Zhang *et al.* 2012). In one instance *Lact. plantarum* was used as the sole bacterial culture in the fermentation of shrimp (Rao *et al.* 2000) which resulted in a DM value of 90%. Zhang *et al.* 2012 used *Lact. plantarum* in combination with protease-producing bacterium *Serratia marcescens* which resulted in a DM of 94%. Isolate Ma22 identified as a *Lactobacillus* species was used in combination with protease-producing C14, identified as *B. subtilis*. The highest demineralization achieved with the combination of these isolates was 97·9% which exceeds the demineralization efficacy of the fermentations carried out by Zhang *et al.* 2012 and shows the efficacy of Ma22 in the extraction of chitin from brown crab.

A study by He *et al.* (2006) involved the recovery of peptides and amino acids from a shrimp-deproteinization fermentation hydrolysate, showing that the products of deproteinization can also be recovered and utilized. Crustacean species contain 30–40% protein (Vani and Stanley 2013), which means that considerable amounts of protein can be obtained from the chitin extraction process of brown crab by fermentation and generate further commercial value to the processing of its waste.

Bacterial isolates obtained from drained sub-peat mineral soil samples were found to be new strains of existing species. The isolates were applied in successive two-step fermentations on a small scale; some used alone, others in combination and were efficient at demineralization of brown crab and the extraction of a crude chitin fraction. All fermentations resulted in a demineralization of >94%. However, the highest demineralization, 98·9% was achieved when isolates identified as *B. cereus* and *Pseudomonas* spp. were used in combination. The transfer of fermentations to a larger scale requires further research for optimization. This may lead to the use of these isolates in the chitin extraction of brown crab waste in an industrial setting.

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Conflict of Interest

No conflict of interest declared.

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