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Production of chitinolytic enzymes by *Serratia marcescens* QMB1466 using various chitinous substrates

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Abstract: The chitinolytic activity of submerged cultures of *Serratia marcescens* QMB1466 in media containing four forms of chitin as the main substrate was investigated via a full factorial design experiment with pH, temperature and substrate concentration as the main parameters. At the optimum conditions (pH 7.0, 32.5 °C and 1.0% (w/v) substrate), bioprocessed chitin (BP), isolated by lactic acid fermentation of prawn shell (*Nephrops* sp), induced a higher level of enzyme activity than untreated prawn shell and colloidal chitin but not that of a chemically isolated chitin (CP). The optimal conditions of pH and temperature were then applied in a bench-top bioreactor and the chitinolytic activity monitored temporally under the influence of higher concentrations of BP and CP. Increasing the concentration of substrate in the bioreactor (>1.0% w/v) was found to inhibit the enzyme activity of the bacteria. The enzyme mixtures in selected 120-h culture supernatants were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and the main proteins characterised by molecular weight. The electrophoretic patterns obtained from cultures from different experiments and by the different chitin substrates showed marked similarity and the main proteins isolated were largely homologous to well-documented chitinases found in the literature. BP chitin was found to be an efficient elicitor of chitinolytic activity from this bacterium and hence is a suitable substrate to employ in an integrated biotechnological process, whereby several commercially applicable products can be obtained from a waste product of the fishing industry.
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Keywords: chitin; chitinolytic enzymes; submerged fermentation; *Serratia marcescens*; waste management

1 INTRODUCTION

Chitin is the second most abundant naturally occurring polysaccharide after cellulose. The structure is similar to that of cellulose with 2-acetamido-2-deoxy-β-D-glucose (*N*-acetyl glucosamine, NAG) monomers attached via β $(1 \rightarrow 4)$ linkages. It also resembles cellulose in that it has very low solubility, low chemical reactivity and functions mainly as a structural polysaccharide.[1] The main industrial source of chitin is from the shells of marine crustaceans, isolated by a harsh chemical method under conditions broadly defined in the literature.[2–4] Several recent studies have aimed to develop a method for producing chitin that is less detrimental to the environment involving lactic acid fermentation of marine crustacean shell waste in solid state,[5,6] submerged fermentation[7] and immobilised cell systems.[8] Bioprocessing in this way allows almost total utilisation of the waste as extraction of proteins and pigments becomes achievable.[9]

Among the considerable amount of applications already existing for chitin, the production of the monomer (NAG) is attracting increasing interest in a number of areas; eg as a neutraceutical for osteoarthritis treatment,[10] in food additives and preservatives[11] and as a fungicide in plant disease control.[12] The depolymerisation of chitin by chemical methods[13,14] as well as via enzymatic hydrolysis by enzymes such as hemicellulase,[15] lysozyme,[15–17] papain,[15] lipase,[15] bromelain[18] and pectinase[19] has been studied extensively. However, the most attention has been given to enzymes that show specificity to chitin, known as chitinases (EC 3.2.1.14). These are naturally occurring enzymes that are synthesised by a vast array of organisms including plants, insects and microorganisms.[20]

The use of microorganisms to process crustacean shell wastes offers a waste management solution and commercial rewards. This has been the motivation for numerous investigations with the use of fungi such

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as *Trichoderma harzianum*,[21] *Verticillium lecanii*,[22] and *Aspergillus* sp[23] and with bacteria such as *Pseudomonas aeruginosa*[24] and *Clostridium paraputrificum*[25] in pilot-scale crustacean chitin-degradation processes. *Serratia marcescens* is considered to be one of the most effective bacteria for the degradation of chitin.[26,27] The chitinase system employed by this species is now well understood due to many thorough studies[28–31] and is believed to consist of five proteins: three chitinolytic enzymes (ChiA, ChiB and ChiC), a chitobiase (chb) and a chitin binding protein (cbp). The chitinases work synergistically as endo- and exo-type chitinases.[32] The former cleave randomly from within the chitin chain while the latter cleave chitobiose, $(GlcNAc)_2$, from chitin and chito-oligosaccharride chain ends. Chitobiases convert GlcNAc dimers into monomers.[27]

S marcescens QMB1466 is a chitinase over-producing mutant that shows considerable chitinolytic activity towards crystalline chitin substrates[26,33] such as those found in crustacean shell. In a series of studies,[34–36] this strain has been used in the development of a bioconversion process of shellfish chitin to single-cell protein. The present study employs this strain for the degradation of chitin substrates, with particular interest in the suitability of bioprocessed chitin as an inducer of chitinolytic activity in a conceptualised, integrated biotechnological process for the conversion of prawn shell (*Nephrops* sp) waste into commercially valuable products.

2 EXPERIMENTAL

2.1 Microorganism and culture media

Serratia marcescens QMB1466 was obtained from The National Collection of Industrial and Marine Bacteria (NCIMB Ltd, Aberdeen). The culture was maintained on Lab-Lemco (Oxiod CM15) Agar slants at 4 °C until required.

The basal culture medium used in both shake flask and bioreactor experiments comprised (w/v): ammonium sulfate (0.100%), magnesium sulfate 7-hydrate (0.030%), potassium dihydrogen orthophosphate (0.136%) and yeast extract (0.050%). In shake flask experiments the media contained either 0.1 mol dm^{-3} sodium phosphate (pH 7.0) or 0.1 mol dm^{-3} sodium acetate (pH 5.5) buffer. Chitin, the main carbon source in the medium, was added as per the experimental detail. Chitin was not added to media used for control experiments.

2.2 Substrate preparation

Untreated prawn shell substrate (PS) was prepared by drying fresh, locally obtained (TH Nicholson, Portavogie, Northern Ireland) prawn shell (*Nephrops* sp) at 40 °C. Commercially available, chemically isolated chitin (CP) was obtained from Sigma (C-7170). Bioprocessed chitin (BP) was prepared by the lactic acid fermentation of fresh prawn shell.[7] The above were ground to a particle size <0.5 mm and dried at 40 °C before use. Batches of colloidal chitin (CC) were prepared thus: 10 g of chitin (Sigma, C-7170) were stirred into 150 cm^3 of 85% phosphoric acid at room temperature to form a thick slurry. The mixture was sonicated for 45 min, after which the complete hydrolysis of chitin was evident. The mixture was poured slowly into a large excess ($\sim$4 dm^3) of cold water, yielding a fine white precipitate. The precipitate was centrifuged (Sanyo Harrier 18/80) at $6460 \times g$ for 10 min and the pellet washed several times with distilled water until the pH was in the neutral range. The final pellet, containing on average 85% (w/w) moisture, was classed as colloidal chitin and stored at 4 °C until required.

2.3 Shake flask experiments

Randomised, full factorial design experiments were performed separately for CC, PS, BP and CP substrates in triplicate. Temperature (25.0, 32.5 and 40.0 °C), pH (5.5 and 7.0) and substrate concentration (0.50% and 1.00% w/v) were the parameters chosen. Volumes of 50 cm^3 of basal medium containing the appropriate amount of chitin in 250 cm^3 conical flasks were inoculated by aseptically transferring a loopful of *S marcescens* cells from agar slants to the medium and incubated in an orbital shaker (New Brunswick Scientific, G25), at the appropriate temperature, at 250 rpm for a period of 120 h. Aseptically drawn samples of the fermentation broth were centrifuged at $9500 \times g$ for 10 min (Eppendorf, Mini-Spin). Analysis of variance of the factorial data was carried out with the aid of Minitab release 14.0 software (Minitab Inc, 2003).

2.4 Bioreactor experiments

Batch fermentations containing various concentrations of BP (0, 1, 2 and 4% w/v) and CP (0, 1 and 2% w/v) substrates were carried out in duplicate in a 5 dm^3 bench-top bioreactor (New Brunswick Scientific, BioFlo 3000) for a period of 120 h, using a working volume of 3 dm^3. Agitation and aeration was set at 150 rpm and 0.5 vvm. The pH was controlled at the optimised value found in shake flask experiments by the addition of 1 mol dm^{-3} NaOH and 1 mol dm^{-3} HCl. Temperature was also set at the optimum found in shake flask experiments. The system was inoculated with 50 cm^3 of 48-h *S marcescens* culture (approx 10^8 cfu per cm^3) containing identical medium to that used in the bioreactor. Aseptically drawn samples of the fermentation broth were centrifuged at $9500 \times g$ for 10 min.

2.5 Measurement of chitinolytic activity

Chitinolytic activity was determined by a modified version of the method of Monreal and Reese.[26] Aliquots of culture supernatant (0.5 cm^3) were added to 1.0 cm^3 of a 1% (w/v) suspension of colloidal chitin in 0.2 mol dm^{-3} sodium phosphate buffer (pH 7.0) and incubated at 37 °C for 30 min. The reaction was stopped by centrifuging the suspension at $9500 \times g$ for 10 min and the concentration of NAG was determined

in the supernatant according to the method of Reissig *et al*.[37] One unit of chitinolytic activity was defined as the amount of enzyme that liberated 1 μmol of NAG per minute at 37 °C.

2.6 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of fermentation broth

Selected samples of fermentation broth were centrifuged (13 000 × *g*, 5 min) and aliquots of the supernatant mixed with an equal volume of 2 × treatment Buffer (0.125 mol dm^{-3} Tris–HCl pH 6.8, 4% (v/v) SDS, 20% (w/v) glycerol, 10% mercaptoethanol). A sample, prior to centrifugation, was treated likewise. After heating at 100 °C for 10 min the samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). This was carried out using a vertical Amersham Pharmacia SE-600 system with 16 cm, 12.5% homogeneous gels of 1.5 mm thickness. A current of 40 mA and voltage of 350 V was applied for 3.5 hs. Gels were subsequently fixed in 7% acetic acid in 40% methanol and stained with Brilliant Blue G—Colloidal Coomassie (Sigma B2025).

Protein bands selected for mass spectrometry analysis were subjected to in-gel digestion as described previously.[7]

2.7 Analysis of residual solid

Homogeneous broth samples of 50.0 cm^3 were briefly centrifuged to precipitate residual substrate from the liquor. The solid pellet was dried at 40 °C to constant mass, which was recorded and expressed as a percentage by mass per unit volume of liquor (% w/v). The ash content of dried substrate after 120 h fermentation was determined gravimetrically after combustion overnight at 650 °C in a muffle furnace. The difference between means was determined by means of an unpaired Student's *t*-test.

3 RESULTS AND DISCUSSION

Submerged fermentation (SmF) was chosen as the preferred processing method in this study over solid-state fermentation (SSF), as has been used in several related investigations.[38–40] Several factors account for this reasoning, such as the greater ease of control of pH, agitation and extraction of enzymes for analysis. SmF is commonly regarded to be more rapid than SSF; however, no marked reduction of fermentation time required to reach maximum enzyme activity was observed in this study when compared with similar studies employing SSF.[38,39]

3.1 Shake flask experiments

All three factors investigated in the factorial design experiment had an effect on the chitinolytic activity induced from *S marcescens*. Additionally, each of the four chitinous substrates employed as elicitors of enzymatic activity, were effective to varying degrees (Table 1). By statistical analysis, pH was affirmed the most influential factor with the best results occurring at pH 7.0, followed by temperature and substrate concentration. Indeed, experiments carried out at the low pH level (pH 5.5) yielded negligible results for chitinolytic activity, with the exception of PS substrate experiments. The calcium carbonate moiety of this material was able to react with the acidic buffer, hence causing the pH of the medium to rise and give false results. The low pH level was tested in this study with regard to the acidic conditions at which bioprocessed chitin is produced.[5–8] It is now apparent that to use a process such as this for chitin processing in conjunction with the fermentative isolation of chitin that neutralisation of the initial substrate would be required. Chitinolytic activity in cultures increased from 25.0 °C to 32.5 °C but the microorganism was intolerant to the high temperature level of 40.0 °C as chitinolytic activity was negligible at this value. Increasing the substrate concentration from 0.50 to 1.00% (w/v) generally resulted in an approximate two-fold increase in activity. The optimal parameters for enzyme production from *S marcescens* were pH 7.0, 32.5 °C and 1.00% (w/v) chitin concentration. These results are in close agreement with those obtained in other studies with respect to pH[33,34,41] and temperature.[26,33]

Table 1. Chitinolyic activity observed in 120-h *S marcescens* cultures under different conditions by full factorial design using various chitinous substrates (data presented are the mean values from experiments carried out in triplicate)

pH	Temp (°C)	Chitin concentration (% w/v)	Chitinolytic activity (U dm^{-3})			
			CC	PS	BP	CP
5.5	25.0	0.50	0.13	7.75	0.06	0.01
5.5	25.0	1.00	0.01	19.71	0.06	0.02
5.5	32.5	0.50	0.03	1.77	0.11	0.00
5.5	32.5	1.00	0.09	2.08	0.14	0.02
5.5	40.0	0.50	0.00	0.08	0.01	0.01
5.5	40.0	1.00	0.00	0.12	0.01	0.00
7.0	25.0	0.50	38.94	9.93	31.31	92.70
7.0	25.0	1.00	20.30	25.06	77.82	112.01
7.0	32.5	0.50	24.28	8.27	83.70	217.86
7.0	32.5	1.00	61.01	16.44	158.37	418.49
7.0	40.0	0.50	0.50	0.10	0.20	0.04
7.0	40.0	1.00	1.56	0.27	0.29	0.03

CC = Colloidal chitin substrate; PS = prawn shell substrate; BP = bioprocessed chitin substrate; CP = chemically isolated chitin substrate.

CP induced the highest chitinolytic activity in the culture medium at each set of conditions (Fig 1(A)), followed by BP, CC and PS. The induced activity appears to be related to the purity of the chitinous substrate. CP allows the bacteria the greatest access to degradation sites. BP, containing small amounts of residual impurity,[7] restricts the chitin available and PS chitin, being intimately bound to proteins and mineral salts, is completely inaccessible to enzymatic attack. The CC substrate, however, induced less activity than both BP and CP, despite being a finely dispersed and readily degraded chitinous substrate. The high

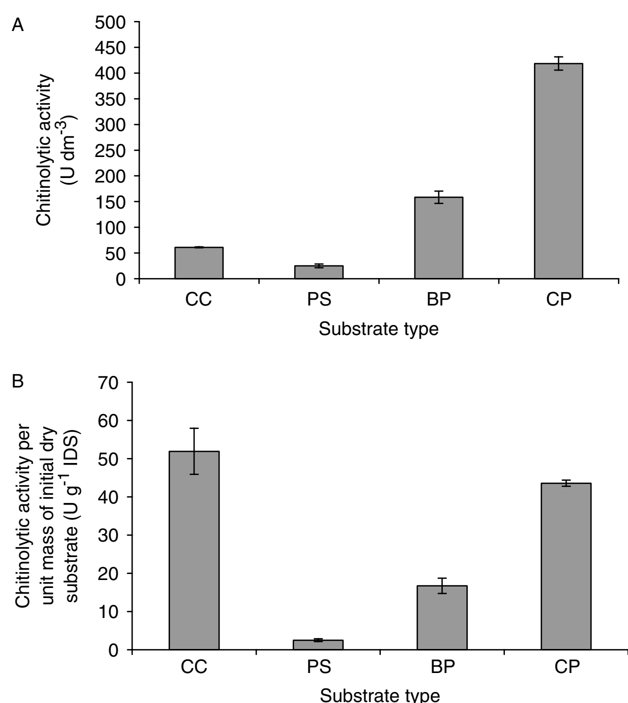

Figure 1. Chitinolytic activity (A) and chitinolytic activity per unit mass of initial dry substrate (B) observed at the optimum conditions by factorial design using various chitinous substrates after a fermentation time of 120 h. Experiments were carried out in triplicate and error bars represent one standard deviation from the mean. CC = Colloidal chitin substrate; PS = prawn shell substrate; BP = bioprocessed chitin substrate; CP = chemically isolated chitin substrate.

moisture content of this material accounts for this occurrence, as is shown in the results (Fig 1(B)). On a dry weight basis, colloidal chitin was the most effective inducer of chitinolytic activity.

3.2 Bioreactor experiments

In general, the results obtained for chitinolytic activity (Fig 2(A)) were lower in magnitude than those from shake flask experiments with corresponding substrate types and concentrations, to which the differing methods of inoculation may be contributory. However, as analysis was not carried out on shake flask cultures before 120 h, this is difficult to ascertain. The scaling up of the process caused some factor to alter, hence the apparent underperformance of the microorganisms. It is believed that the forced aeration set at an arbitrary and relatively high value was responsible for this occurrence. Evidently, the chitinolytic ability of *S marcescens*, defined as an aerobe by the suppliers, does not benefit from high aeration as does the chitinolytic fungus *Verticillium lecanii*.[22]

It is apparent that difficulties arise when attempting to compare, in terms of magnitude, the results of chitinolytic activity with other studies in the literature. The chitinase assay is carried out by various techniques and the definition of chitinolytic activity is yet to be solidly defined in the literature.[21] The study by Reid and Ogrydziak[33] is perhaps the best comparison to make with this work insofar as the methods used for determining and expressing the enzyme activity

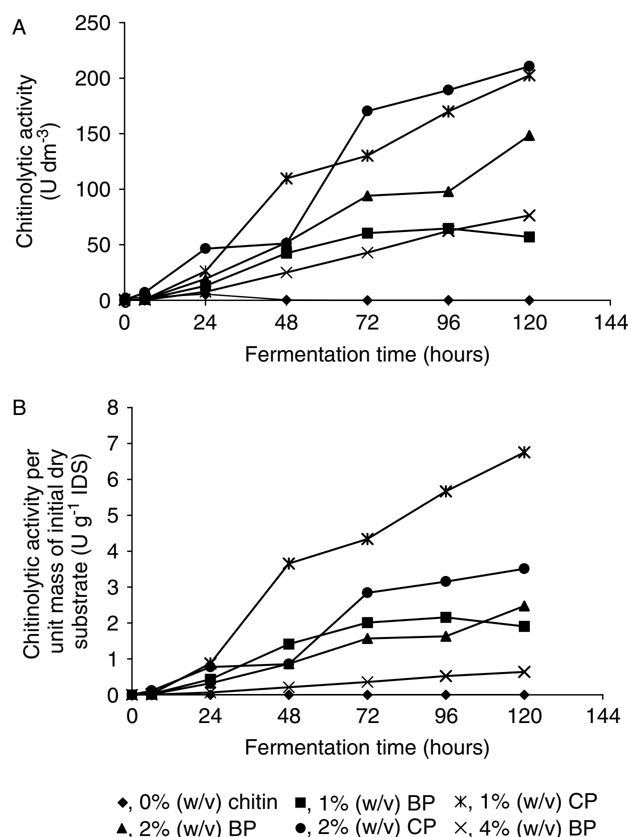

Figure 2. Chitinolytic activity (A) and chitinolytic activity per unit mass of initial dry substrate (B) observed in the bioreactor during the fermentation period. Data points are the mean values of three determinations from two experiments. BP = Bioprocessed chiton substrate; CP = chemically isolated chitin substrate.

are similar. Therefore it is little surprise that the peak results obtained in the bioreactor experiments were similar to those obtained in the aforementioned study[33] ($200\,U\,dm^{-3}$), albeit the peak occurred at a later time due to the use of a more crystalline and less accessible chitin form.

All enzyme activity profiles exhibited increasing trends by the end of the fermentation period, with the exception of those at a substrate concentration of 1% (w/v) BP. Chitin degradation and ash contents among residual BP chitins was also greatest at these concentrations (Table 2). This suggests that the chitin moiety available to the microorganisms

Table 2. Chitin degradation and ash content of residual chitin substrates in bioreactor experiments (data are the mean values and standard deviations of four determinations from two experiments)

Substrate type and concentration	Chitin degradation (%)	Ash content of residual chitin (% w/w)
BP — 1% (w/v)	70.3 ± 1.3	40.90 ± 1.22
BP — 2% (w/v)	62.1 ± 0.8	32.54 ± 1.04
BP — 4% (w/v)	31.8 ± 3.3	17.14 ± 1.35
CP — 1% (w/v)	80.5 ± 1.1	17.47 ± 1.22
CP — 2% (w/v)	74.6 ± 0.9	14.24 ± 0.70

BP = Bioprocessed chitin; CP = chemically isolated chitin.

was consumed to a greater extent than at other concentrations and most likely completely consumed, resulting in the depletion of enzyme activity levels in the culture. Increasing the substrate concentration in the bioreactor did not result in a proportional increase in the peak activity induced from the bacteria as was generally found in the shake flask experiments. There are two possible explanations for this occurrence. Firstly, the inefficiencies introduced in the bioreactor experiments, as previously discussed, may be responsible for the inhibition of enzyme production. Secondly, a substrate concentration of 1% (w/v) in the bioreactor may be a limiting one. It can be seen in Fig 2(B) that these concentrations are much more efficient for each substrate type in terms of the activity produced per mass of substrate.

Chitin degradation at low substrate concentrations was higher than that achieved in other studies that monitored substrate conversion.[21,34,42] CP chitin was degraded to a greater extent than BP chitin, again due to the greater presence of impurities in BP chitin.

3.3 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of fermentation broth

Electrophoretic patterns obtained from the culture broths of three separate bioreactor experiments are shown in Fig 3. The similarity among these patterns is notable, particularly when comparing the most intense bands. It is also worth noting that the similarities occur despite the use of different substrate types and concentrations. The bands circled on the figure were analysed by ion-trap MS/MS. Significant hits were not obtained from the database to allow a confident identification of the selected proteins; however, the molecular weights of the proteins were found to be, in descending order: 102.3, 60.2, 58.0, 41.2, 21.6 kDa. The molecular weights of microbial chitinases range from 20 to 120 kDa with very little consistency.[24] The chitinase system proposed to have been produced in this work compares very closely with other *S marcescens* systems.[27,43,44] There is also similarity with another bacterial system[45] but little homology with fungal systems, which tend to exhibit a much smaller range of proteins.[22,40] From these comparisons the function of some of the enzymes isolated from SDS–PAGE analysis can be estimated, although it is acknowledged that the detection of activity within the protein bands is a much superior method for this purpose. The largest protein found, at 102.3 kDa, is approximate in size to chitobiases commonly found in *S marcescens* cultures. The smallest protein, at 21.5 kDa, has also been frequently reported as a chitin-binding protein and is not responsible for chitinolytic activity.[29] The remaining proteins in the mid-range (60.2, 58.0 and 41.2 kDa) are the main endo- and exo-type chitinases which provide the synergistic action for effective chitin hydrolysis.[20] It is apparent from this work that the *S marcescens* chitinase system is very prominent in the culture broth produced with both BP and CP substrates in the bioreactor experiments.

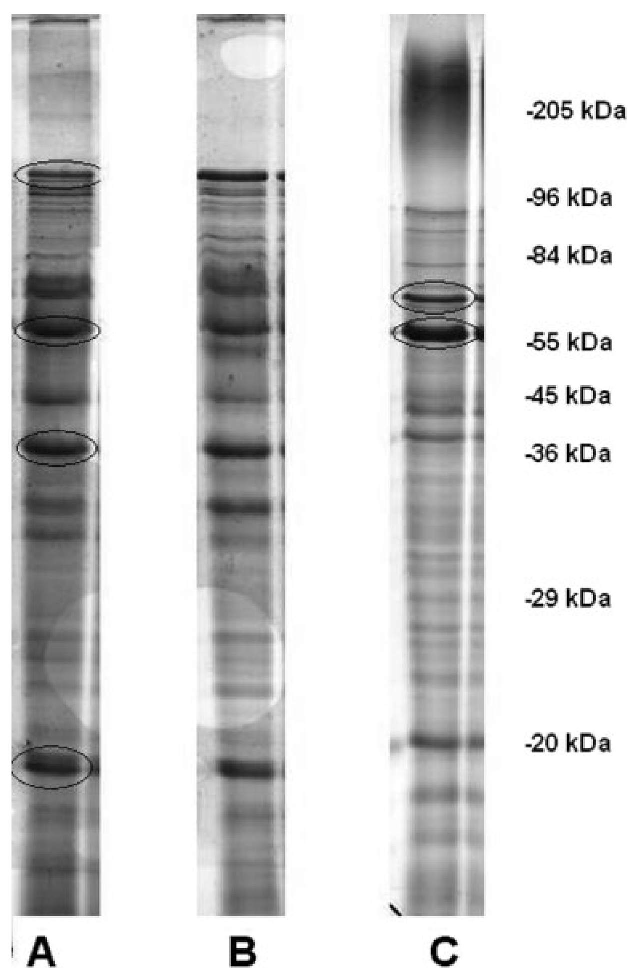

Figure 3. One-dimensional SDS–PAGE of proteins from 120-h fermentation broths containing initial substrate concentrations of 4% (w/v) BP (A), 2% (w/v) CP (B) and 1% (w/v) BP. Protein bands selected for ion-trap MS/MS are circled and positions of molecular weight standards are indicated (BP = bioprocessed chitin substrate; CP = chemically isolated chitin substrate).

4 CONCLUSION

S marcescens QMB1466 has been used for the hydrolysis of insoluble chitin substrates. High levels of activity were induced both in shake flask and stirred tank bioreactor cultures. This work has demonstrated that chitin isolated from a biotechnological process can be used as an efficient inducer of chitinolytic enzymes in a stirred tank bioreactor. The enzymes can then be used to hydrolyse and isolate the chitin monomer, *N*-acetyl glucosamine (NAG), and other oligomers that have many commercial applications. The enzymes are also useful in their own right in substances for plant disease and nematode control. Using this particular strain of bacteria results in the production of a red pigment known as prodigiosin. The extraction of this substance in large amounts in the bioreactor opens exciting possibilities given its potential as an anti-cancer treatment and deserves further attention as a possible by-product of this process.

The integration of this process with the lactic acid fermentation of prawn shell waste offers a complete biotechnological route from waste prawn shell to chitin and NAG, with a number of by-products

being produced as a consequence. Further research should concentrate on the isolation and purification of chitinolytic enzymes produced in the bioreactor and the efficient hydrolysis of chitin.

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