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ORIGINAL PAPER

Bioconversion of α-chitin into *N*-acetyl-glucosamine using chitinases produced by marine-derived *Aeromonas caviae* isolates

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Abstract *N*-Acetyl-D-glucosamine (GlcNAc) is a monosaccharide with great application potential in the food, cosmetic, pharmaceutical, and biomaterial areas. GlcNAc is currently produced by chemical hydrolysis of chitin, but the current processes are environmentally unfriendly, have low yield and high cost. This study demonstrates the potential to produce GlcNAc from α-chitin using chitinases of ten marine-derived *Aeromonas* isolates as a sustainable alternative to the current chemical process. The isolates were characterized as *Aeromonas caviae* by multilocus sequence analysis (MLSA) using six housekeeping genes *(gltA, groL, gyrB, metG, ppsA*, and *recA*), not presented the virulence genes verified *(alt, act, ast, ahh1, aer, aer*A, *hlyA, ascV* and *ascF*G), but showed hemolytic activity on blood agar. GlcNAc was produced at 37 °C, pH 5.0, 2% (w/v) colloidal chitin and crude chitinase extracts (0.5 U mL^{-1}) by all the isolates with yields from 14 to 85% at 6 h, 17–89% at 12 h and 19–93% after 24 h. The highest yield of GlcNAc was observed by *A. caviae* CH129 (93%). This study demonstrates one of the most efficient chitin enzymatic hydrolysis procedures and *A. caviae* isolates with great potential for chitinases expression and GlcNAc production.

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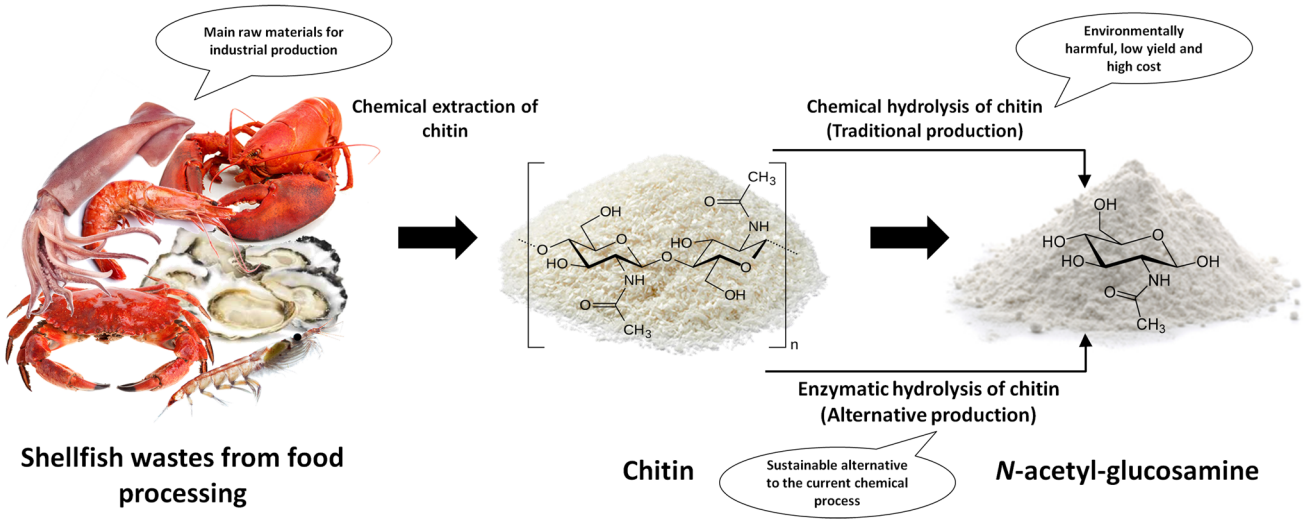
Graphical abstract



Keywords *Aeromonas* · Chitin · Chitinases · Enzymatic hydrolysis · *N*-Acetyl-glucosamine

Introduction

Chitin is one of the most abundant organic compounds found in nature and the main component of the exoskeleton of arthropods, cell walls of fungi and algae (Zobell and Rittenberg 1938). It is insoluble in water, has acrystalline structure and consists of unbranched chains of *N*-acetyl-D-glucosamine (GlcNAc), a monosaccharide with great application potential in the food, cosmetic, pharmaceutical and biomaterial areas (Chen et al. 2010; Liu et al. 2013; Jung and Park 2014).

GlcNAc is biocompatible, biodegradable and is also present in the human body. It is a component of hyaluronic acid, glycoproteins, proteoglycans, glycosaminoglycans and other connective tissue structures (Levin et al. 1961) and can even be found in human breast milk (Kobata and Ginsburg 1969; Miller et al. 1994). Studies indicate that GlcNAc is an important component of biomacromolecular synthesis in the human body (Heim et al. 1989; Shoji et al. 1999), is nontoxic and does not alter blood glucose levels (Levin et al. 1961; Liu et al. 2008). GlcNAc is a valuable pharmacological agent in the treatment of a wide variety of diseases, joint injuries, and inflammatory bowel disease and can be used as a substrate for sialic acid production (Chen et al. 2010). Due to the versatile functions of GlcNAc, this compound is considered an essential ingredient in the formulation of cosmetics for the treatment of wrinkles and reduction of skin hyperpigmentation (Sayo et al. 2004; Bissett et al. 2007; Chen et al. 2008). Studies also have described GlcNAc as a potential pathway for bioethanol production as an alternative to the use of glucose as carbon sources by *Saccharomyces cerevisiae* mutant (Roseman et al. 2010).

GlcNAc has been historically prepared by chemical hydrolysis of chitin, using a strong acid, such as chloric acid (HCl), and carefully selected temperatures (Aam et al. 2010). However, the current processes are environmentally unfriendly, have low yield and high cost. The enzymatic hydrolysis of chitin is an alternative to the chemical process and would not require the use of toxic compounds or generate excessive amounts of wastewater(Chen et al. 2010; Liu et al. 2013; Jung and Park 2014).

Microbial production of GlcNAc has attracted increasing attention due to its advantage as a sustainable alternative to the chemical process (Liu et al. 2013; Jung and Park 2014). Among the microorganisms, marine chitinolytic bacteria show great potential for the production of GlcNAc because of the suggestion that they play a significant role in chitin degradation in the oceans (Zobell and Rittenberg 1938). These bacteria are autochthonous in marine ecosystems, produce chitinases that hydrolyze chitin to biologically useful carbon and nitrogen forms, and consequently, make possible the recycling of chitin in nature (Zobell and Rittenberg 1938; Keyhani and Roseman 1999). The efficiency of these bacteria can be observed in marine sediment because, despite continuous production of chitin in the water column, only traces of this highly insoluble polymer accumulate in marine sediment (Johnstone 1908; Poulicek and Jeauniaux 1989; Alldredge and Gotschalk 1990; Keyhani and Roseman 1999).

Aeromonas is a representative genus of marine chitinolytic bacteria. However, some *Aeromonas* strains have been reported to induce pathogenicity in fish and humans (Janda and Abbott 2010). Three species of *Aeromonas* (*A.*

hydrophila, A. caviae, and *A. veronii*) have been reported to produce systemic infections in humans (Janda et al. 1994; Janda and Abbott 1998). Thus, the potential industrial use of strains belonging to this genus requires an evaluation of their pathogenicity. The most common procedure to examine the potential pathogenicity of *Aeromonas* strains is the identification of the virulence-associated factors. These factors include the genes involved in the production of the major enterotoxins, hemolysins and secretion systems previously described to be involved in strain pathogenicity (Chacón et al. 2003; Sen and Rodgers 2004; Aguilera-Arreola et al. 2005). In addition, the capability of strains to produce hemolytic activity on blood agar is also considered a direct demonstration of the potential pathogenicity of *Aeromonas* strains (Aguilera-Arreola et al. 2007).

In this study, we demonstrate the ability to produce GlcNAc from α-chitin using chitinases of marine-derived *A. caviae* isolates. Multilocus sequence analysis (MLSA) was used for differentiation and characterization of the isolates, and their potential pathogenicity was examined by polymerase chain reaction (PCR) using specific virulence-related genes and by hemolytic activity on blood agar.

Materials and methods

Isolates

Sampling and screening

Marine-derived bacteria were isolated from seawater and zooplankton samples from the coast of São Paulo state, Brazil (Souza et al. 2009). Chitinolytic activity was quantified based on size of clear zones around the colonies on chitin agar plates at 28 °C for 96 h (Fig. 1). Culture medium was prepared with the following composition (per liter): $(NH4)_2SO_4$, 1.0 g; KH_2PO_4, 0.2 g; K_2HPO_4, 1.6 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; NaCl, 0.1 g; $FeSO_4{\cdot}7H_2O$, 0.01 g; $CaCl_2{\cdot}2H_2O$, 0.02 g; colloidal chitin, 10 g; Agar, 15 g (pH 7.0). Colloidal chitin was prepared using α-chitin powder from crab shells (C7170-Sigma-Aldrich Co., St. Louis, MO, USA), according to the method described by Souza et al. (2009). Ten isolates (CH125, CH129, CH147, CH149, CH150, CH151, CH286, CHZ52, CHZ113, and CHZ306) with largest chitin hydrolysis halos were selected for this study.

16S rRNA gene sequencing

Isolates were characterized at the genus level as *Aeromonas* sp. by complete 16S rRNA gene sequencing. PCR reactions were carried out in a final volume of 50 µL using the following components: 1× PCR Buffer (Invitrogen, Carlsbad, USA), 50 mM $MgCl_2$ (Invitrogen), 0.25 mM DNTP (Invitrogen), 0.3 µM 27F and 1525R primers (Lane 1991), 1 U Taq DNA polymerase (Invitrogen) and template DNA (50 ng). Reactions were performed in Mastercycler ep Gradient S Eppendorf thermocycler (Eppendorf, New York, USA) using the following thermal conditions: initial step at 94 °C for 2 min, followed by 30 cycles (denaturation at 94 °C for 30 s, 62.5 for 30 s and extension at 72 °C for 45 s) with a final extension at 72 °C for 3 min. PCR products were sequenced with the BigDye® Terminator v3.1 Cycle sequencing kit (ThermoFisher Scientific, Waltham, USA) using the ABI 3730 DNA Analyzer (Life Technologies, Carlsbad, USA) and compared with sequences available in GenBank at NCBI (National Center for Biotechnology Information) using BLAST (Basic Local Alignment Search Tool).

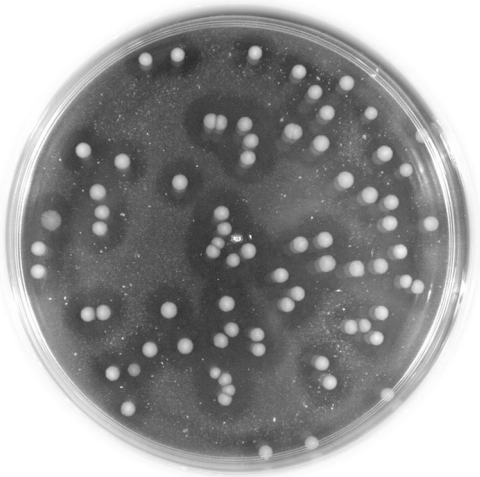


Fig. 1 Experimental demonstration of chitin hydrolysis on agar plates by marine-derived *Aeromonas caviae* CH125. Chitin hydrolysis is indicated by the clear zones around of bacterial colonies

Multilocus sequence analysis (MLSA) and verification of virulence-associated factors

Isolates were characterized at species level by MLSA using six housekeeping genes *(gltA, groL, gyrB, metG, ppsA*, and *recA*) and verifying the presence of virulence genes using nine potential markers *(alt, act, ast, ahh1, aer, aerA, hlyA, ascV* and *ascFG*). Additionally, the hemolytic activity of all strains was verified on blood agar.

For MLSA and virulence genes verification, cells were grown overnight in Luria–Bertani (LB) broth at 28 °C and harvested by centrifugation at 3000×*g* for 5 min at 4 °C. DNA extraction was done using a Wizard Genomic DNA Purification Kit (Promega Co., Madison, WI, USA), according to the manufacturer's instructions.

PCR reactions were carried out in a final volume of 50 µL using the following components: 1× PCR Buffer

(Invitrogen), 50 mM $MgCl_2$ (Invitrogen), 0.25 mM DNTP (Invitrogen), 0.3 µM each primers (Invitrogen) (Table 1), 1 U Taq DNA polymerase (Invitrogen) and template DNA (50 ng). Reactions were performed in Mastercycler ep Gradient S Eppendorf thermocycler (Eppendorf) using the following thermal conditions: initial step at 94 °C for 2 min, followed by 35 cycles (denaturation at 94 °C for 30 s, annealing (Table 1) for 30 s and extension at 72 °C for 2 min) with a final extension at 72 °C for 3 min.

PCR products were purified with the Wizard® SV Gel and PCR Clean-Up System kit (Promega)according to the manufacturer's instructions and sequenced with the BigDye® Terminator v3.1 Cycle sequencing kit (ThermoFisher Scientific) using the ABI 3730 DNA Analyzer (Life Technologies).

Amplicons were compared with nucleotide sequences of *Aeromonas* spp. available in the *Aeromonas* MLST Database (http://pubmlst.org/aeromonas/). For phylogenetic analysis, chromatograms of sequences were analyzed and edited using the Bioedit 7.0.9.0 program (Ibis Biosciences, Carlsbad, USA). After concatenated and aligned, the sequences were analyzed using the MEGA 7.0 program. Phylogenetic trees were constructed using the neighbor-joining statistical method and the Jukes-Cantor

Table 1 Primers, amplicons and annealing temperatures used for amplification of the housekeeping genes and virulence genes in marine-derived *Aeromonas caviae* isolates

Primer	Sequence (5′–3′)	Gene product	Amplicon (bp)	Annealing temp (°C)	References
gltA_F	TTCCGTCTGCTCTCCAAGAT	Citrate synthase I	626	58	Martino et al. (2011)
gltA_R	TTCATGATGATGCCGGAGTA				
groL_F	CAAGGAAGTTGCTTCCAAGG	Chaperonin GroEL	782	56	Martino et al. (2011)
groL_R	CATCGATGATGGTGGTGTTC				
gyrB_F	GGGGTCTACTGCTTCACCAA	DNA gyrase, subunit β	699	59	Martino et al. (2011)
gyrB_R	CTTGTCCGGGTTGTACTCGT				
metG_F	TGGCAACTGATCCTCGTACA	Methionyl-tRNA synthetase	657	57	Martino et al. (2011)
metG_R	TCTTGTTGGCCATCTCTTCC				
ppsA_F	AGTCCAACGAGTACGCCAAC	Phosphoenolpyruvate synthase	619	60	Martino et al. (2011)
ppsA_R	TCGGCCAGATAGAGCCAGGT				
recA_F	AGAACAAACAGAAGGCACTGG	Recombinase A	700	57	Martino et al. (2011)
recA_R	AACTTGAGCGCGTTACCAC				
alt_F	AAAGCGTCTGACAGCGAAGT	Heat-labile cytotonic enterotoxin	320	61	Aguilera-Arreola et al. (2005)
alt_R	AGCGCATAGGCGTTCTCYT				
act_ F	AGAAGGTGACCACCAAGAACA	Cytotoxic enterotoxin	232	56	Kingombe et al. (1999)
act_ R	AACTGACATCGGCCTTGAACTC				
ast_F	TCTCCATGCTTCCCTTCCACT	Heat-stable cytotonic enterotoxin	331	56	Sen and Rodgers (2004)
ast_R	GTGTAGGGATTGAAGAAGCCG				
ahh1_F	GCCGAGCGCCCAGAAGGT GAGTT	Extracellular hemolysin	130	60	Wang et al. (2003)
ahh1_R	GAGCGGCTGGATGCGGTTGT				
aer_F	CCGGAAGATGAACCAGAATAA GAG	Cytolytic enterotoxin	451	57	Chopra et al. (1993), Granum et al. (1998)
aer_R	CTTGTCGCCACATACCTCCTG GCCC				
aerA_F	GCCTGAGCGAGAAGGT	Aerolysin	418	53.5	Heuzenroeder et al. (1999)
aerA_R	CAGTCCCACCCACTTC				
hlyA_F	GGCCGGTGGCCCGAAGAT ACGGG	β-hemolysin	595	57	Heuzenroeder et al. (1999)
hlyA_R	GGCGGCGCCGGACGAGACGGG				
ascV_F	CTCGAACTGGAAGAGCAGAATG	Type III secretion system	577	55	Yamamoto et al. (2000)
ascV_R	GAACATCTGGCTCTCCTTCTC GATG				
ascFG_F	ATGAGGTCATCTGCTCGCGC	Type III secretion system	620	55	Chacón et al. (2004)
ascFG_R	GGAGCACAACCATGGCTGAT				

model. The Bootstrap method with 1000 replications was used as the test of phylogeny.

For hemolytic activity verification, strains were grown overnight in LB broth at 28 °C, and three aliquots (5 µL) were inoculated in blood agar plates. The medium was prepared using tryptic soy agar (TSA) with 5% (v/v) defibrinated sheep blood. Plates were incubated at 28 and 37 °C up to 48 h.

Chitin hydrolysis evaluation

Crude enzyme extracts preparation

Isolates were grown in tubes of 50 mL containing 20 mL mineral broth with colloidal chitin at 28 °C under shaking (180 rpm) for 96 h. The medium for inoculum preparation contained the following composition (per L): $(NH4)_2SO_4$, 1.0 g; KH_2PO_4, 0.2 g; K_2HPO_4, 1.6 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; NaCl, 0.1 g; $FeSO_4{\cdot}7H_2O$, 0.01 g; $CaCl_2{\cdot}2H_2O$, 0.02 g; colloidal chitin, 10 g (pH 7.0).

The crude enzyme extracts were obtained inoculating 2 mL of inoculum in 200 mL of mineral broth (pH 7.0) with 2% (w/v) colloidal chitin and incubated at 28 °C under shaking (180 rpm) for 72 h. The culture supernatant was obtained after centrifugation at 3000×*g* for 5 min at 4 °C, precipitated with 80% ammonium sulfate at 4 °C for 30 min under shaking and centrifuged at 10,000×*g* for 20 min at 4 °C. The precipitates were eluted with 2 mL of 50 mM KH_2PO_4 buffer (pH 5.0) and dialyzed overnight in a regenerated cellulose membrane (MWCO: 6–8 kDa) (Spectrum Labs, Rancho Dominguez, USA) against 50 mM KH_2PO_4 buffer (pH 5.0) at 4 °C. The buffer used for overnight dialysis was removed and changed three times every 2 h for the complete removal of salts.

Total chitinase activities on crude enzyme extracts were determined by measuring the amount of reducing sugar released after chitin enzymatic hydrolysis. The reducing sugars were measured by the dinitrosalicylic acid method (Miller 1959) and one unit of chitinase activity was defined as the amount of enzyme producing 1 µmol of reducing sugars per minute. A standard curve was obtained with commercial GlcNAc (Sigma-Aldrich).

N-Acetyl-glucosamine production and quantification

GlcNAc production reactions were carried out in a final volume of 1 mL containing 2% (w/v) colloidal chitin in 50 mM KH_2PO_4 buffer (pH 5.0) and crude enzyme extract (0.5 U mL^{-1} of total chitinase). Reactions were incubated at 37 °C for 96 h under shaking and stopped at 0, 6, 12, 24, 48, 72 and 96 h after heated at 100 °C for 5 min.

GlcNAc was quantified by high-performance liquid chromatography (HPLC), according to Bassleret al. (1991), using the HPLC Ultimate 3000 System (Dionex, Sunnyvale, CA, USA) with UV Ultimate 3000 diode array detector (Dionex) at 190 nm, Aminex HPX-87H 30 cm × 7.8 mm column (Bio-Rad, Hercules, CA, USA) and 5 mM H_2SO_4 mobile phase with flow rate of 0.8 mL min^{-1}. GlcNAc concentrations were calculated by comparison of peak areas to standard solutions. The dry weight of colloidal chitin was verified and used for conversion calculations of chitin to GlcNAc, according to Nguyen-Thi and Doucet (2016). Calculations considered that 1 mg of chitin could produce a maximal theoretical yield of 1.08 mg of GlcNAc (Eq. 1).

$$\text{GlcNAc yield}\,(\%) = \frac{\text{GlcNAc released (mg)} \times 100}{\text{Initial chitin concentration(mg)} \times 1.08} \quad (1)$$

Results obtained were subjected to one-way analysis of variance (ANOVA) followed by Tukey post-test to determine the significant differences among the yields and productivities. Significant differences were defined as $p < 0.05$.

Results

Multilocus sequence analysis (MLSA) and verification of virulence-associated factors

MLSA was used for *Aeromonas* isolates differentiation and characterization. Fragments of the six selected housekeeping genes *(gltA, groL, gyrB, metG, ppsA*, and *recA*) were PCR amplified and sequenced by using the primers listed in Table 1.

The phylogeny of *Aeromonas* isolates was analyzed by constructing a neighbor-joining tree from the 2600-bp concatenated sequences of the six housekeeping genes (Fig. 2). Results revealed that all isolates are closely related to *A. caviae* species (> 98.5% of similarity). Concatenated phylogenetic tree was compared with the trees of each housekeeping gene constructed independently showing no significant differences among them. Basically, none of the six genes influenced in the topology of concatenated tree and the main cluster divisions were maintained. Intraspecies nucleotide substitution rates (different strains within the same species) of the housekeeping gene fragments were < 2% while interspecies nucleotide substitution rates (strains belonging to different species) were > 3%. The mean G + C contents of these gene fragments were 61% (*gltA*), 61% (*groL*), 63% (*gyrB*), 59% (*metG*), 64% (*ppsA*) and 59% (*recA*).

Nine virulence genes (*alt, act, ast, ahh1, aer, aer*A, *hlyA, ascV* and *ascFG*) were evaluated to examine the potential pathogenicity of *A.caviae* isolates. All isolates were negative for the potential markers selected.

Hemolytic activity isolateswere verified by their ability to form halos of hemolysis on blood agar. This ability is also

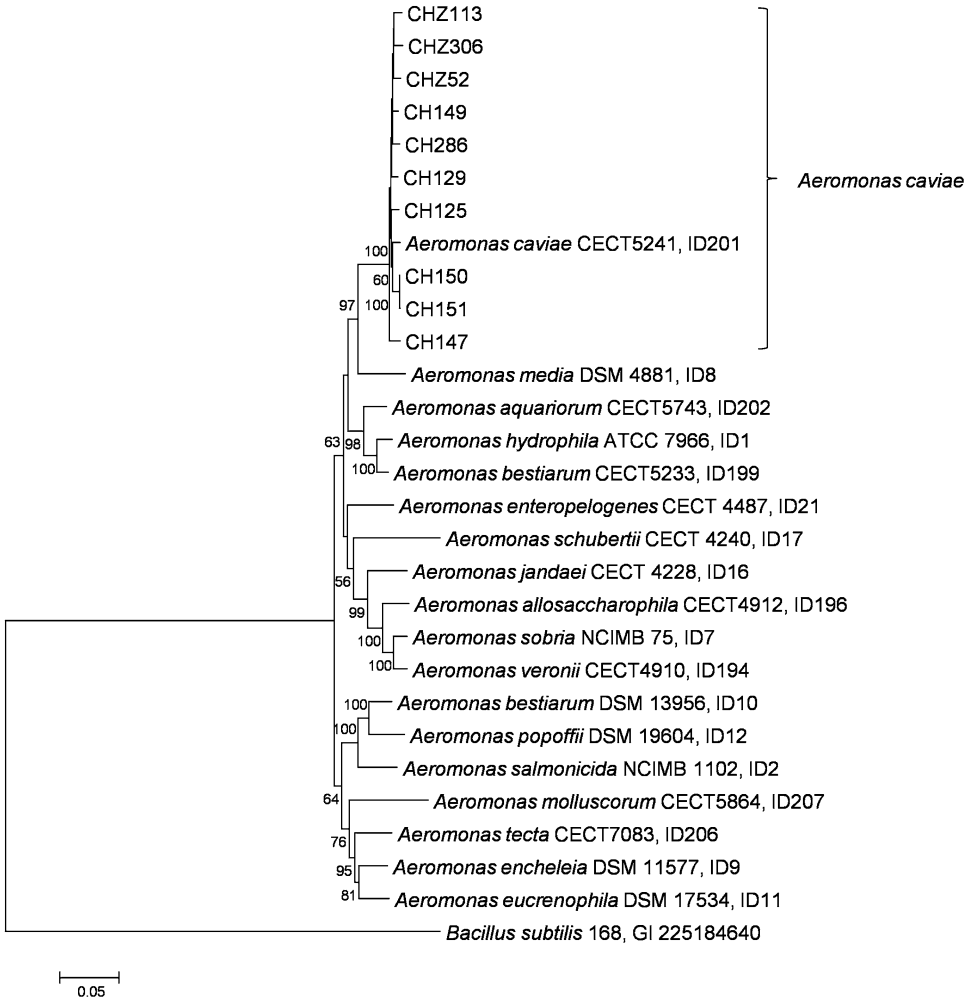


Fig. 2 Concatenated neighbor-joining phylogenetic tree based on *gltA, groEL, gyrB, metG, ppsA* and *recA* gene sequences of marine-derived *Aeromonas caviae* isolates. Numbers at nodes indicate bootstrap values (percentages of 1000 replicates). Phylogeny was performed using nucleotide sequences of type strains from three culture collections: Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM), Colección Española de Cultivos Tipo (CECT) and National Collections of Industrial Food and Marine Bacteria (NCIMB). ID numbers correspond to accession numbers in *Aeromonas* MLST Database

considered a direct demonstration of potential pathogenicity of strains and was evaluated at 28 and 37 °C, environmental and clinical temperatures, respectively.

All isolates showed hemolytic activity at 37 °C after 48 h incubation and eight isolates at 28 °C (Table 2). No strain showed hemolytic activity at 28 or 37 °C with 24 h incubation.

Chitin enzymatic hydrolysis evaluation

To evaluate the GlcNAc production, an enzymatic hydrolysis procedure of colloidal α-chitin was standardized in this study. The HPLC method used was effective and detected GlcNAc with 9.03 min of retention. The dry weight of colloidal chitin (0.74 g L^{-1}) was obtained and used for conversion calculations of chitin to GlcNAc.

Selective production of GlcNAc from colloidal α-chitin was achieved using of all the *A. caviae* isolates. According to Fig. 3, minimum and maximum yields were from 10 to 93% during 96 h of enzymatic reaction, and different isolates had already shown high yields in the first 24 h. At 6 h

Table 2 Hemolytic activity on defibrinated sheep blood agar plates by marine-derived *A. caviae* isolates. Hemolysis was carried out at 28 and 37 °C, environmental and clinical temperatures, respectively

Isolates	Incubation time			
	24 h		48 h	
	28 °C	37 °C	28 °C	37 °C
CH125	−	−	+	+
CH129	−	−	+	+
CH147	−	−	+	+
CH149	−	−	+	+
CH150	−	−	−	+
CH151	−	−	−	+
CH286	−	−	+	+
CHZ52	−	−	+	+
CHZ113	−	−	+	+
CHZ306	−	−	+	+

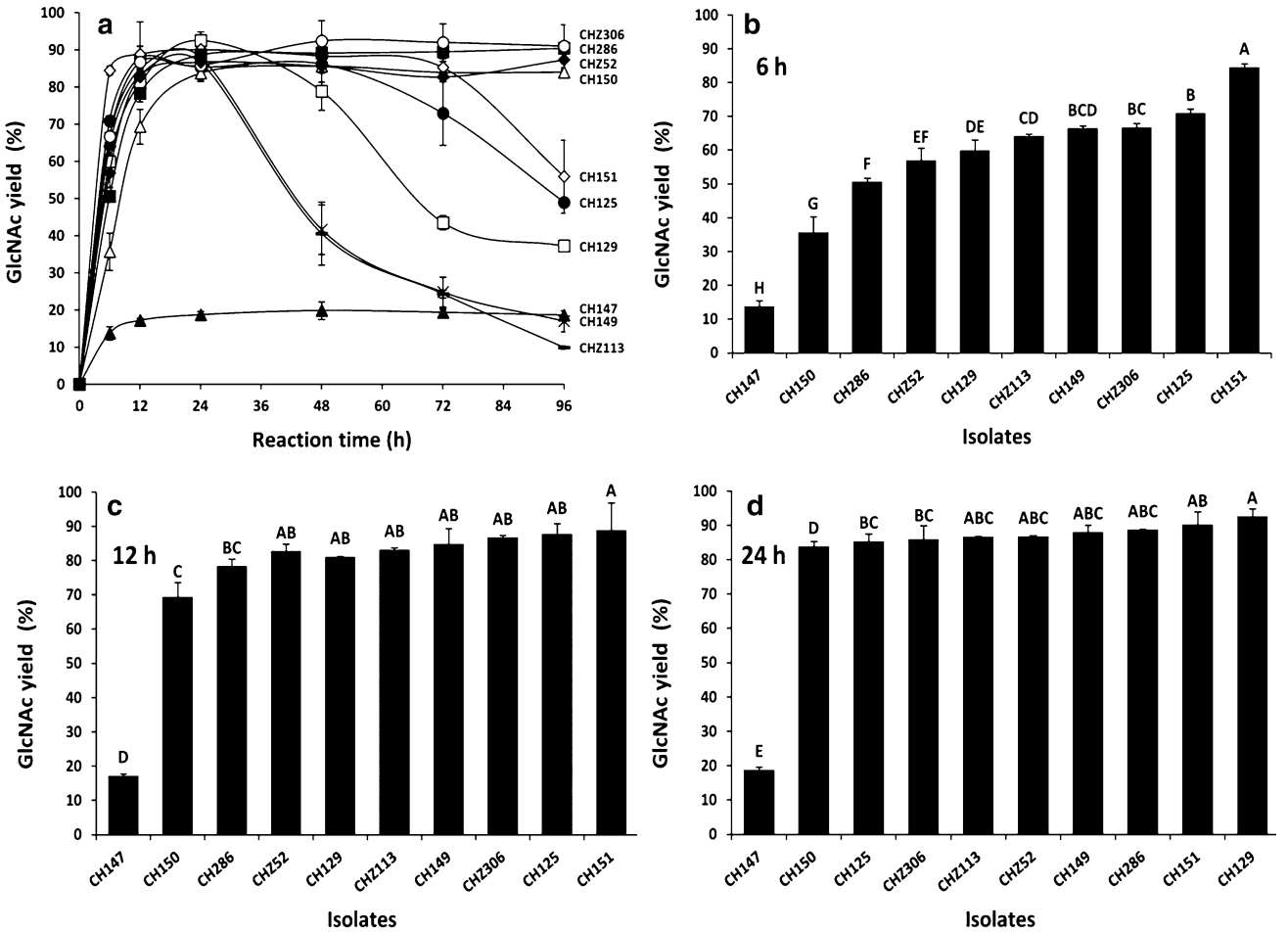


Fig. 3 *N*-Acetyl-glucosamine production from colloidal α-chitin using crude enzyme extracts of ten marine-derived *Aeromonas caviae* isolates. Production up to 96 h of enzymatic reaction (**a**) and analysis of production at 6 h (**b**), 12 h (**c**) and 24 h (**d**). Each data point represents the mean value ± standard deviations of three independent experiments. Different letters mean statistically significant differences according to the Tukey post-test ($p < 0.05$). Legend: [filled circle] CH125, [filled square] CH129, [filled triangle] CH147, [times symbol] CH149, [open triangle] CH150, [open diamond] CH151, [filled square] CH286, [filled diamond] CHZ52, [minus symbol] CHZ113, [open circle] CHZ306

of hydrolysis, for example, the yields were from 14 to 85%, and six isolates (CH125, CH129, CH149, CH151, CHZ113 and CHZ306) showed 60–85% yields of GlcNAc. After 12 h, the yields were from 17 to 89%, and seven isolates (CH125, CH129, CH149, CH151, CHZ113, CHZ306 and CHZ52) showed 81–89% yields. Except for *A. caviae* CH147 (19% yield), all isolates showed 84–93% yields of GlcNAc after 24 h of hydrolysis. The highest yields of GlcNAc were observed by *A. caviae* CH129 (93% at 24 h) and *A. caviae* CHZ306 (92% at 48 h). *A. caviae* CH147 showed the lowest yields (< 20%) at all times verified.

As shown in Fig. 3, two different behaviors were observed during 96 h of GlcNAc production: (1) progressive increase of GlcNAc concentration in the first 24 h, stabilizing until 96 h (CH147, CH150, CH286, CHZ306, and CHZ52); (2) progressive increase of GlcNAc concentration in the first 24 h with a decline after 24, 48 or 72 h (CH151, CH125, CH129, CH149, and CHZ113). All isolates showed higher productivity at 6 h of reaction, with progressive decreasing over 96 h (Fig. 4). The isolated CH151 reached maximum productivity of 112.5 mg L^{-1} h^{-1}.

Discussion

The interest on GlcNAc production has increased thanks to its functional properties and economic interest. However, as chemical processing methods currently face important drawbacks, including low yield, high cost, and environmental pollution, enzymatic processing has shown to be a sustainable alternative to the current chemical process.

Aeromonas demonstrates great potential for application in the enzymatic production of GlcNAc. However, this genus represents a public health concern because some

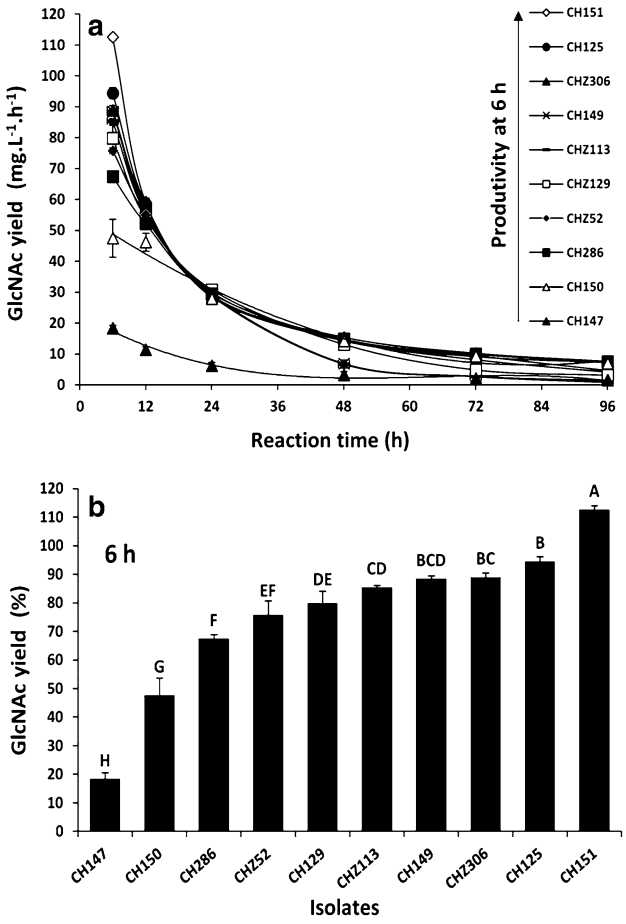


Fig. 4 Productivity of *N*-acetyl-glucosamine production from colloidal α-chitin using crude enzyme extracts of ten marine-derived *Aeromonas caviae* isolates. Productivity up to 96 h of enzymatic reaction (**a**) and analysis of productivity at 6 h (**b**). Each data point represents the mean value ± standard deviations of three independent experiments. Different letters mean statistically significant differences according to the Tukey post-test ($p < 0.05$)

strains have been reported to produce systemic infections in humans and animals (Janda et al. 1994; Janda and Abbott 1998). Thus, MLSA was used to determine phylogenetic relationships among *Aeromonas* isolates in this study. The chosen genes have proven to be excellent molecular markers, characterizing all isolates as *A. caviae* species and showing that the discriminatory power of using these housekeeping genes is relatively higher compared to the 16S rRNA gene. The intra and interspecies nucleotide substitutions observed in this study (< 2 and > 3%, respectively) were in accordance with the observations reported by Janda and Abbott (2010). These authors evaluated results from several phylogenetic investigations (Yáñez et al. 2003; Soler et al. 2004; Küpfer et al. 2006; Nhung et al. 2007) and observed that for most housekeeping genes studied, intraspecies nucleotide substitution rates are < 2%, while interspecies values are typical > 3%.

Although *A. caviae* has been considered one of three *Aeromonas* species (*A. hydrophila, A. caviae* and *A. veronii*) most related to infections in humans (Janda et al. 1994; Janda and Abbott 1998), the strains of this study were negative for the virulence genes checked. As *A. caviae* is a highly diverse species (Aguilera-Arreola et al. 2007), the strains were subjected to additional tests to verify the ability to form halos of hemolysis on blood agar. Our results showed that all the strains have the ability to express hemolysin, contrary to the results obtained by PCR targeting specific virulence-related genes.

Similar results have been observed in studies of virulence in *Aeromonas*. Wolf (2012), for example, observed low frequency of hemolytic genes (10%) in *A. caviae*, but hemolytic activity was detected in approximately 80% of them in blood agar plates. In another study (Castilho et al. 2009), the gene responsible for cytolytic enterotoxin (*aer*), found in 38% of *Aeromonas* verified, was present only in one strain of *A. caviae*. However, an unexpected fragment of 400 bp was observed in 78% of *A. caviae* during the PCR amplifications. This fragment was sequenced and showed homology with several hemolysin genes in *Aeromonas*. These results demonstrate the genetic and phenotypic variability in *Aeromonas* and that the gene identification and hemolysin expression on blood agar should be combined to interpret the potential virulence of *Aeromonas* strains.

Aeromonas is one of the most efficient bacterial groups on chitin hydrolysis. Nevertheless, there are only a few reports on the production of GlcNAc by chitinases from *Aeromonas* sp (Table 3). For example, Sashiwa et al. (2002) produced GlcNAc from different α-chitins (flakes and powder) with 64–77% yields up to 10 days using *A. hydrophila* H-2330 crude enzyme extracts. Kuk et al. (2005) produced GlcNAc from colloidal α-chitin using *Aeromonas* sp. GJ-18 crude enzyme preparations with 83 and 95% yield within 5 and 9 days, respectively. Jamialahmadi et al. (2011) produced GlcNAc from colloidal α-chitin with 79% yield using *Aeromonas* sp. PTCC 1691 crude enzyme extracts after 24 h. In the current study, GlcNAc was produced from colloidal α-chitin using the crude enzyme extracts of marine-derived *Aeromonas* isolates. The several kinds of chitinases on crude enzyme extracts, such as endochitinases, chitobiosidases, and *N*-acetyl-glucosaminidases (data not showed), resulted in efficient production of GlcNAc, with 85% yield and productivity of 112.5 mg L^{-1} h^{-1} within 6 h. The highest GlcNAc yield was obtained after 24 h (93%) and the production ranged from 10 to 93% yield during 96 h of reaction.

The results presented here show that is possible to produce GlcNAc from colloidal α-chitin using the crude enzyme extracts of marine-derived *Aeromonas* isolates. Moreover, the ability to hydrolyze α-chitin is a particularly noteworthy feature for the future development of biocatalytic processes for chitinases production and chitin derivatives,

Table 3 Comparative of some reports of GlcNAc production by microbial enzymes

Organism	Chitin		Enzyme preparation	GlcNAc yield (%)	Production time	References
	Type	Form				
A. hydrophyla H2330	α-Chitin	Flakes and powder	Crude chitinase	64–77	10 days	Sashiwa et al. (2002)
Aeromonas sp. GJ-18	α-Chitin	Swollen	Crude chitinase	83	5 days	Kuk et al. (2005)
				94.9	9 days	
Aeromonas sp. PTCC	α-Chitin	Colloidal	Crude chitinase	79	24 h	Jamialahmadi et al. (2011)
Burkholderia cepacia TU09	α-Chitin	Powder	Crude chitinase	85	7 days	Pichyangkura et al. (2002)
	β-Chitin			85	1 day	
Bacillus licheniformis SK-1	α-Chitin	Powder	Crude chitinase	41	6 days	Pichyangkura et al. (2002)
	β-Chitin			75		
Paenibacillus illinoisensis KJA-424	α-Chitin	Colloidal	Crude chitinase	62.2	24 h	Jung et al. (2007)
Trichoderma reesei	α-Chitin	Regenerated	Cellulases and β-glucanases	86	10 days	Il'ina et al. (2004)
T. viride	α-Chitin	Powder	Crude cellulases	74	19 days	Sashiwa et al. (2003)
Streptomyces coelicolor A3(2)	α-Chitin	Powder	*N*-Acetylhexosaminidase and chitinase C	90	8 h	Nguyen-Thi and Doucet (2016)
A. caviae isolates	α-Chitin	Colloidal	Crude chitinase	14–85	6 h	This study
				17–89	12 h	
				19–93	24 h	

since this form of chitin is highly resistant to degradation due to its crystalline structure and insolubility in water (Mehmood et al. 2010). It is important to mention that the use of colloidal chitin (partially crystallized chitin) as well as the types of chitinases present in the enzymatic extract also has significant influence in GlcNAc production. A colloidal chitin preparation procedure which allows obtaining increasingly decrystallized particles and the use of an enzyme extract with the appropriate amount of endo and exochitinases facilitates enzymatic hydrolysis and GlcNAc production (Hirano and Nagao 1988; Chen et al. 2010).

The decline of GlcNAc concentrations in some tests (CH151, CH125, CH129, CH149, and CHZ113) was a disadvantage of crude enzyme extracts and an important point in the control of GlcNAc production. Data showed that different chitinases of those better characterized on chitin hydrolysis (endochitinases, chitobiosidases, and *N*-acetylglucosaminidases) (Sahai and Manocha 1993; Dahiya et al. 2006) may be present in a relatively high proportion in crude enzyme extracts from chitinolytic bacteria and contribute to a reduction of the final concentration of GlcNAc during long incubation. Chitin deacetylase (Zhao et al. 2010), for example, hydrolyzes the acetyl group of GlcNAc units in glucosamine and acetic acid and could decrease the efficiency of GlcNAc production. Thus, the reaction time is a parameter that requires optimization to maximize GlcNAc yields.

The fact that *A. caviae* isolates have pathogenic potential is not an obstacle for application their crude enzyme extracts on GlcNAc production, since such extracts are intermediates of the process. Moreover, the crude enzyme extracts used in this study were recovered by centrifugation and pre-purified by salt-precipitation and dialysis. These steps can eliminate many remaining contaminants, such as whole bacteria, cell debris, possible toxins, peptides, DNA, pigments and others small molecular compounds. In a study reported by Louise et al. (1999), for example, the authors describe the enzymatic production of GlcNAc from chitin by *Serratia marcescens* QM B1466, a human pathogen often involved in hospital-acquired infections. The system based on five steps (chitinolytic enzymes production, enzyme recovery, pretreatment of chitin, chitin hydrolysis and GlcNAc purification) produced GlcNAc with a purity greater than 98%, and no protein contaminants were detectable. Additionally, a wide variety of methodologies can be applied to use chitinolytic enzymes on GlcNAc production. Potential chitinase-coding genes from these isolates, for example, can be selected, cloned and heterologously expressed in organisms recognized as safe, including *Escherichia coli* K12, *Saccharomyces cerevisiae* and *Pichia pastoris*. Thus, this study represents an initial examination of the chitinolytic potential of marine-derived *A. caviae* strains which is essential for the subsequent application them.

Conclusion

This study demonstrates the potential of GlcNAc production from colloidal α-chitin, using crude enzyme extracts of marine-derived *A. caviae* isolates. This ability is particularly important because this form of chitin is highly resistant to degradation and the production of these extracts is a relatively inexpensive procedure. The genetic and phenotypic diversity, as well as the capacity to express multiple chitinases are characteristics to be considered in studies with *Aeromonas* strains. Thus, it is essential to characterize specific strains and optimize the parameters of chitin derivatives production, such as the type of chitin, chitinases and time of hydrolysis.

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