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BIOTECHNOLOGY AND INDUSTRIAL MICROBIOLOGY - RESEARCH PAPER

Enhanced production of *N*-acetyl-glucosaminidase by *marine Aeromonas caviae* CHZ306 in bioreactor

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Abstract

N-Acetyl-glucosaminidases (GlcNAcases) are exoenzymes found in a wide range of living organisms, which have gained great attention in the treatment of disorders related to diabetes, Alzheimer's, Tay-Sachs', and Sandhoff's diseases; the control of phytopathogens; and the synthesis of bioactive GlcNAc-containing products. Aiming at future industrial applications, in this study, GlcNAcase production by marine *Aeromonas caviae* CHZ306 was enhanced first in shake flasks in terms of medium composition and then in bench-scale stirred-tank bioreactor in terms of physicochemical conditions. Stoichiometric balance between the bioavailability of carbon and nitrogen in the formulated culture medium, as well as the use of additional carbon and nitrogen sources, played a central role in improving the bioprocess, considerably increasing the enzyme productivity. The optimal cultivation medium was composed of colloidal α-chitin, corn steep liquor, peptone A, and mineral salts, in a 5.2 C:N ratio. Optimization of pH, temperature, colloidal α-chitin concentration, and k_La conditions further increased GlcNAcase productivity. Under optimized conditions in bioreactor (i.e., 34 °C, pH 8 and k_La 55.2 h^{-1}), GlcNAcase activity achieved 173.4 $U.L^{-1}$ after 12 h of cultivation, and productivity no less than 14.45 $U.L^{-1}.h^{-1}$ corresponding to a 370-fold enhancement compared to basal conditions.

Keywords *Aeromonas caviae* · Bioproduction · Bioreactor · Chitinase · *N*-Acetyl-glucosaminidase

Introduction

N-Acetyl-glucosaminidases (GlcNAcases), also known as *N*-acetyl-hexosaminidases (EC 3.2.1.52), are exoenzymes belonging to the glycoside hydrolase group [1] and the family of chitinases. They not only catalyze the hydrolysis of *N*-acetyl-D-glucosamine (GlcNAc) and *N*-acetyl-D-galactosamine (GalNAc) from the non-reducing end of oligosaccharides, glycoproteins, glycolipids, and other glycoconjugates [1, 2], but also play a regulatory function in thousands of intracellular proteins, including those found in signal transduction, gene expression, cell cycle, and proteasomal degradation. The role of such enzymes in human physiology and diseases has been subject of extensive studies during the last decades. Their potential in the treatment of disorders related to type 2 diabetes, Alzheimer's disease, different types of cancers [3], and Tay-Sachs' [55] and Sandhoff's [4] diseases, as well as neurodegenerative disorders caused by the accumulation of gangliosides especially in neurons [5], has recently gained a lot of attention among scientific and

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medical communities. Furthermore, different GlcNAcases isoforms have been reported as effective and highly specific tools for the enzymatic production of bioactive compounds like functional carbohydrates and glycomimetics for the food and pharmaceutical industries [6, 7].

Acknowledged as one of the most abundant organic compounds in nature and the main component of the exoskeleton from arthropods and fungal and algal cell walls, chitin, a polyglycan constituted by β (1 → 4)-2-acetamido-2-deoxy-D-glucose, is found in large amounts in seafood processing waste. Every year, 6 to 8 million tons of waste crab, shrimp, and lobster shells are produced globally [8]. Due to its very slow biodegradation, difficult disposal, and accumulation, such a chitin-based biowaste started to raise several concerns regarding its waste management and environmental impact. Aiming to increase competitiveness and expand industrial product family trees, while contributing to the implementation of carbon neutrality and circular economy, in recent decades, a significant number of new green and sustainable processes based on alternative chitin conversion pathways have been developed and proposed that explore a broad range of industrial potential applications [9, 10]. Among the numerous applications claimed for chitinous polymer derivatives, their conversion into value-added products for medical, pharmaceutical, biotechnological, and cosmetic use has aroused much industrial interest. Both chemical and enzymatic hydrolytic methods have been developed to exploit chitin derivatives. However, the acid hydrolytic methods have many industrial and environmental drawbacks, including acid wastes production, low yields, and high costs. On the other hand, given the enhanced functional properties and desirable biological activities of chitin byproducts, its enzymatic hydrolysis has been considered to offer many advantages [11].

Thanks to their role in chitin degradation in ocean ecosystems, marine chitinolytic bacteria are considered owners of great potential for producing chitinases [12]. Due to their ability to grow in wide ranges of temperature and pH, bacterial species belonging to the *Aeromonas* genus, which have a wide phenotypic diversity and are able to synthesize extracellular chitinases using different molecular inducers, are believed to have great biotechnological potential for large-scale GlcNAcases production. Standing as a great chitinase biosynthesis inducer, chitin, either in its α, β, or γ type and in the form of flakes, powder, colloidal matter, or oligomers, has been successfully used in a large number of studies [13]. When compared in terms of inductive efficacy, chitooligosaccharides, oligomers obtained from the hydrolysis of high molecular weight chitin molecules, have shown higher inductive profiles than those of chitin with higher crystalline polymorphic structures [14, 15]. However, colloidal α-chitin, a chemically pretreated chitin form with an enzymatically digestible structure obtained from relatively inexpensive raw materials such as seafood processing waste, is considered the most economically viable alternative [16–18].

This study reports the potential for GlcNAcase production by *Aeromonas caviae* CHZ306, a marine chitinolytic bacterium isolated from zooplankton samples from the coast of São Paulo state, Brazil, provides essential knowledge for the efficient production of GlcNAcases, and fills a gap in the current literature on the development of bioprocesses using chitinolytic bacteria. This enzyme had previously been successfully employed, together with other endochitinases and exochitinases, for the synergistic bioconversion of chitin into GlcNAc [19], an amino-monosaccharide used in the treatment of osteoarthritis and inflammatory bowel disease [16, 20, 21]. Considering the impacts of both growth conditions and culture medium composition on chitinase biosynthesis and activity [22, 23], the influence of different carbon and nitrogen sources, temperature, and pH was investigated either in shake flasks or bench-scale stirred-tank bioreactor, while the volumetric oxygen transfer coefficient (k_La) was optimized in the latter system aiming to maximize the GlcNAcase production yield.

Material and methods

Microorganism and pre-inoculum preparation

Aeromonas caviae CHZ306, a marine chitinolytic bacterium isolated from zooplankton samples from the coast of São Paulo state, Brazil, was used in the present study. The strain was initially grown on colloidal α-chitin agar plates at 28 °C for 96 h, according to the method described by Souza et. al. (2009) [18]. Five bacterial colonies were transferred to 250-mL Erlenmeyer flasks containing 100 mL of colloidal α-chitin broth and incubated at 28 °C for 24 h under orbital shaking (180 rpm). Cryotubes containing 1.0 mL of bacterial culture in 20% glycerol were preserved at − 80 °C and used as pre-inoculum.

Selection of the nitrogen sources for GlcNAcase production

The production of *N*-acetyl-glucosaminidase (GlcNAcase) by *A. caviae* CHZ306 was initially tested using twelve different inorganic and organic compounds as nitrogen sources (Table 1). Each culture medium was prepared with the following composition ($g.L^{-1}$): specific nitrogen source, corresponding to 0.2 g of elemental nitrogen; KH_2PO_4, 0.2; K_2HPO_4, 1.6; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; $FeSO_4.7H_2O$, 0.01; $CaCl_2.2H_2O$, 0.02; and colloidal α-chitin, 10 (pH 7.0). For all tested culture media, the relative proportion of each compound was established based on its elemental composition and desired C:N ratio. The inoculum was prepared by

Table 1 Elemental composition of powdered nitrogen sources tested to select the best culture medium for GlcNAcase production by *Aeromonas caviae* CHZ306

Culture medium	Nitrogen source	Main constituents	Carbon (%)	Nitrogen (%)	C:N ratio[a]
M1	Ammonium sulfate	Nitrogen, hydrogen, sulfur, and hydrogen	0	21	0.7
M2	Ammonium acetate	Carbon, hydrogen, nitrogen, and oxygen	16	18	1.5
M3	Ammonium nitrate	Nitrogen, hydrogen, and oxygen	0	35	0.7
M4	Urea	Carbon, hydrogen, nitrogen, and oxygen	20	47	1.1
M5	Casamino acids	Amino acids	33	11	3.5
M6	Meet extract	Proteins, amino acids, nucleotide fractions, organic acids, vitamins, and minerals	42	13	3.7
M7	Yeast extract	Peptides, amino acids, carbohydrates, and vitamins	39	11	3.9
M8	Bacto peptone	Peptone and amino acids	43	15	3.3
M9	Peptone A	Peptone and amino acids	42	13	3.6
M10	Peptone G	Peptone and amino acids	44	16	3.2
M11	Corn steep liquor	Proteins, amino acids, carbohydrates, organic acids, vitamins, and minerals	38	8	5.2
M12	Tryptone	Peptone and amino acids	44	13	3.8

[a]C:N values represent the final carbon:nitrogen ratios in each culture medium, constituted by substrates and colloidal α-chitin. M1 refers to the conditions of the basal medium, used in our previous studies (results not shown)

addition of 1.0 mL of stock culture in 250-mL Erlenmeyer flasks containing 100 mL of colloidal α-chitin broth and incubation at 28 °C for 24 h under orbital shaking (180 rpm). Submerged cultures were carried out in triplicate adding 1.0 mL of inoculum (approx. 5.0×10^8 CFU L^{-1}) in 250-mL Erlenmeyer flasks containing 100 mL of the different culture media and incubating at 28 °C for 96 h under orbital shaking (180 rpm). Aliquots of 500 µL were collected every 12 h to evaluate cell viability and quantify GlcNAcase activity.

Optimization of physicochemical conditions for GlcNAcase production in shake flasks

The influence of three independent variables, namely, pH, temperature, and colloidal α-chitin concentration, on GlcNAcase activity was investigated in shake flasks using three different factorial designs (Table 2), performed sequentially following the positive gradient of GlcNAcase productivity. To select the optimal initial pH of culture medium, two 2^3 full-factorial designs [54] with five replications at the central point were used. Moreover, a third 3^2 full-factorial design [24] with three replications at the central point was used to evaluate the influence of colloidal α-chitin concentration and temperature, at the optimal initial pH value previously established. Submerged cultures were carried out randomly by addition of 1.0 mL of inoculum suspension, prepared as previously described, in 250-mL Erlenmeyer flasks containing 100 mL of culture medium with the nitrogen sources selected in the Sect. 2.2 (peptone A and corn steep liquor), and subsequent incubation for 96 h under orbital shaking (180 rpm). Aliquots of 500 µL were collected every 12 h

Table 2 Experimental factorial designs used to evaluate the GlcNAcase production by *Aeromonas caviae* CHZ306 in shake flasks and stirred-tank bioreactor

Laboratory-scale set-up	Design	Variables	Levels of variables [a]		
			Lower (− 1)	Center (0)	Higher (+ 1)
Shake flasks	2^3-full factorial	Colloidal α-chitin (%)	1	2	3
		pH	6	7	8
		Temperature (°C)	22	28	34
	2^3-full factorial	Colloidal α-chitin (%)	3	4	5
		pH	8	9	10
		Temperature (°C)	34	38	42
	3^2-full factorial	Colloidal α-chitin (%)	5	6	7
		Temperature (°C)	32	34	36
Stirred-tank bioreactor	3^2-full factorial	Agitation (rpm)	150	250	350
		Aeration (vvm)	0.25	1.00	1.75

[a]Levels of variables do refer to actual values. Coded levels are written between brackets

to evaluate cell viability and quantify GlcNAcase activity. Culture media were prepared with the following composition ($g.L^{-1}$): peptone A, 0.54; corn steep liquor, 1.87; KH_2PO_4, 0.2; K_2HPO_4, 1.6; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; $FeSO_4.7H_2O$, 0.01; and $CaCl_2.2H_2O$, 0.02.

Optimization of physicochemical conditions for GlcNAcase production in stirred-tank bioreactor

GlcNAcase production was then studied in bioreactor cultivation. In this step, the influence of volumetric oxygen transfer coefficient (k_La) on GlcNAcase production was evaluated combining different agitation speed and aeration rate conditions. For this purpose, a 3^2 full-factorial design [24] with three replications at the central point was used (Table 2), and all experiments were performed randomly. The inoculum was prepared by addition of 1.0 mL of stock culture in 500-mL Erlenmeyer flasks containing 200 mL of culture medium and incubation at 34 °C for 16 h under orbital shaking (180 rpm). The culture medium was prepared under optimal conditions, determined in shake flasks, with the following composition ($g.L^{-1}$): peptone A, 0.54; corn-steep liquor, 1.87; KH_2PO_4, 0.2; K_2HPO_4, 1.6; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; $FeSO_4.7H_2O$, 0.01; $CaCl_2.2H_2O$, 0.02; and colloidal α-chitin, 50 (initial pH 8.0). Submerged cultures were carried out adding 200 mL of inoculum in a 3.0-L BioFlo 110 bioreactor (New Brunswick, Edison, NJ, USA) containing 1.8 L of culture medium. Sterilized antifoam Y-30 emulsion (0.002%, w/v) (Sigma-Aldrich, St. Louis, MO, USA) was initially added to avoid the formation of foam, which was continuously controlled during cultivation. During the experiments, temperature was automatically controlled at 34 °C. Dissolved oxygen concentration and pH were measured by electronic probes, and filtered air was continuously bubbled into the medium through a multi-point sparger. The medium pH was not controlled, and aliquots of 3.0 mL were collected over 36 h to evaluate cell viability and quantify GlcNAcase activity. All fermentation media used either in flasks or bioreactor were sterilized in autoclave at 121 °C for 20 min and equilibrated at 34 °C before inoculation.

Analytical methods

Viable cell count

Viable cell count was carried out according to the drop plate method [25]. After serial dilutions, 10 µL of cell suspension was inoculated (triplicate) on colloidal α-chitin agar plates [18, 19] at 34 °C for 96 h. Viable cell count was expressed as colony-forming units per liter ($CFU.L^{-1}$).

GlcNAcase activity assay

GlcNAcase activity was quantified by the chitinase assay kit CS0980 (Sigma-Aldrich), according to the manufacturer's instructions. Briefly, *p*-nitrophenyl-*N*-acetyl-*β*-D-glucosamine (pNP-GlcNAc) was used as a substrate, and one unit of GlcNAcase activity was defined as the amount of enzyme that released 1.0 µmol of *p*-nitrophenol per minute.

Determination of the initial volumetric oxygen transfer coefficient

The initial volumetric oxygen transfer coefficient (k_La) was determined by the dynamic gassing-out (physical) method [26]. Pure nitrogen was bubbled into the non-inoculated medium to remove dissolved oxygen. Agitation speed and airflow conditions were set under the same conditions as those of fermentation experiments. The dissolved oxygen concentration was measured every 5 s throughout the reaeration process employing a sterilizable galvanic electrode connected to a Teflon-silicone-Teflon membrane. The equipment was previously calibrated at atmospheric pressure. The dissolved oxygen mass balance in the liquid phase can be written as:

$$\frac{dC_L}{dt} = k_La\left(C^* - C_L\right) - r_{O_2} = OTR - OUR \quad (1)$$

where C_L is the dissolved oxygen concentration, C^* is the oxygen concentration at saturation in the liquid, OTR is the oxygen transfer rate, and $r_{O2} = OUR$ is the oxygen uptake rate. When $OUR = 0$, the oxygen mass balance in the liquid phase, in the absence of cells, can be simplified to:

$$\frac{dC_L}{dt} = k_La\left(C^* - C_L\right) = OTR \quad (2)$$

The initial k_La value was then obtained from the slope of the straight line describing the oxygen mass variation versus time according to the integrated equation:

$$\ln\left(1 - \frac{C_L}{C^*}\right) = -k_Lat \quad (3)$$

Statistical analysis

The Statistica software 7.0 (Statsoft, Tulsa, OK, USA) was used for experimental designs, data regression, and graphical analysis of the shake flask optimization experiments. The maximum likelihood estimates of the bioreactor model coefficients, as well as their standard errors, *t*-statistic values, and significance scores, were calculated using the system fit package in R [27]. The statistical significance of the regression coefficients was determined by the Fischer's test

for analysis of variance (ANOVA) at a significance level (P) ≤ 0.05, and the extent of variance explained by each model was given by the determination coefficient (R^2). To minimize the error of ANOVA, tests corresponding to the central point of the shake flask optimization experiments were repeated five times. To perform the statistical analysis, the actual values of each independent variable (X_i) were coded according to the equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (4)$$

where x_i are the coded values, X_o the actual value at the central point, and ΔX_i the step change value.To identify the best conditions for GlcNAcase production in the shake flask experiments, the following quadratic model was used:

$$\hat{y}_i = b_0 + \sum b_i x_i + \sum b_{ii} x_i^2 + \sum b_{ij} x_i x_j \quad (5)$$

where $\hat{y}_i$ are the predicted values for each response, while b_o, b_i, b_{ii}, and b_{ij} are the intercept, linear, quadratic, and interaction coefficients, respectively. The Pareto analysis was performed to estimate the effects of the independent variables and their interactions on GlcNAcase production at a confidence level of 95%.

Results

Optimization of culture medium for GlcNAcase production

Culture medium for GlcNAcase production by *A. caviae* CHZ306 was initially tested using twelve different compounds as nitrogen sources, previously analyzed in elemental composition for carbon (C) and nitrogen (N). Indeed, nitrogen supplementation was based on inorganic and organic compounds widely applied in microbial bioprocesses, i.e., M1 (ammonium sulfate); M2 (ammonium acetate); M3 (ammonium nitrate); M4 (urea); M5 (casamino acids); M6 (meat extract); M7 (yeast extract); M8 (bacto peptone); M9 (peptone A); M10 (peptone G); M11 (corn steep liquor); and M12 (tryptone). Based on the obtained results (Fig. 1a), all the culture media proved capable of providing suitable conditions for *A. caviae* CHZ306 growth (10^7 to 10^{10} CFU.L^{-1}) and GlcNAcase production, since the maximum enzyme activity was reached only after 96 h of incubation. Among the different tested nitrogen sources, the medium containing peptone A (M9) and corn steep liquor (M11) in 4.4 and 5.2 C:N ratios, respectively, showed the highest GlcNAcase activities (78 ± 1.0 and 54 ± 2.1 U.L^{-1}, respectively).

In order to detect possible synergistic effects arising from the combination of these two nitrogen sources, additional tests were carried out using them in different proportions, namely, 1:1 (M13), 1:2 (M14), and 2:1 (M15) (w/v) in 4.2, 3.9, and 4.4 C:N ratios, respectively. Compared to individual use of peptone A (M9) and corn steep liquor (M11) (Fig. 1a), none of these combinations allowed improving GlcNAcase activity after 96 h of incubation. However, in all tested proportions, it was observed that the interaction of these two nitrogen sources promoted a significant improvement in the kinetics of GlcNAcase production after 36 h of cultivation (Fig. 1b). In the individual use of peptone A (M9) and corn steep liquor (M11), the production kinetics was only observed after 60 h of cultivation. Particularly, after 60 h of cultivation, GlcNAcase activity using M13, M14, and M15 was 24 ± 0.9, 32 ± 0.4, and 33 ± 0.9 U.L^{-1}, respectively, which represents approximately 2.4- to 3.3-fold activity increases compared to M9 and M11. Compared to the GlcNAcase activity achieved after 96 h in the cultivation performed on the basal medium (M1) (3.7 U.L^{-1}) (Table 3), the M15 medium allowed a 21-fold increase (78 U.L^{-1}), a 4.4 C:N ratio, and exhibited the best production profile among all tested formulations (Fig. 1b).

The statistical significance of these comparisons was assessed using the Bonferroni correction for multiple comparisons. Figure 1c shows the Bonferroni matrix, where each square shows the negative natural log of the p value of each pairwise t-test comparison (calculated using pairwise t-test (p.adjust.method = "bonferroni") function in R and plotted using the package ggplot2). The p value was plotted in the logarithmic scale to facilitate the interpretation of the results. Since -ln (0.05) = 2.996, any value on this matrix above 3 will cross the statistical significance threshold. As shown in Fig. 1c, the GlcNAcase activities at 96 h that resulted from M9 and M15 are statistically significantly larger (based on pairwise comparisons) than those obtained using all remaining nitrogen sources. The only exception was the difference between the GlcNAcase activity obtained at 96 h using M9 and M15 (the two highest ones), whose difference was not statistically significant based on a t-test pairwise comparison.

Optimization of culture conditions for GlcNAcase production in shake flasks

Culture conditions for GlcNAcase production by *A. caviae* CHZ306 were investigated in shake flasks in terms of colloidal α-chitin concentration, pH, and temperature using three factorial experimental designs. Within the condition ranges investigated in the first experimental design, *A. caviae* CHZ306 growth was not significantly influenced by any of the variables tested, reaching viable cell counts around 10^9 to 10^{10} CFU.L^{-1} (results not shown). Despite the absence of any significant effect upon microbial growth, all the independent variables as well as their interactions exerted significant positive effects (Fig. S1 of the Supplementary Material)

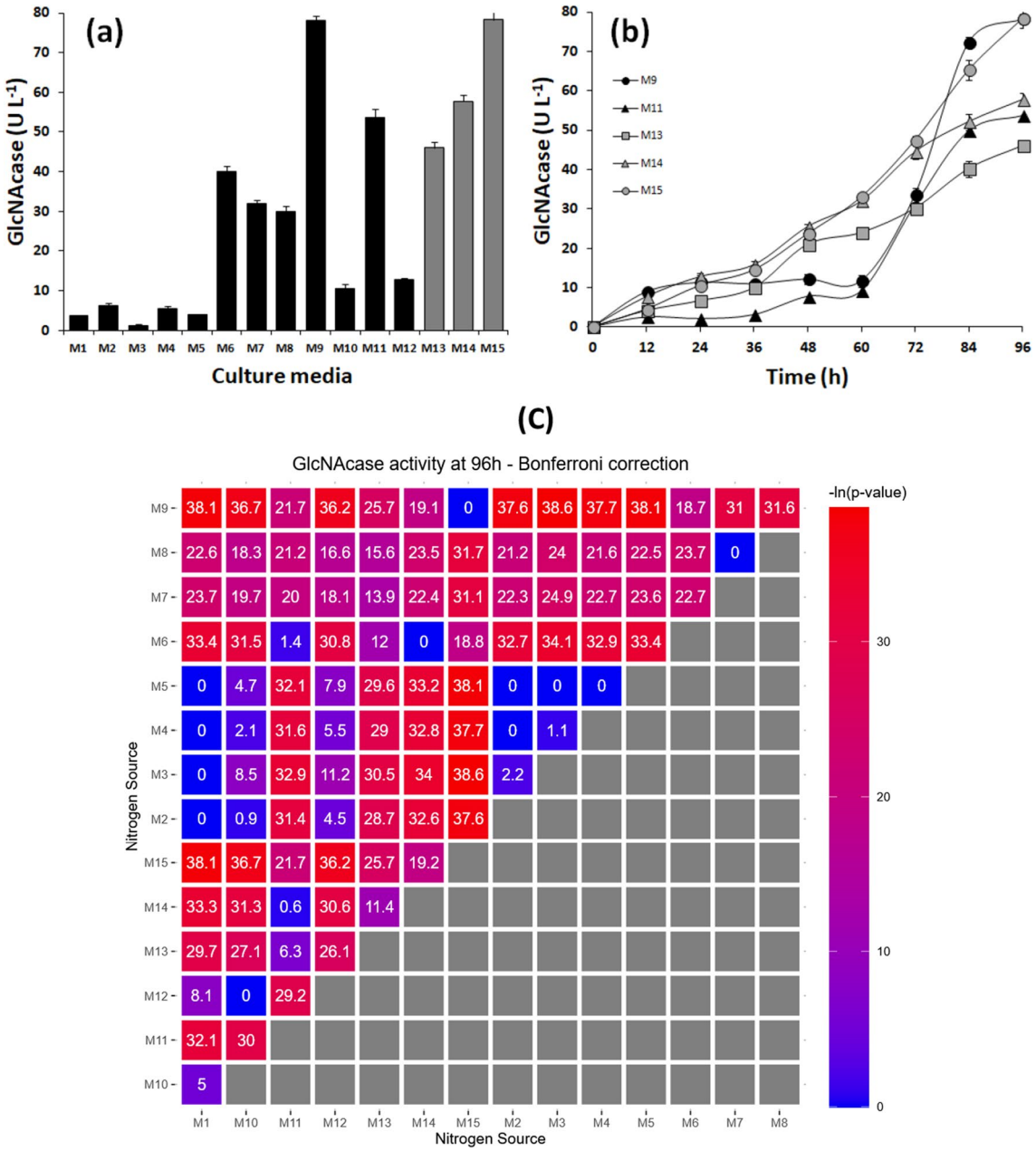

Fig. 1 GlcNAcase production by *Aeromonas caviae* CHZ306 cultivated in the chitin-containing medium under different nitrogen supplementations (viz. M1–M12). **a** GlcNAcase activity at 12 h *A. caviae* CHZ306 cultivation. **b** Time-course profiles of GlcNAcase activity during *A. caviae* CHZ306 cultivation in the medium M9, M11, M13, M14, and M15. M1 (ammonium sulfate); M2 (ammonium acetate); M3 (ammonium nitrate); M4 (urea); M5 (casamino acids); M6 (meat extract); M7 (yeast extract); M8 (bacto peptone); M9 (peptone A); M10 (peptone G); M11 (corn steep liquor); M12 (tryptone); M13 (1:1 (w/w) corn steep liquor:peptone A); M14 (1:2 (w/w) corn steep liquor:peptone A); M15 (2:1 (w/w) corn steep liquor:peptone A). Corn steep liquor and peptone A combinations in different proportions (M13, M14, and M15) are depicted by gray curves and bars. **c** Bonferroni pairwise comparison correction of the GlcNAcase activities at 96 h obtained with different nitrogen sources. Each square represents the negative natural log of the *p* value of the *t*-test pairwise comparison between the GlcNAcase activity obtained using each nitrogen source. Since -ln(0.05) = 2.996, any value on this matrix above 3 will cross the statistical significance threshold

on *A. caviae* CHZ306 GlcNAcase activity over cultivation time (Fig. 2). As shown in Table S1 of the Supplementary Material, where the run numbering and conditions are provided in detail, GlcNAcase activity was in fact progressively increased with the increase in colloidal α-chitin concentration, pH, and temperature. In the run 8, carried out at the highest levels of the three selected variables, not only was an almost 40-fold increase in GlcNAcase activity (141.1 $U.L^{-1}$)

Table 3 Comparison of optimal conditions at each step of GlcNAcase production by *Aeromonas caviae* CHZ306

Variables	Evaluation steps			
	Basal conditions[a]	Nutritional conditions	Physicochemical conditions	
Cultivation system	Shake flasks	Shake flasks	Shake flasks	Stirred-tank bioreactor
Primary carbon source	Colloidal α-chitin	Colloidal α-chitin	Colloidal α-chitin	Colloidal α-chitin
Secondary carbon source	-	Corn steep liquor, peptone A	Corn steep liquor, peptone A	Corn steep liquor, peptone A
Nitrogen source	$(NH_4)_2SO_4$	Corn steep liquor, peptone A	Corn steep liquor, peptone A	Corn steep liquor, peptone A
C:N ratio	0.7	4.4	5.2	5.2
Colloidal α-chitin (%)	1.0	1.0	5.0	5.0
pH	7.0	7.0	8.0	8.0
Temperature (°C)	28	28	34	34
Agitation (rpm)	180	180	180	250
Aeration (vvm)	-	-	-	1.75
k_La (h^{-1})	-	-	-	55.2
Time of max. activity (h)	96	96	36	12
GlcNAcase activity ($U.L^{-1}$)	3.7	78.3	192.5	173.4
GlcNAcase productivity ($U.L^{-1}.h^{-1}$)	0.04	0.82	5.35	14.45

[a]Basal conditions refer to those used in our previous studies (data not shown)

observed, but the production time (48 h) was also even halved (Fig. 2a) compared to basal conditions (Table 3), thus leading to a 75-fold enzyme productivity raise (2.94 $U.L^{-1}.h^{-1}$).

To reach even higher GlcNAcase production levels, higher ranges of temperature (34–42 °C), colloidal α-chitin concentrations (3–5%), and pH values (8–10) were tested according to a second 2^3-experimental design. Whereas colloidal α-chitin concentration continued to exert a statistically significant positive effect (Fig. S1), further increases in temperature and pH were detrimental for enzyme production. As a consequence, the run 11 carried out at colloidal α-chitin concentration of 5%, pH 8.0, and 34 °C ensured the highest GlcNAcase production (192.5 $U.L^{-1}$) after only 36 h (Table S1 and Fig. 2c), corresponding to a 137-fold enzyme productivity raise (5.35 $U.L^{-1}.h^{-1}$) compared to the initial experimental conditions (Table 3).

Based on these results, the initial pH was fixed at this optimum value (8.0) in a third 3^2-experimental design where temperature and colloidal α-chitin concentrations were varied within lower (32–36 °C) and higher (5–7%) ranges, respectively. GlcNAcase production was affected by increasing colloidal α-chitin concentration (6–7%), and despite the positive effect of temperature on GlcNAcase production highlighted by the Pareto chart (Fig. S1), the run 20, which was a replication of the run 11 of the previous design (α-chitin concentration of 5%, pH 8.0, and 34 °C), provided the best results (Table S1); therefore, these conditions were finally selected as the best ones for GlcNAcase production in shake flasks. Under these conditions, the optimum concentration of colloidal α-chitin determinated ensured a C:N ratio in the culture medium (5.2) above the minimum threshold estimated earlier (4.4).

Optimization of culture conditions for GlcNAcase production in stirred-tank bioreactor

The influence of the volumetric oxygen transfer coefficient (k_La) on GlcNAcase production by *A. caviae* CHZ306 was investigated in stirred-tank bioreactor using a 3^2 full-factorial design, by combining different agitation speeds (rpm) and aeration rates (vvm). Figure 3 shows the profiles of GlcNAcase production, cell growth, and dissolved oxygen level (dO_2) versus time.

GlcNAcase production, which varied from 0.53 to 197.10 $U.L^{-1}$ depending on the operational conditions (Table S2 of the Supplementary Material), was the highest after 12 h of cultivation in runs 30, 32, and 33 carried out at k_La in the range of 35–55 h^{-1} (Fig. 3a), while cell growth (10^7 to 10^9 $CFU.L^{-1}$) was poorly affected either by low, medium, or high k_La conditions (Fig. 3b). A qualitatively similar dO_2 profile was observed in all the runs (Fig. 3c), describing an initial decrease in the first 6–12 h of cultivation, corresponding to both the exponential growth phase and the highest GlcNAcase productivities, a subsequent progressive increase, and an almost constant value at the end of cultivation.

Either lower or higher k_La values than the above optimum range negatively affected GlcNAcase production over time. For instance, in the run 28, k_La conditions (8.28 h^{-1}) were able to support cell growth, but not trigger GlcNAcase biosynthesis. On the other hand, runs 35 and 36, carried out at

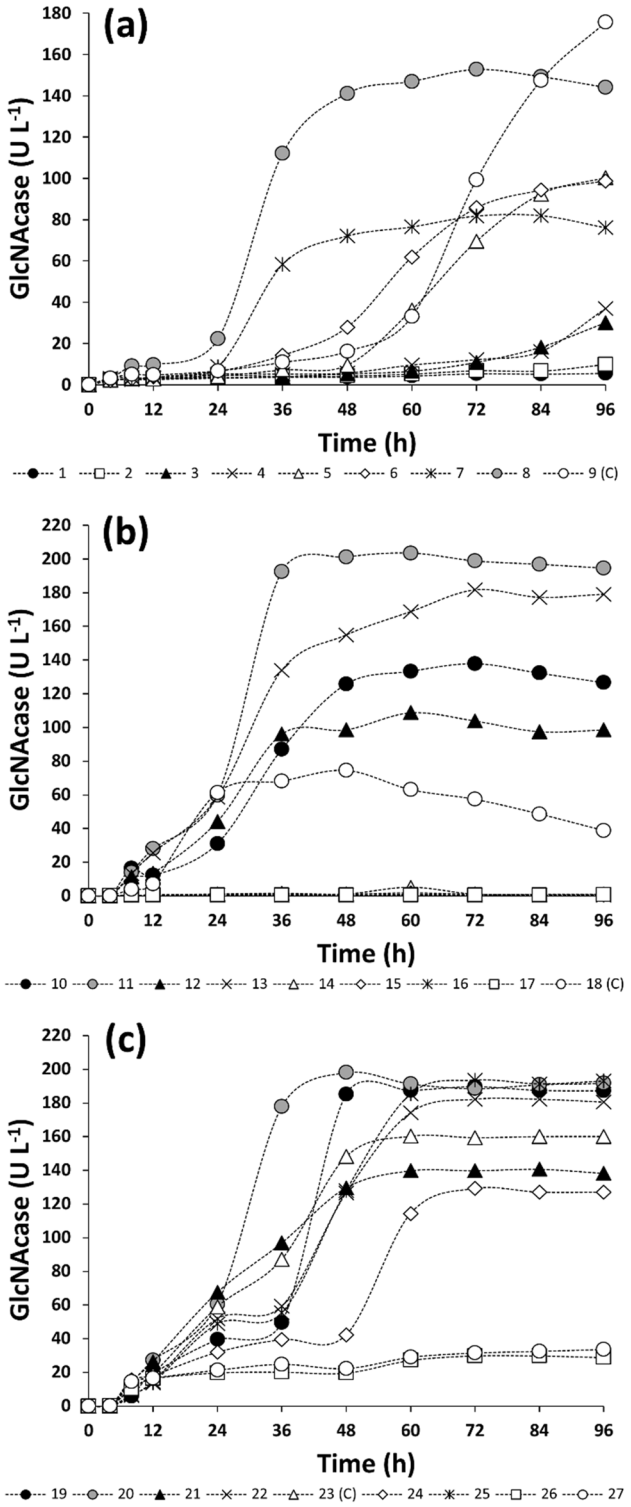

Fig. 2 Influence of colloidal α-chitin concentration, pH, and temperature on GlcNAcase production by *A. caviae* CHZ306 in shake flasks. GlcNAcase production profiles from 1st, 2nd, and 3rd experimental designs are represented in panels **a**, **b**, and **c**, respectively. GlcNAcase productions marked with gray filled circles do refer to the best conditions of each experimental design. Legend numbers are the same as those of runs performed according to the experimental designs (Table S1)

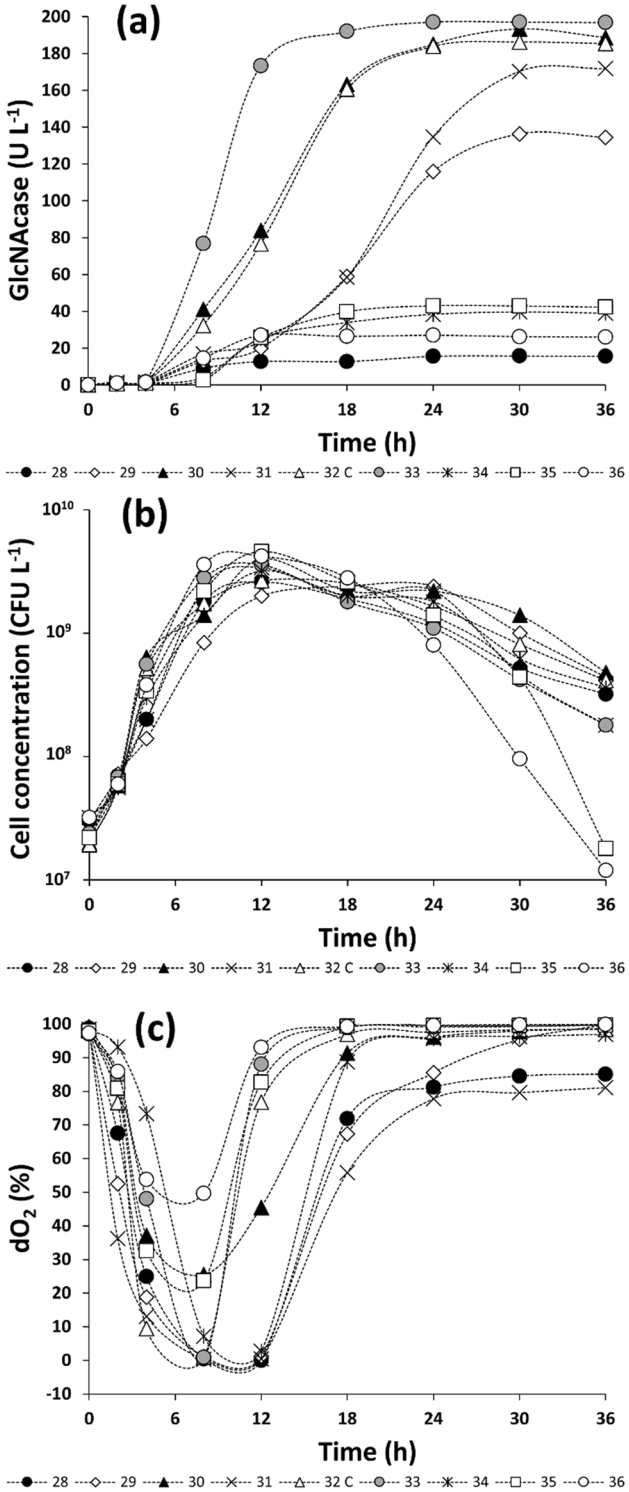

Fig. 3 Influence of the volumetric oxygen transfer coefficient (k_La) on GlcNAcase production by *A. caviae* CHZ306 in stirred-tank bioreactor. Profiles of GlcNAcase production, cell growth and dissolved oxygen (expressed as a percentage of the saturation level) are illustrated in panels **a**, **b**, and **c**, respectively. Profiles marked with gray filled circles do refer to the run 33 that ensured the highest GlcNAcase production

the highest k_La values (70.20 and 96.12 h^{-1}), resulted in poor GlcNAcase production.

On the other hand, the run 33 carried out at 55.20 h^{-1} k_La resulted in the highest GlcNAcase production (173.38 $U.L^{-1}$) after only 12 h (Fig. 3a), corresponding to a 370-fold productivity increase (14.4 $U.L^{-1}.h^{-1}$) compared to the basal conditions (Table 3).

To better visualize the effects of k_La and cultivation time on GlcNAcase productivity in bioreactor (Table S3 of the Supplementary Material), the results obtained were used to construct a response surface model. Due to the large number of k_La levels tested and time points measured, several different options of polynomial functions were evaluated in their capacity to model the productivity values. Although several polynomial functions of orders 3 and 4 fitted the data with an R^2 around 0.8, following the principle of parsimony, the model below was chosen, which allowed to obtain a good fit with the smallest number of free parameters:

$$\ln y = \beta_1 + \beta_2 x_1 + \beta_3 x_1^2 + \beta_4 x_2^2 + \beta_5 x_1^3 + \beta_6 x_2^3$$

where x_1 is the k_La [h^{-1}], x_2 is the cultivation time [h], and y is the productivity [$U.L^{-1}.h^{-1}$].

This model was able to fit reasonably well the complex geometry of data observed with an R^2 of 0.77, while models of higher order tended to overestimate the productivity values at the peaks and underestimate them at the throughs. Table S4 shows the maximum likelihood estimation values of the model coefficients, as well as their standard errors, estimated t-statistic values, and significance scores calculated using the Systemfit package in R [27]. Although the highest experimental productivity was reached after 12 h at a k_La of 55.20 h^{-1}, the optimal productivity predicted by the response surface model is expected to occur after 16.6 h at a k_La of 36.4 h^{-1}.

Discussion

GlcNAcases are exoenzymes with biotechnological potential belonging to the chitinase family and commonly secreted at low concentrations in bacterial culture media. Its biosynthesis in bacteria is influenced by inducers (e.g., chitin and its hydrolysis products), the composition of the culture medium (e.g., carbon and nitrogen sources), and various other physical factors (e.g., temperature, pH, speed agitation, and aeration). However, some of these factors, as well as products of chitin hydrolysis itself, can also repress chitinase biosynthesis [13, 28, 29]. Thus, the choice of the type of inducer, the composition of the culture medium, and the type of culture are determining characteristics in the production of these enzymes.

In the present study, GlcNAcase production by *A. caviae* CHZ306 was explored and discussed based on the most relevant aspects involved in the lab-scale upstream bioprocess. Colloidal α-chitin was used as a low-cost carbon source and inducer of GlcNAcase synthesis, and the most suitable nitrogen source for enzyme production was evaluated using different culture media in shake flasks. The M15 medium allowed a 21-fold increase on GlcNAcase production (78 $U.L^{-1}$) compared to the M1 basal medium (4.0 $U.L^{-1}$) and exhibited the best production profile among all tested formulations (Fig. 1b). Such an increase could probably be due to the higher bioavailability of substrates on M15 medium, such as proteins, peptone, amino acids, carbohydrates, organic acids, vitamins, and minerals, from peptone A and corn-steep liquor. Several studies addressing the improvement of chitinase production using different nitrogen sources (Table 4) unexpectedly demonstrated that some additional carbon sources are more readily assimilable than colloidal α-chitin, thus representing an important contribution for chitinase production. For instance, yeast extract significantly improved chitinase production by *Streptomyces griseorubens* C9 on colloidal α-chitin [30]. A mixture of colloidal chitin, ammonium sulfate, and yeast extract maximized chitinase production by *Bacillus licheniformis* AT6 [31]. The chitinase production by *Bacillus amyloliquefaciens* Z7 was enhanced using chitin, starch, and yeast extract [32], and a mixture of swollen chitin, sucrose, yeast extract, and ammonium sulfate was used to optimize the chitinase production by *Escherichia fergusonii* [33].

Another factor related to the increased production of chitinases could be related to the different C:N ratios between M1 (0.7) and M15 (4.4). Even though several studies have shown that the C:N ratio plays a central role in microbial growth, respiration and biosynthesis [34–38], only few attempts were made to improve chitinase production taking into account the stoichiometric balance between the bioavailability of carbon and nitrogen present in the culture medium and their consumption. However, knowing the elemental cell composition, it is possible to estimate the concentrations of nutrients that will make up the culture medium. Considering that bacterial cells are composed of approximately 50% carbon and 12% nitrogen, under aerobiosis about 50–60% of carbon and nitrogen contained in the culture medium is incorporated into cell biomass, while under anaerobiosis only 10–20%. Based on such considerations, it would be appropriate to prepare a culture medium with a C:N ratio of approximately 4.2, in which 2.3 would be required only for bacterial morphological plasticity under aerobiosis and 0.6 under anaerobiosis. In the present study, as the M1, M2, M3, and M4 culture media had C:N ratios (< 1.5) significantly lower than the nutritional requirements of a bacterial cell

Table 4 Comparative summary of conditions for chitinase production by chitinolytic bacteria

Specie	Carbon source		Nitrogen source		Optimum condition			Reference
	Main	Additional	Main	Additional	pH	Temp. (°C)	Chitin (%)	
A. caviae CHZ306	Colloidal chitin	Corn steep liquor and peptone A	Corn steep liquor	Peptone A	6.0	34	5.0	This study
A. hydrophila HS4	Colloidal chitin	Starch	Malt extract	-	8.0	37	0.3	[39]
A. punctata HS6	Colloidal chitin	Starch	Yeast extract	-	7.0	37	0.3	[39]
Aeromonas sp. JK1	Colloidal chitin	Triton X-100	Ammonium sulfate	-	8.0	30	0.75	[40]
Aeromonas sp. ZD_05	Colloidal chitin	-	Peptone	-	7,0	30	1.0	[41]
B. amyloliquefaciens Z7	Chitin	Starch	Yeast extract	-	6.5	37	0.88	[32]
B. laterosporous MML2270	Colloidal chitin	-	Yeast extract	-	8.0	35	0.3	[44]
B. licheniformis AT6	Colloidal chitin	-	Ammonium sulfate	Yeast extract	7.0	35	0.5	[31]
B. licheniformis NM120–17	Colloidal chitin	Lactose	Casein	-	8.0	30	1.5	[45]
B. thuringiensis NM101–19	Colloidal chitin	Galactose	Casein	-	7.0	30	1.5	[45]
B. thuringiensis R 176	Ball-milled chitin	Rice Straw	Ammonium sulfate	Rice Straw	7.0	37	0.5	[51]
E. fergusonii	Swollen chitin	Sucrose	Ammonium sulfate	Yeast extract	7.0	30	1.0	[33]
S. marcescens ATCC27117	Colloidal chitin	-	Yeast extract	Tryptone	7.5	30	1,5	[52]
S. marcescens KY	Colloidal chitin	-	Yeast extract	Tryptone	6.0–7.0	30	1.5	[52]
S. griseorubens C9	Colloidal chitin	-	Yeast extract	-	5.0–9.0	40	2.0	[30]
S. viridificans	Colloidal chitin	Arabinose	Ammonium sulfate	Yeast extract	6.0	30	1.5	[53]

(Table 1), they led to the lowest GlcNAcase productions (Fig. 1a). Contrarily, the M15 culture medium, which had a C:N ratio (4.4) higher than the above threshold value, was able to meet *A. caviae* CHZ306 nutritional requirements, greatly improving its performance in terms of GlcNAcase production.

Studies have also shown the important effects of pH, temperature, and colloidal α-chitin concentrations on chitinase biosynthesis by different bacterial species (Table 4). The production of chitinases by *A. hydrophila* HS4 [39], *A. punctata* HS6 [39], *Aeromonas*. sp. JK1 [40], and *Aeromonas* sp. ZD_05 [41], for example, was evaluated under different conditions of pH (4–12), temperature (18–55 °C), and colloidal α-chitin concentrations (0.1–3.0%) and showed optimal production conditions of chitinase in ranges of 7.0–8.0, pH; 30–37 °C, temperature; and 0.3–1.0%, chitin concentration. These conditions were further investigated in this study, in which the *A. caviae* CHZ306 growth was not significantly influenced by any of the variables tested. These results are qualitatively in line with those reported in the literature for *Aeromonas* genus growth in wide ranges of temperature (22–37 °C) and pH (4.5–9.0) [42, 43]. As expected, the optimum concentration of colloidal α-chitin determined ensured a C:N ratio in the culture medium above the minimum threshold estimated in the first steps of the study (Table 3). Moreover, as in some previous reports [39, 40, 44, 45], the alkaline pH sustained GlcNAcase production, while temperatures either higher or lower than the optimal one led, as for any enzyme system, to a significant activity decrease. An increase in colloidal α-chitin concentration up to 5% improved GlcNAcase production; however, it appeared to inhibit enzyme synthesis beyond this maximum threshold and over time, probably due to the high concentration of GlcNAc, i.e., the product of GlcNAcase-catalyzed reaction. According to some studies [28, 29], GlcNAc appears to directly or indirectly induce or repress a variety of cellular processes, including chitin utilization, energy metabolism, biofilm formation, and pathogenicity. For instance, in marine-derived *Vibrio parahaemolyticus*, GlcNAc induced and repressed no less than 81 and 55 genes, respectively [29]; in addition, chitinase secretion seems to be unaffected by low GlcNAc levels, but strongly inhibited by high GlcNAc levels [46].

An extensive literature on the operational design of bioprocesses is nowadays available, showing that a scale-up in bioreactors is highly dependent on the ability to transfer O_2 from the gas phase to the liquid phase [47]. Therefore, GlcNAcase production was finally tested in stirred-tank bioreactor, known for providing high performance in bioprocesses, where the influence of the volumetric oxygen

transfer coefficient (k_La) was investigated, by combining different agitation speeds (rpm) and aeration rates (vvm).

Under the best experimental conditions, the balance between agitation speed (250 rpm) and aeration rate (1.75 vvm) expressed in k_La (55.20 h^{-1}) was essential to increase productivity, reducing the production time of GlcNAcase to 12 h. Either lower or higher k_La values than the above optimum range negatively affected GlcNAcase production over time. Lower k_La conditions were able to support cell growth, but not trigger GlcNAcase biosynthesis. Under these conditions, the low agitation speeds and aeration rates, together with the insolubility of colloidal α-chitin, caused cell adhesion to the bioreactor wall. On the other hand, the highest k_La conditions resulted in poor GlcNAcase production, probably due to mechanical stress to cells driven by excess agitation intensity [48] and/or inhibition of chitinase biosynthesis by excess oxygen [49]. Similar results were reported by [50], who evaluated the influence of agitation speed (100, 200, and 300 rpm) and aeration rate (1, 2, and 3 vvm) on the production of *Paenibacillus* sp. CHE-N1 chitinase.

According to the data analysis, as mentioned previously, the highest experimental productivity was reached after 12 h at a k_La of 55.20 h^{-1}, and the optimal productivity is expected to occur after 16.6 h at a k_La of 36.4 h^{-1}. However, given that the response surface model underestimates the productivity at short fermentation times and struggles to fit the abrupt reduction of productivity observed increasing k_La from 55.20 to 70.20 h^{-1}, this reduction was probably caused by mechanical cell damage and excess oxygen. Taking into consideration both experimental data and mathematical modeling, it is likely that the actual optimal operating conditions are at slightly higher k_La and slightly lower times than those predicted by the response surface model, although not too far from them.

Conclusion

Given the functional properties, features, and recognized high technological and economic potential of GlcNAcase, the development of a bioprocess aimed at its large-scale production is of great importance for the consolidation of its current and emerging biotechnological applications. The enhanced production of GlcNAcase by marine *Aeromonas caviae* CHZ306 in bioreactor was successfully attained in the present work. A stoichiometric balance between the bioavailability of carbon and nitrogen in the formulated culture medium with colloidal α-chitin as well as the use of additional carbon and nitrogen sources played a central role in improving the bioprocess, considerably increasing the enzyme productivity. Optimization of physicochemical conditions and k_La values of the bioprocess further increased GlcNAcase productivity. Intermediate cultivation conditions acting synergistically, without exposing the cells to extreme temperature, cell damage, and excess oxygen, were fundamental to achieve such performance. Future efforts should be directed towards controlling the concentration of colloidal α-chitin, because high amounts of its hydrolysis products, such as GlcNAc, can lead to metabolic repression of the synthesis of this enzyme.

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Declarations

Competing interests The authors declare no competing interests.

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