Environmental Science and Pollution Research (2024) 31:45217–45233
https://doi.org/10.1007/s11356-024-34088-x

RESEARCH ARTICLE

Production of a new chitinase from *Nocardiopsis halophila* TN-X8 utilizing bio-waste from the blue swimming crab: enzyme characterization and immobilization

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Received: 23 January 2024 / Accepted: 19 June 2024 / Published online: 3 July 2024

Abstract

In accordance with the framework of the Circular Blue Bioeconomy in the Mediterranean region, the objective of this study was to evaluate the biotransformation of blue swimming crab (*Portunus segnis*) residues obtained from the port of Sfax by an extracellular chitinase produced by *Nocardiopsis halophila* strain TN-X8 isolated from Chott El Jerid (Tozeur, Tunisia). From the analysis of multiple extremophilic *Actinomycetota*, it was determined that strain TN-X8 exclusively utilized 60 g/L of raw blue swimming crab as its carbon and energy source, achieving a chitinase activity of approximately 950 U/mL following a 6-day incubation period at 40 °C. Pure chitinase, designated as ChiA-Nh30, was obtained after heat treatment, followed by ammonium sulfate fractionation and Sephacryl® S-200 column chromatography. The maximum ChiA-Nh30 activity was observed at pH 3 and 75 °C. Interestingly, compared with cyclohexamidine, ChiA-Nh30 showed a good antifungal effect against four pathogenic fungi. Furthermore, when using colloidal chitin as substrate, ChiA-Nh30 demonstrated a higher degree of catalytic efficiency than the commercially available Chitodextrinase®. In addition, ChiA-Nh30 could be immobilized by applying encapsulation and encapsulation-adsorption techniques. The kaolin and charcoal used acted as excellent binders, resulting in improved ChiA-Nh30 stability. For the immobilized ChiA-Nh30, the yield of *N*-acetyl-D-glucosamine monomers released from 20% (*w*/*v*) blue swimming crab residues increased by 3.1 (kaolin) and 2.65 (charcoal) times, respectively.

Keywords *Portunus segnis* waste · Circular economy · *Actinomycetota* · Chitinolytic enzyme · Chitin · Enzyme reuse · Antifungal activity

Introduction

Tunisia has a 1350 km Mediterranean coastline, including a national maritime area and lagoons of 80,000 km^2 and 105,200 ha, respectively. Benefiting from a long, sandy coastline sloping slightly towards the sea, the area of Sfax has been the site of significant fishing activity. The fishing port of Sfax in Tunisia is one of the major ports in Africa.

Since 2014, the invasive African blue swimming crab, a crustacean species, reached Tunisian waters. It has proven to be devastating to fish stocks and fishing hardware. Like the Islamic State of the Levant and Iraq, Tunisian fishermen have named it "Daesh" because of its destructiveness and invasiveness (Rabaoui et al. 2021). Moreover, the crabs are not consumed in Tunisia and are not part of traditional Tunisian cuisine. Therefore, they are discarded in large quantities and have a negative impact on fauna and flora, especially in the port of Sfax. However, biotechnologists from Tunisia consider the blue swimming crab as a potential opportunity (Mechri et al. 2019; Hamdi et al. 2020) through the extraction and valorization of the bioactive compounds in this species, such as chitin, peptides, minerals, lipids, and carotenoids. It is of particular interest that the African blue swimming crab is mainly composed of 15 to 40% chitin (Rabaoui et al. 2021; Jabeur et al. 2020).

Glycosyl hydrolases (GH) 18 and 19, or chitinases, are categorized as *endo*-, *exo*-, or *N*-acetyl-D-glucosaminidases (Bouacem et al. 2018; Mohamed et al. 2019; Yahiaoui

Responsible Editor: Ta Yeong Wu

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et al. 2019; Asmani et al. 2020; Laribi-Habchi et al. 2020; Pentekhina et al. 2023), and they hydrolyze various important chitin structures. Chitin has a high molecular weight and is divided into three forms: α-, β-, and γ-chitin, all of which are made up of a series of β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc) (Dahiya et al. 2022; Lucas and Rode 2023; Vakkachan et al. 2023; Zhou et al. 2023). Chitinases are extensively distributed in prokaryotic micro-organisms (Vakkachan et al. 2023). The *Actinomycetota* comprises the most significant group of chitin decomposers, with chitinolytic machinery that is considered valuable for converting chitinous wastes (Wang et al. 2023). However, the purification and characterization of chitinases of extremophilic *Actinomycetota* has, so far, been the subject of only limited studies.

In this study, the effectiveness of the extremophilic, chitinolytic *Nocardiopsis halophila* strain TN-X8 in the fermentation of blue swimming crab *Portunus segnis* Forskål (1775), harvested in the port of Sfax, has been examined. This was followed by the biochemical characterization and immobilization of the novel chitinase, ChiA-Nh30, produced by this strain. The ChiA-Nh30 hydrolytic potential on colloidal chitin and anti-fungal efficacy was also assessed.

Materials and methods

Biological material, microorganisms, and chitinase production

The swimming crab, *Portunus segnis*, was transported on ice after being collected from the fishing port of Sfax, Tunisia (Jabeur et al. 2022). For the experimental work, the crabs were ground into a homogeneous, fine powder as previously described (Jabeur et al. 2020; Yao et al. 2024).

A variety of fungal strains, including *Phytophthora infestans* (Mont.), *Geotrichum candidum* Link, *Penicillium digitatum* (Pers.) Sacc., and *Aspergillus niger* (Tiegh.), obtained from the Microbial Biotechnology Laboratory at the Faculty of Sciences, Oujda, Morocco, were used in this study. These fungal strains were cultivated on Potato Dextrose Agar (PDA) medium at 25 °C for 4 days.

The culture media used were International *Streptomyces* Project (ISP) medium 2, chitinase detection agar (CHDA), Sabouraud (Asmani et al. 2020; Laribi-Habchi et al. 2020), and the TN-X8-OCP medium. The latter medium was composed of blue crab powder at 60 g/L.

Soil samples were collected from several extreme regions of Tunisia and stored at 23 ± 2 °C until use. The isolation and the screening of the strains on the CHDA medium were carried out as previously described (Bouacem et al. 2018; Laribi-Habchi et al. 2020). The isolate, TN-X8, obtained from the Chott El Jerid soil sample, Hazoua, Tozeur, Tunisia (conductivity, 232 ms/cm; water content, 14.62; temperature, 28 °C; and pH, 8.45), displayed a large clear chitin-zone of hydrolysis (with a ratio of 5), and was therefore retained for further study. Strain TN-X8 was cultivated in TN-X8-OCP media in 500-mL Erlenmeyer flasks at 40 °C and with shaking at 190 rpm.

Identification of strain TN-X8

The morphological properties of the TN-X8 were investigated following the recommendations of Shirling and Gottlieb (1966) and Bergey's bacteriology recommendations (Wilmotte 1994).

The physiological and molecular identification of strain TN-X8 was carried out using the instructions provided by the manufacturer (bioMérieux, Marcy-l'Étoile, France), and 16S rRNA gene sequencing was performed as previously described by the authors (Mechri et al. 2022).

Chitinase assay

Chitinase activity was quantified by analyzing the amount of GlcNAc freed from colloidal chitin (Laribi-Habchi et al. 2020): 0.5 mL of enzyme solution was mixed with 0.5 mL of glycine–HCl (100 mM and 3 mM $CoSO_4$, pH 3; buffer A) containing colloidal chitin (10 mg/mL) and incubated at 75 °C. The reaction mixture was treated for 10 min, placed on ice, and centrifuged to remove insoluble colloidal chitin. The release of reducing sugars was determined by using the DNS assay. One unit of chitinase activity is defined as the amount of the enzyme that produces 1 μmol of reducing sugar, as GlcNAc, per minute under the above conditions.

ChiA-Nh30 purification

A 500 mL of clear supernatant was removed from the strain TN-X8 culture, heat-treated for 30 min at 70 °C, and subsequently fractionally precipitated by ammonium sulphate (40–85%). Next, the precipitate was centrifuged, then solubilized in 50 mM MOPS buffer (pH 3; buffer B), and dialyzed overnight against repeated changes of buffer B. The clear supernatant was loaded onto a Sephacryl S-200 HR column (2.5 × 150 cm) pre-equilibrated with buffer B. Protein content (A280 nm) and chitinase activity (500 nm) were determined. The pooled fractions containing ChiA-Nh30 activity were concentrated (Amicon 10-kDa) for further chitinase characterization.

Protein determination and analytical techniques

The Bradford's method was used to determine the protein concentration (Bradford 1976). The ChiA-Nh30 molecular mass was determined using 12% SDS-PAGE (Laemmli

1970). The chitin azure zymography was performed on a 4% stacking gel with 1 mg/mL of heat-denatured chitin azure incorporated into the 10% separation gel, as previously described (Laribi-Habchi et al. 2012; Laribi-Habchi et al. 2015, 2020), but with slight modifications. Electrophoresis was performed at a constant current of 25 mA. After electrophoresis, the gel was immersed with 100 mL of refolding glycine–HCl buffer at pH 3, with the addition of 3 mM $CoSO_4$ and 1% Triton X-100, for 12 h at 75 °C, to replace the SDS and separation buffer in the gel. The gel was washed with distilled water and then stained with a fluorescent brightener (Calcofluor white M2R) at a concentration of 0.01% (*w*/*v*) in 50 mM Tris–HCl buffer at pH 8. After 5 min, the brightener solution was removed, and the gel was washed with distilled water. Lytic zones were revealed by UV illumination with a transilluminator after staining for 5 min with 0.01% (*w*/*v*) Calcofluor white M2R.

The protein band of ChiA-Nh30 was cut from an SDS-PAGE gel, transferred, and then sequenced, using an ABI Automated Protein Sequencer (Model 473A), to determine the N-terminal residues.

ChiA-Nh30 immobilization and physico-chemical analysis of beads

Carrier_ChiA-Nh30 preparation and beads formation

The immobilization of ChiA-Nh30 was carried out with the encapsulation procedure, using either 2% (*w*/*v*) sodium alginate (SA) alone or combined with one of the organic supports, i.e., carboxymethyl cellulose (CMC) (1%, *w*/*v*), agar (Ag) (1%, *w*/*v*), or κ-carrageenan (CA) (0.8%, *w*/*v*). Alternatively, the encapsulation-adsorption procedure was used with 2% (*w*/*v*) SA combined with one of the inorganic supports, i.e., white clay (WC) (2%, *w*/*v*), kaolin (KA) (2%, *w*/*v*), or charcoal (CH) (1%, *w*/*v*).

The SA was purchased from FLUKA® Buch (Switzerland). Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) provided the CMC and Ag. The KA was supplied by Central Drug House (P) Ltd (CDH®, India), and the CH (1%, *w*/*v*) was provided by BBI Life Sciences (Asia–Pacific). The WC and kaolin were collected from Djebel Bouamrane (Gafsa, Tunisia).

The encapsulation of the ChiA-Nh30 enzyme in SA was carried out by mixing 300 µL of the ChiA-Nh30 enzyme (10 U) with 700 µL of 2% (*w*/*v*) SA solution, prepared as described by Mechri et al. (2022). Where the SA was combined with another support, the ChiA-Nh30 enzyme (300 µL, 10 U) was added to one of the organic or inorganic carriers using the optimized concentrations mentioned above and incubated at 4 °C for 2 h. Following addition to the SA, the mixture was homogenized for one hour at 4 °C with continuous shaking.

The ChiA-Nh30 enzyme was mixed with each one of these carriers, and the ChiA-Nh30 beads were prepared as detailed elsewhere (Mechri et al. 2022).

FE-SEM, FTIR, and XRD analyses

The free and immobilized ChiA-Nh30 and the empty beads (without carriers) were analyzed by FE-SEM (JEOL-JSM7001F scanning electron microscope), ATR-FTIR (Jasco 4700-ATR spectrophotometer), and XRD-6000 spectrometry (SHIMADZU, Kyoto, Japan) (Mechri et al. 2022).

ChiA-Nh30 biochemical characterization

Temperature effect

The temperature effect on the free ChiA-Nh30 was determined at 40–100 °C. The thermal stability of ChiA-Nh30 in its immobilized and free forms was determined for 36 h at 80 °C, using buffer A and with or without 3 mM $CoSO_4$.

pH effect

The effect of pH on the ChiA-Nh30 activity was analyzed between pH 2 and 10 with different buffers (100 mM) at 75 °C (Bouacem et al. 2018; Asmani et al. 2020; Laribi-Habchi et al. 2020). The pH stability was assessed by incubating immobilized and free forms of ChiA-Nh30 at pH 7 over a 48-h time period at 60 °C.

ChiA-Nh30 kinetic characteristics

The kinetic constants for ChiA-Nh30 in its free and immobilized forms, Chitodextrinase®, a commercial enzyme produced by Sigma St. Louis (MO, USA), and ChiA-Ba43 were determined, as detailed previously (Bouacem et al. 2018; Mohamed et al. 2019; Yahiaoui et al. 2019; Asmani et al. 2020; Laribi-Habchi et al. 2020), on colloidal chitin as a natural substrate, under the optimal conditions for each chitinase.

Potential application of ChiA-Nh30 in its free and immobilized forms

Immobilized ChiA-Nh30 beads recycling

The operational stability of the ChiA-Nh30-SA, ChiA-Nh30-SA-CA, ChiA-Nh30-SA-Ag, ChiA-Nh30-SA-CMC, ChiA-Nh30-SA-KA, ChiA-Nh30-SA-CA, and ChiA-Nh30-SA-WC beads were tested over several cycles (Mechri et al. 2022). During the recycling, the beads were washed with deionized water to remove excess reaction residues. The recycled encapsulated ChiA-Nh30 and encapsulated-adsorbed ChiA-Nh30 beads were then used for the next cycle. The initial enzymatic activity of the encapsulated and encapsulated-adsorbed ChiA-Nh30 beads was taken as 100%.

Degradation of blue crab chitin by free and immobilized ChiA-Nh30

The degradation of 20% (*w/v*) blue crab chitin, prepared as previously described by the authors (Jabeur et al. 2022), was evaluated using free ChiA-Nh30, ChiA-Nh30-SA, ChiA-Nh30-SA-CA, and ChiA-Nh30-SA-KA, maintained for 10 days at 50 °C in 1000-mL Erlenmeyer flasks with shaking at 120 rpm. Subsequently, samples were taken from each Erlenmeyer flask every 12 h and were incubated for 15 min at 90 °C to render each ChiA-Nh30 form inactive. Then, 3 mL of 3,5-dinitrosalicylic acid (DNS) was added, and the samples were incubated for 10 min at 100 °C. Finally, 20 mL H_2O was added, and the samples were centrifuged at 15,000 rpm for 10 min. The absorbance of the samples was taken at 550 nm. The following formula was used to determine how much chitin had been degraded:

$$\text{Degree of degradation}(\%) = \left[V_t/V_\infty\right] \times 100$$

where V_t is the GlcNAc content at time t (μg/mL) and V_∞ is the GlcNAc content at time zero (μg/mL).

Antifungal activity

The antifungal activity of ChiA-Nh30 was determined using a modified version of the protocol described by Raymaekers et al. (2020). The fungal spores were harvested from the an agar culture using a sterile solution consisting of physiological water and glycerol (in a ratio of 9:1). Subsequently, the fungal concentration of each target strain (*Phytophthora infestans*, *Geotrichum candidum*, *Penicillium digitatum*, and *Aspergillus niger*) was determined and adjusted to 10^5 spores/mL using a UV–visible spectrophotometer set at 600 nm. Antifungal activity was assessed using the well diffusion method, as outlined previously (Abouloifa et al. 2021). Petri dishes with a diameter of 90 mm were utilized for this purpose, with 100 μL of distilled water for the negative control and 100 μL of cycloheximide (1 mg/mL, *w/v*) for the positive control introduced into each 6 mm diameter well. The agar plates were kept at 4 °C for 30 min and then incubated in an oven at 25 °C for 18 h. To ensure the accuracy of the results, all tests were performed in triplicate. The antifungal activity is estimated by measuring the clear zones (halos), in mm, that form around the wells by using a caliper. A test sample is considered active if the diameter of the inhibition zone is greater than 8 mm (Aboul Ela et al. 1996).

Results and discussion

Screening for chitinase activity

As part of the Italian-Tunisian project, ARIBiotech C-5–2.1–41, CUP I55F21003350006 (https://www.aribiotech.eu/), there is a constant search for innovative approaches for the sustainable exploitation of marine biowastes and the development of biotechnology in Tunisia.

In line with the objectives of this project and with cross-border initiatives to promote the use and production of marine by-products, raw *Portunus segnis* (Forskål 1775) powder was used as the sole energy and carbon source for the production of chitinase (950 U/mL following 6 days of incubation at 40 °C). This optimized chitinase production medium (TN-X8-OCP) was subsequently used to produce chitinase by an actinobacterium strain, TN-X8.

The present study aimed to contribute information on using blue crab powder as an inexpensive and readily available complex substrate, reducing the cost of chitinase production. Moreover, using this biowaste could be an ecological solution for the port of Sfax and, therefore, of economic importance to Tunisia.

In the current study, chitinase-producing *Actinomycetota*, isolated from different extreme Tunisian ecological niches, were tested for their chitinase-production ability. Five of the seventeen isolates exhibited a production ratio higher than 3, with the highest ratio of 5, on CHDA media at pH 7.4. The ratio of the clear zone diameter and that of the colony served as an indicator for selecting strains with high chitinase production ability. Among the five chitinolytic strains isolated, strain TN-X8 demonstrated the greatest degree of activity (~ 150 U/mL) and, consequently, was selected for further study.

Next, the chitinase activity was optimized using the classical method "one-factor-at-a-time (OFAT)", which involves changing one independent variable while fixing the other parameters at certain levels. Under optimized conditions, the chitinase yield of 950 U/mL was 6.33-fold higher than that obtained using the initial conditions (150 U/mL).

Identification of strain TN-X8

Strain TN-X8 is an aerobic, non-acid-fast, Gram-positive, catalase-positive *Actinomycetota* with nocardioform substrate mycelia and aerial mycelia bearing long chains of spores. Additionally, strain TN-X8 can grow at 50 °C and within a pH range of 6 to 9. It also requires the presence of NaCl for its growth, suggesting that it is halophilic (Table 1). The characteristics presented in Table 1 indicate that both TN-X8 and IQ-H3^T strains are similar. These phenotypic characteristics are consistent with other members of the genus *Nocardiopsis*.

To confirm strain TN-X8's assignment as a *Nocardiopsis* species, a 16S rRNA gene phylogenetic tree was created (Fig. 1). Both GenBank and EzTaxon identified that strain TN-X8 is most closely related to *Nocardiopsis halophila* IQ-H3^T (GenBank accession no.: MT704623).

From the phylogenetic tree, the bootstrap investigation of all groups showed that node values ranged between 51 and 100%, indicating their robustness. The neighboring identified *Nocardiopsis* strains were *Nocardiopsis halophila* DSM 44464^T (GenBank accession no.: AJ421018) and *Nocardiopsis chromatogenes* YIM 90109^T (GenBank accession no.: AY619715).

ChiA-Nh30 purification

Over the past decade, chitinase-driven biocatalysis has become a vital technological tool for transforming chitinous materials obtained from the blue crab (Rodriguez-Kabana et al. 1989). This field of research has been significantly enhanced, partly due to work on enzyme purification.

A crude enzyme solution was obtained from a TN-X8 culture, with a chitinase activity of 950 U/mL, that was consecutively purified following the steps detailed in Materials and Methods Section (Table 2). A 74.28-fold purification of the enzyme was attained, with a recovery of approximately 42% and a specific activity of 81,375 U/mg. SDS-PAGE analysis of ChiA-Nh30 showed a single band of approximately 30-kDa (Fig. 2B). The chitinase activity of ChiA-Nh30 in a chitin azure-zymogram was visible, with a clear band observed against the fluorescent brightener (Calcofluor white M2R) stain (Fig. 2C). With this technique, an exact and sensitive identification of the protein bands showing chitinase activity is possible. Compared to ChiA-Ba43 (Asmani et al. 2020) and ChiA-Pt70 (Yahiaoui et al. 2019), the ChiA-Nh30 specific activity was lower but superior to those of ChiA4 (Gao et al. 2018), ChiA-AR17 (Harvinda et al. 2023), and ChiA-Si40 (Laribi-Habchi et al. 2020). ChiA-Nh30 was identified as monomeric with a molecular mass different from several previously described chitinases, namely ChiA-Pt70 (Yahiaoui et al. 2019), ChiA-Si40 (Laribi-Habchi et al. 2020), SaChiA4 (Gao et al. 2018), and ChiA-Mt45 (Mohamed et al. 2019).

ChiA-Nh30 N-terminal sequencing

The ChiA-Nh30 enzyme from *N. halophila* TN-X8 was shown to have the following 19 N-terminal amino acid residues: YILTCYFTNWGQYRPGLTIYFPTNI.

The homogeneity of this sequence indicated that the enzyme was isolated in its purest form. It was seen to be very similar to other chitinase sequences in the GH18 family: ChiA-SA (89.47% identity), ChiA-SP (87.50% identity), ChiA-SG (84.21% identity), and ChiA-CH18 (78.95% identity) (Table 3). These results strongly suggest that ChiA-Nh30 is closely related to proteins in the GH18 family.

ChiA-Nh30 immobilization and physico-chemical analysis of beads

Screening of different immobilization supports

Enzymes that have been immobilized are fixed to an insoluble and inert substance that can be utilized repeatedly. In addition, such enzymes are known to be more stable and resistant to changes in temperature or pH.

The variation in the recovery of activity between encapsulation and encapsulation-adsorption approaches may be due to the great mechanical stability of the inorganic supports, thus permitting adsorption and diminished ChiA-Nh30 release from the carrier (Mechri et al. 2022). Hence, the ChiA-Nh30 high recovery activity can be explained by the mechanical strength, hygroscopic qualities, and abrasion resistance of charcoal and kaolin, which are inorganic supports with large surface areas, large pore volumes, high adsorption, and multiple functional groups in addition to the gelling and emulsifying qualities of sodium alginate. The ChiA-Nh30-SA, ChiA-Nh30-SA-KA, and ChiA-Nh30-SA-CA immobilized forms generated in this study were successfully shown to be potential candidates for the generation of GlcNAc monomers from the raw blue swimming crab, *Portunus segnis*.

The immobilization of ChiA-Nh30 was carried out with the use of seven different supports, i.e. sodium alginate (SA), sodium alginate-carboxymethyl cellulose (SA-CMC), sodium alginate-agar (SA-Ag), sodium alginate-charcoal (SA-CA), sodium alginate-white clay (SA-WC), sodium alginate-kaolin (SA-KA), and sodium alginate-κ-Carrageenan (SA-κ-CA) (Table 4). Ideal immobilization (100% activity retained) was attained when SA was used alone or combined with CA or KA. Moreover, ChiA-Nh30 displayed 78, 69,

Table 1 Phenotypic characteristics of strain TN-X8 (GenBank accession no.: MT704623) and *Nocardiopsis halophila* IQ-H3^T (GenBank accession no.: AJ421018)

Phenotypic characteristics		Strain TN-X8	*Nocardiopsis halophila* strain IQ-H3^T
Aerial mycelium		+	+
Synnemata		-	-
Utilization of:	L-Arabinose	+	+
	Inositol	+	+
	Melibiose	ND	ND
	Rhamnose	+	+
	Xylose	+	+
Acid from:	Adonitol	ND	ND
	Arabinose	+	+
	Galactose	-	-
	Glycerol	ND	ND
	Inositol	+	+
	Lactose	-	-
	Mannitol	+	+
	Mannose	-	-
	Melibiose	ND	ND
	L-Rhamnose	+	+
	Sucrose	-	-
	D-Xylose	+	+
Decarboxylation of:	Lactate	ND	ND
	Malonate	ND	ND
	Oxalate	ND	ND
	Propionate	ND	ND
	Lactate	+	+
	Malonate	-	-
	Oxalate	+	+
	Lactate	ND	ND
Decomposition of:	Casein	+	+
	Tyrosine	-	-
	Tween 40	+	ND
	Tween 80	+	+
	Nitrate Reductase	ND	ND
	Urea	+	+
Growth at:	5% NaCl	+	+
	10% NaCl	+	+
	15–20% NaCl	+	+
	10 °C	ND	ND
	30 °C	+	ND
	40 °C	+	ND
	42 °C	+	-
	45 °C	+	-
	50 °C	+	ND
	Major menaquinones	10/6, 10/8	10/6, 10/8

+, All strains are positive; -, all strains are negative; *ND* no data

61, and 37% recovery activity when immobilized on SA-κ-SA-CA, SA-WC, SA-Ag, and SA-CMC, respectively.

Physico-chemical analysis of ChiA-Nh30 beads

The FT-IR spectrum, the XRD spectra, and FE-SEM micrographs visibly confirmed the modifications in the bead

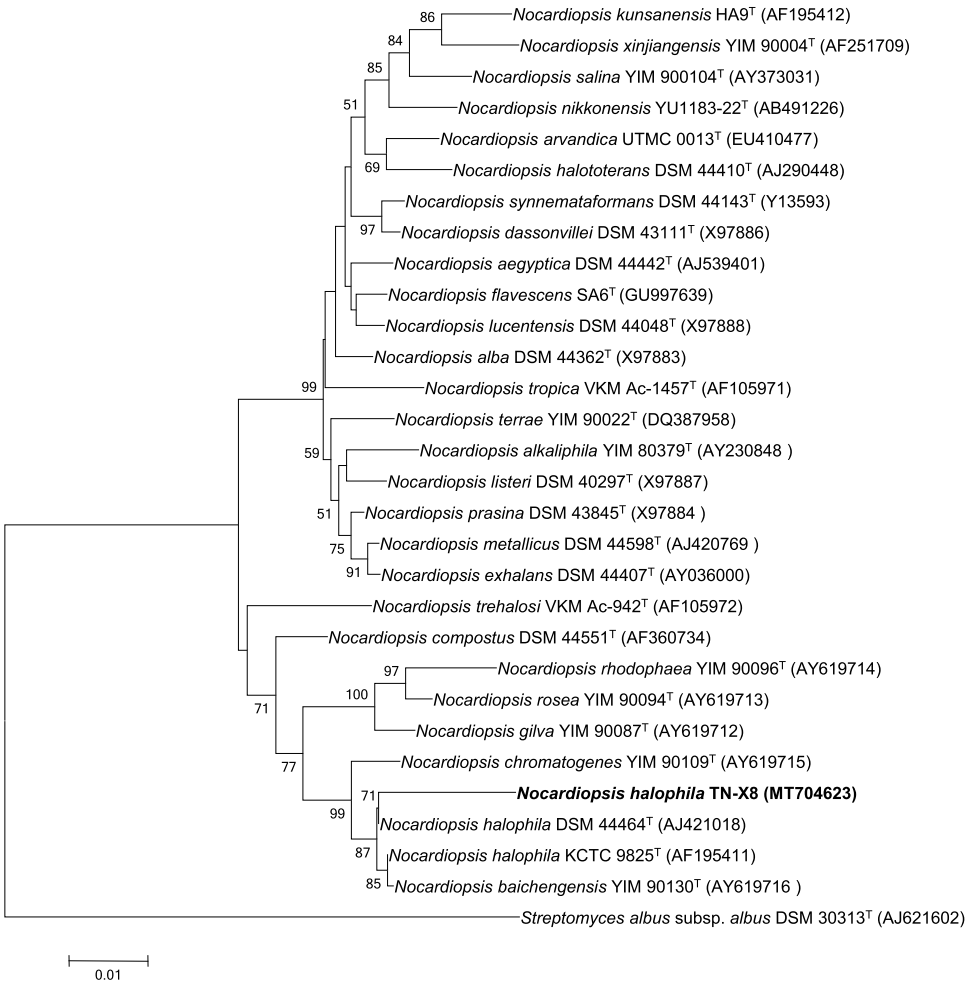

Fig. 1 Phylogenetic tree showing the relationship of strain TN-X8 to other species within the genus *Nocardiopsis* based on 16S rRNA gene sequences (GenBank accession no.: MT704623). The sequence of *Streptomyces albus* subsp. *albus* DSM 30313^T (GenBank accession no.: AJ621602) was randomly selected as an outgroup. Bar, 0.01 nt substitutions per base. Numbers at nodes (> 50%) show support for the internal branches within the tree obtained by bootstrap analysis (percentages of 100 bootstraps)

Table 2 Purification stages of ChiA-Nh30, an acido-thermostable chitinase from *Nocardiopsis halophila* TN-X8

Step of purification	Full of activity (units)[a,b] × 10^3	Full of protein (mg)[a,c]	Specific activity (U/mg of protein)[a]	Activity recovery rate (%)	Purification factor (*n*-fold)
Crude preparation (500 mL)	3100 ± 63	2830 ± 63	1095.40	100	1
Heat treatment (30 min at 70 °C)	2542 ± 50	1155 ± 22	2200.86	82	2.00
$(NH_4)_2SO_4$ fractionation (40–85%)-Dialysis	1953 ± 39	69 ± 2	28,300.34	63	25.83
Gel filtration chromatography Sephacryl S-200 HR	1302 ± 24	16 ± 1	81,375	42	74.28

[a]Assays were reproduced three times, and ± SE are indicated

[b]ChiA-Nh30 units are defined as the quantity of chitinase liberating 1 μmol of Glc-NAc per min at pH 3 and 75 °C

[c]The protein concentration of ChiA-Nh30 was evaluated using Bradford's method (Bradford 1976)

surface morphologies of the supports (SA, SA-CA, and SA-KA) and of the three immobilized forms (ChiA-Nh30-SA, ChiA-Nh30-SA-CA, and ChiA-Nh30-SA-KA).

ATR-FTIR spectroscopy validated the complexation between the enzyme ChiA-Nh30 and the SA, SA-CA, and SA-KA supports following enzyme immobilization (Fig. 3A).

The FTIR spectrum obtained for free ChiA-Nh30 displayed characteristic peaks in the vicinity of 1633 and 3313 cm^{-1}, which correspond to a stretching vibration of C = O in the amide group and to the stretching band of the NH_2 group, respectively. The 1029 cm^{-1} peak corresponds to a stretching vibration of the C-N bond.

The FTIR spectrum applied to the empty SA beads (SA without ChiA-Nh30) displayed two major peaks at 1596 and

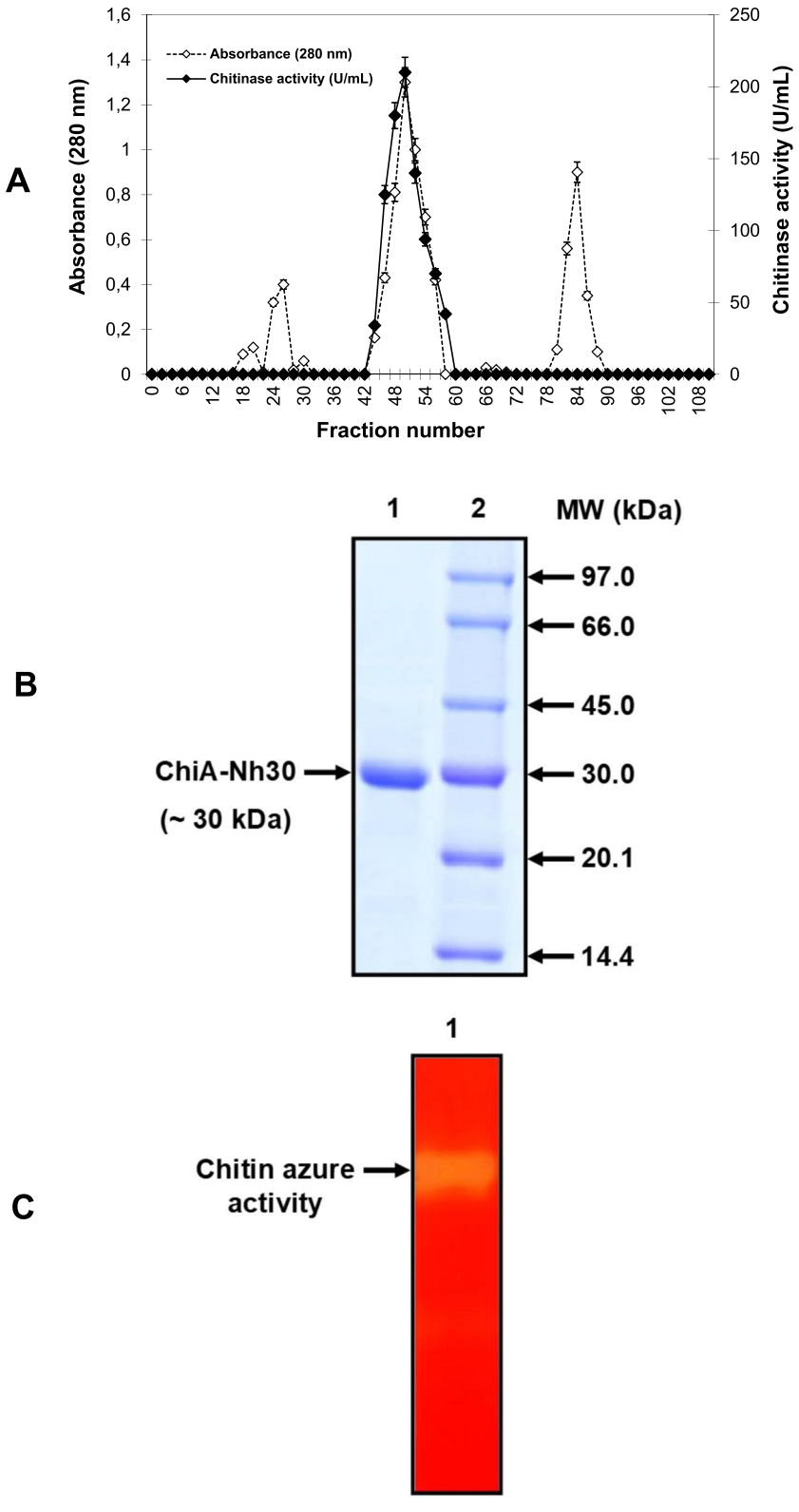

Fig. 2 **A** Chromatography of ChiA-Nh30 enzyme on Sephacryl-S200 HR. **B** SDS-PAGE of the ChiA-Nh30 enzyme. **C** Chitin azure-zymography qualitative technique of the ChiA-Nh30 enzyme stained with a fluorescent brightener (Calcofluor white M2R)

1411 cm^{-1}, respectively, allocated to the asymmetric and symmetric carboxyl groups. This spectrum confirms the presence of two signals in the vicinity of 1013 to 934 cm^{-1}, which are associated with the COO- group vibration, which is characteristic of the ring of a polysaccharide.

In addition, a vibration band representing a C-H group is located near 2331 cm^{-1}, and the spectrum also contains

Table 3 Comparison of the ChiA-Nh30 N-terminal sequence to known chitinases

Chitinase	Origin	N-terminal amino acid[a]	Sequence identity (%)[b]
ChiA-Nh30 (This work)	*Nocardiopsis halophila* TN-X8	GPREKINLGYFTDWGAYGR	-
ChiA-SA (NEC93844)	*Streptomyces albidoflavus* SID9939	GPREKINLGYFTEWGVYGR	89.47
ChiA-SP (WP_219570462)	*Streptomyces poriferorum* DSM 111306^T	– – –DKINLGYFTDWGVYGR	87.50
ChiA-SG (WP_189399987)	*Streptomyces gougerotii* ATCC 10975^T	GPGEKINLGYFTEWGVYGR	84.21
ChiA-CH18 (WP_033298381)	*Streptomyces atroolivaceus* IFO 12741^T	– – –DKINLGYFTDWGVYGR	78.95

[a]Amino acid sequences were obtained from BLASTP

[b]Amino acid residues different from those of ChiA-Nh30 are highlighted

The protein ID or GenBank accession number is provided in brackets

a broad peak near 3442 cm^{-1}, which can be allocated to the hydroxyl group (OH^-) elongation (Papageorgiou et al. 2010).

The FTIR spectrum applied to ChiA-Nh30-SA shows a new peak near 1114 cm^{-1}, which matches the ChiA-Nh30 and SA interaction. In addition, the two peaks associated with the asymmetric and symmetric COO^- groups shifted from 1596 and 1411 cm^{-1} towards 1607 and 1428 cm^{-1}, respectively, with an increase in the transmission proportions, demonstrating that there is a strong interaction between ChiA-Nh30 and SA at these groups.

The spectrum analyses for SA-KA without ChiA-Nh30 and SA-KA-ChiA-Nh30 showed that the peaks corresponding to free ChiA-Nh30 vanished compared to the immobilized form. Indeed, the peaks corresponding to the asymmetric and symmetric COO^- grouping have shifted from 1596 and 1411 cm^{-1} to 1634 and 1421 cm^{-1} with an increase in the transmission percentages, especially for the 1029 cm^{-1} band. This coincides with C-N bond stretching, which shows a strong interaction between ChiA-Nh30 and the hybrid SA-KA complex at these groups.

In addition, a shift in the peak of the OH group from 3252 to 3378 cm^{-1} is noted, as well as the appearance of two new peaks in the immobilized form around 1400 and 900 cm^{-1}. The spectrum of the SA-CA without ChiA-Nh30 displays two peaks at 3429 and 1080 cm^{-1}, corresponding to the OH and C–OH bonds, respectively, following the stretching of the phenolic groups of SA-CA.

The peaks observed in the region between 1490 and 1700 cm^{-1} are attributed to the C = C bonds of the pyrone groups and the C = O bonds of the carboxylic groups, respectively, corresponding to CA.

For SA-CA-ChiA-Nh30, the FTIR analysis showed three new peaks around 2950, 1308, and 602 cm^{-1}, resulting from the interaction between ChiA-Nh30 and the SA-CA hybrid support.

In addition, a substantial increase in the transmission percentages of the peaks between 1590 and 1020 cm^{-1} was observed. Moreover, the characteristic peak of the OH^- group of SA moved from 3442 to 3240 cm^{-1}. Similarly, a displacement was recorded for the distinctive peaks of the asymmetric and symmetric COO-, from 1596 and 1411 cm^{-1} to 1590 and 1408 cm^{-1}.

The XRD spectra showed that all the peaks were taller after the ChiA-Nh30 immobilization and that SA, SA-KA, and SA-CA crystalline structures were constantly preserved

Table 4 ChiA-Nh30, an acido-thermostable chitinase from *Nocardiopsis halophila* TN-X8, was immobilized through encapsulation and the use of encapsulation-adsorption carriers

Supports	Unbound ChiA-Nh30 (U/g carrier)	Immobilized ChiA-Nh30 (U/g carrier)	Immobilization yield (%)[a]	Recovery activity (%)[b]
Encapsulation				
ChiA-Nh30-SA	0	203.30	30.57	100
ChiA-Nh30-SA-κ-CA	2.55	82.34	10.94	78
ChiA-Nh30-SA-Ag	0.51	91.31	13.73	61
ChiA-Nh30-SA-CMC	0	82.44	12.39	37
Encapsulation-adsorption				
ChiA-Nh30-SA-KA	0	363.17	54.61	100
ChiA-Nh30-SA-CA	0	235.18	35.36	100
ChiA-Nh30-SA-WC	0	90.41	13.60	69

[a]Immobilization yield (%) = (loaded ChiA-Nh30 − unbound ChiA-Nh30)/loaded ChiA-Nh30 × 100

[b]Recovered activity (%) = immobilized ChiA-Nh30/(added ChiA-Nh30 − unbound ChiA-Nh30) × 100

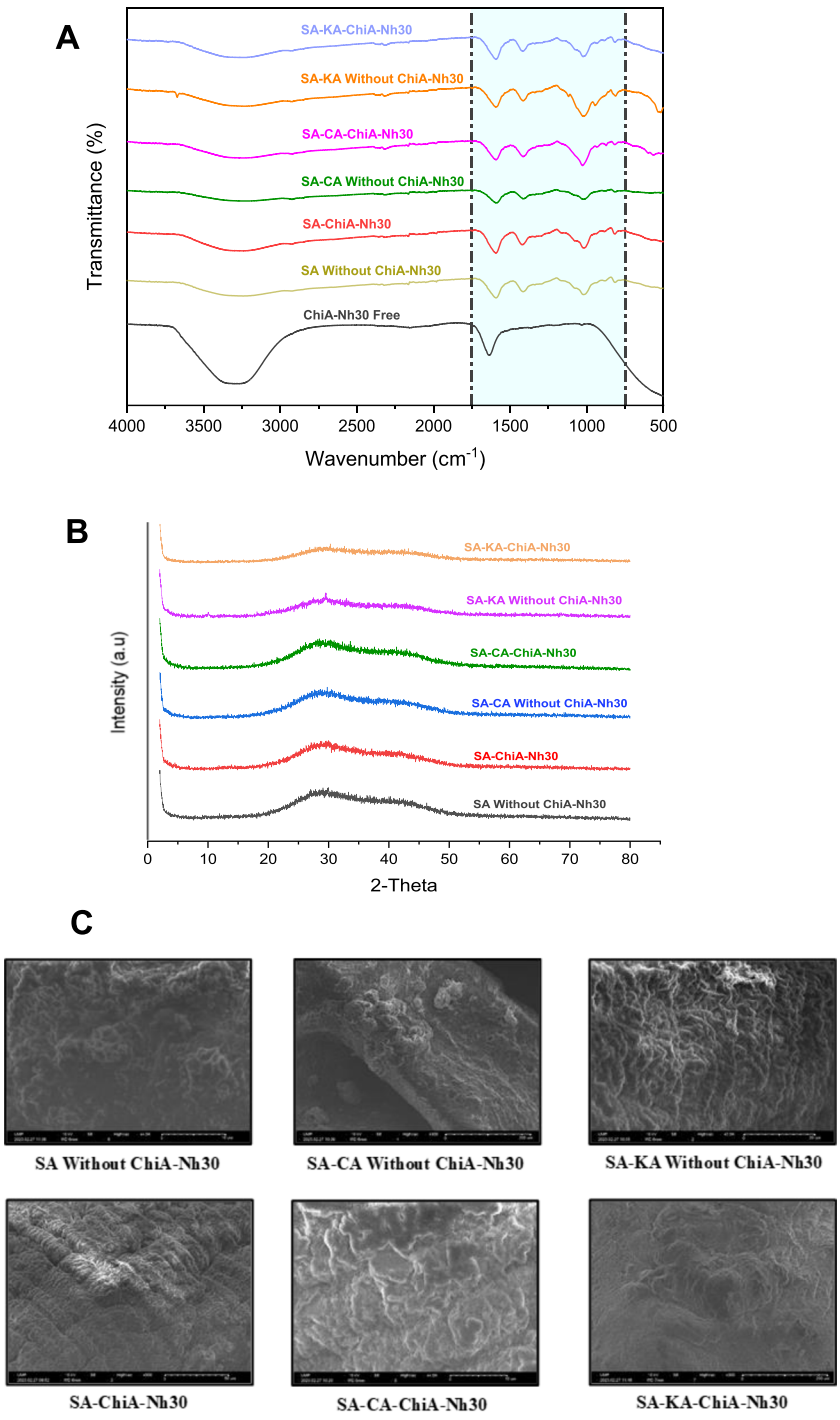

Fig. 3 ATR-FTIR spectra (**A**), XRD patterns (**B**), and FE-SEM images (**C**) of ChiA-Nh30-SA, ChiA-Nh30-SA-KA, and ChiA-Nh30-SA-CA immobilized forms. The OriginLab OriginPro software 2021b v9.8.5.204 (SR1) was employed to produce the ATR-FTIR patterns. Match! software (version 3.10.2.173) was used to produce the XRD patterns

after ChiA-Nh30 immobilization procedures (Fig. 3B). These findings were confirmed by FE-SEM analyses (Fig. 3C).

The FTIR results also confirmed that the ChiA-Nh30 immobilized forms were effective for recycling the ChiA-Nh30 enzyme. The XRD spectra matched the ATR-FTIR observations and demonstrated that the rigid structure of the SA, SA-KA, and SA-CA supports were retained for the ChiA-Nh30 immobilization. The FE-SEM micrographs allowed for the visualization of immobilized ChiA-Nh30; where the enzyme was present, the surface of the beads resembled coarse mesh structures. Additionally, the beads without ChiA-Nh30 have large pores, while the pores in ChiA-Nh30 beads appear to be occupied by the protein.

Biochemical characterization of free and immobilized ChiA-Nh30

Temperature and pH effect

The free ChiA-Nh30 had a maximum activity at 65 °C and pH 3 (Fig. 4A–B). When the Co^{2+} (3 mM) was added, the maximum activity was displaced to 75 °C. ChiA-Nh30 slowly loses activity above 85 °C. No temperature and pH optima change was observed for ChiA-Nh30 in its free and immobilized forms when different immobilization carriers were used.

The ChiA-Nh30-SA-KA beads were more stable, with a half-life ($t_{1/2}$) of 20 h compared to the ChiA-Nh30-Free, ChiA-Nh30-SA, and ChiA-Nh30-SA-CA beads, which had a $t_{1/2}$ of 4, 6, and 16 h, respectively, at pH 7 (Fig. 4C). The hybrid support SA-KA thus offers good stability to the modification of the charges carried by the ionizable groups at an alkaline pH.

The immobilization via encapsulation-adsorption resulted in a slight improvement in temperature stability. The ChiA-Nh30-SA-KA beads showed greater stability ($t_{1/2}$ = 32 h) at 80 °C when compared to the free ChiA-Nh30, ChiA-Nh30-SA, and ChiA-Nh30-SA-CA beads, which showed $t_{1/2}$ values of 16, 18, and 28 h, respectively.

The hybrid carrier SA-KA provided greater stability for ChiA-Nh30 activity in relation to the thermal agitation of the ChiA-Nh30 atoms brought on by the increase in temperature. This behavior may be the consequence of immobilization, which lessens the steric interference that the pH and temperature changes cause in the ChiA-Nh30 protein and its substrates.

Kinetic factors

The K_m and k_{cat} for ChiA-Nh30 were determined to be 0.105 mg substrate/mL and 54,250 s^{-1}, respectively (Table 5). When colloidal chitin was used as a substrate, ChiA-Nh30 exhibited a $k_{cat}/K_{m,}$ 1.2928.38-fold higher than that of ChiA-Ba43 and Chitodextrinase®, respectively (Fig. 5).

The increase in K_m and the decrease in V_{max} following immobilization may be explained by the mass transfer resistance and decreased flexibility of the ChiA-Nh30 protein to the substrate in the immobilization carriers, as well as the

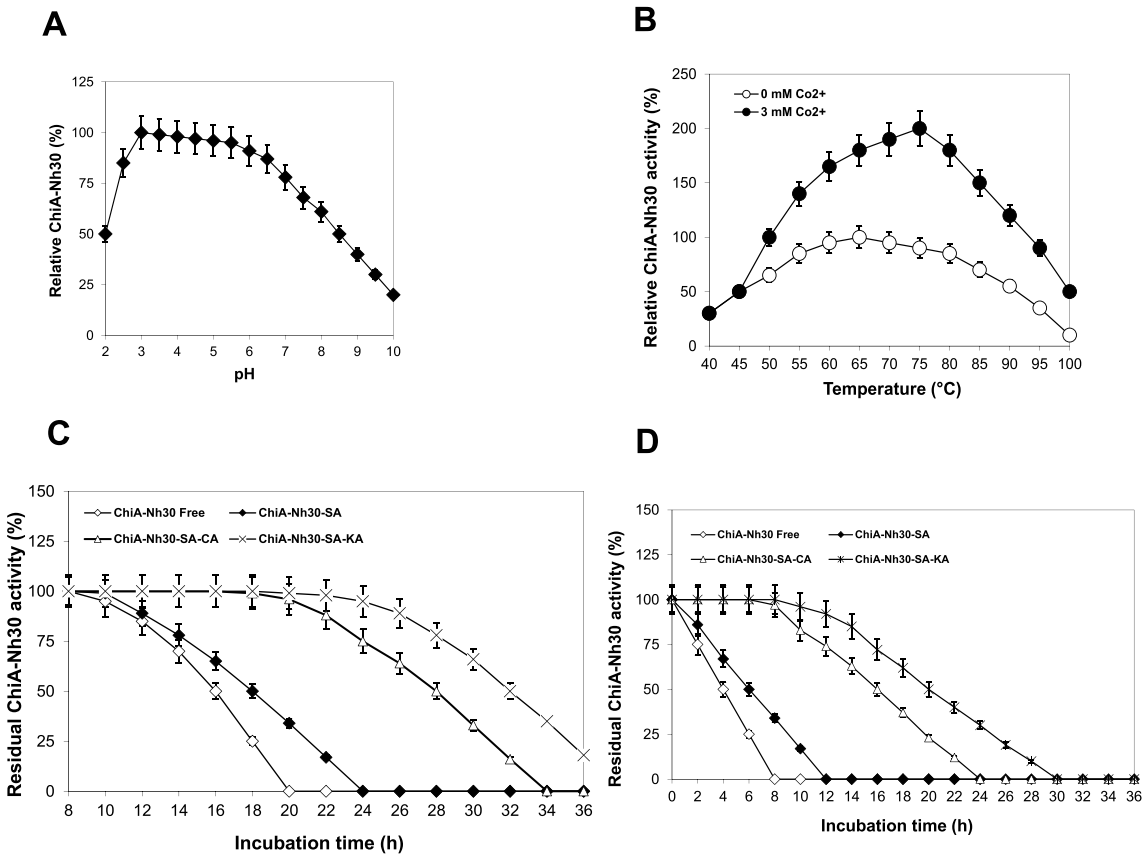

Fig. 4 Effect of pH on free ChiA-Nh30 activity (**A**). Effect of temperature on the activity of free ChiA-Nh30 in the presence and absence of Co^{2+} (**B**). Stability at pH 7 (**C**) and 80 °C (**D**) for the free and immobilized forms of ChiA-Nh30 tested in this study

Table 5 Kinetic parameters of chitinases when using 40 g/L colloidal chitin as a substrate

Enzymes	Wavelength (nm)	K_m (mg/mL)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat}/K_m $(mg/mL)^{-1}$ s^{-1}
ChiA-Nh30	550	0.105 ± 0.01	81,375 ± 610	54,250	516,667
ChiA-Ba43	550	0.201 ± 0.01	120,000 ± 681	80,000	398,100
Chitodextrinase®	550	0.457 ± 0.05	12,056 ± 125	8037	17,586

[a]Assays were performed in buffer A at 75 °C

Values are reported with ± SE and represent the means of at least three replicates

decreased or insufficient accessibility of the substrate to the enzyme active site (Table 6).

Consequently, fewer substrate molecules are required to be converted to a product per unit of time due to the increase in V_{max} following immobilization. This could result from the immobilized ChiA-Nh30 being released from the carrier and the ChiA-Nh30 enzyme leaking from the beads during repeated washings. This immobilized ChiA-Nh30 reusability criterion could further decrease the processing costs and, thus, make it viable for bioengineering applications.

Biotechnological potential of ChiA-Nh30

Reutilization of the immobilized ChiA-Nh30 beads

The residual activity of ChiA-Nh30-SA, ChiA-Nh30-SA-CA, and ChiA-Nh30-SA-KA was examined for multiple cycles (Fig. 6).

With their distinct reusability, the three immobilized forms can be extracted from the reaction mixture and used again in a subsequent reaction. In particular, the ChiA-Nh30-SA beads retained more than 5% of their initial activity for seven cycles in a row (Fig. 6A). The ChiA-Nh30-SA-KA and ChiA-Nh30-SA-CA were effective for nine consecutive cycles, retaining more than 15% of the initial activity (Fig. 5C-6B), although activity gradually declined after each cycle. In the second, third, fourth, fifth, sixth, seventh, eighth, and ninth cycles, respectively, the ChiA-Nh30-SA-CA beads displayed 88, 76, 62, 54, 46, 35, 24, and 15% of residual ChiA-Nh30 activity.

Degradation of blue crab chitin by ChiA-Nh30

The elimination of chitin in the absence of any enzymatic activity is explained by the fact that chitin is held together by electrostatic forces and hydrogen bonds that can be separated by thermal treatment. The outcomes show that immobilized ChiA-Nh30 can be a valuable tool for biodegrading crustacean waste. The ChiA-Nh30 biodegradation abilities are anticipated to contribute to producing highly valuable biotransformation products.

GlcNAc monomer production from 20% (*w*/*v*) blue crab chitin, using ChiA-Nh30 in free and immobilized forms, showed that the use of ChiA-Nh30-SA, ChiA-Nh30-SA-CA, and ChiA-Nh30-SA-KA improved the production of GlcNAc monomers by 2.25, 2.65, and 3.1 times, compared to the resulting content from the action of free ChiA-Nh30 (Fig. 6D).

ChiA-Nh30 antifungal effect

Since the ChiA-Nh30 can degrade chitin, a major component of fungi and yeast cell walls, the chitinolytic enzyme adds value as a potential antifungal agent. It is, therefore, not surprising that glycosidase enzymes are futuristic biocatalytic tools with vast biotechnological applications (Singh and Arya 2019; Laribi-Habchi et al. 2020; Patil et al. 2023). The biological approach focused on using chitinases in the fight against pathogenic fungi and yeasts is a new and promising research area.

Antifungal activity of the chitinase was tested against the fungal strains indicated, and its activity was compared

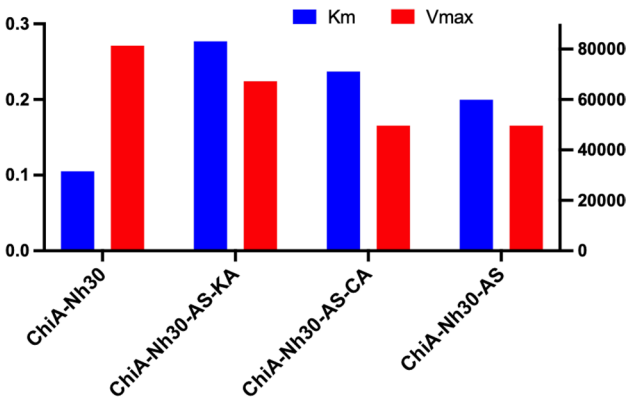

Fig. 5 Kinetic parameters of ChiA-Nh30-SA, ChiA-Nh30-SA-KA, and ChiA-Nh30-SA-CA for hydrolysis of colloidal chitin as substrate

Table 6 Kinetic parameters of free ChiA-Nh30, ChiA-Nh30-AS-KA, ChiA-Nh30-AS-CA, and ChiA-Nh30-AS when using colloidal chitin as substrate

Enzymes	K_m (mg/mL)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat}/K_m (mg/mL)$^{-1}$ s^{-1}
Free ChiA-Nh30	0.105 ± 0.01	81,375 ± 610	54,250	516,667
ChiA-Nh30-AS-KA	0.277 ± 0.03	67,250 ± 507	44,833	161,852
ChiA-Nh30-AS-CA	0.237 ± 0.02	49,641 ± 370	33,094	143,055
ChiA-Nh30-AS	0.199 ± 0.01	36,982 ± 281	24,655	123,894

[a]Assays were performed in buffer A at 75 °C

Values are reported with ± SE and represent the means of at least three replicates

to cycloheximide, the positive control. The results revealed that chitinase exhibits varying levels of inhibitory efficacy against distinct fungal strains (Fig. 7).

In the present study, the antifungal effectiveness against various fungal strains, notably *P. infestans* (Mont.) de Bary, (1876) (Fig. 7A), *G. candidum* Link: Persoon, anamorph, 204307™ (Fig. 7B), *P. digitatum* (Pers.) Sacc., 1881 (Fig. 7C), and *A. niger* (Tiegh.) 1867 (Fig. 7D), was evaluated as shown in Table 7.

For *P. infestans*, the inhibition diameter was 17.9 mm, slightly lower than that of the control cycloheximide, which showed an inhibition diameter of 19.1 mm; for *G. candidum*, an inhibition diameter of 16.2 mm was observed with chitinase, while cycloheximide showed a larger inhibition diameter of 35.6 mm. Similarly, an inhibition diameter of 18.6 mm was observed against *P. digitatum* when chitinase was used, while the control showed a higher inhibition diameter of 30.3 mm. An inhibition diameter of 18.6 mm with chitinase was also observed against *A. niger*, while

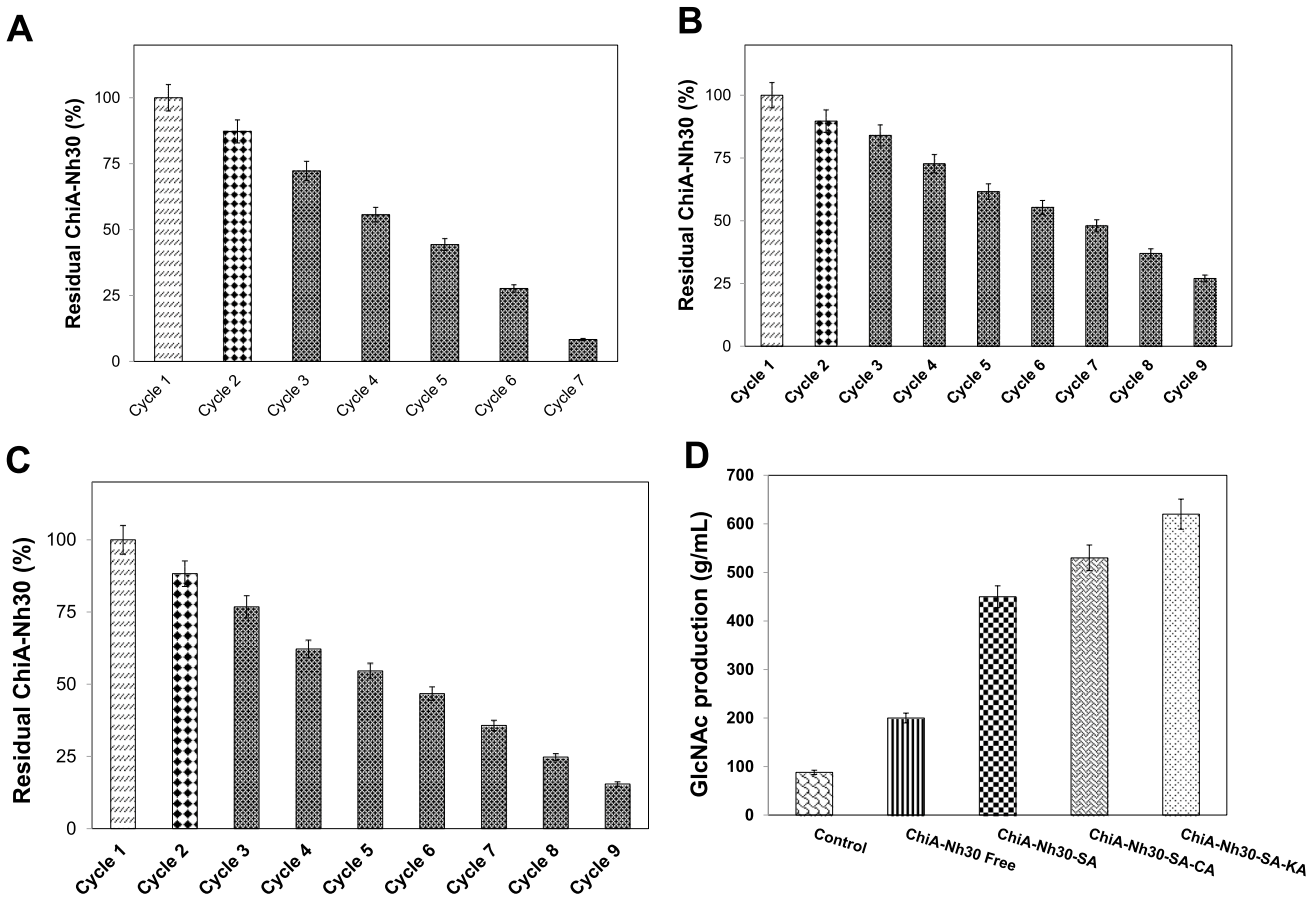

Fig. 6 Efficiency of ChiA-Nh30-SA (**A**), ChiA-Nh30-SA-KA (**B**), and ChiA-Nh30-SA-CA (**C**) in recycling. Generation of GlcNAc monomers from the residues of the blue swimming crab, *Portunus segnis* (**D**)

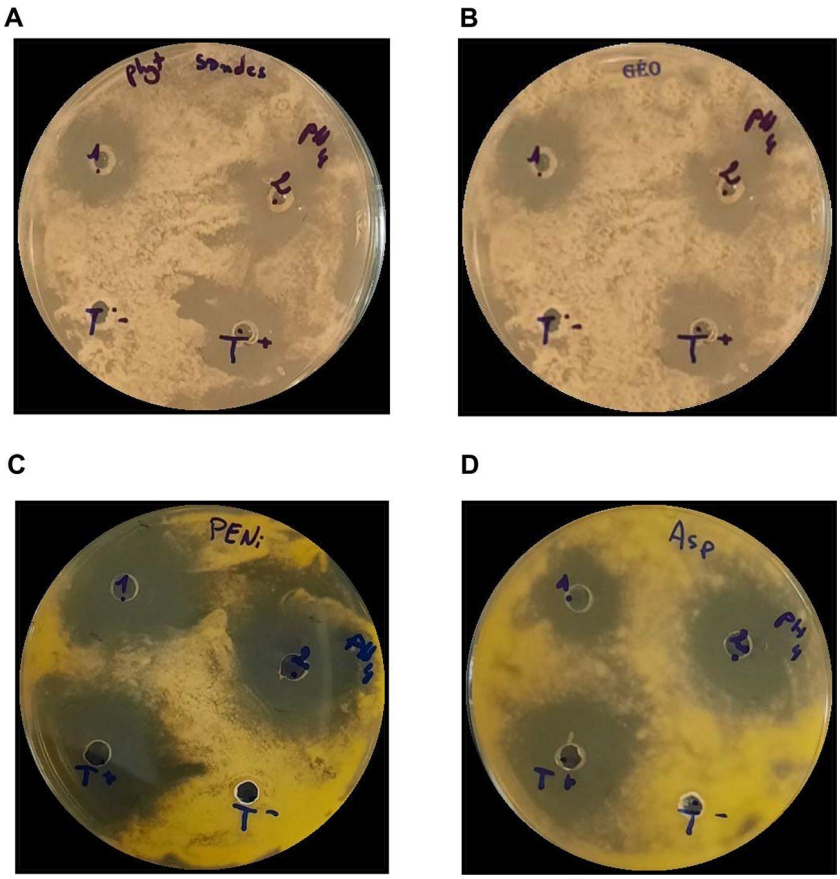

Fig. 7 Inhibition diameters of ChiA-Nh30, water (negative control), and cycloheximide (positive control) against fungal strains. *Phytophthora infestans* (Mont.) (**A**), *Geotrichum candidum* Link (**B**), *Penicillium digitatum* (Pers.) Sacc. (**C**), and *Aspergillus niger* (Tiegh.) (**D**)

the control showed a slightly larger inhibition diameter of 22.9 mm. More information is required to understand the ChiA-Nh30 characteristics/functions relationship better. This is very important for developing functional chitinases that could have specific biotechnological uses, such as in preparing pharmaceutically important chitooligosaccharides (COS) and potential antifungal products.

Table 7 Inhibition diameters of ChiA-Nh30 and cycloheximide against fungal strains

Fungal strains	Inhibition diameters (mm)	
	ChiA-Nh30	Cycloheximide[a]
Phytophthora infestans (Mont.)	17.9 ± 0.3	19.1 ± 0.5
Geotrichum candidum Link	16.2 ± 0.5	35.6 ± 0.7
Penicillium digitatum (Pers.) Sacc	18.6 ± 0.1	30.3 ± 0.4
Aspergillus niger (Tiegh.)	18.6 ± 0.1	22.9 ± 0.2

[a]Cycloheximide (Sigma Aldrich, USA) served as the experiment positive control

Conclusion

The results obtained during this study demonstrate that the chitinase from *N. halophila* TN-X8 exhibits several unique properties of significant industrial value, notably its acidic pH and temperature stability. The ChiA-Nh30 was successfully immobilized through the use of encapsulation and encapsulation-adsorption techniques. The ChiA-Nh30-SA-KA and ChiA-Nh30-SA-CA beads exhibited the best stability and activity. The immobilized forms can be used for more than nine cycles, highlighting the economic viability of the ChiA-Nh30. The kaolin and charcoal supports are inexpensive, easy to use, non-toxic, and heat resistant. ChiA-Nh30-SA-KA and ChiA-Nh30-SA-CA can be used directly for the hydrolysis of chitin and to produce GlcNAc monomers. Accordingly, future research will involve sequencing the genome of strain TN-X8 to focus on genes that encode for other enzymes of industrial and biotechnological significance.

Acknowledgements The writers would like to thank Miss Z. Gharbi (Higher Institute of Biotechnology of Sfax, University of Sfax, Tunisia) and Miss O. Rayachi (National Engineering School of Sfax, University of Sfax, Tunisia) for their kind technical support. Additionally, we would like to thank Prof. A. Talhaoui and the technical team at Mohammed First University at the FSO Analytics Platform for their

helpful support with the FE-SEM, ATR-FTIR, and XRD analyses. The authors are grateful to Associate Professor M. Le Roes-Hill from the Applied Microbial and Health Biotechnology Institute at the Cape Peninsula University of Technology (Bellville, South Africa) and Associate Professor V. James from the Polytech Lyon at the Université Claude Bernard Lyon 1 (Villeurbanne-Lyon, France) for their assistance with proofreading and editing in English.

Author contribution S.M.: conceptualization, methodology, formal analysis, data curation, writing-original draft, methodology, formal analysis, data curation. S.M., F.J., B.B., S.M., M.I.Y., N.M., A.A., F.M., S.V., P.D'A., and C.D.B.: writing-review and editing, methodology, formal analysis. A.A., S.S., M.L.R.-H., and B.J.: conceptualization, writing-review, editing. S.S. and B.J.: supervision. All authors have read and agreed to the published version of the manuscript.

Funding This work was conducted within the European RDI Project "ARIBiotech – Alliance de Recherche et d'Innovation en Biotechnologie bleue pour la valorisation des déchets marins" (ARIBiotech C–5–2.1–41 and CUP I55F21003350006), coordinated by Prof. Saloua Sadok (INSTM), and co-financed by the European Neighbourhood Instrument (ENI) Cross-Border Cooperation (CBC) Italy-Tunisia IEV CT 2014–2020, the Multilateral Project Partenariats Hubert Curien (PHC)-Maghreb 2020 Program of the French Ministry of Foreign Affairs and Ministry of Higher Education, Research, and Innovation (FranMaghZYM 2020–2024, code Campus France: 43791TM, code PHC: 20MAG01) coordinated by Prof. Bassem Jaouadi (CBS), and by the Tunisian Ministry of Higher Education and Scientific Research (General Directorate for Scientific Research) and the Moroccan Ministry of Higher Education, Scientific Research and Innovation.

Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on request.

Declarations

Ethics approval and consent to participate Not applicable.

Competing interests The authors declare no competing interests.

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