Improving efficiency and sustainability of chitin bioconversion through a combination of *Streptomyces* secretomes and mechanical-milling

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ABSTRACT

Chitin, particularly α-chitin, is the most abundant and highly recalcitrant form, fortified by an intricate network of hydrogen bonds. Efficient valorization of α-chitin requires a mild pre-treatment and enzymatic hydrolysis. *Streptomyces* spp. secrete chitin-active CAZymes that can efficiently tackle the recalcitrant problem of chitin biomass. To better understand the potential of *Streptomyces* spp., a comparative analysis was performed between the novel isolate, *Streptomyces* sp. UH6 and the well-known chitin degraders, *S. coelicolor* and *S. griseus*. Growth studies and FE-SEM analysis revealed that all three *Streptomyces* spp. could utilize and degrade both α- and β-chitin. Zymogram analysis showed expression of 5-7 chitinases in the secretomes of *Streptomyces* strains. The chitin-active-secretomes produced by *Streptomyces* sp. UH6 and *S. griseus* were optimally active at acidic pH (pH 4.0 and 5.0) and 50°C. Time-course degradation of α- and β-chitin with the secretomes generated *N*-acetyl-*D*-glucosamine (GlcNAc) and *N,N*-diacetylchitobiose [$(GlcNAc)_2$] as the predominant products. Further, the highly crystalline α-chitin was subjected to pre-treatment by ball-milling, which reduced the crystallinity from 88% to 56.6% and increased the BET surface area by 3-folds. Of note, the activity of all three *Streptomyces* secretomes was improved by a mild pre-treatment, while *Streptomyces* sp. UH6 secretome displayed improved GlcNAc and $(GlcNAc)_2$ yields by 14.4 and 9.6-folds, respectively. Overall, our results suggest that the *Streptomyces* chitin-active-secretomes, particularly *Streptomyces* sp. UH6, can be deployed for efficient valorization of chitin biomass and to establish an economically feasible and eco-friendly process for valorizing highly recalcitrant α-chitin.

Keywords: α-Chitin, *Streptomyces*, Chitin-Active-Secretome, Ball-Milling, $(GlcNAc)_{1-2}$

1. INTRODUCTION

Chitin is a naturally occurring homopolymer consisting of N-acetyl-D-glucosamine (GlcNAc) monomers linked together by β-1,4-glycosidic bonds. It is the second most abundant biopolymer found in nature, surpassed only by cellulose. Chitin plays a crucial structural role in the exoskeleton of arthropods and crustaceans, as well as in the cell wall of fungi.[1] Chitin biomass can be processed through mechanical, chemical or biological means (via microbes or enzymes) to produce chitooligosaccharides (CHOS). CHOS are highly sought-after commercially due to their biodegradable, biocompatible, and non-toxic properties.[2] The monosaccharide GlcNAc and disaccharide, *N,N*-diacetylchitobiose [$(GlcNAc)_2$] are particularly important among CHOS, owing to their diverse biological applications. GlcNAc

is widely used in medicine for its anti-bacterial, anti-oxidant, and anti-tumor properties, and as a clinical drug for rheumatism and rheumatoid arthritis.[3] It can also promote the synthesis of synovial fluid and increase skin hyaluronic acid content.[3] Further, it is also used as food additive and for seed treatments and foliar applications in agriculture.[3] Of note, GlcNAc is also used in the production of various valuable nitrogen-containing chemicals.[3] $(GlcNAc)_2$ or chitobiose, on the other hand, has been shown to have implications in medicine and therapeutics, such as protection against acute hepatotoxicity induced by CCl_4-induced,[4] high anti-oxidant and DNA protection activities,[5] amelioration of metabolic dysfunction in type 2 diabetes mice,[6] and alleviation of oleic acid-induced lipid accumulation.[7] Given the diverse commercial applications of GlcNAc and chitobiose, it is imperative to maximize their production through efficient and sustainable chitin valorization approaches.

However, the main obstacle for efficient valorization of chitin biomass is its high crystallinity and resistance to degradation due to other intimate contaminants. Chitin occurs naturally in two primary polymorphic forms, α- and β-chitin, with the former being the most prevalent and crystalline form. The high degree of crystallinity in α-chitin is due to two factors: tightly packed alternating parallel and anti-parallel chains and the presence of both inter- and intra-sheet hydrogen bonds.[1, 8] While employing enzymatic methods for α-chitin valorization is environmentally friendly, the enzyme load required to facilitate efficient hydrolysis would be substantial, resulting in high process costs. To overcome this limitation and develop new technologies for efficient chitin valorization, integrated approaches such as mechano-enzymatic,[9] chemo-enzymatic[10] or a combination both are in high demand. These approaches mainly involve mild pre-treatment of crystalline chitin using mechanical (such as milling) or chemical (such as ionic liquids) methods, followed by enzymatic hydrolysis to improve overall CHOS yields, particularly GlcNAc and $(GlcNAc)_2$. [9, 10]

Ball-milling is a dry mechanical method used to amorphize crystalline chitin substrates. In ball-milling, the substrate is mixed with balls of varying sizes (made of high hardness materials such as zirconium or stainless steel) in a specific ratio within a sealed chamber. The chamber rotates at a set rpm, leading to a reduction in particle size and crystallinity of the substrate due to frictional impact between the substrate, the balls, and the chamber walls.[11] Although there are reports of using a combination of ball-milling and enzymatic hydrolysis (mainly with mono-component enzymes),[9, 12] it would be interesting to investigate the effectiveness of this method when using the "chitin-active-secretomes" of microbial origin. Employing such chitin-active-secretomes (rich in chitin-active CAZymes) will avoid the costs associated with producing mono-component recombinant enzymes.

When considering sustainability and environmentally friendly methods, biological, specifically enzyme-catalyzed chitin degradation is the most preferred approach. In nature, microorganisms, particularly bacteria, degrade chitin using chitinolytic enzymes for nutrition. Enzymatic chitin degradation involves a synergistic interplay between different chitinolytic enzymes, such as lytic polysaccharide monooxygenases (LPMOs), chitinases, and β-N-acetylglucosaminidases.[9, 13-15] *Streptomyces,* the largest genus in the phylum Actinobacteria, is gram-positive and can degrade complex polysaccharides, including chitin and thus play a key role in the nature's carbon cycle.[16, 17] In general, *Streptomyces* harbours genomes of larger size (>5 Mb), which encode a plethora of carbohydrate-active enzymes (CAZymes) for degradation of complex polysaccharides.[17] Among the known *Streptomyces* species, chitinolytic enzymes from *S. coelicolor*[18-21] and *S. griseus*,[14, 22, 23] have been well-studied. Recently we reported *Streptomyces* sp. UH6, a novel strain isolated from the University of Hyderabad campus, has a promising chitin-degrading/modifying enzyme machinery.[17] The genome of *Streptomyces* sp. UH6 comprises a diverse range of chitin-active CAZymes, including five chitinases (four GH18 and one GH19), four AA10 LPMOs, and four β-*N*-acetylglucosaminidases (four GH20 and one GH3).[17] The secretome of *Streptomyces* sp. UH6 showed high activity towards highly crystalline chitin substrates, indicating the potential for efficient valorization of such substrates.[17] Further, exploration of the *Streptomyces* sp. UH6 'chitin-active-secretome' could yield valuable insights into chitin degradation and its applications.

In this study, we investigated the chitinolytic potential of *Streptomyces* sp. UH6, compared to the well-studied *Streptomyces* spp., *S. coelicolor* and *S. griseus*. Growth and chitinase activity studies confirmed the ability of all the three *Streptomyces* spp. to effectively utilize chitin biomass, and field emission-scanning electron microscopy (FE-SEM) analysis confirmed the deformations in chitin particles. On the other hand, zymogram analysis confirmed the expression of chitinase isozymes in the 'chitin-active-secretomes' of the *Streptomyces* spp. Time-course degradation of α- and β-chitin was performed to understand the CHOS profile and overall yields generated. Further, the α-chitin substrate was subjected to pre-treatment by ball-milling and the milled substrates were characterized to understand the changes in physical properties after pre-treatment. Additionally, the milled substrates were subjected to hydrolysis using the secretomes of *Streptomyces* spp., thereby confirming our hypothesis that 'chitin-active-secretomes' can be effectively utilized for chitin biomass valorization.

2. EXPERIMENTAL

2.1. Bacterial strains, chemicals and reagents

Streptomyces sp. UH6 was isolated from soil samples collected at the University of Hyderabad campus located in Hyderabad, India at coordinates 17°27'22"N, 78°18'54"E.[17] While, the strains, *Streptomyces griseus* DSM 40236 and *Streptomyces coelicolor* A3(2) were procured from DSMZ (Braunschweig, Germany). Both α- and β-chitin were purchased from Mahtani Chitosan, located in Gujarat, India. Colloidal and glycol chitin were prepared as previously reported.[2] All chemicals and reagents were purchased from HiMedia Laboratories and Sisco Research Laboratories Pvt. Ltd., Mumbai, India, unless otherwise specified.

2.2. Growth of *Streptomyces* species on α- and β- chitin substrates

To investigate chitin utilization, the three *Streptomyces* species were cultured in M9 minimal media containing 47.7 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.5 mM NaCl, 9.4 mM NH_4Cl, 1 mM $MgSO_4$ and 0.3 mM $CaCl_2$. The media was enriched with 1X trace elements and vitamins (1 mM each of biotin and thiamine) and supplemented with 0.5% α- or β-chitin as the sole carbon source. Prior to the main experiment, each *Streptomyces* species was pre-cultured in R-2A broth for 48h at their optimal growth temperatures. Later, the fully grown cultures were harvested and washed with sterile milliQ water and resuspended in 1X M9 medium. The optical density of the resuspended cultures was measured at 600 nm and adjusted to 0.1. A 1% inoculum was added to the chitin containing minimal media and cultured at their respective optimal growth temperatures. Samples were collected at different time points over a 10-day period to estimate the total cellular protein, which is an indicator of growth, and extracellular chitinase activity. All experiments were conducted in biological triplicates.

2.2.1. Estimation of total cellular protein

Growth of the *Streptomyces* spp. on different forms of chitin substrates was examined by estimating the total cellular protein from the culture pellet collected at different time points as reported previously.[2] To these cell pellets, 0.2N NaOH was added and boiled at 120°C for 10 min to lyse the cells. This was followed by centrifugation at 12000 rpm for 15 min at 4°C, and the total cell protein was estimated using standard Bradford's method, as instructed in the manufacturer's protocol (Sigma Aldrich, USA).

2.2.2. Estimation of chitinase activity for the secretomes

Chitinase activity was measured by Schales' assay as described earlier[24], with slight modifications. A 200 µL of reaction mixture consisting of 50 mM sodium acetate pH 5.5, 0.5% colloidal chitin and 50 µL culture supernatant was incubated for 1 h at 37°C and 800 rpm shaking. The samples were centrifuged at 10000 rpm for 15 min at 4°C and 100 µL of the

supernatant was boiled with 300 µL Schales' reagent (0.5 M sodium carbonate and 0.5 g/L potassium ferricyanide). Absorbance was measured at 420 nm. Quantification of reducing sugars was done using a GlcNAc standard curve. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugar per hour.

2.3. FE-SEM analysis to track deformities in chitin flakes

Direct visualization of α- and β-chitin degradation by the three *Streptomyces* species was performed using FE-SEM. Sample preparation protocol and FE-SEM analysis parameters were maintained similar to those reported by Mukherjee et al., (2020)[2], with slight modifications. For each strain, three experimental setups were created, including two control setups with untreated α- and β-chitin separately, and one test setup with *Streptomyces* spp. grown in M9-minimal media supplemented with α- or β-chitin at their optimal growth temperatures. Samples were collected for FE-SEM analysis at 96 h and 72 h for α- and β-chitin, respectively. All samples were harvested by centrifugation at 10,000 rpm for 3 min at 4°C, resuspended in 2.5% glutaraldehyde, and incubated at 4°C overnight for pre-fixation. The samples were then dehydrated using a progressive ethanol washing (10, 30, 50, 70, 90 and 100% [v/v]) and placed on a clean glass coverslip attached to two-sided magnetic tape on a stub. A single chitin flake (untreated or treated) was analyzed using Quorum Q150T ES sputter, and gold coating was done for 120 s before FE-SEM analysis was performed using the Merlin Compact (model no. 20-59) of Carl Zeiss, Germany. The parameters for high resolution imaging were set at working distance of 2-20 µm and 5 and 3 kV accelerating voltage, and the magnification range varied from 500-10,000X.

2.4. Semi-native PAGE analysis of secretomes to identify chitin-active CAZymes

The secretomes of the three different *Streptomyces* spp. under investigation were collected over α- and β- chitin, at their respective optimal activity times. To visualize the chitinase isozymes in the secretome fractions, a zymogram analysis was performed using 12% SDS-PAGE containing 2.5% glycol chitin. A 10 µL sample of culture supernatant was mixed with 4 µL of loading dye (1M Tris-HCl pH 6.8, 10% SDS, 50% Glycerol, 0.05% bromophenol blue and 14.3 M β-mercaptoethanol) and boiled at 80°C for 10 min before electrophoresis. Analysis was conducted on samples collected from at least three different experiments. After electrophoresis, the gels were washed at least three times with 50 mM Tris Cl (pH 8.0) containing 0.1% Triton X-100 and twice with 50 mM Tris Cl (pH 8.0) and then incubated in the same buffer at 37°C overnight. The gels were stained with Calcofluor-white M2R dye (Sigma Aldrich, USA) for 20 min and washed thoroughly with double distilled water before examination for the presence of bands under UV transilluminator.

2.5. Determination of optimal conditions for efficient saccharification

Streptomyces spp. were grown in M9-minimal medium with 0.5% β-chitin until peak chitinase activity was reached. The resulting secretomes were obtained by centrifugation at 8000 rpm for 20 min at 4°C, and their concentration was measured by Bradford's method. The secretomes were then stored at -20°C for subsequent characterization experiments.

2.5.1. pH and temperature optima

Optimal pH for the secretomes was determined using various 50 mM buffers within a pH range of 2.2 to 10.0. The buffers used were glycine-HCl (pH 2.2-3.0), sodium citrate (pH 3.0-6.0), sodium acetate (pH 4.0-5.5), sodium phosphate (pH 6.0-8.0), Tris-HCl (pH 7.2-9.0), and glycine-NaOH (pH 9.0-10.0). Each reaction mixture contained a particular buffer and 50 µL of respective secretome, and was incubated with 0.5% colloidal chitin at 37°C and 800 rpm shaking. Meanwhile, the optimal temperature for the secretomes was measured at a pH determined in the previous experiment, with temperatures ranging from 10 to 80°C. Chitinase activity was measured using Schales' assay as described the section 2.2.2, with all reactions performed in triplicate and proper controls in place.

2.5.2. Thermal stability of the chitin-active-secretomes

Thermal stability experiments were performed at temperatures corresponding to the point of maximal activity. The secretomes were subjected to incubation at 45°C and 50°C for various time intervals, and the residual chitinase activity was measured using the Schales' assay as described the section 2.2.2. To ensure accuracy, all reactions were conducted in triplicate and included appropriate controls.

2.6. Pre-treatment of shrimp shell α-chitin by ball-milling

The shrimp shell α-chitin was subjected to pre-treatment through ball-milling using a SPEX Sample Prep Mixer/Mill 8000 D. In this process, 2.5 g of α-chitin was mixed with 25 g of stainless-steel balls of varying diameters, maintaining a ball to substrate ratio of 10:1. The milling procedure was carried out for 30 and 60 min, respectively, at a speed of 1060 cycles per min, resulting in the production of two milled powders named BM-30 and BM-60. The structural properties of the α-chitin from shrimp shells were analyzed using various techniques to assess the effects of ball-milling.

Powder X-ray Diffraction (pXRD) analysis:

The pXRD measurements were carried out on a PANalytical X'Pert^3 powder diffractometer using Cu-Kα radiation with a wavelength of 1.54 Å, in a 2θ range of 5^o-40^o and with a step size of 0.0167^o. The crystallinity index (I_{CR}) of the samples was calculated using the intensities of

the (110) and (I_{am}) peaks at 2θ values of ~20° (representing the maximum intensity) and ~16° (corresponding to the amorphous halo contribution), according to the following equation[25]:

$$I_{CR}\ (\%) = [(I_{110} - I_{am})/I_{110}] \text{ X } 100$$

Fourier Transform Infrared Spectroscopy (FTIR) analysis:

The samples were uniformly compressed with KBr to create pellets/disks for FTIR analysis on a PerkinElmer Frontier FTIR/FIR Spectrometer. The spectra were obtained using the attenuated total reflection method and recorded in the range of 4000 cm^{-1} to 400 cm^{-1}.[25]

BET analysis:

The surface area of the pre-treated chitin samples was determined using a surface area analyzer, the Quantachrome autosorb iQ, Germany. The samples were subjected to degassing at 60°C for 24 h under a N_2 atmosphere to remove any moisture adsorbed on the solid surface. The specific surface area was estimated using the BET surface area (S_{BET}) method.[25]

2.7. Time-course degradation of crystalline chitin substrates

The time-dependent hydrolysis of crystalline chitin substrates, α- (native and pre-treated) and β-chitin (10 mg/mL) was carried out using 5 U of chitin-active-secretomes. All reactions were performed in triplicate under optimal conditions at 45°C and 1000 rpm. Aliquots were collected at different time points between 1-72 h and filtered using a 96-well filter plate (0.45 µm filters; Merck Millipore, USA) operated by a Millipore vacuum manifold. The products were analyzed using HPLC (Shimadzu, Japan) equipped with Shim-pack GIST NH2 column (5 µm, 4.6 X 250, Shimadzu, Japan) at 210 nm, through isocratic elution with 70% acetonitrile at a flow rate of 0.7 mL/min. The oligomers were identified and quantified using the respective CHOS standards essentially as described previously.[26]

3. RESULTS & DISCUSSION

In a previous study, we reported that the genome of *Streptomyces* sp. UH6 possessed a complete chitin-active CAZyme repertoire.[17] The isolate's ability to degrade various chitin substrates, especially the crystalline chitin substrates α- and β-chitin, was also validated. The chitinase activity on α- and β-chitin was found to be higher than that on colloidal chitin, suggesting that the chitinolytic enzymes in the secretome of *Streptomyces* sp. UH6 prefer highly crystalline chitin substrates. To gain deeper understanding of the chitinolytic potential of *Streptomyces* sp. UH6, we performed a comparative study with two well-studied species, *S. coelicolor* A3(2) and *S. griseus*.

3.1. All three *Streptomyces* spp. were able to utilize crystalline chitin substrates

The growth of the studied *Streptomyces* spp. was analyzed on M9 minimal medium supplemented with α- and β-chitin as the sole carbon source. Samples were collected at regular intervals up to 10 days to analyze the total cellular protein and extracellular chitinase activity. *Streptomyces* sp. UH6 showed slower growth on α-chitin compared to β-chitin, with maximum growth at 216 h and 96 h, respectively (Fig. 1A and B). *S. griseus* had similar growth rates on α-chitin, but slower growth on β-chitin (maximum growth observed at 168 h). *S. coelicolor* had the slowest growth among the three, with maximum growth at 240 h on both substrates. Notably, *Streptomyces* sp. UH6 displayed the highest total cellular protein content at the peak growth time point on both the substrates.

The extracellular chitinase activity followed the same pattern as growth studies, with *Streptomyces* sp. UH6 exhibiting the highest activity on both the substrates (Fig. 1C and D). However, activity on α-chitin was lower than on β-chitin, possibly due to the high crystalline nature of α-chitin hindering chitinase accessibility and reducing saccharification efficiency.

3.2. FE-SEM imaging of α- and β-chitin deconstruction by *Streptomyces* spp.

FE-SEM visualization showed that *Streptomyces* sp. UH6, *S. coelicolor*, and *S. griseus* colonized and perforated the surface of α- and β-chitin, resulting in de-fibrillation. While, the untreated α-chitin displayed a smooth surface (Fig. 2A), the treated samples exhibited visible colonization by the respective *Streptomyces* spp., accompanied by perforations and de-fibrillation (Fig. 2B-D). The perforations and de-fibrillation were more intense in α-chitin for *Streptomyces* sp. UH6 (Fig. 2B). β-chitin was degraded much faster and intensely by the *Streptomyces* spp. While the untreated β-chitin had a smooth surface (Fig. S1A), dense colonization of the strains and perforations were observed upon treatment (Fig. S1B-D), leading to almost complete degradation at 96 h. Direct surface attachment and colonization were the primary mechanism for crystalline chitin degradation, as observed in the FE-SEM analysis, which is in agreement with the growth studies and previous findings with *Paenibacillus* sp. LS1.[2]

3.3. *Streptomyces* species can produce 'chitin-active-secretomes'

The secretomes produced by three *Streptomyces* spp., collected over α- and β-chitin, were resolved using semi-native PAGE containing 2.5% glycol chitin, to understand the expression pattern of different chitinase isozymes. For secretomes collected over α-chitin, six isozymes were detected for *Streptomyces* sp. UH6, while *S. coelicolor* and *S. griseus* showed five and four isozymes, respectively. In the case of secretomes collected over β-chitin, five isozymes were observed for *Streptomyces* sp. UH6, while *S. coelicolor* and *S. griseus* displayed six and

seven isozymes, respectively. The isozymes detected were approximately within the range of 20-70 kDa (Fig. 3).

Previously, zymogram analysis of secretomes produced by *Paenibacillus* sp. LS1, collected over different chitin substrates, revealed a higher expression of chitinase isozymes on β- and colloidal chitin, as compared to α-chitin.[2] A similar observation was made in the current study, where the expression of the chitinase isozymes was higher in β-chitin than α-chitin for the secretomes produced by *Streptomyces* sp. UH6 and *S. griseus* (Fig. 3). However, no significant difference in the expression of chitinase isozymes was observed on both substrates for the secretome produced by *S. coelicolor*. The expression of six chitinase isozymes on α-chitin by *Streptomyces* sp. UH6 indicates the potential of the isolate to utilize recalcitrant forms of chitin.

3.4. Biochemical characterization of the chitin-active-secretomes

The process of chitin saccharification is greatly affected by reaction conditions, such as pH and temperature. Therefore, to optimize the chitin valorization process and improve the production of chitooligosaccharides, the influence of pH and temperature on the chitinase activity of the secretomes produced by *Streptomyces* sp. UH6, *S. coelicolor*, and *S. griseus* was thoroughly investigated.

3.4.1. The chitin-active-secretomes were optimally active in acidic pH and 50°C

The optimal pH for chitinase activity of the secretomes produced by *Streptomyces* sp. UH6, *S. coelicolor* and *S. griseus* was investigated under different buffer conditions within the pH range of 2-10 (Fig. 4A). The secretomes from *Streptomyces* sp. UH6 and *S. griseus* showed optimal activity in 50 mM sodium acetate, pH 4.0, while the secretome from *S. coelicolor* was optimally active at pH 5.0 of the same buffer (Fig. 4A). *Streptomyces* sp. UH6 secretome retained up to 70% activity throughout the acidic pH range of 2.0-6.0, while *S. griseus* secretome retained up to 80% activity within the pH range of 5.0-7.0, with a sharp decline to ≤60% at pH below 4.0. Chitin-active-secretome from *S. coelicolor* retained up to ~80% was activity at pH 4.0, but a sharp decline in activity was observed below pH 4.0 and above pH 5.0 (Fig. 4A).

All three chitin-active-secretomes showed optimal activity at 50°C (Fig. 4B). They retained up to ~90% activity at 45°C and 55°C, except for *Streptomyces* sp. UH6 secretome, which retained ~80% activity at 55°C (Fig. 4B). The optimal parameters observed for the secretomes of *Streptomyces* spp. suggest the presence of chitinases that are active under acidic conditions and could potentially promote efficient saccharification of chitin under such conditions. It is noteworthy that similar observations were made in previous studies with *Paenibacillus* sp. LS1 and *Aeromonas* sp. GJ-18, whose secretomes showed optimal activity

in 50 mM sodium acetate buffer, pH 4.0, and at 50°C and 10 mM sodium acetate buffer, pH 5.0, and 45°C, respectively.[2, 27]

3.4.2. The chitin-active-secretomes are relatively stable at 45°C

The secretomes produced by *Streptomyces* sp. UH6 and *S. coelicolor* retained chitinase activity up to 70% even after 120 min of pre-incubation at 45°C. Similarly, the secretome produced by *S. griseus* retained up to 65% of chitinase activity under the same conditions (Fig. 4C). However, at the optimal temperature of 50°C, *Streptomyces* sp. UH6 secretome retained up to 60% of chitinase activity upon pre-incubation up to 120 min. The secretome from *S. coelicolor* was also stable at 50°C, retaining up to 70% activity after 120 min of pre-incubation (Fig. S2). However, the secretome from *S. griseus* showed sharp decrease in activity, dropping to nearly 60% after only 20 min of pre-incubation at 50°C. The activity further decreased up to 50% after 120 min of pre-incubation at 50°C (Fig. S2). Previously, secretome produced by *Paenibacillus* sp. LS1 was reported to be stably active at 45°C, retaining up to 53% activity after 90 min of pre-incubation, compared to its optimal temperature, 50°C where chitinase activity declined up to 33% after only 20 min pre-incubation.[2] Our observations indicated that the three secretomes were stably active at 45°C, and therefore all the further assays were performed at this temperature.

3.5. *Streptomyces* sp. UH6's chitin-active-secretome effectively breaks down chitin

The ability of the three *Streptomyces* secretomes to degrade α- and β-chitin substrates was compared over a time-dependant reaction. $(GlcNAc)_2$ was the primary product formed in all three cases, followed by GlcNAc. Trace amounts of $(GlcNAc)_3$ and $(GlcNAc)_5$ were only detected in case of reactions with *Streptomyces* sp. UH6 secretome (data not shown). From α-chitin, the secretomes produced by *Streptomyces* sp. UH6, *S. coelicolor* and *S. griseus* generated total CHOS [GlcNAc+$(GlcNAc)_2$] concentrations of 0.79 mM, 0.62 mM and 0.52 mM, respectively (Fig. 5A and B). Conversely, from β-chitin, the total CHOS generated were 6.2 mM, 4.2 mM and 2.9 mM, respectively (Fig. 5C and D). Notably, *Streptomyces* sp. UH6 secretome generated a higher concentration of CHOS from both crystalline chitin substrates than the other two, indicating its efficiency in chitin biomass valorization. This can be attributed to the chitinolytic enzyme machinery encoded by *Streptomyces* sp. UH6, which comprises extracellularly secreted chitinases, β-*N*-acetyl-glucosaminidases and AA10 LPMOs (those consisting of an N-terminal signal peptide).[17] However, the low CHOS yields by the *Streptomyces* secretomes from α-chitin can be attributed to the high crystallinity of the substrate. Due to anti-parallel polymeric chain arrangements and intrinsic hydrogen bonding within and among the polymeric chains, α-chitin, although most abundant yet recalcitrant form

of chitin.[8] Hence, pre-treating α-chitin prior to enzymatic hydrolysis could be useful in reducing the crystallinity of the substrate, and thereby enhancing the activity of chitin-active-secretomes.

3.6. The process of ball-milling induced alterations in the structure of α-chitin.

To improve the substrate accessibility and enzymatic hydrolysis, α-chitin was pre-treated through ball-milling. Typically, ball-milling breaks down the interchain hydrogen bond network within chitin polymer, inducing alterations in the overall chitin structure and reducing the polymer's crystallinity.[3] The structural properties of the substrate before and after pre-treatment were analyzed by FT-IR, pXRD and BET analysis and were compared between un-milled α-chitin (UM α-chitin) and the two ball-milled substrates, BM-30 and BM-60. FT-IR analysis revealed several changes in the spectra of the ball-milled substrates compared to the UM α-chitin. The disappearance of a small peak between the wavenumbers 1619 and 1654 cm^{-1} indicated loss of amide-I bond in the ball-milled substrates (Fig. 6A). Similar observations were made in previous studies regarding changes in the 1640-1660 cm^{-1} region of the FT-IR spectra after ball-milling chitin substrates. [9, 12] Additionally, a flattening of the peak between 3259 and 3440 cm^{-1} indicated the loss of N-H and O-H stretching bonds, respectively (Fig. 6A). Furthermore, the decrease in peak intensity between 1029 and 1068 cm^{-1} indicated that the ball-milling induced considerable breakage of the C-O stretching bonds, corresponding to the glycosidic linkages in the chitin (Fig. 6B). Previously, loss of N-H and O-H stretching bonds and C-O stretching bonds were observed for solid shrimp shell waste after ball-milling.[28] While several changes were observed in the spectral diagrams of the ball-milled substrates compared to the UM α-chitin, these changes did not heavily distort the overall spectral sketch of chitin.

The pXRD analysis revealed five crystal reflection peaks for all the substrates i.e., 020, 021, 110, 120 and 101 (Fig. 6C). Sharp peaks at ~9^{o} (020) and ~19^{o} (110) in the UM α-chitin indicated a compact structure of the substrate, which is usually not accessible for direct enzymatic hydrolysis. A significant decrease in the intensities of the above-mentioned peaks was observed for both ball-milled substrates, with BM-60 showing the maximum decrease (Fig. 6C). Further, the crystallinity index (C.I.) was calculated, revealing a substantial decrease in the crystallinity of the milled chitin substrates. While UM α-chitin showed a C.I. of 88.3%, the substrates BM-30 and BM-60 showed a decrease in the C.I. up to 72% and 56.6%, respectively (Table 1). Previously, ball-milling powdery chitin for 4 h at 380 rpm led to a decrease in C.I. from 86.9% to 52.2%.[12] In another study, a C.I. of 40% from 94% was achieved

for crab α-chitin, when ball-milled at 800 rpm for 30 min.[9] However, in both cases, the authors used of 10 mm diameter zirconia balls.

The BET surface areas (S_{BET}) of the resulting ball-milled chitin substrates were also examined and compared against the UM α-chitin. The S_{BET} was 6.3, 10.6 and 18.6 m^2/g for UM, BM-30 and BM-60, respectively (Fig. 6D). This showed ~1.7 and 3-fold increase in surface area for substrates BM-30 and BM-60, respectively, compared to UM α-chitin (Table 2). The increase in BET surface area could be due to the decrease in compactness of the α-chitin structure as a result of hydrogen bond breakage between chitin chains by ball-milling.[29] Previously, shrimp chitin powder ball-milled for 60 min at 1060 cycles per minute had an S_{BET} of 36.5 m^2/g, however, the S_{BET} value for unmilled chitin was not provided.[30] The results together indicate substantial changes in structural parameters of the UM α-chitin after pre-treatment with ball-milling. These alterations in the chitin structure might be helpful in promoting improved enzymatic hydrolysis by the *Streptomyces* chitin-active-secretomes.

3.7 Pre-treatment of α-chitin with ball-milling enhanced its valorization by *Streptomyces* chitin-active-secretomes

With this study we compared CHOS production from ball-milled α-chitin substrates using secretomes from the three *Streptomyces* species. The results showed that ball-milling followed by enzymatic hydrolysis significantly increased both GlcNAc and $(GlcNAc)_2$ yields compared to UM α-chitin. The secretome produced by *Streptomyces* sp. UH6 exhibited the most significant hydrolytic activity. For example, the GlcNAc yield from UM α-chitin was only 0.32 mM after 72 h, whereas a substantial increase up to 1.6 mM was observed for BM-30 and up to 4.6 mM for BM-60 (Fig. 7A). Similar observations were made for $(GlcNAc)_2$, which increased from 0.47 mM to 2.3 mM and 4.5 mM from BM-30 and BM-60, respectively (Fig. 7B). These results suggest that ball-milling induces structural changes in α-chitin, such as increased surface area and decreased crystallinity (particularly for BM-60), which improve the hydrolytic efficiency of the chitin-active-secretomes produced by *Streptomyces* sp. UH6.

The secretomes produced by *S. coelicolor* and *S. griseus* also showed improvement in GlcNAc and $(GlcNAc)_2$ yields from the ball-milled α-chitin substrates (Fig. 7C-F). However, the yield obtained using the *Streptomyces* sp. UH6 chitin-active-secretome was higher compared to the other two. Interestingly, the *Streptomyces* sp. UH6 chitin-active-secretome employed over BM-60 produced nearly equal concentrations of both GlcNAc and $(GlcNAc)_2$ (4.6 mM and 4.5 mM, respectively), highlighting its clear advantage over the other two secretomes for efficient hydrolysis of ball-milled α-chitin to produce value-added CHOS.

Previously, it was reported that ball-milling of crab α-chitin improved hydrolysis by *Serratia marcescens* chitinases, both individually and in combination with *Sm*CBP21.[9] The authors achieved up to 0.8 mM of $(GlcNAc)_2$ from the ball-milled crab α-chitin, compared to less than 0.05 mM for the unmilled substrate after 48 h, resulting in almost a 16-fold increase in yield. Additionally, combined hydrolysis of ball-milled powdery chitin by a chitinase and β-*N*-acetylglucosaminidase from *P. barengoltzii* increased overall chitin conversion to GlcNAc by 3.2-fold compared to the unmilled substrate.[12] While these studies utilized purified mono-component enzymes, we used chitin-active-secretomes produced by *Streptomyces* species for valorization of the α-chitin substrates, which makes our method economically feasible. We found that the combination of BM-60 and chitin-active-secretome from *Streptomyces* sp. UH6 was the most preferred for enhanced production of both GlcNAc and $(GlcNAc)_2$ in equal concentrations. Our results are comparable with the previously reported methods, with up to 14.4- and 9.6-fold higher GlcNAc and $(GlcNAc)_2$ yields, respectively from BM-60 compared to the UM α-chitin using the chitin-active-secretome from *Streptomyces* sp. UH6.

Interestingly, while *S. griseus* and *S. coelicolor* A3(2) have a higher number of chitinases and LPMOs in their CAZyme profiles compared to *Streptomyces* sp. UH6,[17] the latter is able to degrade both unmilled and milled α- and β-chitin much more efficiently. This efficient chitin degradation by the chitin-active-secretome of *Streptomyces* sp. UH6 may be attributed to the extracellular secretion of chitinases and LPMOs.[17] According to the genome analysis of *Streptomyces* sp. UH6, all the chitinases (both GH18 and GH19) and the LPMOs encoded have an N-terminal signal peptide, indicating their extracellular secretion.[17] Moreover, the higher production of GlcNAc by the *Streptomyces* sp. UH6 secretome may be due to the action of the β-*N*-acetylhexosaminidase containing a signal peptide (peg.2875). It could also be possible that the chitinolytic enzymes produced by *Streptomyces* sp. UH6 are strong degraders of chitin as compared to those secreted by *S. griseus* DSM 40236 and *S. coelicolor* A3(2). However, further studies are needed to validate these observations through thorough comparative molecular characterization of these chitin-active CAZymes.

4. CONCLUSIONS

A novel mechano-enzymatic approach that combines ball-milling with naturally produced bacterial secretomes for bioconversion of crystalline α-chitin to GlcNAc and $(GlcNAc)_2$ has been demonstrated. The ball-milling process significantly decreased the crystallinity and increased the surface area of the normally compact α-chitin, enabling its enzymatic hydrolysis with the secretome produced by *Streptomyces* sp. UH6 to produce both GlcNAc and $(GlcNAc)_2$

in equal concentrations. This eco-friendly method does not involve any harsh chemicals and is cost-effective due to the use of naturally produced chitin-active-secretomes instead of mono-component recombinant enzymes, which require expensive isolation and purification procedures. Furthermore, this method could aid in the production of platform chemicals, such as GlcNAc and $(GlcNAc)_2$ for various value-added products. *Streptomyces* sp. UH6's chitin-active-secretome showed a clear advantage over those produced by *S. coelicolor* and *S. griseus* in valorizing both unmilled and milled α-chitin as well as β-chitin, indicating the superior efficiency of its chitin-active CAZymes. Despite encoding fewer chitin-active CAZymes in its genome than *S. coelicolor* and *S. griseus*, *Streptomyces* sp. UH6 is a valuable target for chitin valorization to GlcNAc and $(GlcNAc)_2$ on an industrial scale.

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Table 1:

Substrates	I_{110} (~20°)	I_{am} (16°)	I_{CR}
UM α-chitin	52198	6083	88.3%
BM-30	32228	9052	71.9%
BM-60	25711	11170	56.6%

Table 1. Crystallinity Index (C.I.) of the unmilled and ball-milled α-chitin substrates as determined from powder XRD. I_{110} denotes the maximum intensity of diffraction for the (110) plane at $2\theta = 20°$ and I_{am} denotes the intensity of the diffraction at the amorphous region at $2\theta=16°$ approximately.

Table 2:

Substrates	S_{BET} (m2/g)	Fold change of S_{BET}
UM α-chitin	6.3	-
BM-30	10.6	1.69
BM-60	18.6	2.97

Table 2. S_{BET} analysis of the unmilled and ball-milled α-chitin substrates

Fig. 1:

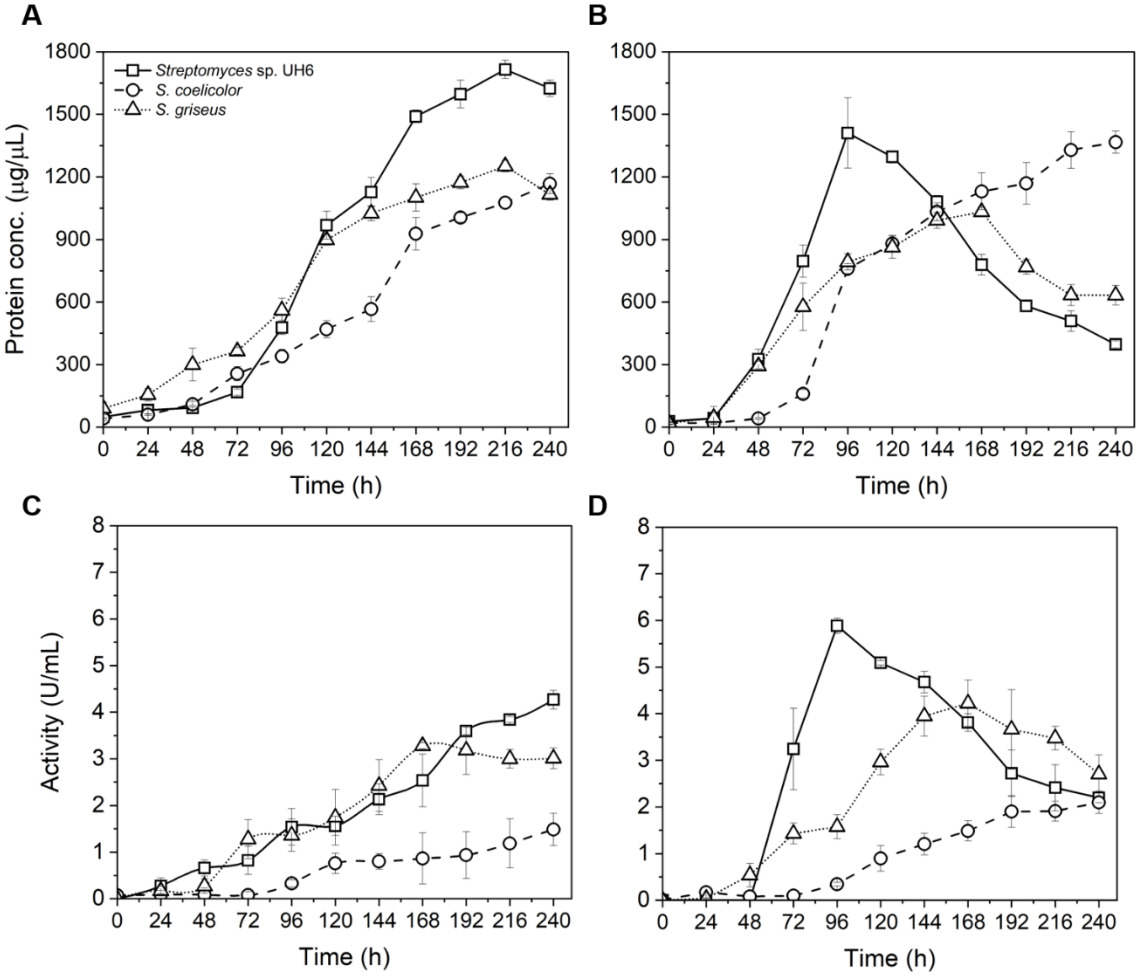

Fig. 1. Cell growth and activity analysis of the *Streptomyces* spp. on α- and β-chitin. Cell growth was analyzed in terms of total cellular protein concentration (µg/µL) on α- (A) and β-chitin (B). Extracellular chitinase activity (U/mL) for the secretomes collected over α- (C) and β-chitin (D) was estimated using Schales' assay. The experiment was performed in biological triplicates and error bars represent standard deviation.

Fig. 2:

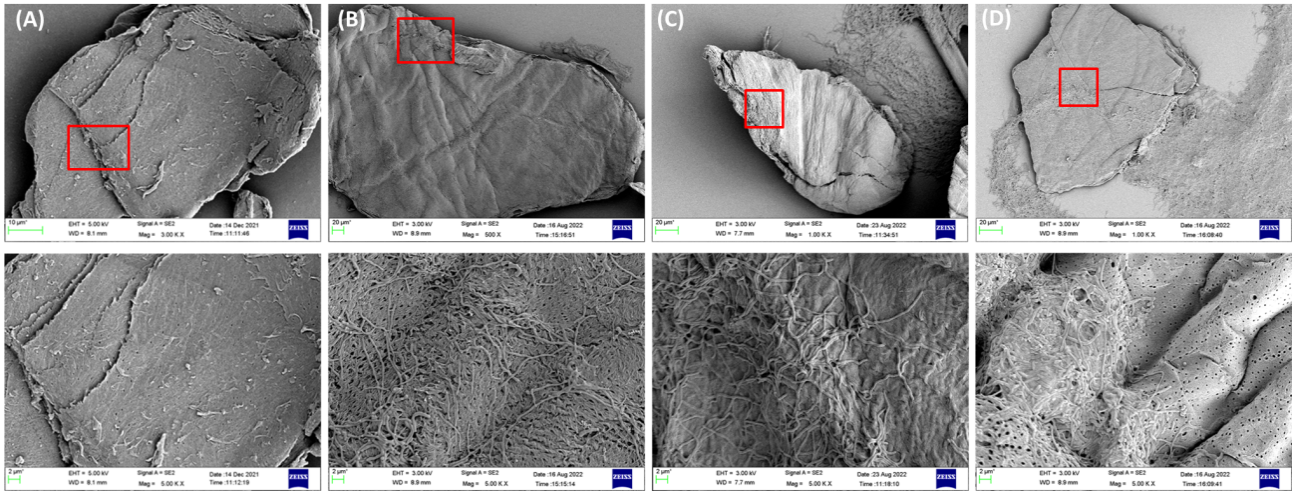

Fig. 2. FE-SEM micrographs showing the degradation of α-chitin by the *Streptomyces* spp. (A) untreated α-chitin particle; (B, C, D) α-chitin treated with *Streptomyces* sp. UH6, *S. coelicolor* and *S. griseus*, respectively. The upper panel represents the untreated and treated chitin particles, while the lower panel represents the close-up images of the target area (shown in red frame). Scale bar for the images in the upper panel is 20 µm and those in the lower panel is 2 µm.

Fig. 3:

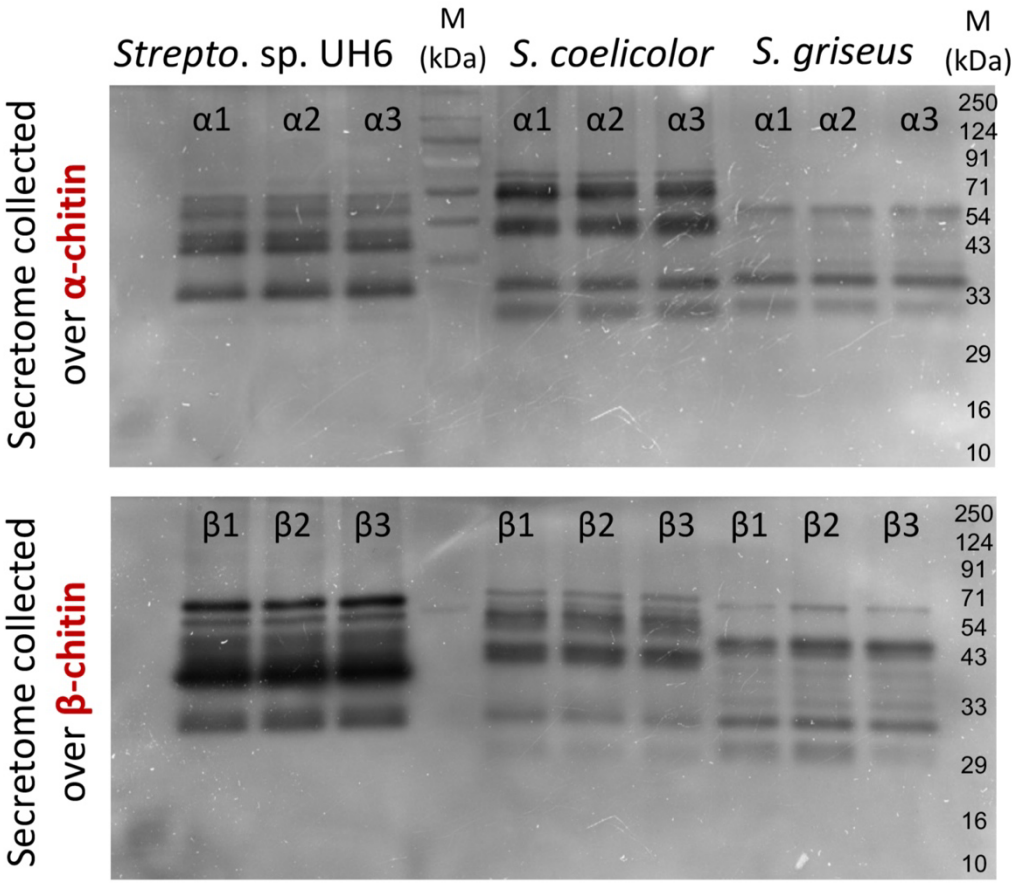

Fig. 3. Semi-native PAGE of *Streptomyces* spp. secretomes using glycol chitin as substrate. Each expression band in the gel is considered as one chitinase isozyme. Upper panel represent chitinase isozymes detected in the secretomes collected over α-chitin, and lower panel represent chitinase isozymes detected in the secretomes collected over β-chitin as substrate. Lanes 1-3, 5-7 and 8-10 represents secretomes produced *Streptomyces* sp. UH6, *S. coelicolor* and *S. griseus*, respectively; lane 4 is protein marker (in kDa) in both panels. The labels α1-3 and β1-3 represents biological replicates of the secretomes collected over α- and β-chitin, respectively.

Fig. 4:

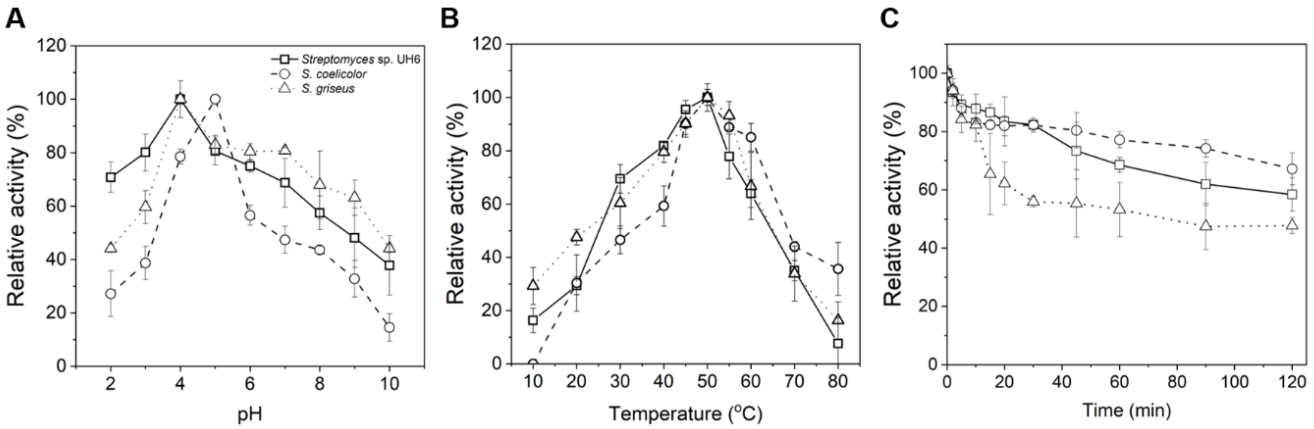

Fig. 4. Optimal conditions and thermal stability: (A) Optimal pH of the *Streptomyces* secretomes was measured using different buffers of 50 mM strength at a pH range of 2-10. (B) Optimal temperature was measured at a temperature range of 10-80°C under the optimum pH conditions. (C) The secretomes were pre-incubated at 45°C for different time-intervals and the residual activity was estimated using Schales' assay. All assays were performed in triplicates and error bars indicate standard deviation.

Fig. 5:

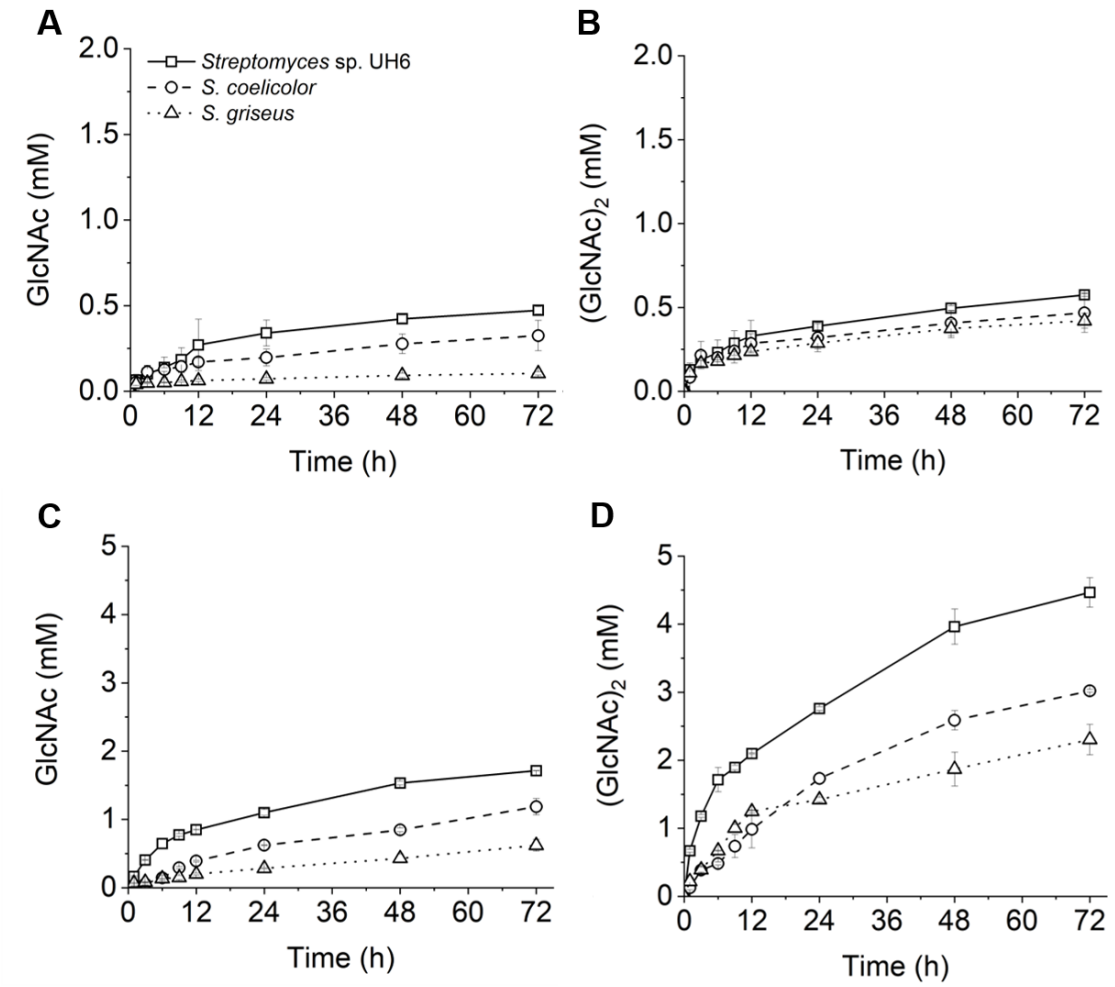

Fig. 5. Time-course degradation of α- (A and B) and β-chitin (C and D) by the secretomes produced by *Streptomyces* sp. UH6, *S. coelicolor* and *S. griseus*. All experiments were performed in biological triplicates and the error bars represent standard deviation.

Fig. 6:

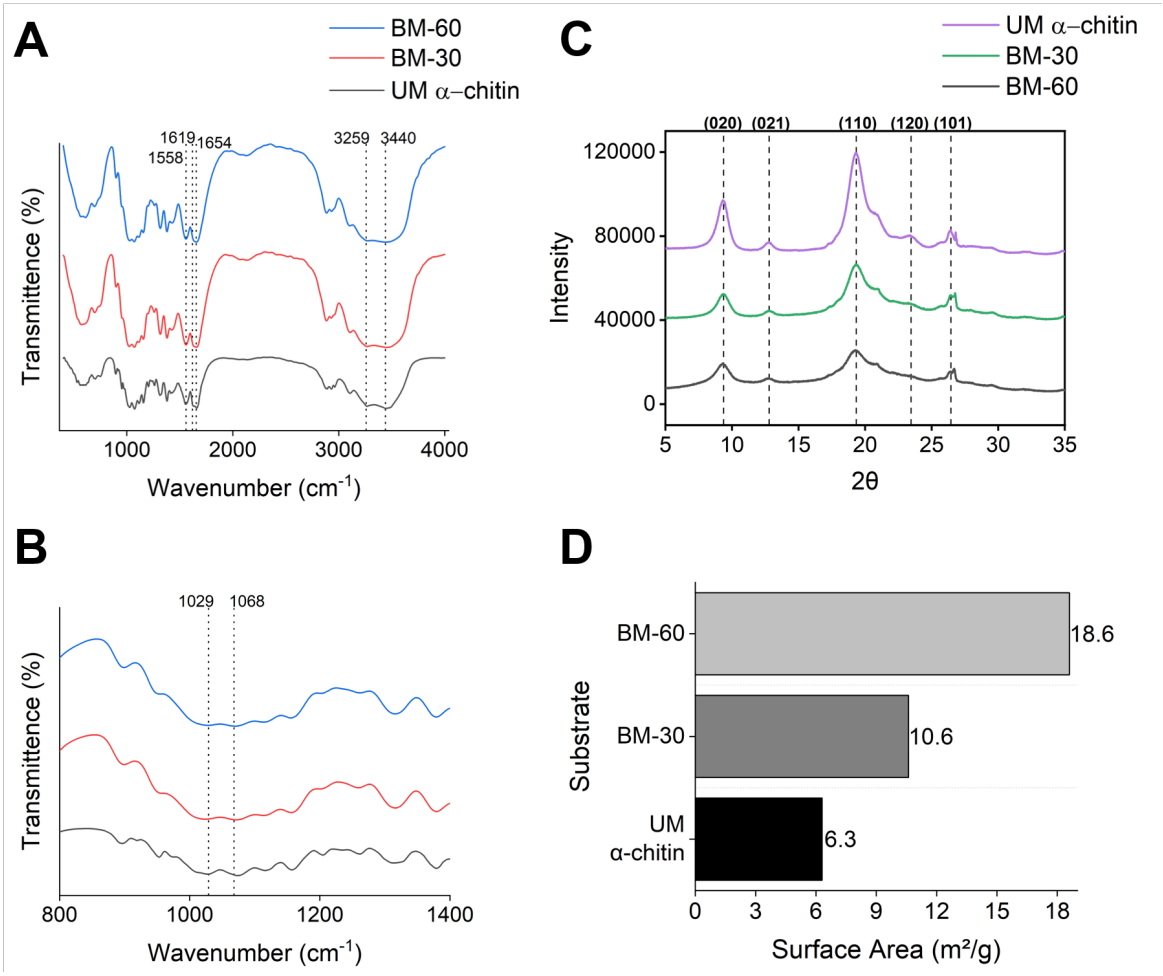

Fig. 6. Characterization of unmilled and ball milled α-chitin substrates by FT-IR (A and B), pXRD (C) and BET analysis (D). UM α-chitin denotes unmilled α-chitin, BM-30 and BM-60 denotes α-chitin ball-milled for 30 and 60 minutes, respectively.

Fig. 7:

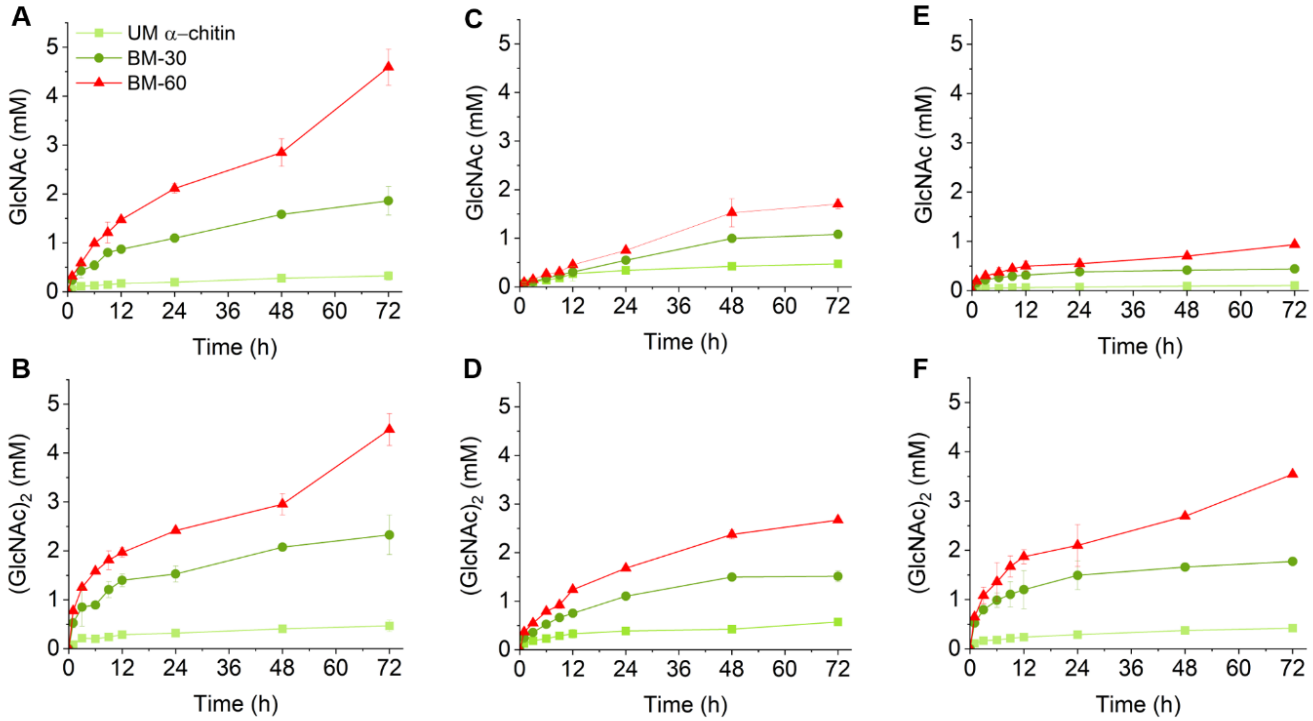

Fig. 7. A comparative analysis of GlcNAc and $(GlcNAc)_2$ production from unmilled α-chitin (light green) and the ball-milled α-chitin substrates, BM-30 (green) and BM-60 (red) using chitin-active-secretomes produced by *Streptomyces* sp. UH6 (A and B), *S. coelicolor* (C and D) and *S. griseus* (E and F). All experiments were performed in biological triplicates and the error bars represent standard deviation.