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# GENOME MINING REVEALS CHITIN DEGRADATION POTENTIAL OF *Streptomyces parvulus* VCCM 22513

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## ABSTRACT

The genus *Streptomyces* is not only known as a natural producer of antibiotics but also a prolific source of chitinolytic enzymes that digest recalcitrant chitin to chitooligosaccharides. However, only a few reports have used whole-genome sequencing to study chitin degradation of *Streptomyces* to date. In the present study, out of 22 *Streptomyce*s strains, *Streptomyces parvulus* VCCM 22513 produced the highest chitinase activity. Time courses of incubation revealed that the maximum chitinase (0.91 ± 0.04 U/mL) of this strain was observed after 96 hours in the yeast extract salts medium supplemented with 10.0 g/L colloidal chitin. Additional genomic analysis of VCCM 22513 was also conducted to discover the genomic information related to chitin degradation. The VCCM 22513 genome consists of 341 carbohydrate-active enzyme coding genes divided into 6 families including glycoside hydrolase (134 genes), carbohydrate-binding module (88 genes), glycosyl transferase (87 genes), carbohydrate esterase (18 genes), polysaccharide lyase (7 genes), and auxiliary activity (7 genes). Further genome mining revealed the presence of 10 chitinases, 4 lytic polysaccharide monooxygenases, and 14 β-N-acetylhexosaminidases, which mainly contribute to the degradation of chitin polymers. This is the first report revealing the mechanism underlying the chitin degradation of *S. parvulus*. Future investigations are required to characterize chitinolytic enzymes found in this study for the bioeconomic production of high-quality chitooligosaccharides from chitin food wastes.

**Keywords:** CAZy, chitin degradation, chitinases, chitooligosaccharides, genomic analysis, *Streptomyces parvulus*.

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## INTRODUCTION

Chitin is made up of N-acetylglucosamine (GlcNAc) linked by β-(1,4)-glycosidic bonds and serves as the second largest renewable resource on Earth (Xu et al., 2020). Chitin has various applications such as wastewater treatment, wound healing, functional food, drug delivery, and dietary fiber. It is distributed abundantly in the outer skeleton of crabs, insects, lobsters, and especially shrimps (Jha et al., 2016; Doan et al., 2021). In Vietnam, shrimp processing generates massive amounts of such waste, estimated to be more than 200,000 metric tons (wet weight) per year, causing pollution in coastal areas (Si Trung & Bao, 2015). However, only a small portion of the shrimp waste is used to make chitin and chitosan.

Recently, chitooligosaccharide (COS) has attracted increasing interest from scientists worldwide due to its applications in medicine, food, and pharmacology. COS can be obtained by treatment of chitin or chitosan with acid hydrolysis, enzymatic degradation or both (Xu et al., 2020). Bioconversion using enzymes is preferred due to its high purity and low environmental pollution. Bacterial chitinases (EC 3.2.1.14) hydrolyzing chitin to produce fully acetylated COS stand out as promising candidates. Chitinase can be classified into 3 groups of glycosyl hydrolase (GH) 18, 19, and 20. GH18 chitinases are abundant in various bacteria from different habitats, while GH19 chitinases are only found in a few bacterial strains, such as *Pseudoalteromonas rubra* and *Streptomyces alfalfa* (Kim et al., 2021). Among bacteria, many members of the genus *Streptomyces* including *Streptomyces plicatus*, *Streptomyces lividans*, *Streptomyces virdificans*, *Streptomyces halstedii*, *Streptomyces aureofaciens*, and *Streptomyces griceus* can effectively degrade chitin (Jha et al., 2016). Especially, *Streptomyces diastaticus* CS1801 isolated from shrimp paste could degrade both untreated crab shell waste and colloidal chitin to COS (Xu et al., 2020). In addition, chitinases from *Streptomyces* spp. also showed strong antifungal activity against phytopathogenic fungi, which are widely applied in agriculture (Ekundayo et al., 2022).

Whole-genome sequencing is a cost-effective and comprehensive approach to investigate chitinolytic enzymes from bacteria (Quach et al., 2022a; Quach et al., 2022c). The genome of *Curtobacterium* sp. GD1 isolated from leaves of conventionally grown soybean was sequenced by the Illumina platform to discover its chitinolytic activity in response to chitin substrates (Dimkić et al., 2021). Despite of 3,063 *Streptomyces* genomes available on GenBank (NCBI), only one research had used whole-genome sequencing to fully decipher the chitin degradation potential of *S. diastaticus* CS1801 (Xu et al., 2020). Our earlier work exploited adaptive responses of *S. parvulus* VCCM 22513 to survive in *Bruguiera gymnorrhiza* using intensive genomic analysis (Quach et al., 2022b). In this study, we shed light for the first time on chitin degradation activity and its related genomic information in *S. parvulus* VCCM 22513. These findings provided the additional genomic basis for chitin decomposition from *Streptomyces* and a solid foundation for industrial COS production.

## MATERIALS AND METHODS

### Materials

Twenty-two *Streptomyces* spp. from different environments were provided by VAST-Culture Collection of Microorganisms, Institute of Biotechnology, Vietnam Academy of Science and Technology. The draft genome sequence of *S. parvulus* VCCM 22513 was retrieved from Genbank (NCBI) under accession number JAJVLA000000000.

Chitin was extracted from white leg shrimp *Litopenaeus vannamei* waste collected from Dai Phat company, Ca Mau province by using Alcalase and ColoProtease P200, followed by chemical treatment to remove proteins and mineral residues (Valdez-Peña et al., 2010). The obtained chitin had 0.86% protein and 0.51% ash.

### Screening and assessment of chitinase activity

Colloidal chitin was prepared from in-house produced chitin using the previously reported method (Wu et al., 2009). In brief, 10

g of chitin was added to 60 mL HCl and rotated for 1–2 h at room temperature. Once completely dissolved, the resulting solution was poured into 400 mL of cold deionized water to obtain the precipitates of colloidal chitin. The mixture was centrifuged at 7,000 × *g* for 10 min at 4 $^{o}$C and then washed 3 times with sterile water. Colloidal chitin solution was neutralized to a pH of 5.0 with 1 M NaOH followed by centrifugation at 7,000 × *g* for 10 min and then washed 2 times with sterile water. The resulting colloidal chitin was air-dried and kept at 4 $^{o}$C as a pellet for chitinase activity assay.

Twenty-two strains were grown in yeast extract salts medium (yeast extract, 0.5 g/L; $K_2HPO_4$, 2.0 g/L; $MgSO_4.7H_2O$, 1.0 g/L; and $FeSO_4.7H_2O$, 0.1 g/L; pH 6.0) supplemented with 10.0 g/L colloidal chitin at 30 $^{o}$C for 4 days. Chitinase from cell-free supernatant was measured based on the release of reducing saccharides from colloidal chitin using N-acetyl-D-glucosamine (GlcNAc) as a standard (Doan et al., 2021). Briefly, 200 µL of the reaction solution, consisting of 100 µL of 1% (w/v) colloidal chitin prepared in 100 mM sodium acetate buffer (pH = 5.0) and 100 µL of cell-free supernatant, was incubated at 37 $^{o}$C for 30 min. Then, 300 µL of dinitrosalicylic acid (DNS) reagent was added to the reaction solution. After that, the mixture was heated at 100 $^{o}$C for 10 min and centrifuged at 13,000 rpm for 10 min to remove the precipitates. Then, 200 µL of the clear solution was measured at 515 nm using a microplate reader. Besides, the standard curve for N-acetyl-D-glucosamine was generated with a regression equation of y = 0.1143x – 0.062 and $R^2$ = 0.988. One unit (U) of chitinase is defined as the amount of enzyme which releases 1 µM N-acetyl-D-glucosamine in 1 mL of the reaction mixture under standard assay conditions.

### Genome annotation

*S. parvulus* VCCM 22513 was sequenced with the Illumina Miseq sequencing platform (Illumina, California, USA) and deposited to Genbank (NCBI) as described previously (Quach et al., 2022b). The whole genome sequence of strain VCCM 22513 was re-annotated using Rapid Annotations via Subsystem Technology (RAST) and Pathosystems Resource Integration Center (PATRIC) platforms (Overbeek et al., 2014; Wattam et al., 2017).

### CAZy carbohydrase analysis

The carbohydrate-active enzymes (CAZymes) of *S. parvulus* VCCM 22513 were identified using the CAZymes database (http://www.cazy.org/). Briefly, the genome sequence of *S. parvulus* VCCM 22513 was submitted to the dbCAN meta server (https://bcb.unl.edu/dbCAN2/) integrating three tools HMMER (E-value < 1e-15), DIAMOND (E-value < 1e-102) and eCAMI (important k-mer number ≥ 5) with the screening parameter E-value < $1e^{-5}$ (Vu et al., 2021). All obtained genes were classified into 5 groups including glycoside hydrolase (GH), carbohydrate-binding module (CBM), glycosyl transferase (GT), polysaccharide lyase (PL), carbohydrate esterase (CE), and auxiliary activity (AA).

### *In silico* analysis of chitin-degrading enzymes

Genes involved in chitin degradation were determined by the CAZymes database and crosschecked with the annotations from the Cluster of Orthologous Groups (COG) using EggNOG-mapper v1.0.3 (Tatusov et al., 2000). All obtained sequences were analyzed by the InterProScan web server (https://www.ebi.ac.uk/interpro/) and NCBI batch CD-search online tool (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) to identify functional and conserved domains.

## RESULTS AND DISCUSSION

### Screening of chitinolytic actinomycetes

Twenty-two *Streptomyces* spp. were screened for their chitinase activity on yeast extract salts medium supplemented with 10.0 g/L colloidal chitin. It turned out that 16 of 22 strains showed extracellular chitinolytic activity ranging from 0.12–0.83 U/mL. Among them, *S. parvulus* VCCM 22513

showed the highest chitinase activity (0.83 ± 0.08 U/mL) and then was selected for further studies.

### Chitinase production from *Streptomyces parvulus* VCCM 22513

The chitinase production of *S. parvulus* VCCM 22513 on the yeast extract salts medium was explored. *S. parvulus* VCCM 22513 did not produce chitinase after 24 h of incubation (Fig. 1), which could be due to the enrichment of cells. Prolonging incubation time was subjected to a significant increase of chitinase activity. At 48 h, chitinase activity was 0.08 ± 0.02 U/mL. Of note, the highest chitinase production was observed after 96 h (0.91 ± 0.04 U/mL), followed by a slight decrease after 120 h (0.66 ± 0.05 U/mL). These indicated that colloidal chitin was degraded by chitinase from *S. parvulus* VCCM 22513. Chitinase from *Streptomyces rubiginosus* isolated from the rhizosphere of *Gossypium* sp. was shown to produce the highest chitinase of 2,790 U/mL after optimization (Jha et al., 2016), which was around 3-fold higher than our study. Thus, optimization of chitinase production of VCCM 22513 could be an interesting subject for future study.

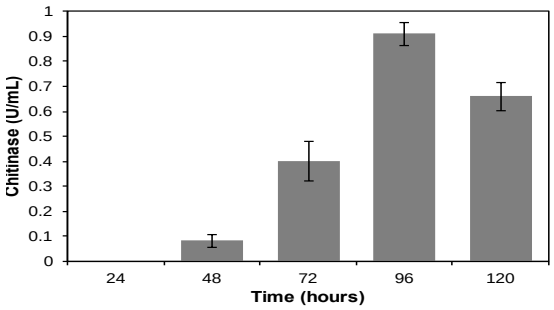


*Figure 1.* Chitinase production of *Streptomyces parvulus* VCCM 22513 determined at different incubation periods

### CAZyme identification in the *Streptomyces parvulus* VCCM 22513 genome

To confirm the chitin-degrading genotype leading to the observed phenotype, genomic analysis of *S. parvulus* VCCM 22513 was performed. The genome of *S. parvulus* VCCM 22513 consists of a linear chromosome with a size of 7,688,855 bp and 72.1% GC content, assembled into 62 contigs of which 6782 CDSs were predicted (Fig. 2).

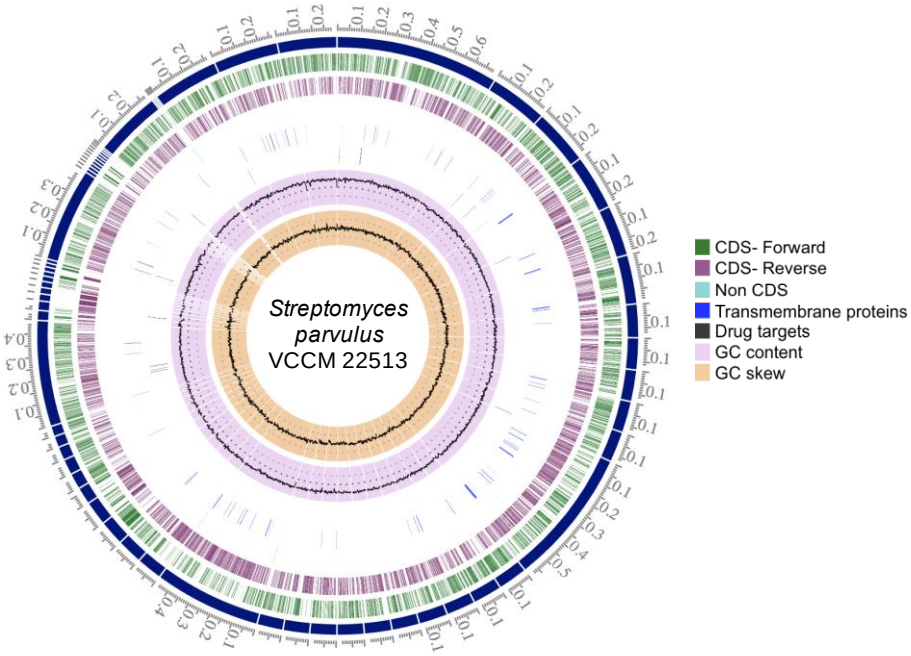


*Figure 2.* Circular map of *Streptomyces parvulus* VCCM 22513 genome

Since CAZymes are essential to degrade polysaccharides, the CAZymes of the VCCM 22513 were predicted using dbCAN2 with at least two databases (HMMER, DIAMOND or eCAMI). The result showed that VCCM 22513 genome contained 341 CAZy genes belonging to one or more CAZy domains (Fig. 3). The dominant enzyme family was GH with a total of 134 genes, followed by 88 CBMs, 87 GTs, 18 CEs, 7 PLs and 7 AAs. Similarly, the genome of chitin-degrading *Streptomyces diastaticus* CS1801 also comprised 254 CAZy genes including 90 GHs, 54 GTs, 53 CEs, 29 AAs, 22 CBMs, and 6 PLs (Xu et al., 2020).

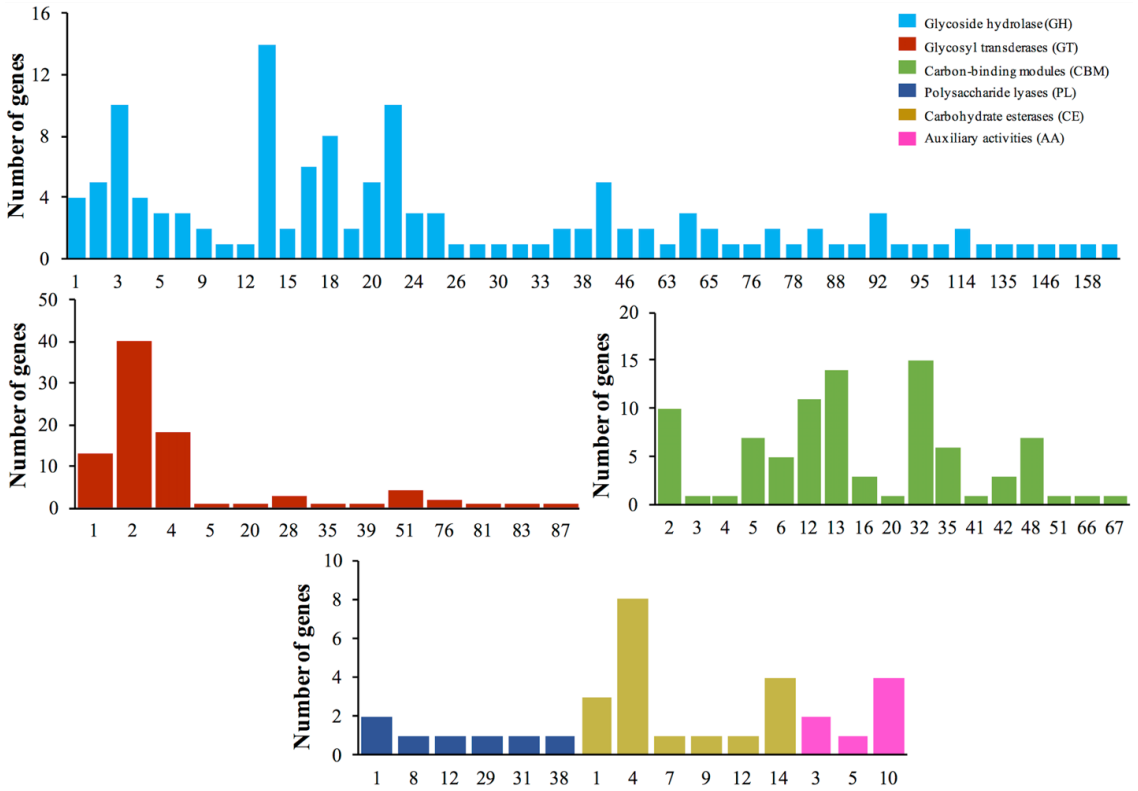


*Figure 3.* Distribution of different CAZyme families in the VCCM 22513 genome

Regarding the GH family, there were 50 different GH sub-families of which GH20 (5 genes), GH18 (8 genes), GH23 (10 genes), and GH3 (10 genes) are amongst the most abundant GH sub-families (Fig. 3). Since GH18 is known to contain chitinases that hydrolyze the β-1,4 bonds of chitin to produce oligomeric, dimeric, or monomeric GlcNAc (Suma & Podile, 2013), the presence of 8 GH18 genes strongly indicated bioconversion of chitin in strain VCCM 22513. In addition, 2 genes in the VCCM 22513 genome were found belonging to the GH19 sub-family which is a bifunctional family of chitinases (Abady et al., 2022). Thus, the presence of GH18 and GH19 highlighted the potential of strain VCCM 22513 in degrading chitin.

On the other hand, 88 GTs belonging to 13 sub-families were predicted in the genome. The major sub-family was GT2 (40 genes), followed by GT4 (18 genes), and GT1 (13 genes) (Fig. 3). Given that GTs contribute to the biosynthesis of disaccharides, oligosaccharides, and polysaccharides (Coutinho et al., 2003), they might not involve in chitin degradation. Similar to GTs, PLs represented by 5 sub-families also did not contribute to chitin hydrolases. In contrast, CBMs that support chitinase binding to

insoluble chitin and substrate accessibility (Paulsen et al., 2016) existed in the VCCM 22513 genome with CBM32, CBM13, CBM12, and CBM2 being the most abundant. In the case of CEs, CE4 was the most predominant category in the VCCM 22513 genome which functioned as chitin deacetylase responsible for the conversion of chitin to chitosan. Among 7 AAs, 4 AA10 genes encoding for lytic polysaccharide monooxygenases (LPMOs) may enhance chitinase activity during the degradation process (Gutiérrez-Román et al., 2014).

**Genome mining of chitin-degrading genes**

Further genomic analysis revealed the presence of 10 genes encoding chitinases, including *orf_138, orf_437, orf_652, orf_924, orf_1232, orf_1248, orf_3487, orf_4484, orf_4832,* and *orf_5074* (Table 1). All predicted chitinases belonging to either GH18 or GH19 sub-families contained a signal peptide at the N-terminal indicating that these enzymes are secretory. Notably, *orf_652* and *orf_5074* shared 46% and 37% sequence similarities with *chiA* of *Bacillus cereus*, respectively, while *orf_924* showed 74% identity with *chi40* of *Streptomyces thermoviolaceus* OPC-520. Prediction of the functional domains revealed that genes *orf_652*, *orf_924*, and *orf_5074* consisted of either CBM2 or CBM16 at the N-terminal and a fibronectin type III (Fn3) located in the middle region. Since Fn3 domains are frequently found in chitinases (Zhong et al., 2015), this domain might contribute to the degradation of insoluble and crystalline chitin. Besides, 6 out of 10 chitinase genes consisted of either CBM2 or CBM16 domain might enhance binding affinity to chitin substrates (Paulsen et al., 2016). In contrast, *orf_437*, *orf_1232*, *orf_3487*, and *orf_4484* were predicted to be chitinases with a single domain (Table 1), among them *orf_1232* displayed the highest similarity (72%) with chitinase from *S. thermoviolaceus* OPC-520. A previous study showed that there were only 9 chitinases encoded in the *S. diastaticus* CS1801 genome (Xu et al., 2020), which was lower than that of *S. parvulus* VCCM 22513.

Apart from that, four LPMOs (*orf_94*, *orf_1544*, *orf_1766*, *orf_4485*) belonging to the AA10 subfamily were identified in the VCCM 22513 genome (Table 1). Of these, only *orf_1766* had a CBM2 domain. The four LPMOs shared sequence similarity of 78%, 57%, 50% and 56% with TfAA10A of *Thermobifida fusca*, CbpD of *Pseudomonas aeruginosa* PAO1, ScLPMO10C of *Streptomyces coelicolor* A3(2), and JdLPMO10A of *Jonesia denitrificans*, respectively. LPMOs enhance chitinase activity by binding to the chitin chains and cleaving the glycosidic bonds via an oxidative mechanism (Jensen et al., 2019).

Genome mining further revealed the presence of 14 genes encoding β-N-acetylhexosaminidase that degrade chitin oligosaccharides into monomers (Table 1). However, only a few β-N-acetylhexosaminidase was found to degrade chitin, which are listed in the BRENDA database (http://www.brenda-enzymes.info/index.php4). For example, β-N-acetylhexosaminidase produced by *Vibrio harveyi* hydrolyzed colloidal chitin due to a binding pocket containing four N-acetylglucosamine binding subsites (Wang et al., 2019).

Taken together, both phenotypic and genomic analyses proved the capability of degrading chitin of *S. parvulus* VCCM 22513. It inferred that VCCM 22513 degraded colloidal chitin using endochitinase that hydrolyzes the insoluble chitin to yield water-soluble oligomers, especially $(GlcNAc)_2$. The second pathway is attributed to exochitinase which cleaves chitin biopolymers into GlcNAc. Thirdly, VCCM 22513 could utilize β-N-acetylhexosaminidase with unique enzymatic activity to directly hydrolyze colloidal chitin. Alternatively, β-N-acetylhexosaminidase with no activity on chitin only hydrolyzes $(GlcNAc)_2$. Thus, the degradation of chitin by β-N-acetylhexosaminidases will be a topic worth exploring in the future study.

*Table 1.* Detected genes encoding enzymes involved in chitin degradation in *Streptomyces parvulus* VCCM 22513

| Predicted function | EC | Predicted genes | Domains |
|---|---|---|---|
| Chitinase | 3.2.1.14 | *orf_138* | SP(1-38) - CBM2(34-142) - GH18 (194-476) |
| | | *orf_437* | SP(1-29) - GH18(53-311) |
| | | *orf_652* | SP(1-36) - CBM16(40-150) - Fn3(176-259)-GH18 (273-589) |
| | | *orf_924* | SP(1-31) - CBM2(35-131) - Fn3(142-229) - GH18(239-602) |
| | | *orf_1232* | SP(1-38) - GH18(52-410) |
| | | *orf_1248* | SP(1-40) - GH18(78-708) - CBM5(735-780) |
| | | *orf_3487* | SP(1-35) - GH18(51-307) |
| | | *orf_4484* | SP(1-30) - GH19(44-248) |
| | | *orf_4832* | SP(1-31) - CBM5(36-80) - GH19(94-296) |
| | | *orf_5074* | SP(1-36) - CBM16(39-152) - Fn3(183-266) - GH18(277-567) |
| LPMO | 1.14.99.53 | *orf_94* | SP(1-29) - AA10(30-199) |
| | | *orf_1027* | SP(1-31) - AA10(31-169) |
| | | *orf_1544* | SP(1-33) - AA10(34-211) |
| | | *orf_1766* | SP(1-35) - AA10(35-225) - CBM2(265-354) |
| β-N-acetyl hexosaminidase | 3.2.1.52 | *orf_156* | SP(1-32) - GH20b(47-147) - GH20(173-497) |
| | | *orf_187* | SP(1-41) - GH3(58-599) |
| | | *orf_533* | GH3(44-706) - Fn3(735-805) |
| | | *orf_1027* | GH3(39-329) |
| | | *orf_1948* | GH3(37-651) - Fn3(688-757) |
| | | *orf_3928* | GH3(39-328) |
| | | *orf_4446* | GH20b(8-135) - GH20(136-480) |
| | | *orf_4670* | SP(1-31) - GH3(51-606) |
| | | *orf_5527* | GH20b(70-188) - GH20(192-519) |
| | | *orf_5360* | GH3(37-705) - Fn3(741-811) |
| | | *orf_6020* | GH20b(6-133) - GH20(131-483) |
| | | *orf_6133* | GH20b(27-70) - GH20(81-312) |
| | | *orf_6193* | GH3(76-638) - Fn3(676-746) |
| | | *orf_6497* | SP(1-55) - GH3(107-734) - Fn3(767-838) |

*Note:* SP: signal peptide; Fn3: fibronectin type-3 domain; GH20b: N-terminal non-catalytic GH20 domain. Numbers in bracket represent the starting and ending amino acid positions.

## CONCLUSION

The present study is the first to decipher chitinase production of *S. parvulus* VCCM 22513 at genotypic and phenotypic aspects. The highest chitinase production of *S. parvulus* VCCM 22513 was observed after 96 h when colloidal chitin was supplemented as sole carbon. Genomic analysis revealed the presence of 10 chitinases, 4 LPMOs, and 14 β-N-acetylhexosaminidase, significantly contributing to chitin degradation. Further study is needed to clone and characterize chitinases and β-N-acetylhexosaminidases for the degradation of chitinous materials in biotechnological applications.

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