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CHITINASE CHRONICLES: UNVEILING EVOLUTION, MECHANISMS, CLONING, AND APPLICATIONS

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ABSTRACT

Chitin, the second most ubiquitous polysaccharide (after cellulose) is found in the shells of crustaceans, insects, and some fungi and in the internal structures of many vertebrates. *Chitinases* catalyze the cleavage of β-1,4 glycosidic linkages in chitin and contribute to the generation of carbon and nitrogen in the ecosystem. *Chitinases* can be used to control pests and diseases in plants. *Chitinases* can also enhance plant growth by breaking down chitin in the soil and making nutrients more available to plants. *Chitinases* can be used to break down chitin in agricultural and industrial waste to produce biofuels. *Chitinases* can also be used to develop new anti-fungal drugs to treat fungal infections. In the food industry *chitinases* can be used to extract protein from shrimp and other crustacean shells, that can be used as a food ingredient. Further, *chitinases* can be used in the textile industry to improve the texture of the fabric and waste management strategies. Overall, *chitinases* have a wide range of potential applications in various industries, and ongoing research is exploring new applications for this versatile enzyme. Conceivable outcomes of a few possible utilizations of *chitinase*s make it an intriguing objective polymer for protein engineering. Hence, this article focuses on properties of *chitinase*, its mechanism of action, cloning strategy for commercial production and various associated applications. Here, *in-silico* agarose gel analysis of the PCR product with 1Kb maker has been carried out after cloning.

Keywords: *Chitinase*, Chitin, Bacteria, Evolution, Mechanism, Cloning, Diversity

SIGNIFICANCE

- *Chitinase* holds significant industrial applications due to its ability to break down chitin, a sturdy biopolymer found in the exoskeletons of insects and crustaceans.
- *Chitinase* also plays a pivotal role in waste management by aiding in the degradation of chitin-rich materials like shrimp shells, contributing to environmentally friendly waste disposal.
- *Chitinases* versatility and eco-friendly nature make it a valuable enzyme in diverse industries.
- *Chitinase* TA cloning is significant as it enables the efficient insertion of PCR-amplified DNA fragments into vectors. It simplifies molecular biology research, aiding in gene manipulation, protein expression, and functional studies.

ABBREVIATIONS

- *Bt - Bacillus thuringiensis*
- bp – Base pairs
- GH – Glycoside Hydrolase
- Kda – Kilo daltons
- PRP – Pathogenesis Related Proteins
- PCR – Polymerase Chain Reaction
- ROS – Reactive Oxygen Species
- BOD – Biological Oxygen Demand
- ML – Maximum Likelihood
- JTT – Jones-Taylor-Thornton
- *K. lactis – Kluyveromyces lactis*
- ssDNA – Single Stranded Deoxyribonucleic acid
- dsDNA – Double Stranded Deoxyribonucleic acid
- TBE – Tris Borate Ethylenediamine tetra acetic acid
- Tm – Melting temperature
- SCP – Single Cell Protein
- *S. violaceusniger – Streptomyces violaceusniger*
- *S. thermoviolaceus – Streptomyces thermoviolaceus*
- *T. harzianum - Trichoderma harzianum*
- *R. oligosporus - Rhizopus oligosporus*

BACKGROUND

Enzymes are the proteins that increase the rate of chemical reactions in living bodies. They perform anabolic and catabolic activities. Enzymes are specific to their substrate, pH and temperature and, like catalysts, participate in many metabolic reactions. Enzymes have been studied for a long time about their tremendous potential. The research field in enzyme engineering is becoming more accessible for the creation of novel enzymatic functionalities **(Chen and Arnold 2020; Oyeleye and Normi 2018)**. *Chitinases* offer great biotechnological promise in various fields like health, and food and agricultural technology (figure 1) due to their catalytic action.

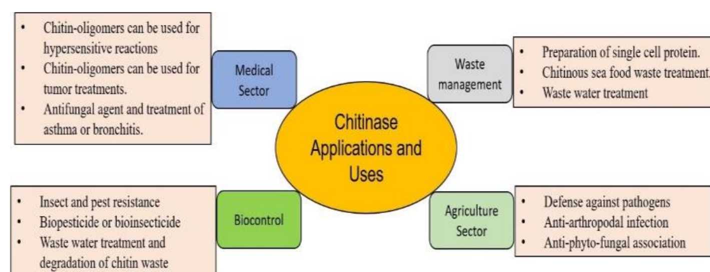

Figure 1 Various industrial applications of *Chitinase* **(Taokaew and Kriangkrai 2023; Mahajan *et al.,* 2023)**

Chitinases hydrolyze *β*-1,4 linkage of N-acetyl glucosamine present in chitin chains of different sizes (20 kDa to 90 kDa) **(Bhattacharya *et al.,* 2007; Daulagala 2021)**. **Glycoside hydrolases**, a prevalent enzyme hydrolyzes glycosidic bond present between two carbohydrates molecules or a carbohydrate having non-carbohydrate part. Due to clear correlation between amino acid sequences and folding, glycoside hydrolases have been categorized into several families. The ***Glycoside hydrolases*** classification explains structural characteristics of enzymes and aids in the discovery of evolutionary connections. Additionally, this classification offers a quick method for determining the relationships and structural details between members of enzyme family and their associated substrate specificity **(Cantarel *et al.,* 2009; Schomburg and Schomburg 2010)**.

The present article focusses on the importance and various environmental applications. The article also depicts industrial applications of *chitinase* including its commercial production. The phylogenetic analysis of *chitinase* has been conducted to retrieve the vulnerability and ancestral homology. An effective cloning strategy has been designed for commercial production of *chitinase*.

Several research articles on *chitinase* effectivity, mechanism of action etc., are available but none of them has its ancestral study and an experimental organism for study. To understand a particular biomolecule, one should consider an experimental organism, so that complete ancestral, structural, and analytical studies can be carried out. A considerable research gap was identified regarding *chitinase* categories, ancestral relationship, and commercial production. To study the said research gap, *Bacillus thuringiensis* was used as an experimental organism and the findings are illustrated in this article.

CLASSIFICATION OF *CHITINASE*: TYPES AND FAMILIES

Endochitinases and *exochitinases* are the two major subcategories of chitin-lytic enzymes. *Exochitinases* are additionally grouped in two classes, the enzymes *chitobiosidases*, which catalyze the gradual release of *di-acetylchitobiose* from the N-terminal non-reducing end, and *N-acetylglucosaminidases*, which break down the oligomeric products using *endochitinases* into N-acetyl glucosamine (GlcNAc) monomers **(Nayak *et al.,* 2021)**. *Chitinases* from different species are classified into families 18, 19, and 20 based on similarities in amino acid composition **(Henrissat 1999; Franceus *et al.,* 2021)**. *Chitinases* from viruses, bacteria, fungi, mammals, and some plant species belongs to family 18. Family 18 is made up of many conserved amino acids repetition units with 8 parallel sheets that create a barrel-shaped down-helix, which in turn forms a ring that faces outward **(Gooday 1999; Singh *et al.,* 2021)**. Most plant *chitinase*s beside *Streptomyces chitinase*s are members of family 19 **(Hart *et al.,* 1995)**. Family 18 and family 19 *Chitinases* have different 3D structures due to varied amino acid sequences. They have therefore developed from several forebears, according to this. The enzyme *N-acetylglucosaminidases* from family 20, is present in humans, fungi, and bacteria. Chitolectins is another protein family (GH18) that includes *chitinases* and their homologous proteins. However, they still include extremely conserved residues necessary for attachment of oligosaccharide and 3D structural stability. Chitinolectins lack the essential active-site residue (glutamate), which supplies the proton needed for hydrolytic activity. *Chitinases* are often categorized into either GH18 or GH19 *glycoside hydrolase* families, each of which has a unique structure and catalytic process **(Ohno *et al.,* 1996; Chen *et al.,* 2020)**. The plants, nematodes, and certain bacteria were found to have the majority of GH19 *chitinases* **(Grover 2012)**. Recent studies indicate that the GH48 and GH20 protein families also contain *chitinase* activity **(Kubota *et al.,* 2004; Fujita *et al.,* 2006; Orlando *et al.,* 2021)**. By hydrolyzing N-acetylglucosamine, the *N-acetyl-D-glucosaminidases* of GH20 family breakdown chitin (GlcNAc) **(Mark *et al.,* 2001)**.

Chitinase family 18 has a lot of repeating conserved amino acids. It is made up of an eight-stranded core of parallel sheets, a barrel oriented downward with helices, and ultimately a ring facing outward **(Gooday 1999)**. The transglycosylation products of *Trichoderma harzianum Chit42*, *Chit33* and *Aspergillus fumigatus ChiB1* has already been reported **(Martinez *et al.,* 2012)**. The amino acid sequence of enzyme *chitinase*s, belonging to two separate families are not identical, including their entirely different 3D structures and molecular processes. They most likely descended from several forebears in this way. The GH family 18 *chitinases* possibly catalyze trans-glycosylation processes **(Orlando *et al.,* 2021)**.

EVOLUTIONARY ANALYSIS OF *BACILLUS THURINGIENSIS* (INSECTICIDAL) *CHITINASE*

The relevance of evolution to microbial waste control agents primarily depends on two wide-ranging categories: effectiveness and risk. The impact of microevolution on chitin waste management efficacy has focused on the evolution of chitin metabolizing microflora **(Cory and Franklin 2012; Chakravarty and Edwards 2022)**.

The yearly chitin production in aquatic environment is around 1011 tons **(Das *et al.,* 2012; Senevirathna *et al.,* 2022)**. *Chitinases* synthesized by chitin-lytic bacteria degrades chitin into significant pharmaceutical end products like, N-acetyl glucosamine and chito-oligosaccharides, which are feasible substitutes of chemical methods presently being employed to achieve this goal. Enzymatic hydrolysis is the greatest solution to this issue because chemical-based remediation can cause environmental deterioration.

Bt-Chitinase usage in chitin waste management approaches, like chitin augmentation to inundate entomological control, with due consideration of *chitinase* evolutionary principles that may influence the success rate of above strategies. Moreover, the byproducts of *chitinases* action over chitin are water soluble possessing anti-cancerous, anti-microbial and immuno-boosting activities **(Kim *et al.,* 2022)**.

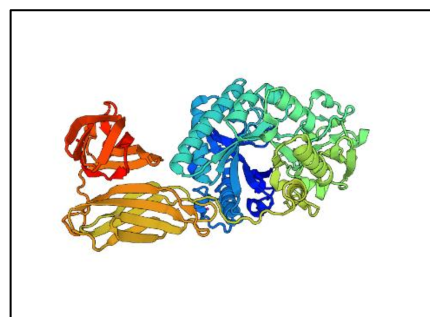

Figure 2 3D structure of *Bacillus thuringiensis chitinase* enzyme retrieved from genbank of NCBI

To perform evolutionary study, the *chitinase* amino acid sequence of *Bacillus thuringiensis* (Bt) is retrieved from genbank of NCBI with accession number ABO63962.1, the 3D structure of which is represented in figure 2. The evolutionary history (figure 3) was inferred by using MEGA-X software through Maximum Likelihood (ML) method based on the Jones-Taylor-Thornton (JTT) matrix-based model **(Jones *et al.,* 1992)**. The JTT model is based on empirical amino acid exchange matrices calculated from protein alignment databases that consider the average amino acid patterns of the set of data under study. The ML method is employed for generation of dendrogram and estimation amino acid substitution for any sort of microbial species. The best phylogeny and branch lengths were discovered together with the amino acid substitute matrix that produced the maximum probability for an aligning of sequences linked by single phylogenetic tree. By utilizing all the information included across the sequences at all homology levels and employing a model which specifically permits multiple modifications to take place on a single branch at any location inside an alignment. This ML technique overcomes the challenges associated with the counting approaches. The ML estimation method utilizes sequence data information more efficiently with no systematic error and generates a single phylogenetic tree. The evolutionary history of the examined taxa is represented by the bootstrap consensus tree estimated from 500 repetitions **(Felsenstein 1985)**. Branch collapse occurred for partitions repeated in fewer than 50% of bootstrap repetitions. In the bootstrap test, the proportion of duplicate trees in which the linked taxa clustered together is displayed next to the branches **(Felsenstein 1985)**. The analysis involved 51 amino acid sequences. All positions with less than 95% site coverage were eliminated. There was a total of 674 positions in the final dataset. Evolutionary analyses were conducted in MEGA-X **(Kumar *et al.,* 2018)**. Due to differences in amino acid position and number, the bootstrap values have a short range of variations. The dendrogram also depicts that the GH family of *chitinase* strongly relates with the *Bacillus* species and subspecies.

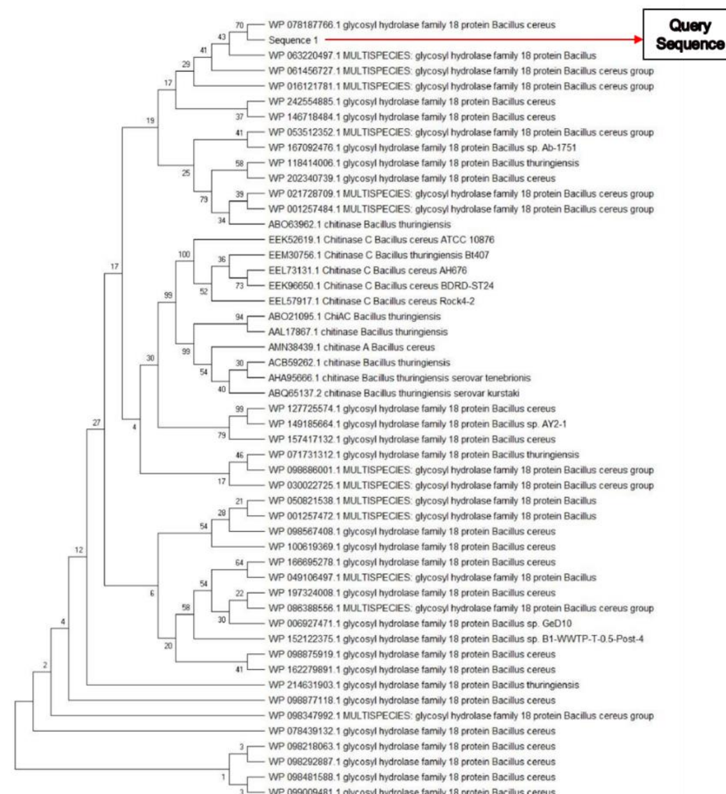

Figure 3 Phylogenetic tree (Neighbour-joining) based on *Chitinase* amino acid sequences of *Bacillus thuringiensis (Bt),* showing closest relationship with

glycosyl hydrolase family 18 protein of *Bacillus cereus*. The numbers in parenthesis indicate bootstrap values (based on 1,000 re-samplings). Bar, 0.02 nucleotide substitutions/position

MULTIPLE *CHITINASE* PRODUCTION

The *S. marcescens* generates three distinct *chitinase*s of GH family 18, is an example of a bacterial species that has been documented to produce several *chitinase*s **(Watanabe *et al.,* 1997)**. The *Streptomyces coelicolor* have 13 types of *chitinase*s distributed in GH 18 and 19 families **(Kawase *et al.,* 2006)**. The *Paenibacillus* sp. Str. FPU-7 secretes seven different *chitinase*s **(Yang *et al.,* 2016)**, moreover, certain fungus has been found to secrete over 20. It is thought that lateral gene transfer from one organism to another causes a single organism to produce several *chitinase*s **(Itoh *et al.,* 2016)**. It is believed that the many *chitinase*s released by these organisms provide structural and nutritive roles or work in concert to efficiently hydrolyze chitin for carbon and nitrogen source. Moreover, we must concentrate on the identification of other microbial species having the property of multiple *chitinase* production that belongs to different families.

PHYTO-*CHITINASE*

Plants are highly equipped with a variety of defense mechanisms to protect themselves against the attack of pathogens. *Chitinase*s are constitutively present in stems, seeds, flowers in a plant and tubers. *Chitinase*s in plants are developmentally controlled and tissue specific. Plant derived *chitinase*s have different amino acid series and are sorted in 5 or 6 classes. Globular domains are found in class I, II, and IV enzymes' central structure. While 8 (eight) α-helices and 8 (eight) β-strands form the class III and V of plant *chitinase*s. The class III enzymes carry out hydrolysis of β-1, 4-glycosidic linkage by an inverting mechanism, and the Class IV enzymes through retaining mechanism **(Fukamizo *et al.,* 2003; Poria *et al.,* 2021)**.

Plant synthesizes *chitinase*s as PRP, that regulates plant self-defense in response to phytopathogens attack, or by elicitors contact, such as chito-oligosaccharides **(Singh *et al.,* 2021)**. In comparison to the *chitinases* produced by insects, those produced by plants frequently have lower molecular weights.

Numerous environmental issues are linked to the use of chemicals to remove plant diseases. The two primary culprits behind plant diseases are fungal and insect infestations. Nevertheless, *chitinase* oversees hydrolyzing chitin because it is the main structural component of insects and fungi. Thus, there is a great deal of interest in *chitinase* synthesis to produce biopesticides or defense proteins, such as in genetically modified plants, as well as antibacterial compounds **(Khokhani *et al.,* 2021)**.

Although there is no apparent connection between the production of *chitinase* and the generation of oxygen radicals (ROS), various studies have shown that the two processes cooperate to protect plant vegetation against anthropogenic effects.

BACTERIAL *CHITINASES*

During the previous years, *chitinase*s have gained increased consideration because of its wide applications (Table 1). Chitin is broadly dispersed in nature as a major component of crab's shell and shrimps, exoskeletons of insects, and cell wall of various fungi. The structural similarity of chitin with cellulose (presence of acetamide group ($NH.CO.CH_3$) at C-2 position in chitin), makes it highly vulnerable like cellulose in nature.

Table 1 Bacterial sources of *chitinase* and their properties

Bacteria Name	Chitinase Type	Potential Usage	Reference
Streptomyces griseus HUT 6037	Endochitinase C-1 and C-2	Antifungal biocontrol agent	**Mitsutomi *et al.,* 1995**
Streptomyces lydicus WYEC108	Endochitinase	Antifungal biocontrol agent	**Yuan *et al.,* 1995**
Streptomyces violaceusniger XL-2	Endochitinase	Fungi *Phanerochaete chrysosporium*	**Shekhar *et al.,* 2006**
Bacillus thuringiensis	Endochitinases (*chiA74*),	larvicidal activity & Insecticidal activity	**Regev *et al.,* 1996**
Serratia marcescens	Endochitinase (Chi60), exochitinase (Chi50)	Chitinolytic activity	**Xie *et al.,* 2021**
Bacillus cereus 6E1	Exochitinase (Chi36)	Chitinolytic activity	**Wang *et al.,* 2001**
Bacillus amyloliquefaciens V656	Endochitinase	Antifungal activity	**Yuan *et al.,* 1995**
Burkholderia gladioli CHB101	Family 19 chitinase (ChiB),	Antifungal activity	**Shimosaka *et al.,* 2001**

Bacteria utilize *chitinase* to break down GlcNAc-containing macromolecules (chitin) to access their source of nutrients. The development of many marine bacteria ecologically depends on the *chitinase* activity. *Chitinases* produced by bacteria range in size from 20 to 60 kDa, which is smaller than insect *chitinases* (40 to 85 kDa) and comparable to plant *chitinases* (25 to 40 kDa) **(Kotb *et al.,* 2023)**. However, they also depend on the bacteria from which they were derived. Microbial *chitinases* normally work in a wide variety of pH and temperature settings **(Kotb *et al.,* 2023)**. The optimal temperatures for the endochitinases isolated from *S. violaceusniger* and *S. thermoviolaceus* OPC-520 are 28^0C and 80^0C, respectively. While the *chitinase* isolated from *Stenotrophomonas maltophilia* C-3 has a pH range of 4.5 to 5.0 and the *chitinase* of *S. thermoviolaceus* OPC-520 has a high pH range of 8.0 to 10.0 **(Nayak *et al.,* 2021)**.

FUNGAL *CHITINASE*

Fungi producing *chitinase* degrades chitin for its own defense for survival and to obtain nutrients saprophytically **(Homthong *et al.,* 2016)**. Fungal *chitinase* is synthesized from *chitinase* genes, that are induced by the presence of chitin **(Deng *et al.,* 2007)**. Moreover, excessive varieties of *chitinase* are found among fungal species, producing different *chitinase* having distinct catalytic properties (Table 2).

Table 2 Fungal *Chitinase* and Their Activities

Microorganisms	Protein/Gene	Activity	Reference
Histoplasma capsulatum	Cts5	Exochitinase/ Chitobiase	**Goughenour *et al.,* 2021**
Aspergillus niger	Cfu1	Exochitinase	
Aspergillus fumigatus	ChiA1	Endochitinase	
Saccharomyces cerevisiae	Cts1	Endochitinase	
Beauveria bassiana	Chit1	Endochitinase	
Kluyveromyces lactis	Cts1	Endochitinase	
Candida albicans	Chit2	Endochitinase	

Based on similarities in the amino acid arrangement of the catalytic site *chitinases* were categorized under the glycosyl hydrolase system **(Henrissat 1999; Nakamura *et al.,* 2021)**. Because they are found in bacteria, fungi, yeast, viruses, plants, and mammals, the family 18 *chitinases* have a diverse evolutionary history. Members of family 19 are nearly entirely found in plants **(Banerjee and Mandal 2019)**. In the glycohydrolase family of proteins, family 18, has the majority of fungal chitinases **(Jiménez-Ortega *et al.,* 2022)**. *Chitinases* from family 18 have a multi-domain organization as one of their traits **(Jiménez-Ortega *et al.,* 2022)**. The N-terminal signal peptide region, catalytic domain, ser/thr-rich region, chitin-binding domain, and C-terminal expansion region are the five domains (regions) that make up a typical fungal *chitinase* structure. The final three domains are absent from the majority of fungal *chitinases*. Because naturally occurring *chitinases* lacking these domains (regions) are nevertheless enzymatically active, it suggests that these domains are not necessary for *chitinase* activity. May these domains help stabilize the enzyme's overall structure and catalytic site.

Each domain of the fungal *chitinase* structure serves a different metabolic purpose. Except for *R. oligosporus chitinase* III, **(Takaya *et al.,* 1998)** and *T. harzianum* Chi18-2, Chi18-3, and Chi18-7 **(Seidl-Seiboth *et al.,* 2014)**, all the decoded fungal *chitinases* have a signal peptide added before the N-terminal region of the final protein **(Sharma *et al.,* 2022)**. The signal peptide facilitates the enzyme's secretion

and is further detachable by signal peptidases once the protein has passed across the membrane. The *chitinase* that lack a secretory signal sequence are demonstrated to be intracellular, and their role in morphogenesis is expected **(Jeong *et al.,* 2023)**.
Apart from N-terminal signal peptide region and catalytic domain, the structure of some fungal *chitinase*s reported to have ser/thr rich region and chitin-binding domain, such as *endochitinase* of *S. cerevisiae*, CTS1, *K. lactis* KlCts1p, *T. reesei* Chi18-13 and Chi18-16 and *R. ologosporus chitinase* I and *chitinase* II **(Koutb *et al.,* 2021)**. To produce mature protein, the ser/thr rich region of fungal *chitinase* is often glycosylated post-translationally.

MECHANISM OF *CHITINASE* ACTION ON CHITIN

*Chitinase*s *chitodextrinase*, *glucosaminidase*, *glycanohydrolase* are hydrolytic enzymes that break down glycosidic bonds of chitin **(Singh *et al.,* 2021; Abulikemu *et al.,* 2021)**. As discussed above, *endochitinase* and *exochitinases* are the two primary groups of chitin-lytic enzymes. The group of *chitinase*s known as *endochitinases* randomly cleaves at internal chitin polymer sites to produce low molecular weight multi-subunit glucosamine residues **(Rathod *et al.,* 2015)**. The mechanism of *chitinase* action to derive fructose-6-phosphate that can be utilized as energy source is given in figure 4.

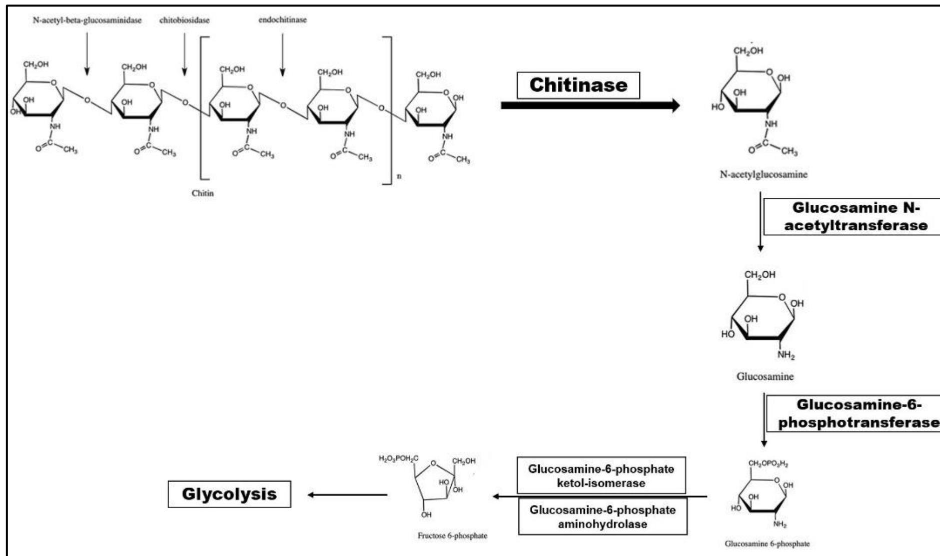

Figure 4 Mechanism of *chitinase* action on its substrate (chitin). The figure shows that *chitinase* performs a catalytic reaction with other two enzymes and leads to the production of Fructose – 6 – phosphate that is further utilized in glycolysis **(Beier and Bertilsson 2013; Stoykov *et al.,* 2015)**

Based on the specified site of action the enzyme commission (EC) has classified the chitinolytic enzymes into endo- and exo-acting enzymes. *Chitinases* (E.C 3.2.1.14), *endo-chitodextrinases* (EC 3.2.1.202), and *chitosanases* (EC 3.2.1.132) are endo-acting enzymes. Chitin is cleaved randomly along the inner chain by *chitinases* (EC 3.2.1.14), to produce water soluble oligomers of N-acetylglucosamine. The *endo-chitodextrinase* (EC 3.2.1.202), hydrolyzes chitodextrins and release N, N′-diacetylchitobiose, with slight quantities of N, N′, N″-triacetylchitotriose **(Poria *et al.,* 2021)**.

THE TA CLONING STRATEGY FOR COMMERCIAL PRODUCTION OF *CHITINASE*

To predict the industrial production of *chitinase* enzyme Snap gene tool was used. To perform the desired task reverse translation of the amino acid sequence (ABO63962.1) was carried out. The reverse translation was performed using Uniprot online tool that resulted in ssDNA. The ssDNA was then replicated to dsDNA by using Snap gene tool and then the resulted dsDNA was cloned by TA cloning method using Snap gene tool. At first the template (dsDNA) specific primers were designed having Tm – 60^{0}C and the PCR was performed using forward primer – AGATCACAAAAATTTACACTGCTGCTGCT (29mer) and reverse primer – TTGCTTCCCAGCCGCC (16mer). The PCR performance indicates that the desired inserted sequence can be identified by PCR after the TA cloning execution *invitro*. Now, the desired dsDNA sequence was inserted into double stranded pT7Blue T-vector of 2888 bp size that resulted in 3763 bp of recombinant vector (figure 5). The complete cloning history is given in figure 6.

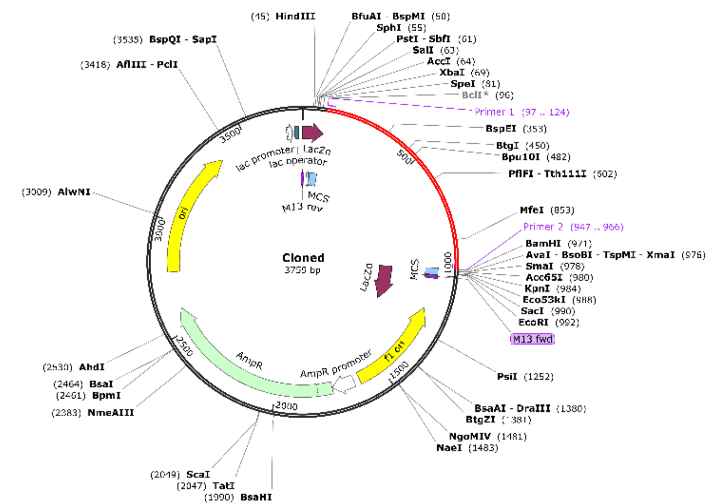

Figure 5 *In silico* TA cloning of the reverse translated dsDNA of *chitinase* amino acid sequence **(ABO63962.1)**. The dsDNA is inserted into the **pT7Blue T-vector** expression vector where the red region indicates the gene coding for *chitinase,* and the black colored region represents the vector. The inserted dsDNA sequence under control of lac operator with T7 promotor. The various restriction sites are also indicated with their restriction enzyme name

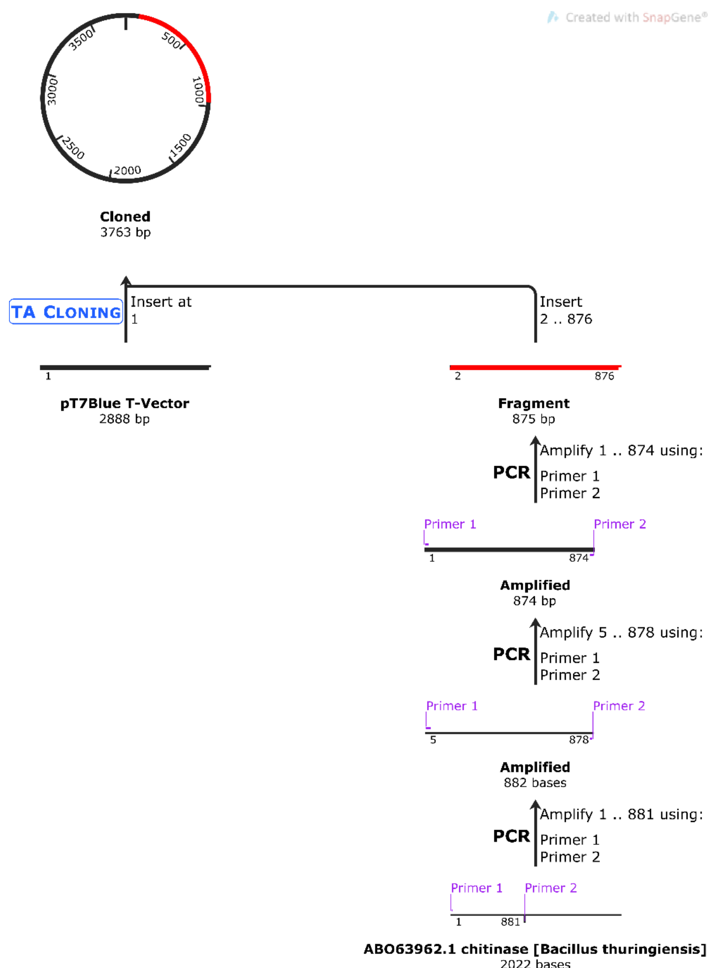

Figure 6 The TA cloning methodology flowchart with PCR primer lengths and amplified products length

The respective TA cloned product was then analyzed 1% agarose in TBE buffer (figure 7) with pH – 7.1 ± 1. The migration of supercoiled DNA relative to linear DNA depends on the gel buffer. The lane – 1 in gel image shows 874 bp of PCR product size with 3'A or T overhangs, the lane – 2 shows 2888 bp of digested vector and lane – 3 depicts 882 bp of PCR amplified dsDNA sequence. The size of the amplicon was compared with a molecular marker of 1Kb size as shown in lane – MW in figure 7. The resulted recombinant dsDNA can be cloned and expressed in BL-21(DE3) – Dam+Dcm– EcoK1–. The BL-21 strain of *E.coli* can be used to express pT7Blue T-vector expression vector, by using a suitable culture medium in a fed batch bioreactor.

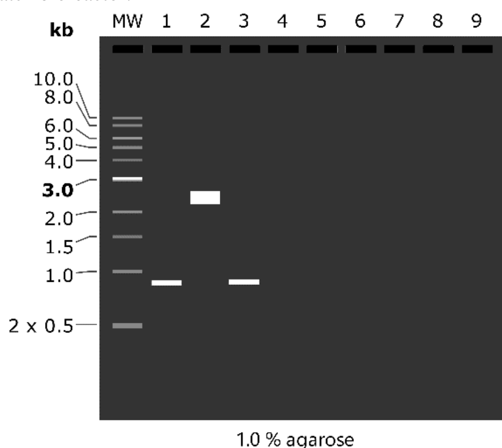

Figure 7 Image of Agarose Gel Electrophoresis (AGE), with 1Kb marker, 1% agarose gel in 1x TBE buffer

INDUSTRIAL USES OF *CHITINASES*

The *chitinase* mediated chitin hydrolysis produces such monomers that have significant industrial importance. *Chitinase*s can be a vital tool in waste management as they can break down chitinous waste into smaller, simple molecules that can enter rivers in a safe manner therefore solving environmental problems (**Rathod *et al.*, 2015**). In the food industry *chitinases* have a significant role. Earlier it was publicized that *Planococcus rifitoensis, chitinase* was used as a biocontrol agent for fungus called *Botrytis cinerea* (grey mould) which decay citrus fruits (**Essghaier *et al.*, 2010; Essghaier *et al.*, 2021**). Also, research has demonstrated that several microbial populations employ *chitinases* as an energy source (**Gomaa *et al.*, 2021**).

If fish waste from the sea food business enters rivers directly, it becomes a dangerous pollutant because it contains a lot of chitin. It raises BOD and requires a lot of demineralization, including acidic activity, as well as deproteinization (**Suryawanshi and Eswari 2021**). Recombinant *chitinases* reduce river pollution by creating chito-oligomers from chitinous waste, that are employed in a variety of biotechnological applications. Chito-oligomers are non-toxic, water-soluble substances that have antibacterial and anticancer properties as well as the ability to boost animal immunity (**Ibrahim *et al.*, 2016**). Additionally, *chitinase* turns chitinous waste into biofertilizers, boosting crop productivity (**Mishra *et al.*, 2016; Leite *et al.*, 2021**).

Chitinases are utilized in an indirect manner to estimate the biomass levels of fungi in soil. High amounts of *chitinase* in the soil indicate high quantities of fungi there (**Sharma *et al.*, 2022**). When examining the fungal populations in the necessary soil environment, this factor may be crucial (**Dukariya and Kumar 2020**).

In the food industry, *tannase* is used to break down the tannins in fruit juices like pomegranate, cranberry, etc. The fungus *Aspergillus niger* produces *tannase*, which binds to its cell wall and reduces the enzyme's total production (**Shao *et al.*, 2020**). The cell wall of this fungus may also be broken down using *chitinase*, which could increase the amount of *tannase* produced (**Barthomeuf *et al.*, 1994; Gezaf *et al.*, 2021**). In a recent investigation of chitinolytic activity of *chitinase Stenotrophomonas maltophilia* portrayed the highest enzymatic activity than other selected bacterial isolates (**Gonfa *et al.*, 2023**).

Hydrolytic products (chito-oligomers) of *chitinase* have a variety of industrial applications like, SCP production, pharmaceutically considerable chito-oligosaccharides, including antibacterial, antifungal, antihypertensive, and food quality-improving properties (**Gonfa *et al.*, 2023**).

AGRICULTURAL USES OF *CHITINASES*

*Chitinase*s act as biocontrol agent in plants as a part of its defense mechanism. The Phyto-*Chitinase* acts against fungal invaders and harmful insects having chitinous exoskeletons (**Banerjee and Mandal 2019**). Due to lack of a sophisticated immune system like humans, plants depend on inherited defense mechanisms like *chitinases* for protection against phyto-pathogens (**Dukare *et al.*, 2021**). A lot of research on overexpressing *chitinase*s was conducted previously in the transgenic plants to protect them from fungal pathogens, as *Chitinase* can break down the pathogens cell wall which is made-up of its substrate chitin (**Prasad *et al.*, 2013; Moosa *et al.*, 2018; Kumar *et al.*, 2018; Eslahi *et al.*, 2021; Yang *et al.*, 2020**). This has major significance in agriculture because crop losses due to pathogen attack can cost 200-300 billion US dollars annually (**Thakur *et al.*, 2021**). Most methods to protect crops involve chemicals like pesticides or fungicides which are harmful to living organisms and the environment, so *chitinase* offers a safe and natural mode of protection. It is also important to consider that crops like tobacco, tomatoes, cabbage, etc., can be saved that are usually affected by fungal pathogens. A recent study suggests that beside chemical pesticides, chitinase has demonstrated its ability to effectively suppress a range of harmful fungi and insect pests (**Mahajan *et al.*, 2023**).

PHARMACEUTICAL USES OF *CHITINASES*

In the pharmaceutical sector, chemotherapeutic medications, and allergies, *chitinase* serve a variety of purposes. Proteins with *chitinase* activity have been found to be biomarkers for cutaneous allergies and asthma in humans. Moreover, the increased immunological activity of Th-2 cells in the bronchioles is directly related to asthma. An earlier (2001) study exposed a link that a *chitinase*-like-protein upregulates CD4+ Th-2 cells in mouse due to a specific interleukin signaling (**Shekhar *et al.*, 2006; Sharma *et al.*, 2022**). It was the first time that an unknown link of *chitinase* with inflammation in the bronchioles due to asthma was explored and established (**Regev *et al.*, 1996**).

The chitooligosaccharide produced can also be employed in therapies for cancer due to their anticancer action, like that of earlier outlined *chitinase* antifungal activity in treatment of fungal infection (**Nayak *et al.*, 2021; Sharma *et al.*, 2022**). N-acetyl glucosamine's anti-inflammatory qualities make it a potential component of innovative and potent treatments for bacterial and fungal infections. If we can investigate functional relevance, substrate utilization qualities, and action mechanisms, *chitinases* might possibly serve as chemotherapeutic targets for many additional illnesses (**Xie *et al.*, 2021**). Chitinases also offer supportive statistics for their potential use as a biomarker in Th-2 associated alveolar infection, like that of *TKL-40* and *AMCase* (**Shekhar *et al.*, 2006; Regev *et al.*, 1996**).

CONCLUSION

The diverse family of *chitinases* have a unique molecular geometry, substrate selectivity, and catalytic mechanism. It is important to study and identify the *chitinase*-substrate specificity, as it could reveal its relationship with specific

substrate types, physiological roles and it can be re-designed to utilize chitin for generating novel products of industrial use.

Looking forward, *chitinase* has many significant applications in agricultural sector, industries, and pharmaceuticals. So, it is worth producing different *chitinase*s at a reasonable cost, using recombinant DNA technology. Their use as biological control agents against phyto-pathogens is by far their considerable successful application, but further investigation will bring out their complete potential.

Recombinant techniques can manufacture *chitinases* in large quantities for commercial use. Protein engineering might be used to create *chitinase*s with the necessary functionality to satisfy the demands of *chitinase*s for treating various disorders. It is critical to choose reactors carefully that promote the enhanced synthesis of *chitinase*s with the right parameters. It is mandatory to use appropriate carbon sources since they have a significant effect on the production of enzymes. Aeration and agitation rate have little bearing on the synthesis of *chitinase*. Therefore, scaling up is challenging without complete knowledge of the necessary characteristics. Not much research has been conducted using airlift and bubble column reactors.

Biological systems do not assemble chitin, indicating that the main factor governing chitin's own degeneration and turnover is its *de-novo* production. However, the prospects for chitin's synthesis of new biomass or its transformation into inorganic components are very different. There is little knowledge about the variables governing this discrepancy. The structure of the substrates and the bacterial population that uses chitin may also affect the substrate's preference for hydrolyzed metabolites. Habitat structure may influence the overall characteristics of the inheriting chitin-using population and may also influence the pace of chitin mineralization. This might have significant ramifications for carbon (C) and nitrogen (N) cycling in food webs i.e., via carbon or nitrogen removal because of mineralization and volatilization.

FUTURE PROSPECTS

Chitinase as a hydrolyzing enzyme has various applications in environmental, therapeutic, and industrial biotechnology sectors. High yield or improved catalytic activity are two ways that *chitinase*s might be used economically and practically. *Chitinase* engineering research is mostly focused on increasing its catalytic activity. Site-directed mutagenesis is a significant strategy that allows the mutation of specific amino acid moieties in chitin-binding catalytic site. For instance, the glutamic acid found in the active region of *chitinase* contributes significantly to the catalytic efficiency by serving as a proton contributor for breaking glycosidic bonds. Therefore, any modification close to glutamate improves the effectiveness of the enzyme.

Another approach can be directed evolution. Seeds that have been *chitinase*-treated are found to be more resistant to fungal infestations. To generate *chitinase*s with wide activity, directed evolution might be investigated. The function of fungal *chitinase*s in their interaction with plants, however, is not well understood. To comprehend the mechanism and function of fungal *chitinase* during plant pathogenesis, further study is thus necessary.

Chitinase produced by microbes and insects, has numerous applications in waste management, SCP production, biocontrol, etc. The use of *chitinase* as a biocontrol agent in agriculture has important implications since it offers a safe substitute for hazardous chemical-based pest and insects control agents.

For industrial scale *chitinase* production, submerged fermentation is the preferred method, producing higher yields; however, this method needs more investigation, particularly on heat transfer and downstream processing issues, prior to it being considered commercially viable.

Immobilized *chitinase* offers great potential by reducing the time and cost of waste elimination. But identifying a perfect cross-linker to develop immobilized *chitinase* enzyme formulation remains a major difficulty. Due to the wide range of applications the demand for *chitinase*s is anticipated to increase soon.

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