Bioresources and Bioprocessing
a SpringerOpen Journal

REVIEW

Open Access

Bacterial chitinase: nature and perspectives for sustainable bioproduction

Qiang Yan[1] and Stephen S Fong[1,2*]

Abstract

Concurrent advances in a number of fields have fostered the development of bioprocesses for biochemical production. Ideally, future bioprocesses will meet the demands of commercial chemical markets in an economical fashion while being sustainable through the use of renewable starting materials. A number of different renewable and abundant biopolymers (e.g., cellulose, hemicelluloses, lignin, and chitin) are potential starting material for sustainable bioprocesses, but a broad challenge remains on how to efficiently depolymerize these biopolymers to generate monomeric sugars that can be metabolized by industrial microorganisms or other useful building block chemicals. Indeed, a variety of specialty chemicals may be able to be generated from these various monomers. This review focuses on the biopolymer chitin and discusses research and knowledge relevant to chitin degradation and potential chemical products that can be made from chitin degradation products.

Keywords: Chitin; Chitinase; Biopolymer; Bioprocessing; Renewable; Sustainability

Introduction

Recent advances in biotechnology have fostered the development of bioprocesses to use engineered microorganisms to produce value-added chemicals. Chemicals produced through bioprocesses range from bulk commodity chemicals such as organic acids and biofuels to high-value specialty chemicals such as isoprenoid drugs. For specialty chemicals with high monetary value per unit, the emphasis has been discovery and production feasibility. For other chemicals where economic pressures constrain industrial scale processes, one area of focus has been on utilizing cheap, abundant starting materials [1, 2].

Biobased degradation of lignocellulosic biomass (cellulose, hemicelluloses, and lignin) was a logical focus of initial research efforts as cellulose is the most abundant available carbon substrate and potential mechanisms for breaking down lignocellulosic material should be identifiable from cellulolytic organisms. As the world's second most abundant carbon substrate, chitin has many similarities to cellulose: it is highly abundant in nature (10^{10}–10^{11} tons year^{-1}), it is a homopolymer of a simple

sugar (*N*-acetylglucosamine), and it requires specialized enzymes for degradation (chitinase), shown in Fig. 1. Due to these similarities, improvement of chitinase production has the potential to enhance the biorefinery industry by broadening the range of practicable biomass conversion and lowering the environmental impact of chemical-based industries. While there is now knowledge being gained on chitinases, challenges still remain related to economical use of chitinases at industrial scale. For example, since crustacean shells are not soluble in standard aqueous media, the direct degradation and separation of α-chitin from shrimp and crab shells (and/or microbial cell walls) poses a significant challenge. Discovery of new enzymes that function in the extreme environment are important for this purpose. It can be projected that focused efforts on understanding and developing methods for breaking down chitin will be needed [3].

In this review, we focused on structural and functional properties of chitin with an emphasis on mechanisms for chitin degradation by bacterial chitinases. More specifically, modes of action for identified classes of chitinases, experimental assays, and prospects for metabolic engineering targets of chitin degradation products will be presented.

* Correspondence: ssfong@vcu.edu
[1]Department of Chemical and Life Science Engineering, School of Engineering, Virginia Commonwealth University, West Hall, Room 422, 601 West Main Street, P.O. Box 843028, Richmond, VA 23284-3028, USA
[2]Center for the Study of Biological Complexity, Virginia Commonwealth University, Richmond, VA, USA

Springer

Chitin structure

Chitin, poly(β-(1 $\rightarrow$ 4)-*N*-acetyl-D-glucosamine (GlcNAc)), consists of *N*-acetylglucosamine by β-(1 $\rightarrow$ 4) glycosidic bond. In nature, there are two different crystalline forms, α and β. α-Chitin is by far the most abundant crystalline form where the linear chains of GlcNAc unit are arranged in an antiparallel manner. On the other hand, β-chitin consists of parallel chains of GlcNAc units [4]. As of the author's knowledge, it has not been possible to obtain β-chitin either from solution or by in vitro biosynthesis.

Bacterial chitinase family

Chitinases are encoded in a wide range of organisms including viruses, bacteria, fungi, insects, higher plants, and animals for different purposes such as nutrition, morphogenesis, and defense against chitin-containing pathogens [5–7]. Based on amino acid sequence similarity, chitinolytic enzymes are grouped into glycosyl hydrolase (GH) families numbered 18, 19, and 20. Bacterial chitinases are mainly from family 18, although some *Streptomyces* chitinases are from family 19. Family 20 includes the β-*N*-acetylhexosaminidases from bacteria [8, 9].

Bacterial GH 18 chitinases are classified into three subfamilies A, B, and C (again, based upon sequence homology). It should be noted that the nomenclature of bacterial chitinases does not follow this classification; for example, chitinase B from *Serratia marcescens* belongs to subfamily A while *Bacillus circulans* chitinase D is classified in subfamily B [7]. Subfamily A chitinases have the presence of a third domain corresponding to the insertion of an $\alpha + \beta$ fold region between the seventh and eighth $(\alpha/\beta)_8$ barrel [10]. None of the chitinases in subfamilies B and C have this insertion. Several chitinolytic bacteria that possess chitinases belonging to different subfamilies like *S. marcescens* and *B. circulans* WL-12 are reported [11, 12].

Chitinases structure

Chitinolytic bacteria generally produce multiple chitinases derived from different genes. Many chitinolytic bacteria produce only family 18 chitinases, whereas other bacteria such as the *Streptomyces* species produce family 19 chitinases in addition to family 18 chitinases [13].

Catalytic domain

GH18 chitinases catalytic domains have a $(\beta/\alpha)_8$ TIM-barrel fold with crucial catalytic residues being located on β-strand number 4 [11]. High-resolution 3D structures of bacterial chitinases from *B. circulans* (ChiA1) have been revealed by X-ray crystallography [14]. The catalytic domain of ChiA1 consists of a deep substrate-binding cleft on the top of its $(\beta/\alpha)_8$-barrel structure. A mechanism has been suggested by a hydrophobic stacking interaction between the aromatic residues in this cleft and bound oligosaccharide [15, 16]. Watanabe et al. reported that mutations of W433 and Y279 showed drastically reduced hydrolytic activity against substrates including crystalline chitin, colloidal chitin, and $(GlcNAc)_5$. W433 is considered to interact with −1 GlcNAc through a hydrophobic stacking interaction, holding this moiety at this position during the catalytic reaction. Y279 has been assumed to assist the formation of an oxazolinium ion intermediate [17]. In addition to the amino acid residues in the substrate-binding cleft, W122 and W134, two tryptophans, on the surface of CatD, are aligned on the extension of the nonreducing end side of the bound $(GlcNAc)_7$. Mutations of both sites reduced the hydrolyzing activity against highly crystalline β-chitin microfibrils [16].

Chitin-binding domain

GH18 chitinases are usually multi-modular. For example, ChiA1 from *B. circulans* WL-12 contains a C-terminal chitin-binding domain and two fibronectin III-like domains (FnIII-D-1 and FnIII-D-2) [18]; *S. marcescens* contains an N-terminal chitin-binding domain with a fibronectin III domain (ChiA), or a C-terminal chitin-binding domain (ChiB), or a C-terminal FnIII domain coupled with a downstream chitin-binding domain (ChiC) [11]. The properties and roles of the chitin-binding domain have been addressed in several studies but remain partly unresolved. The typical mechanisms of a chitin-binding domain function to facilitate correct positioning of the catalytic domain, to contribute to processive action, and to facilitate local decrystallization of the substrate. Several studies have confirmed that the presence of these domains increases substrate affinity as well as the efficiency of chitin hydrolysis, especially for more crystalline chitin.

Fibronectin III (FnIII) domains

The FnIII domain is one of the most commonly found motifs in animal proteins, and it has been proposed that bacterial FnIII domains were acquired from animals by horizontal gene transfer. This domain shares barely detectable sequence identity with FnIII modules between ChiA from *S. marcescens* and ChiA1 from *B. circulans* [19, 20]. For example, the FnIII domain from *S. marcescens* ChiA consists of aromatic residues that contribute to substrate binding and substrate hydrolysis [21]. In contrast to the N-terminal domain of ChiA from *S. marcescens*, the structurally characterized FnIII domain of ChiA1 from *B. circulans* does not have exposed aromatic residues on its surface [19]. Therefore, this domain does not appear to be directly involved in chitin binding. However, it is proposed that FnIII domains are important for enzyme activity, probably by affecting the overall enzyme structure and the spatial localization of the catalytic domain and the chitin-binding domain.

Chitinase processivity

Some of the challenges associated with insoluble chitin are disruption of crystal packing and guiding single-polymer chains into the catalytic center. Generally, processive degradation is thought to improve catalytic efficiency because single-polymer chains are prevented from re-associating with the insoluble material between catalytic cycles, thus reducing the number of times the enzyme has to carry out the energetically unfavorable process of gaining access to a single chain. The substrate-binding sites in processive chitinases and cellulases are lined with aromatic residues, in particular tryptophan residues. These residues are thought to facilitate processivity by functioning as a flexible and hydrophobic sheath along which the polymer chain can slide during the processive mode of action. The hydrophobic interactions are nonspecific and engage large interaction surfaces, thus providing strong but fluid binding while avoiding tight binding at any species sites that could hamper the sliding movement. A study by Horn et al. focused on using the gene *chib* from *S. marcescens* strain BJL200 to study chitinase processivity. They found that the loss of processivity in the W97A mutant was accompanied by a 29-fold increase in the degradation rate for single-polymer chains. The result showed that processivity comes at a large cost in terms of enzyme speed [22].

Chitinase activity assay

To explore the diversity of organisms and to suggest enzymes involved with chitin degradation, it is useful to have a sensitive, fast, and accurate assay to test and compare chitinase activity in a system-independent manner. Chitinase activity has been assayed by various methods. The most commonly used methods are viscosimetric, colorimetric, and radiochemical methods [23–25].

In the viscosimetric method, assessment of chitinase activity can be accomplished by monitoring the viscosity change of solubilized chitin derivatives, such as ethylene glycol chitin, carboxymethyl chitin, and 6-*O*-hydroxypropyl-chitin [26]. The viscosimetric method is more sensitive to endochitinases than other methods and is strongly affected by the degree of polymerization of the substrates as well as the ionic strength and pH of the solutions. Another disadvantage of the viscosimetric method is that the use of native unmodified substrate is highly preferred compared to the use of surrogate substrates that are chemically modified [23, 26].

The colorimetric method is based upon the determination of *N*-acetyl-D-glucosamine monomers released from colloidal chitin upon exposure to some oxidizing reagents. The most commonly used methods for measuring reducing sugar content are the 3,5-dinitrosalicylic acid (DNS) method and the ferricyanide-based Schales' procedure [27, 28]. The reduction of inorganic oxidants such as ferricyanide or cupric ions by the aldehyde/hemiacetal groups of the reducing sugar ends leads to color change that can be measured spectrophotometrically. However, there are certain drawbacks about these methods: (1) insensitivity at low range of sugar concentrations, (2) lack of specificity, and (3) difficulty in use in high-throughput screening [29–31].

Improvements to the colorimetric assay have been made by replacing the chemical oxidizing reagents. Chitin azure is prepared by covalently linking a suitable dye to colloidal chitin. When chitin azure is depolymerized, clear zones appear around colonies that are easier to visualize than the halos formed on chitin plates [26]. On the other hand, Ferrari et al. reported use of an oxidase to release hydrogen peroxide upon the oxidation of chitinase-produced hydrolytic products. The hydrogen peroxide produced can be monitored using a second enzyme, horseradish peroxidase (HRP), and a chromogenic peroxidase substrate. The detection limit is lower, and there is no requirement for harsh conditions for the development of the signal [29].

The third method is the radiochemical method, in which tritium-labeled chitin is incubated with the chitinase solution and chitinase activity is assayed by determining the radioactivity of the water-soluble chito-oligosaccharides formed [25]. This method could be regarded as the most sensitive assay method, but this method requires a radioactive substrate and specialized equipment.

With advances in mass spectrometry-based detection methods, a rapid and sensitive method based on the simultaneous detection of substrate and products by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry (MS) was also applied to assay chitinase activity. The MALDI-TOF MS-based assay requires 2 μl of total volume containing only 1 ng of enzyme and 2 μg of oligosaccharide substrate. This method provides potential for the high-throughput screening of chitinase inhibitors [32]. Chitinase activity can also be detected in gels after electrophoresis by zymography staining by incorporating an assay substrate into the gel during casting. After electrophoresis, the chitinase activity band can be visualized by staining gel with 0.1 % Congo red or a fluorescent brightener [33, 34].

Heterologous expression and purification of chitinases in *E. coli*

The regulation of recombinant protein expression is a complex system consisting of interaction elements, such as promoters, ribosome binding sites (RBS), and transcription terminators. Owing to developments in synthetic biology, these problems have been addressed to rely on genetic parts tested in a combinatorial manner. Recombinant enzyme production systems are now promising platforms for efficient industrial chitinase production. Cloning of chitinase genes has been carried out for studying aspects of
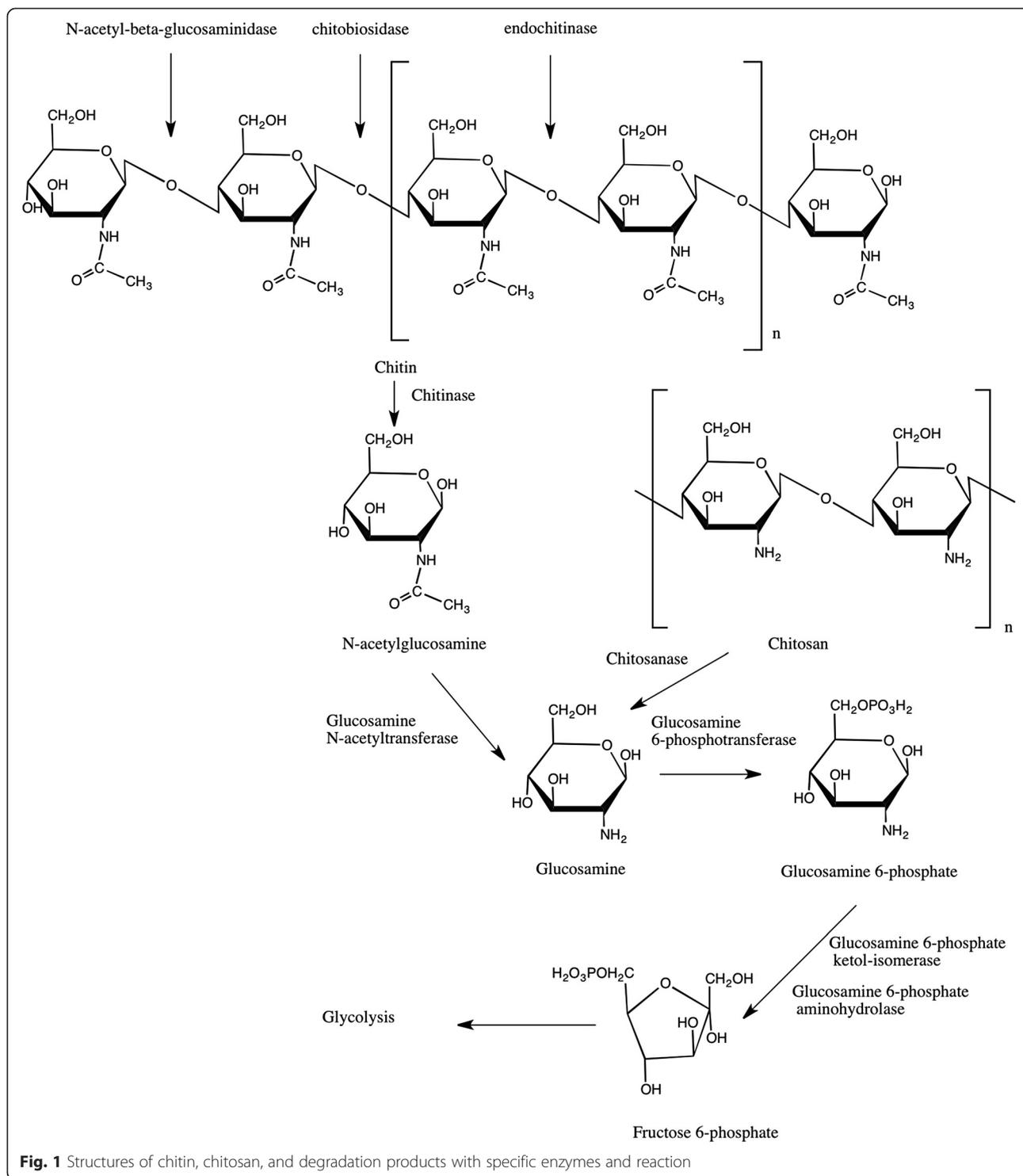
Fig. 1 Structures of chitin, chitosan, and degradation products with specific enzymes and reaction

chitinase sequence variations, functional characteristics, changes in enzyme induction pattern, and for enzyme engineering. Chitinase genes from different bacteria have been cloned and expressed mainly in *Escherichia coli*, shown in Table 1.

Enzymes used for industrial applications can usually be prepared and used in their crude, nonpurified form. The use of chitinases in chitin oligosaccharide preparation usually does not require purification of the enzyme, but chitinases in their purified form are required to study the biochemical properties, structure-function relationships, and their biotechnological applications. The purification of chitinases from bacterial sources in most cases has involved classical

Table 1 Recent studies of chitinase heterologous expression in *E. coli*

E. coli	Chitinase genes	Type of chitinase	Chitinase organism	Specific activity[a] (U/mg)	References
E. coli BL21 (DE3)	*chiA*	Exochitinase	*Bacillus halodurans* C-125	3.92	[55]
E. coli Top10	*chiA*	Endochitinase	*Bacillus licheniformis* DSM 8785	306,600	[56]
E. coli BL21 (DE3)	CV2935	Endochitinase	*Chromobacterium violaceum* ATCC 12472	22,260.5	[57]
E. coli M15	*chiS* and *chiL*	–	*Bacillus pumilus* SG2	1410 and 888	[58]
E. coli TOP10	*HschiAl*	Endochitinase	*Halobacterium salinarum* CECT 395	16.48	[59]
E. coli BL21 cd+	ORF BAB65950	–	*Sulfolobus tokodaii*	4500	[60]
E. coli BL-21-Rosetta	ChiA74	Endochitinase	*Bacillus thuringiensis*	75.23	[61]
E. coli M15	VhNag1	β-*N*-Acetylglucosaminidase	*Vibrio harveyi* 650	15,000	[62]
E. coli M15	VhNag2	β-*N*-Acetylglucosaminidase	*Vibrio harveyi* 650	105,000	[62]
E. coli X11-Blue	ArchiA	–	*Arthrobacter sp.* TAD20	–[b]	[63]
E. coli X11-Blue	ArchiB	–	*Arthrobacter sp.* TAD20	–	[63]

[a]Specific enzyme activity was shown from reports
[b]Data not shown

enzyme purification methods. These methods involve removal of microbial biomass from the culture broth, selective precipitation/concentration by $(NH_4)_2SO_4$, or solvents, or polyethylene glycol. The concentrated chitinase is further subjected to chromatography, commonly gel filtration, ion exchange, or affinity techniques.

Potential metabolic engineering targets

Just as cellulose is viewed as a renewable starting carbon source for a variety of biochemical products, chitin and chitosan can also be renewable carbon sources. A variety of potential biochemical products can be made based on the monomers of chitin degradation, shown in Fig. 2 http://www.aureliagloves.com/product_details_distinct.php.
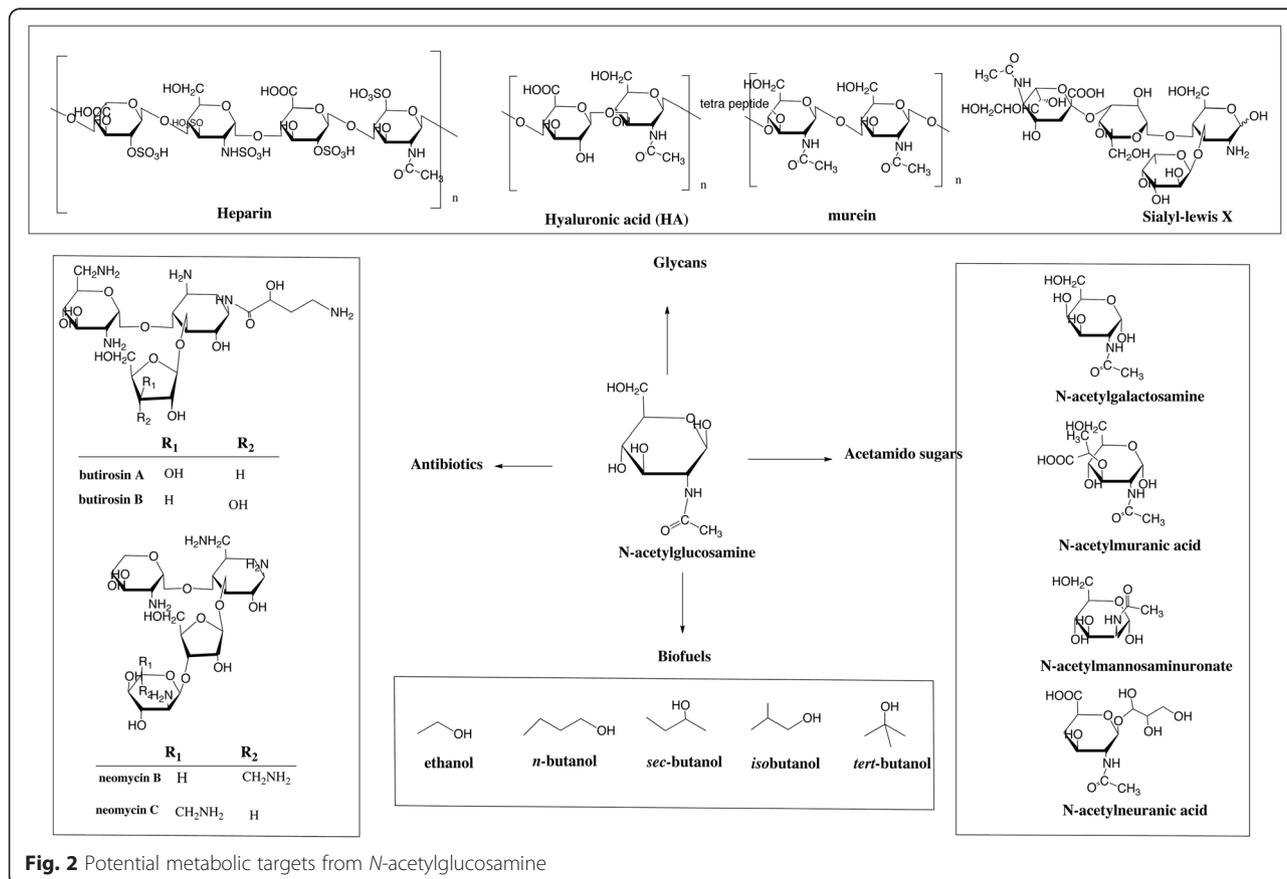

Fig. 2 Potential metabolic targets from *N*-acetylglucosamine

Glycan biosynthesis

In addition to being a structural component of homogeneous polysaccharides like chitin, GlcNAc is also a constituent of heterogeneous polysaccharides, such as murein and hyaluronic acid (also called hyaluronan or hyaluronate, HA). Murein is the basic component of the bacterial cell wall and consists of cross-linked peptide chains with repeating GlcNAc and muramic acid residues [35, 36].

HA is a linear heteropolysaccharide that is composed of repeating D-glucuronic acid and GlcNAc residues. HA is a major component of extracellular matrix and is extensively distributed in connective, epithelial, and neutral tissues [37, 38]. HA performs numerous roles in cell motility, inflammation, and cancer metastasis [39, 40].

Heparin, which is produced by basophils and mast cells, consists of a variably sulfated repeating disaccharide unit and acts as an anticoagulant. The major repeating disaccharide unit of keratin sulfate, also called keratosulfate, is composed of galactose and sulfated GlcNAc. Keratin sulfate is distributed in the cornea, cartilage, and bone and usually acts as a cushion in joints to absorb mechanical shock [41, 42].

Sialyl lewis is a tetrasaccharide carbohydrate that is usually attached to O-glycans on the surface of cells. It is known to play a vital role in cell-to-cell recognition processes. It is also the means by which an egg attracts sperm—first to stick to it, then bond with it, and eventually form a fetus. There are two types of sialyl-lewis, X and A. Sialyl-lewis X is [NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc]. Defective synthesis of the sialyl-lewis X antigen results in immunodeficiency (leukocyte adhesion deficiency type 2) [43, 44].

Acetamido sugars

GlcNAc is the precursor of modified acetamido sugars, such as *N*-acetylgalactosamine, sialic acid, *N*-acetylmuramic acid, *N*-acetylmannosaminuronate and several 6-deoxy-2-acetamido sugars, including bacillosamine and pseudaminic acid, which are core components of bacterial N-linked and O-linked glycans, respectively [45].

N-acetylneuraminic acid (Neu5Ac) is the most common sialic acid and exists as >40 structural derivatives in mammalian and avian species. Sialic acids are found at the end of sugar chains connected to the surfaces of cells and proteins, serving as receptors for influenza virus-mediated endocytosis. The guanidyl derivative of Neu5Ac, zanamivir, has been chemically synthesized as a novel pharmaceutical agent to produce neuraminidase inhibitors which are used to treat influenza infections.

Because the Neu5Ac content in natural products is too low for the isolation of Neu5Ac with sufficient recovery and purity, novel processes had to be developed for the industrial production of this compound [46].

Enzymatic synthesis of Neu5Ac from *N*-acetylmannosamine (ManNAc) and pyruvate using Neu5Ac aldolase as a catalyst has been reported. However, because ManNAc is very expensive, methods for the preparation of ManNAc from inexpensive GlcNAc have been developed. Kang et al. designed an artificial Neu5Ac biosynthetic pathway through intermediate *N*-acetylglucosamine 6-phosphate by adding two genes *slr1975* and *GNA1* into *E. coli*. By repressing the feedback inhibition of glucosamine-6-phosphate synthase, increasing the accumulation of *N*-acetylglucosamine and pyruvate, and blocking the catabolism of NeuAc, the NeuAc yield reached 7.85 g/L in fed-batch fermentation [47].

Antibiotic biosynthesis

Aminoglycoside antibiotics have been widely used clinically since the first use of streptomycin as an effective antibiotic in the treatment of tuberculosis. These antibiotics consist of a central aminocyclitol ring, such as streptamine, streptidine, or 2-deoxystreptamine (2-DOS) [48].

Neomycin is a 4,5-disubstituted aminoglucoside antibiotic. It is typically used as a topical preparation, such as Neosporin, because it is not absorbed from the gastrointestinal tract and has been used as a preventive measure for hepatic encephalopathy. By killing bacteria in the intestinal tract, it keeps ammonia levels low and prevents hepatic encephalopathy, especially prior to GI surgery. The first glycosylation step of neomycin involved in the formation of pseudodisaccharides is the addition of *N*-acetylglucosamine to 2-DOS. NeoM from the neomycin gene cluster was first characterized as the UDP-*N*-acetylglucosamine glycosyltransferase.

Butirosin is a relatively new aminoglycosidic antibiotic complex active in vitro and in vivo against various pathogenic gram-positive and gram-negative bacteria. Butirosin includes two isomers, butirosin A and butirosin B, which differ only in the configuration at one carbon atom in the pentose moiety.

Perspectives

Discovery of new enzyme with better properties

Expanding the throughput of genetic and biochemical assays holds great potential for exploring biodiversity in environments where chitin degradation is known to occur. Metagenomics has promoted the genetic and bioinformatic screening of chitin-degrading genotypes of microbial communities retrieved from plant phytopathogen-suppressive soil and marine soil [49, 50]. Exploring the diversity in chitin degradation environments may also reveal novel enzymatic activities for hydrolysis of chitin. Vaaje-Kolstad et al. showed that CBP21 (CBP for chitin-binding protein), produced by the chitinolytic bacterium *S. marcescens*, is an enzyme that catalyzes cleavage of glycosidic bonds in crystalline chitin, thus opening up the inaccessible

polysaccharide material for hydrolysis by normal glycoside hydrolases. The products were identified as chitin oligosaccharides with a normal sugar at the nonreducing end and an oxidized sugar, 2-(acetylamin)-2-deoxy-D-gluconic acid (GlcNAcA), at the end. This is an oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides [51].

Improving chitinase property by directed evolution

Thanks to recent advances in functional genomics, numerous new enzymes have been discovered from various sources. However, in most cases, naturally occurring enzymes are not optimized for practical applications due to the difference between the cellular environment and industrial settings. One of the most successful approaches is to engineer existing enzyme to be compatible with the ideal industrial process via directed evolution. Directed evolution involves four main steps including choosing a parent protein, creating a mutant library based on the parent protein, identifying variants with improved target properties, and repeating the entire process until achieving the desired function, shown in Fig. 3. This approach is superior to iterative mutagenesis techniques because it provides a mechanism to separate, randomly, beneficial and deleterious mutations.

Unlike the random mutagenesis method, which only requires limited prior knowledge of the parent protein, the success of designing a smart library relies heavily on three major factors: (1) understanding of the consequences of mutations on protein folding and activity, (2) structure-function relationships of the parent protein, and (3) methods and programs for identifying "key beneficial" mutations. Directed evolution has been widely used in cellulose and lignin degradation [52]. For example, engineering efforts have been focused on endo-glucanases and β-glucosidases, whose activities can be assayed in a high-throughput manner with the help of artificial substrates that were soluble or chromogenic. For endoglucanases, carboxymethyl cellulose (CMC) was widely used as a model substrate. A petri dish plate assay can be performed where the hydrolysis of CMC can be visualized by the clear zone after staining with Congo red. The size of the clear zone can be correlated to the enzyme activity. Based on an assay such as this, endoglucanase variants with improved activity or thermal/pH tolerance were identified by screening libraries created by error-prone PCR and/or DNA shuffling [53]. Currently, there only exist a few studies focused on directed evolution of chitinases. One of the challenges is devising high-throughput screening methods on activities towards the insoluble cellulosic substrates because artificial substrates do not necessarily show improved hydrolysis of natural substrates. Fan et al. generated a library of variants of a fungal chitinase after three rounds of error-prone PCR and DNA shuffling. The chitinase variants were expressed in *E. coli* using a prokaryotic signal peptide, PelB, for efficient secretion. Chitinase variants were selected based on colloidal chitin clearance on agar plates. An additional chitinase activity assay showed two variant chitinases with increased activity [54].

As more chitinase structural and functional knowledge is gained, directed evolution has the potential to become a powerful and commonly used tool in chitinase protein engineering. With the development of ultrahigh-throughput screening methods based on drop-based microfluidics and fluorescence-activated cell sorting (FACS), the capability
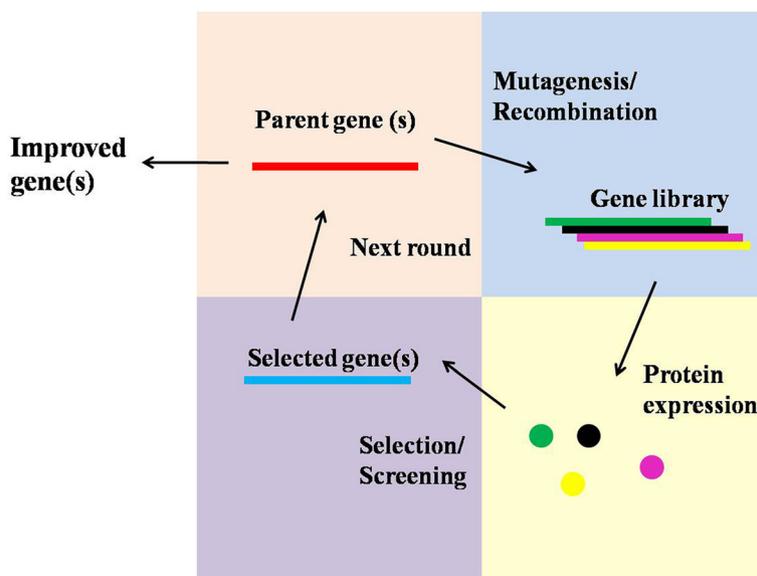
Fig. 3 Diagram of directed evolution

of screening large libraries has been greatly enhanced. Novel ultrahigh-throughput methods will enable us to perform directed evolution of a target protein with limited knowledge more efficiently, which will in turn provide more insights of the target protein and improve the desired property for industrial applications.

Conclusions

Chitin biomass is an abundant, renewable feedstock for the production of fuels and high value-added chemicals, if an efficient and affordable conversion technology can be established to overcome its recalcitrance. Recent advances in biotechnology enable many bacterial chitinases to be cloned, expressed, purified, and characterized. Knowledge and methods relevant to chitin degradation and potential chemical products have been reviewed. Further development of chitin degradation will require continued discovery of a new enzyme, improvement of the quality of the enzyme, and high-throughput chitinase assay. Future metabolic pathway will be constructed based on the acetylglucosamine from chitin degradation.

References

1. Hasunuma T, Okazaki F, Okai N, Hara KY, Ishii J, Kondo A (2013) A review of enzymes and microbes for lignocellulosic biorefinery and the possibility of their application to consolidated bioprocessing technology. Bioresour Technol 135:513–522
2. Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtlzapple M, Ladisch M (2005) Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol 96:673–686
3. Eijsink VGH, Vaaje-Kolstad G, Vårum KM, Horn SJ (2008) Towards new enzymes for biofuels: lessons from chitinase research. Trends Biotechnol 26:228–235
4. Rinaudo M (2006) Chitin and chitosan: properties and applications. Prog in Polym Sci 31:603–632
5. Harti L, Zach S, Seidl-Seiboth V (2012) Fungal chitinase: diversity, mechanistic properties and biotechnological potential. Appl Microbiol and Biotechnol 93:533–543
6. Aam BB, Heggset EB, Norberg AL, Sorlie M, Vårum KM, Eijsink VGH (2010) Production of chitooligosaccharides and their potential applications in medicine. Mar Drugs 8:1482–1517
7. Adrangi S, Faramarzi MA (2013) From bacteria to human: a journal into the world of chitinases. Biotechnol Adv 31:1786–1795
8. Li H, Greene LH (2010) Sequence and structural analysis of the chitinase insertion domain reveals two conserved motifs involved in chitin-binding. PLOS one 5(1):e8654–e8664
9. Watanabe T, Kanai R, Kawase T, Tanabe T, Mitsutomi M, Sakuda S, Miyashita K (1999) Family 19 chitinases of *Streptomyces* species: characterization and distribution. Microbiology 145:3353–3363
10. Watanabe T, Kobori K, Miyashita K, Fujii T, Sakai H, Uchida M, Tanaka H (1993) Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. J Biol Chem 268:18567–28572
11. Vaaje-Kolstad G, Horn SJ, Sorlie M, Eijsink VGH (2013) The chitinolytic machinery of Serratia marcescens—a model system for enzymatic degradation of recalcitrant polysaccharides. FEBS J 280:3028–3049
12. Hashimoto M, Ikegami T, Seino S, Ohuchi N, Fukada H, Sugiyama J, Shirakawa M, Watanabe T (2002) Expression and characterization of the chitin-binding domain of chitinase A1 from *Bacillus circulans* WL-12. J Bacteriol 182:3045–3054
13. Dahiya N, Tewari R, Hoondal GS (2006) Biotechnological aspects of chitinolytic enzymes: a review. Appl Microbiol Biotechnol 71:773–782
14. Toratani T, Kezuka Y, Nonaka T, Hiragi Y, Watanabe T (2006) Structure of full-length bacterial chitinase containing two fibronectin type III domains revealed by small angle X-ray scattering. Biochem Biophys Res Commun 348:814–818
15. Watanabe T, Ariga Y, Sato U, Toratami T, Hashimoto M, Nikaidou N, Kezuka Y, Nonaka T, Sugiyama J (2002) Aromatic residues within the substrate-binding cleft of *Bacillus circulans* chitinase A1 are essential for hydrolysis of crystalline chitin. Biochem J 376:37–244
16. Watanabe T, Ishibashi A, Ariga Y, Hashimoto M, Nikaidou N, Sugiyama J, Matsumoto T, Nonaka T (2001) Trp122 and Trp134 on the surface of the catalytic domain are essential for crystalline-chitin hydrolysis by *Bacillus circulans* chitinase A1. FEBS Lett 494:74–78
17. Watanabe T, Ariga Y, Sato U, Toratani T, Hashimoto M, Nikaidou N, Kezuka Y, Nonaka T, Sugiyama J (2003) Aromatic residues within the substrate-binding cleft of *Bacillus circulans* chitinase A1 are essential for hydrolysis of crystalline chitin. Biochem J 376:237–244
18. Watanabe T, Suzuki K, Oyanagi W, Ohnishi K, Tanaka H (1990) Gene cloning of chitinase A1 from *Bacillus circulans* WL-12 revealed its evolutionary relationship to *Serratia* chitinase and to the type III homology units of fibronectin. J Biol Chem 265:15659–15665
19. Jee JG, Ikegami T, Hashimoto M, Kawabata T, Ikeguchi M, Watanabe T, Shirakawa M (2002) Solution structure of the fibronectin type III domain from *Bacillus circulans* WL-12 chitinase A1. J Biol Chem 277:1388–1397
20. Watanabe T, Ito Y, Yamada T, Hashimoto M, Sekine S, Tanaka H (1994) The roles of the C-terminal domain and type III domains of chitinase A1 from *Bacillus circulans* WL-12 in chitin degradation. J Bacteriol 176:4465–4472
21. Uchiyam T, Katouno F, Nikaidou N, Nonaka T, Sugiyama J, Watanabe T (2001) Roles of the exposed aromatic residues in crystalline chitin hydrolysis by chitinase A from *Serratia marcescens* 2170. J Biol Chem 276:41343–41349
22. Horn SJ, Sikorski P, Cederkvist JB, Vaaje-Kolstad G, Sørlie M, Synstad B, Vriend G, Vårum KM, Eijsink VGH (2006) Costs and benefits of processivity in enzymatic degradation of recalcitrant polysaccharides. Proc Natl Acad Sci USA 103:18089–18094
23. Akira O (1998) Viscosimetric assay for chitinase. In: Willis A, Wood STK (eds) Methods in enzymology. Academic Press, New York
24. Boller T (1998) Colorimetric assay for chitinase. In: Willis A, Wood STK (eds) Methods in enzymology. Academic Press, New York
25. Enrico C (1998) Assay for chitinase using tritiated chitin. In: Willis A, Wood STK (eds) Methods in enzymology. Academic Press, New York
26. Howard MB, Ekborg NA, Weiner RM, Hutcheson SW (2003) Detection and characterization of chitinases and other chitin-modifying enzymes. J Ind Microbiol Biotechnol 30:627–635
27. Zhang PYH, Himmel ME, Mielenz JR (2006) Outlook for cellulose improvement: screening and selection strategies. Biotechnol Adv 24:452–481
28. Eveleigh DE, Mandels M, Andreotti R, Roche C (2009) Measurement of saccharifying cellulose. Biotechnol Biofuels 2:21–28
29. Ferrari AR, Gaber Y, Fraaije MW (2014) A fast, sensitive and easy colorimetric assay for chitinase and cellulose activity detection. Biotechnol Biofuels 7:37–44
30. Shen CR, Chen YS, Yang CJ, Chen JK, Liu CL (2010) Colloid chitin azure is a dispersible, low-cost substrate for chitinase measurements in a sensitive, fast, reproducible assay. Biomol Screening 15:213–217
31. Horri SJ, Eijsink VGH (2004) A reliable reducing end assay for chito-oligosaccharides. Carbohydr Polym 56:35–39
32. Price NPJ, Naumann TA (2011) A high-throughput matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry-based assay of chitinase activity. Anal Biochem 411:94–99
33. Thadathil N, Velappan SP (2014) Recent developments in chitosanase research and its biotechnological applications: a review. Food Chem 150:392–399
34. Toratani T, Shoji T, Ikehara T, Suzuki K, Watanabe T (2008) The importance of chitobiase and N-acetylglucosamine (GlcNAc) uptake in N,N

'-diacetylchitobiose [(GlcNAc)2] utilization by *Serratia marcescens* 2170. Microbiol 154:1326–1332

35. Suginta W, Chenark D, Mizuhara M, Fukamizo T (2010) Novel β-N-acetylglucosaminidases from *Vibrio harveyi* 650: cloning, expression, enzymatic properties, and subsite identification. BMC Biochem 11:40–51

36. da Silva AF, García-Fraga B, López-Seijas J, Sieiro C (2014) Characterization and optimization of heterologous expression in Escherichia coli of the chitinase encoded by the chiA gene of *Bacillus halodurans* C-125. Process Biochem 49:1622–1629

37. Songsiriritthigul C, Lapboonrueng S, Pechsrichuang P, Pesatcha P, Yamabhai M (2010) Expression and characterization of *Bacillus licheniformis* chitinase (ChiA), suitable for bioconversion of chitin waste. Bioresour Technol 101:4096–4103

38. Lobo MDP, Silva FDA, Landim PGC, Cruz PR, de Brito TL, de Medeiros SC, Oliveira JTA, Vasconcelos IM, Pereira HDM, Grangeiro TB (2013) Expression and efficient secretion of a functional chitinase from *Chromobacterium violaceum* in *Escherichia coli*. BMC Biotechnol 13:46–60

39. Ghasemi S, Ahmadian G, Sadeghi M, Zeigler DR, Rahimian H, Ghandili S, Naghibzadeh N, Dehestani A (2011) First report of a bifunctional chitinase/lysozyme produced by *Bacillus pumilus* SG2. Enzyme Microb Technol 48:225–231

40. García-Fraga B, da Silva AF, López-Seijas J, Sieim C (2014) Functional expression and characterization of a chitinase from the marine archaeon *Halobacterium salinarum* CECT395 in *Escherichia coli*. Appl Microbiol Biotechnol 98:2133–2143

41. Staufenberger T, Imhoff JF, Labes A (2012) First crenarchaeal chitinase found in *Sulfolobus tokodaii*. Microbiol Res 167:262–269

42. Casados-Vázquez LE, Avila-Cabrera S, Bideshi DK, Barboza-Corona JE (2014) Heterologous expression, purification and biochemical characterization of endochitinase ChiA74 from *Bacillus thuringiensis*. Protein Expression Purif Doi: 10.1016/j.pep.2014.11.015

43. Lonhienne T, Mavromatis K, Vorgias CE, Buchon L, Gerday C, Bouriotis V (2011) Cloning, sequences, and characterization of two chitinase gene from the Antarctic *Arthrobacter* sp. strain TAD20: isolation and partial characterization of the enzymes. J Bacteriol 183:1773–1779

44. Ashry ESHE, Aly MRE (2007) Synthesis and biological relevance of N-acetylglucosamine-containing oligosaccharides. Pure Appl Chem 12:2229–2242

45. Chen JK, Shen CR, Liu CL (2010) N-acetylglucosamine: production and applications. Mar Drugs 8:2493–2516

46. Dube B, Lüke HJ, Aumailley M, Prehm P (2001) Hyaluronan reduced migration and proliferation in CHO cells. Biochim Biophys Acta 1538:283–289

47. Chien LJ, Lee CK (2007) Hyaluronic acid production by recombinant *Lactococcus lactis*. Appl Microbiol Biotechnol 77:339–346

48. Chien LJ, Lee CK (2007) Enhanced hyaluronic acid production in *Bacillus subtilis* by coexpressing bacterial hemoglobin. Biotechnol Progr 23:1017–1022

49. Danishefsky I, Steiner H, Bella A, Friedlander A (1969) Investigations on the chemistry of heparin: VI position of the sulfate ester groups. J Biol Chem 244:1741–1745

50. Bhavanandan VP, Meyer K (1966) Mucopolysaccharides: N-acetylglucosamine- and galactose-6-sulfates from keratosulfate. Science 151:1404–1405

51. Tsai M, Takeishi T, Thompson H, Langley KE, Zsebo KM, Metcalfe DD, Geissler EN, Galli SJ (2001) Induction of mast cell proliferation, maturation, and protein synthesis by the rat c-kit ligand, stem cell factor. Proc Natl Acad Sci USA 88(14):6382–6386

52. Ugorski M, Laskowska A (2002) Sialyl Lewis (a): a tumor-associated carbohydrate antigen involved in adhesion and metastatic potential of cancer cells. Acta Biochim Pol 49:303–311

53. Polley MJ, Phillips ML, Wayner E, Nudelman E, Singhal AK, Hakomori S, Paulson JC (1991) CD62 and endothelial cell-leukocyte adhesion molecule 1 (ELAM-1) recognize the same carbohydrate ligand, sialyl-Lewis x. Proc Natl Acad Sci USA 88:6224–6228

54. Piacente F, Bernardi C, Marin M, Blanc G, Abergel C, Tonetti MG (2013) Characterization of a UDP-N-acetylglucosamine biosynthetic pathway encoded by the giant DNA virus Mimivirus. Glycobiology. doi:10.1093/glycob/cwt089

55. Vimr E, Lichtenteiger C (2002) To sialylate, or not to sialylate: that is the question. Trends Microbiol 10:254–257

56. Kang JH, Gu PF, Wang Y, Li YK, Yang F, Wang Q, Qi QS (2012) Engineering of an N-acetylneuraminic acid synthetic pathway in *Escherichia coli*. Metab Eng 14:623–629

57. Song MC, Kim E, Ban YH, Yoo YJ, Kim EJ, Park SR, Pandey RP, Sohng JK, Yoon YJ (2013) Achievements and impacts of glycosylation reactions involved in natural product biosynthesis in prokaryotes. Appl Microbiol Biotechnol. doi:Doi:10.1007/s00253-013-4978-7

58. Hjort K, Presti H, Elväng A, Marinelli F, Sjöling S (2014) Bacterial chitinase with phytopathogen control capacity from suppressive soil revealed by functional metagenomics. Appl Microbiol Biotechnol 98:2819–2828

59. Schmeisser C, Steele H, Streit WR (2007) Metagenomics, biotechnology with non-culturable microbes. Appl Microbiol Biotechnol 75:955–962

60. Vaaje-Kolstad G, Westereng B, Horn SJ, Liu Z, Zhai H, Sørlie M, Eijsink VGH (2010) An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. Science 330(6001):219–222

61. Wang M, Si T, Zhao HM (2012) Biocatalyst development by directed evolution. Bioresour Technol 115:117–125

62. Liang C, Firoroni M, Rodríguez-Ropero F, Xue Y, Schwaneberg U, Ma Y (2011) Directed evolution of a thermophilic endoglucanase (Cel5A) into highly active Cel5A variants with an expanded temperature profile. J Biotechnol 154:46–53

63. Fan YH, Fang WG, Xiao YH, Yang XY, Zhang YJ, Bidochka MJ, Pei Y (2007) Directed evolution for increased chitinase activity. Appl Microbiol Biotechnol 76:135–139