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Research Progress of Cellulase

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Abstract Cellulase is a complex enzyme that can decompose cellulose into glucose, and it could effectively treat cellulose waste. In this paper, it aims to explore development status and research progress of cellulase, and introduce concept and action mechanism of cellulase, research situation of cellulase in molecular aspect, application of cellulase, and development of cellulase is also prospected.

Key words Cellulase, Research progress, Molecular modification

1 Introduction

Since the discovery of cellulase in the digestive tract of snail in 1906, microbial degradation of cellulose has attracted considerable attention[1]. After cellulose is degraded by cellulase, economic and rich raw materials for production could be generated, thereby turning waste into treasure, and saving resources. The earth is rich in cellulose (accounting for 40% of total earth biomass), with wide distribution, and it is reproducible. Therefore, cellulase has attracted more and more attention.

Only a very small part of cellulosic substances in nature such as straw, rice stalk, and waste paper are used, and most of them are burnt, causing environmental pollution. Oil and coal resources are also depleting. So, sufficient utilization and effective transformation of cellulose have key significance for solving current energy crisis, food shortage and environmental pollution. Cellulase could decompose cellulose into glucose, thereby sufficiently and effectively using cellulose. The advantages of cellulase degrading cellulose contain high specificity, mild reaction condition and little environmental pollution. Therefore, action mechanism and structure modification of cellulase gradually become research focuses in recent years. But action mechanism of cellulase is not entirely clear yet, and production ability of cellulase is lower, with certain distance from actual application. At present, domestic and foreign scholars use fermentation method to produce cellulase, and then cellulase is applied into biomass energy, food, feed, washing and other industries. It not only solves reuse of cellulose but also obtains considerable economic benefits.

2 Research contents

2.1 Concept and action mechanism of cellulase

2.1.1 Concept of cellulase. Cellulase is general term for a group of enzymes that degrade cellulose into glucose. It is not a monomer enzyme, but a multi-component enzyme system with synergistic effect, namely a complex enzyme.

2.1.2 Action mechanism of cellulase. The primary structure of cellulase molecule is similar, that is to say, it is composed of spherical catalytic domain, linkage area and cellulose binding domain (CBD). Its advanced structure is more complex, and enzyme molecules with the same source have different advanced structures. For example, *Clostridium cellulovorans* contains two polycellulases, and each cellulase contains nine subunits with the same component, in which activity of one cellulase is four times of the other[2], which illustrates complexity of cellulase composition.

Cellulase is composed of many hydrolytic enzymes with high synergistic activity. Cellulase is mainly divided into three kinds: (i) exogenous glucanase (also called C1 enzyme, abbreviated as CBH from fungi and Cex from bacteria); (ii) endoglucanase (also called Cx enzyme, abbreviated as EG from fungi and Len from bacteria); (iii) β-glucanase (BG for short).

Endogenous glucanase cuts the amorphous region of cellulose polymer in a random form, and its sensitivity to end-to-end bond is less than that to interbond, and its main product is small molecular cellulose with non-reducible end such as cellulose dextrin, cellobiose and cellotriose. The exogenous glucosidase can cleave the glycoside bond from the reduced or non-reduced end of the cellulose chain, thereby producing soluble fibrin dextrin and cellobiose, and it plays a leading role in degradation process of natural cellulose. Cellobiase hydrolyzes cellobiose and short-chain cell-oligosaccharide to produce glucose[3].

The most widely accepted action mechanism of these three enzymes is synergistic theory. Firstly, Cx enzyme plays a role in the interior of cellulose polymer, and cuts non-crystalline part of cellulose, thereby producing new end. Then, C1 enzyme uses cellobiose as the unit, and hydrolyzes it from the end. Finally, BG enzyme degrades cellobiose to glucose[4].

2.2 Research situation of microorganisms producing cellulase There are three types of cellulase-producing species: microorganism, animal and plant. Cellulase widely exists in plant, plays the role of hydrolyzing cell wall, and joins in fruit ripening and pedicle shedding. But cellulase content is very low in plant, and its extraction is difficult. There is also cellulase in animal that is different from those produced by microorganism, such as termite and *Pomacea canaliculata*. Cellulase is mainly extracted from mi-

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croorganism (bacteria, actinomycetes, fungi, *etc.*) and animal, and microbial fermentation is an effective way to produce cellulase on a large scale.

Cellulase content is low in actinomycetes, and its research is also less. Recently, an actinomycete which could degrade cellulose thoroughly was obtained by Dalian Polytechnic University. Via identification of systematic taxonomy and comparison of molecular biology, the strain was identified as a new strain and was named *Streptomyces cellulolyticus*, and it has been listed in the new bacteria catalogue of the International Association of Microbiology[5].

Yield of cellulase in bacteria is also lower, and they mainly produce endoglucanase. Cellulase produced by most of bacteria does not have activity on crystalline cellulose. More importantly, the enzymes produced are intracellular or easily adsorb on cell walls, and very few could be secreted out of cells, with very difficult extraction and purification. So, it is seldom used in industry.

Filamentous fungi are microbial strains with many advantages of producing enzymes: produced cellulase could secrete to extracellular space, which is favorable for purification and extraction, with high efficiency, and the structure of enzyme system is more reasonable; meanwhile, many other enzymes could be generated, such as hemicellulase, pectinase, and amylase. Seen from systematic mass production and application of cellulase, it is very meaningful to study the enzyme production of filamentous fungi. Now, filamentous fungi are the main microbial strains used to produce cellulase, containing *Trichoderma*, *Aspergillus* and *Penicillium*, especially *Trichoderma reesei* and its closely related strains. It has three kinds of enzymes degrading natural cellulose, namely EG, CBH and BG, and stable genetic character. Its produced cellulase system is complete, and its anti-metabolic repression ability is strong. Producing activity of enzyme is high, and it is easy to be extracted. Moreover, the strain is safe and nonpoisonous. Therefore, *T. reesei* is recognized as the most valuable cellulase-producing strain in industry.

3 Study of cellulase in molecular aspect

3.1 Study of cellulase gene In the 1980s, DNA recombinant technology has been used to clone and identify cellulase gene in microorganism, and extracellular cellulase genes encoding Eng and Exg were widely studied[6]. The Hong Kong University of Science and Technology started to study cellulase-producing bacteria since 1988, and the cloned genes encoding Eng and Exg in *Cellulomonas fimi* were successfully introduced in multiple hosts[7]. Since *Fibrobacter* had higher secretion ability of Cel, and had certain correlation with *C. fimi*[8], synergistic reaction of Cel and Eng, Exg in *Fibrobacter* was mainly studied in the laboratory.

Kim *et al.* [9] cloned and transfected gene encoding heat-resistant endoglucanase from thermophilic bacteria into *E. coli*. Via expression and detection, it showed that the endoglucanase had the maximum enzyme activity at 80℃, and its activity could last for 2 h at 100℃. Hakamada *et al.* [10] purified and studied alkaline glucan endonuclease with higher isoelectric point in *Bacilluis circulans*, and carried out PCR analysis on its gene. The obtained structure gene contained a single open reading box, and encoded 407 amino acids, in which containing a signal peptide containing 30 amino acids.

Taking *E. coli* as host cell, Cai Yong *et al.* [11] cloned and expressed protein with cellulase activity from *Bacillus*. When studying homologous recombination of cellulase and xylanase in 2009, Rahman *et al.* [12] proposed that promoter and terminator could be used to construct expression vector to prepare homologous and heterologous protein under ideal situation. Via fluorescent protein labeling, the determination of transcription activity of promoter could reach cellular level[12].

Kitagawa *et al.* [13] introduced endogenous glucanase gene and plasmid from *Clostridium thermocellum* into yeast cell via gene recombination technology. Compared with wild strain, activity of endogenous glucanase in the obtained deletion strain was improved somewhat. After classification of deleted strain, it was proved that β-glucosidase produced by heterologous strain had very high activity via the test.

Random and fixed-point integration type of gene expression vectors were constructed in East China University of Science and Technology in 2011[14]. The two kinds of genes could be introduced into *T. reesei* via *Agrobacterium*, and the practicability of the constructed carrier could be detected via red marker gene[15]. It was found that random integrative type was more suitable for randomized recombination test, while fixed-point integration was suitable for homologous recombination. The research was favorable for studying gene function and expression in filamentous fungi.

Anthony *et al.* [16] firstly obtained β-glucosidase of glycoside hydrolase family 1 from *Fibrobacter* in 2012 (new cellobiase gene was obtained from *Fibrobacter*, and the gene encoded β-glucosidase). In prior research and report, β-glucosidase encoded by cellobiase gene of *Fibrobacter* was member of glycoside hydrolase family 3. Now, *E. coli* or yeast cell was taken as host, and cellulase gene was expressed in many bacteria or fungi.

Li Wang *et al.* [17] thought that it could obtain complete information of cellulase gene by constructing DNA library and cDNA library in prior period of cellulase gene research and colony. With the progress of technology, cellulase gene could be obtained by artificial synthesis and selective amplification from macrogenome.

These research results could provide ideas for artificially constructed cellulase applied in industrial production.

3.2 Molecular modification technology of cellulase Molecular modification technology of cellulase indicates that base mutation in encoding region of cellulase at DNA level causes change in space structure of cellulase. The technology mainly contains two kinds. One is that properties of cellulase are improved by changing space structure or chemical bond of enzyme. The other is that yield of cellulase is improved by changing transcription factor, promoter, and codon.

3.2.1 Molecular modification technology of improving properties of cellulase. The technology mainly contains site-directed mutagenesis, error-prone PCR and DNA shuffling.

(i) Site-directed mutagenesis. Michael Smith invented site-directed mutagenesis and won the 1993 Nobel Prize in chemistry. Site-directed mutagenesis could control mutation, which is favorable for studying the relationship between structure and function of protein, and is convenient for optimizing and transforming gene.

Tang Zizhong *et al.* [18] conducted site-directed mutagenesis of endoglucanase gene in mutant strain with high enzyme activity, and No. 91 lysine was changed into glutamic acid, and No. 369 lysine was changed into arginine. Three kinds of engineering bacteria were constructed, and they were respectively glutamic acid, arginine, and glutamic acid/arginine. Test results showed that thermal stability of endoglucanase produced by the three kinds of engineering bacteria was improved somewhat. When treated for 1 h at 70℃, residual enzyme activity of glutamic acid improved by 24% than that of original enzyme, while residual enzyme activity of arginine improved by 18% than that of original enzyme, and residual enzyme activity of glutamic acid/arginine improved by 29% than that of original enzyme.

(ii) Error-prone PCR. By adding manganese ion and changing reaction system, reproduction of target gene reduces fidelity, and then beneficial mutant is screened. Error-prone PCR has some advantages that site-directed mutagenesis does not have. That is to say, it does not need to know the sequence of the target gene in advance, which is favorable for studying unknown protein of gene sequence.

Zhang Yang[19] used the technology for directed mutation of *Thermobifida fusca*, and two mutant strains were obtained finally. Sequencing results showed that the two mutant strains both had mutations of three amino acids; activity of cellulase in the two mutant strains was improved by 2.19 and 1.88 times respectively, and the optimal temperature was improved by 10℃ and 5℃ respectively, while the optimal pH respectively rose by 1.0 and 2.0.

(iii) DNA shuffling. Stemmer invented DNA shuffling in 1994, which was also called sexual PCR. The technology has many advantages: improving probability of mutation; repeatable screening; having homologous sequences with large fragment, and they can exchange with each other.

Bai Yu[20] took endoglucanase I gene in *T. fusca* as transformation object, and used DNA shuffling technology for mutation of wild *egI* gene. Finally, a strain with 23.75 U/mL of enzyme activity was obtained, and its enzyme activity improved by 10 times than original strain.

3.2.2 Molecular modification technology improving cellulase yield. (i) Promoter. Strong promoter is a promoter with high affinity to RNA polymerase, and it can produce large amounts of RNA by transcription. Strong promoters commonly used at present contain lactose operon (lacP), tryptophan operon (trpP) and polyhedrin gene promoter from *E. coli*.

To improve expression of target gene in prokaryotic cell, Chen Ke *et al.* [21] linked target gene with T7 promoter and lipoprotein promoter, and then it was introduced into expressing host cells. Results showed that two kinds of expression host cells improved expression amount of cellulase to different degrees.

(ii) Transcription factor. Transcription factor could affect gene expression to some extent by joining in transcription of regulatory genes, and it is a kind of protein.

Zhang Jiwei[22] found transcription factor regulating expression of cellulase in *Trichoderma reesei*. After transcription factor was knocked out by molecular means, expression ability of cellulase obviously declined in *T. reesei*. When transcription factor was continuously activated, expression of cellulase gene in *T. reesei* obviously improved than original strain.

(iii) Codon. Zhou Guangqi *et al.* [23] used a *Penicillium decumberns* mutant strain lacking dub gene for research. Results showed that activities of three kinds of enzymes expressed by mutant strain were all improved somewhat, and activities of exogenous glucanase, endoglucanase and xylanase respectively improved by 2.04, 1.71 and 2.06 times than original strain. It illustrated that the gene may join in expression process of cellulase.

3.3 Application and significance of cellulase

3.3.1 Application in food industry. Cellulose is the main component of cell wall in plant, while the edible agricultural by-products are generally inclusions of plant. Permeability of cell wall could be increased by cellulase, which is conducive to extraction of nutrients such as protein and glucose in inclusions, and is convenient for food processing.

Cellulase could be used in fermentation of low-salt solid soy sauce. Salt-tolerant cellulase could be properly added in enzymatic hydrolysis process, which could make cell membrane of soybean and other raw materials soften and expand, and release protein and carbohydrate in cell. It shortens brewing time, and improves yield and quality[24]. Dosage of cellulase is only 0.012 5%, and chromaticity in soy sauce is improved by 4.2%, while utilization rate of raw material improves by 8.1% than that without cellulase.

In the processing of fruits and vegetables, cooking and acid-base treatment are often used to quickly soften and expand plant tissue, which could destroy the aroma of fruits and vegetables and lose nutrients[24]. In recent years, low-temperature cellulase is often used to prepare dehydrated vegetables. After treated by cellulase, drying and dewatering are conducted, which could avoid excessive loss of nutrients.

3.3.2 Application in feed industry. Cellulase is widely used in feed industry. Cellulose is abundant in most feeds, and except that some ruminants can break down cellulose, other animals do not have this ability. Cellulase is a kind of new feed additives. Cellulase and cellulase-producing bacteria can convert part of the roughage, such as wheat straw, wheat bran, corn cob, *etc.*, into sugar, bacterial protein and fat, which is easy for animals to absorb, reduces crude fiber content in feed, and improves nutritive value. Main effects are as below.

Number of microorganism with cellulase in reactive animal is

limited, while some monosalic animals, such as chicken and pig, can not digest cellulose. Cellulase could improve utilization rate of crude fiber as a kind of endogenous enzyme. It destroys cell wall of plant and decreases nutrient loss. Cellulase, hemicellulase and pectinase destroy cell wall via synergistic reaction, making inclusions dissolve. Using degradation effects of amylase and protease, absorption rate of nutrients and utilization rate of polysaccharide are improved. Cellulase could increase diffusion of endogenous enzyme in animal and enzyme-substrate contact area, and decrease mucus of gastrointestinal contents in animal, which is favorable for good digestion and absorption of feed.

3.3.3 Application in washing industry. Cellulase has a long history as an enzyme preparation in the washing industry, and is deeply studied in recent years. In 1980, Some developed countries such as Europe began to study enzymatic detergents. In 1987, a Japanese company introduced a new type of "biological detergent" with cellulase for the first time and began to produce alkaline cellulase in industrialized level. Alkaline cellulase generally could not act on crystalline cellulose and is not affected by detergents and other additives. 90% of the pollutants adhere to cotton fibers, and alkaline cellulase acts on non-crystalline region, which could effectively soften and hydrolyze a gelatinous structure formed by cellulose molecule, water and dirt[25], and wash out dirt. Moreover, the strength and apparent polymerization degree of cotton and linen fabrics will not change significantly[26]. Alkaline cellulase could well adapt to alkaline condition of detergent. Therefore, alkaline cellulase has very good application prospect in detergent industry.

3.3.4 Application in biological energy. In the 21st century, energy competition is the most fierce, and all countries in the world are striving to develop new energy. The United States recognized the advantages of ethanol fuel as early as the 20th century, and forced to add 10% of ethanol in fuel[27]. Liu Yu[28] isolated and purified cryogenic cellulase from products of Antarctic strain, and found that the cryogenic cellulase could degrade cellulose into ethanol via synchronized saccharification and fermentation experiment, and specific performance of it resisting to low temperature effectively solved the problem that the temperature of yeast growth was not consistent with the optimum temperature of cellulase activity in synchronized saccharification and fermentation process. After screened Antarctic strain producing cellulase, Gao Cong[29] obtained cryogenic cellulase, which could degrade kelp cellulose into monosaccharide fermented and used by yeast, and it could be produced into ethanol. It developed new raw material for preparing cellulosic ethanol. Besides used to prepare fuel ethanol, cellulase also could be used to produce biogas by fermentation. Since there are still many limitations in the preparation of fuel ethanol from cellulose, its preparation has not yet reached the level of large-scale industrialization[30].

4 Research prospect of cellulase

Up to now, the screening of cellulase-producing strain and the research and modification of cellulase gene have achieved some results, but it is far from meeting demand. It is still a key problem to be solved in the future to obtain cellulase-producing strain with high yield and enzyme activity. Most of the studies are obtaining cellulase-producing strains from terrestrial microorganism, and more in-depth research and development are needed in the field of ocean. The preparation of cellulase engineering bacteria is still difficult. In view of the existing problems of cellulase, future research focuses are as below. First, screen strain with high enzyme activity, and sufficiently use molecular biology to modify it, to improve stability and enzyme activity. Second, explore application of cellulase in extreme condition, and excavate its potential market. Third, enhance study on functional amino acids of various groups of cellulases, and grasp their action mechanisms, to effectively play role of cellulase. With the rapid development of biochemistry, bioinformatics, modern molecular biology and other disciplines, application of cellulase will be perfected gradually, to improve its market-oriented application.

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(To page 82)

Citrus grandis. Therefore, how to detect the diseased tree early is challenging. At present, laboratory identification method is mainly used by experts and scholars in the research. It is difficult for *Citrus grandis* growers, even major growers, to detect *Citrus grandis* HLB disease by laboratory identification. Because of the long time, the indicator identification method has not been found to be used by growers in our investigation, and has also become a method used by experts and scholars. In this paper, it is recommended that the field identification method should be combined with the detection method of "HLB disease detection reagent", which has practical significance in *Citrus grandis* production.

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(From page 77)

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