

Molecular Aspects of β -Galactosidase Production System in *Aspergillus* Genomes

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Abstract

Lactose utilizing enzymes of filamentous fungi is applied in food industry for hydrolyzing the β - (1, 4) linkage between galactose and glucose in lactose. Nevertheless, a less attention has been made on exploitation of fungi for lactase production due to cellular and metabolic complexity. The exploitation of cellular complexity for strain improvement has been a challenging goal for applied biological research because it requires the coordinated understanding of multiple cellular processes. We found the key enzymes lactose permease, β -galactosidase, galactokinase, galactose oxidase, lactase and galactose-1-phosphate uridylyltransferase, which are suggested to utilize lactose from the environment. Betaine aldehyde dehydrogenase was indentified in the genomes of *A. clavatus* and *A. terreus*, which is concerned to develop halotolerance in these organisms. Our study also suggested that phylogenetic and structural resemblances of proteins and or enzymes involved in lactose utilization pathway of some *Aspergillus* genomes. As a result of this study, a proposed lactose utilization pathway of *Aspergillus* genomes was reconstructed by bioinformatics approach. Thus, quantification of the metabolic network of an organism offers insights into possible ways of developing mutant strain for better productivity of an extracellular lactase. Recent focus has been on new genome-wide modeling approaches in functional genomics, which aim to take full advantage of genome sequence data, transcription profiling, proteomics, metabolite profiling and system biology for strain improvement.

Keywords: Metabolomics; Lactose; *Aspergillus*; Halotolerant; Galactosidase; Systems Biology

Introduction

Today, steady fast progress is being made in the application of genetic and molecular approaches to the production of industrial enzymes, currently a \$

750 market worldwide and increasing in volume by 5-10% per year. Filamentous fungi are extensively utilized for production of extracellular enzymes, including cellulases, proteases, amylases, pectinases and lipases. Improved production of cellulase from mutants of *Aspergillus niger* and *Trichoderma viride* by random-induced mutagenesis and recombinant strains of *A. niger* and *T. viride* by protoplast fusion technique have already been succeeded¹⁻⁵. Genetic transformation experiments have also been carried out in *A. oryzae*, *A. niger* with *A. nidulans* for production of protease and glucoamylase⁶⁻⁹. Genes encoding for glucose oxidase, (β -glucosidase, and glucoamylase) have also been identified in many *Aspergillus* genus⁶. In filamentous fungi, the productivity of cellulase in *Trichoderma* sp, invertase in *A. niger* and *Rhizopus* sp. have been improved by the induction of 2-deoxy- β -D-glucose resistance mutants that are less sensitive to catabolite repression¹⁰⁻¹³. To our knowledge, however, the production of β -galactosidase from genetically modified halotolerant *Aspergillus* genus has not been applied yet. The β -Galactosidase (β -D-galactoside galactohydrolase or lactase; EC.3.2.1.23) is widely distributed in nature, which hydrolyze β - (1, 4) linkage between galactose and glucose in lactose. The worldwide production of which is approximately 5.75 million metric tons per year. A commercial lactase preparation is obtained from *A. niger*, *A. oryzae*, as well as from yeast *Kluyveromyces marxianus*, var. lactis, *K. fragilis*, or *Candida pseudotropicalis*¹⁴⁻²¹. The lactase from filamentous fungi is more heat stable than yeast lactase and has a broader pH optimum and greater acid stability, being able to operate well below pH 6.5¹⁷. Despite the promising biotechnological importance; molecular genetics of halo-tolerant filamentous fungi have received little attentions which are mainly due to the lack of a sexual cycle^{22,23}.



Molecular Aspects of β -Galactosidase Production System in *Aspergillus* Genomes

Halotolerant mechanism of filamentous fungi

Halotolerant microorganisms can grow in very high NaCl concentration (0.5-2.0M) without denaturation of proteins. The proteins themselves appear halo-tolerant in that they are only stable in solvents of high NaCl concentration in which proteins from more familiar physiological environments are likely to aggregate, precipitate or even unfold, depending upon the type of salts^{24,25}. In certain industrial organic synthesis, enzymes are required to function in the presence of a high NaCl concentration of substrate; at low water activities. Enzymes from natural organisms are often inactive in these situations but clearly those from extremely halotolerant microorganisms may be used. Halophilic proteins show unique molecular adaptations. These include the presence of a large excess of acidic amino acids and small amounts of hydrophobic amino acids. The low content of hydrophobic amino acids is offset by a high content of the "borderline hydrophobic amino acids" serine and threonine. In addition, compatible solutes seem to be more efficient than others in protecting enzymes from the harmful effects of exposure to high NaCl concentration and other stressful treatments²⁵⁻²⁹.

The β -galactosidase yield from halophilic bacteria is significantly low and rare, therefore other halotolerant fungi especially *Aspergillus* species are selected for the genetic improvement program. The most general mechanism in halotolerant fungi is to accumulate sugars or sugar derivatives that do not interfere with the regulation of normal metabolic pathways. The most common compatible solutes in fungi are glycerol, which is characteristic of the most stress tolerant fungi^{26,27}. Other osmolytes mannitol, trehalose, sucrose, glucosyl glycerol, and arabitol are also contributed to the osmotic potential of fungi. Glycerol is formed via reduction of dihydroxy acetone phosphate, sucrose and trehalose are produced by coupling of UDP-glucose with fructose-6-phosphate and glucose-6-phosphate respectively, and biosynthesis of glucosyl glycerol is based on the reaction of ADP-glucose with glycerol^{28,29}.

Genetic manipulation of filamentous fungi

The biotechnology potential is increasing exponentially with the isolation of new organisms, the

identification of novel compounds and pathways, and the molecular and biochemical characterization of cellular components³⁰. Wild-type strains isolated from nature usually produce a low level of enzymes. A great deal of effort and resources is therefore committed to improve enzyme producing strains with desirable traits to meet commercial requirements. The genus of *Aspergillus* is well suited for production of extra cellular enzymes from other fungal species. Production in *Aspergillus* species could be beneficial if the natural fungal host that produced the desired enzymes at low levels was not amenable to fermentation nor genetic manipulation, or if it produced undesirable side products in addition to the desired enzyme product. Increasing the productivity of the initial isolates requires a program of genetic improvement. Specialized production strains have been developed for large scale fermentation and manufacture of industrial enzymes^{1,10}. Customarily, the development of production strains has involved classical mutagenesis of wild-type isolates followed by screening or selection for specific improvements in productivity. Yield and specific properties of the currently marketed enzyme products could be improved by application of molecular genetics. For those products that have commercial possibilities, a combination of rigorous biochemical analysis and molecular genetics could accelerate both our understanding of these enzymes as well as commercial development^{30,31}.

Considerable refinement in mutation and selective techniques have occurred in recent years and today, protoplast fusion is one of the most powerful and promising techniques for the genetic manipulation of industrial fungi. The aim of the cell fusion of fungi is principally the generation of fungi expressing new and novel products, increasing the yield of products, or combining the desirable attributes of several organisms into one. Both protoplast fusion and mutational studies are possible with little knowledge of the genetics and biochemistry of the metabolic pathway leading to end products. Protoplast fusion can of course also be used as a reliable way to initiate heterokaryon formation in filamentous fungi even when natural mechanisms are available. Somatic crosses have the disadvantages in the industrial context



Molecular Aspects of β -Galactosidase Production System in *Aspergillus* Genomes

that they tend to efficiently randomize the genomes of the two partners^{1,10}. The development of DNA-mediated transformation systems for several *Aspergillus* species has also sparked interest in rapid improvement of enzyme production strains through recombinant DNA technology^{6,19,32}. It can be used to alter the genotype, so that the phenotype exhibits cellular prosperities that are beneficial for the organisms utilized in industrial processes. This area is a very broad classification that describes any alternations in the cellular operation that leads to an improvement in an industrial strain. The improvements in cellular processes that are not directly involved in the formation of a product can have a significant influence on the industrial value of a strain. Additionally, unexpected results are common when metabolic enzymes are over expressed or repressed. Manipulating the cellular genotype to produce specific changes in the metabolic network outweighs we can rationally design the manipulations so that the desirable quantities are imparted to the organisms^{6,30}.

Development of genetic marker in filamentous fungi

A very useful type of biosynthetic alternation in either cell wall or cell membrane permeability is common mechanism of resistance to toxic chemicals. Such permeability changes may sometimes lead to increased productivity, presumable through increased rate of export from the cell³⁰. Hence, the genetic background of this strain is best suited for the expression of productivity-improvement or cost-reducing mutation. A very useful technique of rational screen is to use a toxic analogue of biosynthetic intermediate to select for resistant mutants. Such mutants may be resistant due to over production of the natural intermediate and thus either lead directly to increased productivity, or provide a genetic background in which further productivity improvements are more likely to be exposed^{33,34}. If drug resistant markers are chosen wisely, the drug resistant mutants themselves could be used as valuable input to the screening program^{2,6}. Addition of DG (2-deoxy-D-glucose) to the media inhibits growth of both yeast and filamentous fungi. Since DG is readily phosphorylated by hexokinase or glucokinase and the accumulated DG-6-phosphate inhibits early glycolytic enzymes as well as the incorporation of glucose into

cell wall polysaccharides. The mechanisms of resistance to DG are generally known to be classifiable into at least three types. The first type is that a new phosphatase specific for DG-6-phosphate. The second type is that resistance results from a defect of hexokinase or glucokinase. The third is that resistance results from alternation in the transport system for glucose. If drug resistance markers are chosen wisely, the drug resistant mutants themselves could be used as valuable input to the screening program^{2,12}.

Experiments for genetic analysis are often limited by the non-availability of suitable markers in a particular organism. The traditional technique of induced random mutations, followed by the selection, is still a major tool, as it can bring about desired alternation in the genes. Such mutations also form the basis of strain improvement strategies and have not been eliminated by the new procedures of molecular biology. Any resistance mutation causing alternation in cell wall or cell membrane structure is potentially capable of allowing increased export of metabolites, but some selective agents seem to be more successful than others¹⁰. The selection of polyene antibiotic-resistant mutants has been well documented as a strain improvement technique in fungi. A transformation system for *A. niger* has been developed using the *A. nidulans* gene 'amdS' for dominant selection, and there is growing evidence to suggest that inter-specific genes between various filamentous fungi and *A. nidulans* is possible^{2,10}.

Current advances of systems biology in filamentous fungi

A more comfortable and interesting cafe can result from industrialization. In order to develop profitable manufacturing, developing nations need a good match of products to excising or potential markets. Biotechnology opens fascinating possibilities in helping to fulfill this condition. Although new method for enzyme hydrolysis of whey and other clearly products are fast approaching commercialization, it may be best to wait until nations with adequate venture capital optimize the technology. Developing nations should address the opportunities presented by the coming availability of less expensive fermentation feed stocks. They should initiate research and development on fermentation products that match



Molecular Aspects of β -Galactosidase Production System in *Aspergillus* Genomes

well their individual development needs. Thus we should make it as a low cost bio-product to our society and will make a bridge to our nation so as to raising our economy³⁵.

Systems biotechnology is emerging so rapidly that no single nations can explore all the promising ideas. A developing country could embark on exciting new research and development and assume a leadership position in certain area³⁰. Solvay Enzymes, Tokyo and Waller stein, USA are manufacturing thermo-stable lactase from *A. oryzae* and *A. niger*, respectively. Microbia has extensive experience in the optimization of fungal processes for the production of statins such as lovastatin and compactin. Processes employing their best statin-producing strains have displayed product yields (YP/S, g product g-1 carbohydrate) approaching 40% of the theoretical yield in appropriately managed fermentations, levels rarely attained in secondary metabolite fermentations. Applications of association analysis, regulator engineering and robust genetic selections to improve lovastatin production in *A. terreus* have been described elsewhere. These approaches have been combined with traditional metabolic engineering and mutation and selection methods, both to enhance existing commercial processes and to develop competitive strains from wild-type isolates^{6,10,30}.

Application of fungal lactase in dairy industry

The cheese-manufacturing industry produces large quantities of whey as a by-product of which lactose represents 70-75% of the whey solids. The hydrolysis lactose by lactase converts whey into more useful food ingredients. Lactose, the main sugar in milk and whey, is hydrolyzed by lactase to glucose and galactose. The enzymatic cleavage of lactose overcomes some disadvantages, like the moderate solubility and lower sweetness of lactose compared with galactose and glucose and its partial indigestibility; normally encountered in the food industry with use of lactose containing products. Therefore depending upon which conditions are required (with regard to temperature, pH, ionic strength, substrate, salt, etc.) a variety of lactases with suitable properties are desirable^{14,16,18}. Lactase derived from mesophilic fungi is relatively thermo-stable (50-55°C). The purpose salting is to aid in whey removal,

shrink the curd, slow down acid development and check undesirable form of bacteria. Under this circumstance, either microbial sources or enzyme sources should be potentially stable even at high concentration of NaCl during the whey syrup processing from cheese or dairy waste management. It created a problem that lactase should be somewhat halotolerant when it applied in a treatment plant containing whey waste. It is also possible if the salted whey is treated with halotolerant *Aspergillus* species properly. The lactase hydrolyzed products (whey syrup, permeate syrup, milk) can serve as ingredients in baked goods, canned fruit, candies, yogurt, ice cream, and other foods. The ready availability of lactose free milk would make more nutritional dairy products available to lactose intolerant people, especially lactose-deficient individuals, who make up a large percentage of the world's population. The cell suspension containing lactose is used to hydrolyze of whey which is subsequently concentrated to produce sweet whey syrup. Lactase is also used for prevention of lactose crystallization in whey concentrate. The introduction of measured doses of lactase solution into a sterilized milk line followed piping to an aseptic filling machine would permit post process supplementation of milk products. This technology would like-wise permit the production of more digestible, lactose free dairy products, besides also increasing the sweetness of the food products. In recognition of the number of waste sites contaminated with organic pollutants plus heavy metals around the world, and safety hazards and cost involved in clean-up using physiochemical means, the potential use of genetically modified microorganisms is an important and exciting product. The application for the lactase system is in the modification of whey from cheese wastes and is also used for dairy wastes management and recycling processes^{3,17,20}.

Lactose utilization pathway in halotolerant *Aspergillus* genomes

We have isolated some halotolerant *Aspergillus* strains from the soil contaminated with dairy industrial waste (Figure 1). By using data mining and a combined multiple functional annotation approaches, we have identified key genes responsible for lactose assimilation and halotolerance in the different types



Molecular Aspects of β -Galactosidase Production System in *Aspergillus* Genomes

Table 1. List of identified genes involved in lactose utilization metabolism in *Aspergillus* genomes

Accession	Protein name	Organism
Lactose metabolism		
EDP56947	MFS Lactose permease	<i>A. fumigatus</i> Af293
EAW08924	MFS Lactose permease	<i>A. clavatus</i> NRRL 1
EAW06436	Carboxylic acid transport protein	<i>A. clavatus</i> NRRL 1
AAC60538	β -galactosidase	<i>A. niger</i>
AAY21925	β -galactosidase	<i>A. phoenicis</i>
CAD24293	β -galactosidase	<i>A. candidus</i>
EAW11155	β -galactosidase, putative	<i>A. clavatus</i> NRRL 1
XP_660805	Hypothetical protein AN3201	<i>A. nidulans</i> FGSC A4
XP_663992	Hypothetical protein AN6388	<i>A. nidulans</i> FGSC A4
XP_001727461	Hypothetical protein	<i>A. oryzae</i> RIB40
EAW09607	Galactokinase	<i>A. clavatus</i> NRRL 1
XP_001215438	Galactose oxidase	<i>A. terreus</i> NIH2624
EAW14017	Galactose-1-phosphate uridylyltransferase	<i>A. clavatus</i> NRRL 1
XP_001208787	Galactose-1-phosphate uridylyltransferase	<i>A. terreus</i> NIH2624
ABL07484	Lactase	<i>A. niger</i>
Halotolerance system		
EAW15085	Betaine aldehyde dehydrogenase	<i>A. clavatus</i> NRRL 1
XP_001210106	Betaine aldehyde dehydrogenase	<i>A. terreus</i> NIH2624
XP_001396859	Hypothetical protein An15g03210	<i>A. niger</i>
CAK42396	Unnamed protein product	<i>A. niger</i>
CAK39302	Unnamed protein product	<i>A. niger</i>
CAK46906	Unnamed protein product	<i>A. niger</i>
XP_001398203	Hypothetical protein An16g09060 (Osmolyte)	<i>A. oryzae</i>
CAK47102	Unnamed protein product (Osmolyte)	<i>A. niger</i>
CAK46958	Unnamed protein product	<i>A. niger</i>

Molecular Aspects of β -Galactosidase Production System in *Aspergillus* Genomes

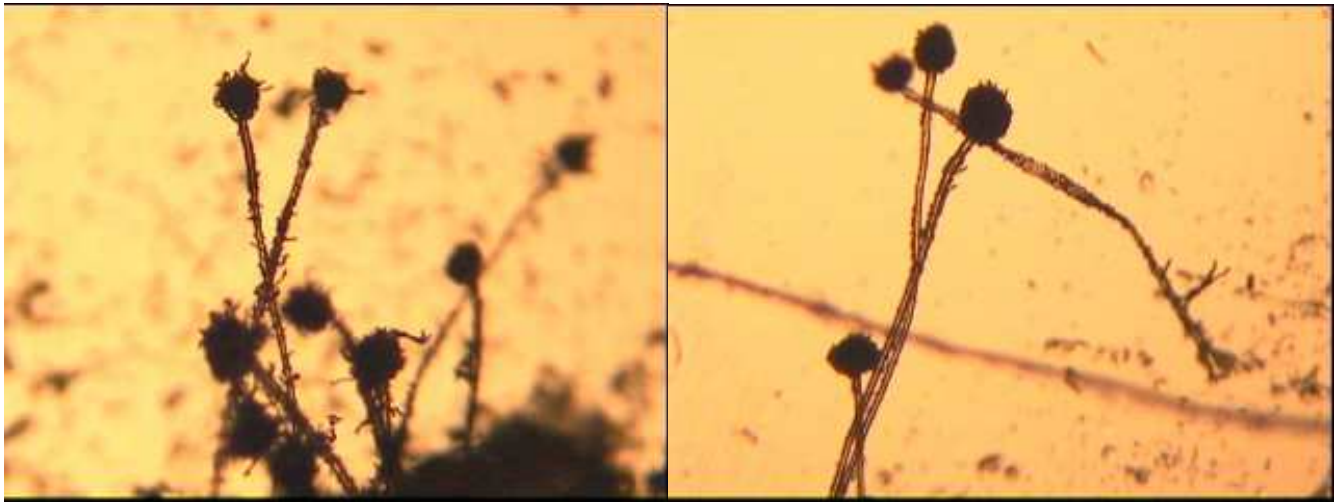


Figure 1. Conidial morphology of halotolerant *Aspergillus* strains isolated from soil contaminated with dairy wastes.

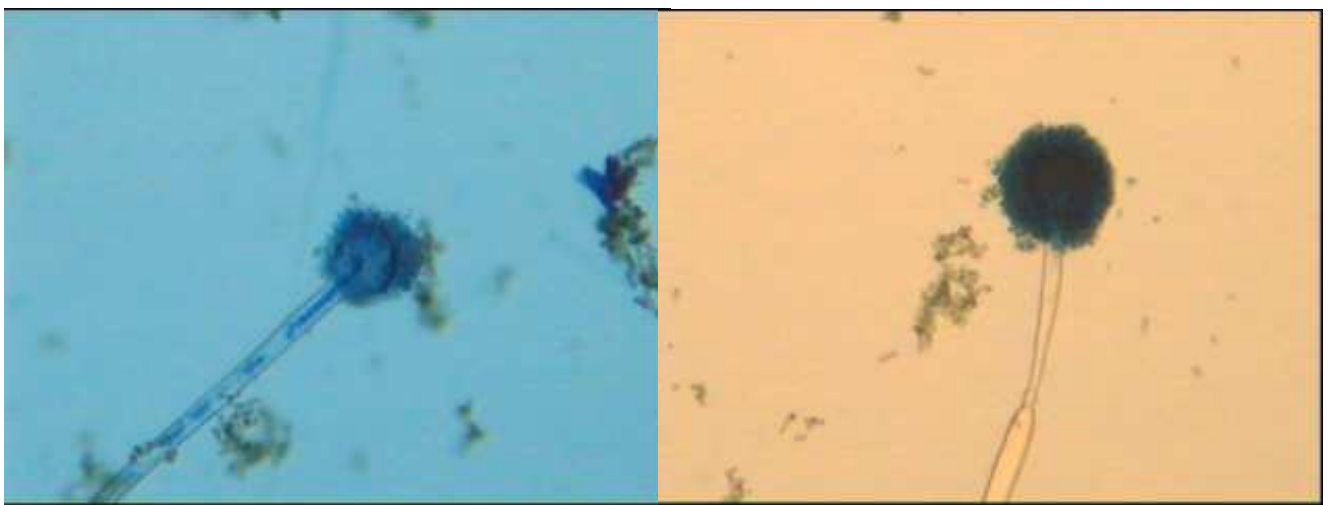


Figure 2. Proposed lactose utilization pathway in *Aspergillus* genomes

Molecular Aspects of β -Galactosidase Production System in *Aspergillus* Genomes

of *Aspergillus* genomes. It showed that β -galactosidase, galactose epimerase, UDP-galactose-4-epimerase, monosaccharide transporter, carboxylic acid transport protein, and major facilitator superfamily proteins were identified in *Aspergillus* genomes. They showed closed phylogenetic relationship with genes related to lactose pathway of bacterial genomes. Similarly, tryptophane synthase (betaine synthase) and cytochrome P450 hydroxylase (halo-tolerant protein), responsible for growing this genus under saline environment were also related with halobacterial proteins (Table 1). On 3D structure homology patterns analyses, most of binding sites and active sites of the hypothetical proteins were corresponded to PDB structure of key enzymes involved in this pathway. Accordingly, we reconstructed metabolic pathway for lactose utilization in the genomes of *Aspergillus* as shown in Figure 2. This proposed pathway can be used to alter the genotype so that the phenotype exhibits cellular prosperities that are beneficial for the organisms utilized in industrial processes.

Conclusion

The different native strains of *Aspergillus* and *Streptomyces* genera have already been isolated and exploited for the purpose of producing extracellular enzymes through solid state fermentation or submerged fermentation^{4,5,36-39}, but, the potential of their industrial usages are comparatively lower than genetically modified fungal strains. Thus, the objective of an industrial strain improvement program should be produce and detect genetically altered cultures that give increased productivity, or in some other way reduce production cost. The genetically modified strains would be helpful to improve the productivity and to minimize the cost of β -galactosidase from native strains. In fermentation industry, the genetically modified *Aspergillus* species (halotolerant) can improve the productivity of lactase enzyme if salted whey or other dairy wastes are used as fermentation substrates (as lactose). This will make a more economy to the industrialists and will generate it as a low cost product. Advances in profiling methods, molecular genetics and the availability of genome sequence information for a wide variety of microbes have led to the development of integrated and flexible strain

improvement platforms to rationally design microbes for the efficient production of commercially valuable metabolites. Thus, these approaches often still benefit from considering the advantages that conventional mutation and screening efforts have to offer. Molecular genetics research should be urged to investigate the new and potential industrial strains for enzyme manufacture so as to improve the quality as well as the quantity of the products.

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Molecular Aspects of β -Galactosidase Production System in *Aspergillus* Genomes

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Molecular Aspects of β -Galactosidase Production System in *Aspergillus* Genomes

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