

Reprint from

ISSN 1550-9087

VOLUME 1 NUMBER 1
SPRING 2005

Industrial Biotechnology

Gene discovery and protein production technology

An integrated system to discover, develop, and
manufacture enzymes and other proteins

Richard P. Burlingame¹ and Ray Chandra²

1. Executive Director, R&D

2. VP, Marketing, Biotechnology Systems,

1.,2. Dyadic International, Inc., Jupiter, FL

*Corresponding Author: Ray Chandra

genomics@dyadic-group.com

Phone: (561) 743-8333 ext. 16

Fax: (561) 743-8343

Dyadic International

140 Intracoastal Pointe Drive, Suite 404

Jupiter, FL 33477

Summary

Until recently, it has been difficult to functionally screen for useful genes from eukaryotes using high-throughput robotic systems. Often, even if the gene is found with one host organism, it is necessary to switch to another host for product development and commercial-scale production of functionally active proteins and enzymes. For novel enzymes and other proteins derived from eukaryotic DNA, no currently used host system fulfills all of the requirements necessary for functional HTS screening, product development, and large-scale manufacturing using the same host cell line.

This article covers an integrated gene discovery and expression platform that is expected to satisfy all of the critical needs for protein and enzyme product discovery, development, and production for commercial purposes. These include the abilities to: (i) rapidly



Figure 1. Robotic systems, shown at Dyadic's Netherlands subsidiary, are used for eukaryotic gene discovery by functional expression. (Top) Caliper LifeSciences' Allegro™ system is used for seeding microtiter plates with gene expression libraries, and for compression and replication of the libraries. After incubation of 96- or 384-well plates for growth and gene expression, the replicate (daughter) plates are assayed for proteins of interest using Caliper's Staccato™ system shown at bottom.

identify viable product leads; (ii) produce sufficient quantities of enzymes/proteins for laboratory and application testing for new industrial uses, as well as for preclinical and clinical tests of new drug candidates; and (iii) develop cost-effective commercial manufacturing processes for new products that are not capital cost- or manufacturing capacity-limited. The same fungal host is used in this platform for all phases of the process, from high-throughput robotic screening for target gene discovery to product manufacturing. By eliminating inherent bottlenecks in process from enzyme and protein discovery, to market launch, this system is expected to result in decreased incompatible leads, increased probability of success in R&D, and accelerated product development.

Introduction

Enzymes and other proteins play an increasingly important role in the discovery and development of new products and processes for not only pharmaceuticals, but also for industrial products in the food, animal feed, pulp and paper, textiles, personal care products, agriculture, and diagnostics industries. Because of the functional roles of proteins and enzymes in the cells of all living organisms, they represent a large fraction of all biologically active molecules and represent the entire spectrum of biological activities. They bring about complex chemical changes, transforming substances one into another, many of which are of significant therapeutic and industrial value. Often, enzymes also simplify produc-

tion processes for fine chemicals and pharmaceutical intermediates where chemical synthesis would be difficult and economically not viable. As a result, there is an accelerating demand for novel proteins and enzymes to satisfy unmet needs of diverse industries. This demand is further enhanced, as enzymes are highly cost-effective and environment-friendly, reducing demand for harsh chemicals and products made from petroleum. As a result, there has been a crying need for an integrated system, based on a single host organism, to discover and develop products from the DNA of eukaryotic organisms, which represent an enormous gene pool that remains largely unexplored.

Currently, there are a variety of systems available to perform discovery, development, and manufacturing steps individually, although not comprehensively. However, some of these systems are limited with regard to their abilities to discover and express genes from a large portion of the Earth's gene pool. Others are impractical for commercial production of many proteins, because of restrictive conditions necessary for growth and cultivation of the host cells or the inability of those systems to produce functional proteins economically at large-scale. For example, bacteria are unable to process introns or largely unable to glycosylate proteins/enzymes. Accordingly, they are unable to express many genes from eukaryotic genomic DNA sources. Yeast, on the other hand, hyperglycosylate and are very limited in their ability to splice introns.

Similarly, mammalian cells and insect cells are difficult to cultivate, and produce relatively low levels of expressed proteins. Their growth media are expensive and capital costs involved are high, so their use is limited to high-value proteins, such as biotherapeutics. However, there too the pressure is increasing on the pharmaceutical industry to find more efficient, lower cost production methods for drugs. While filamentous fungi are very efficient in producing large amounts of proteins, they are, for the most part, unsuitable for growth in the microtiter wells used in the high-throughput environment due to high viscosity in culture. This high viscosity can also lead to operational difficulty in large-scale industrial fermentations.

Taking these issues into account, Dyadic International, Inc., in collaboration with TNO Quality of Life based in Zeist, The Netherlands, is developing a novel system that is expected to allow gene discovery, gene expression, gene evolution, and manufacturing in a single fungal host, *Chrysosporium lucknowense* (C1), which can be cultured at low viscosity in microtiter wells as well as in large, industrial-scale fermentors.

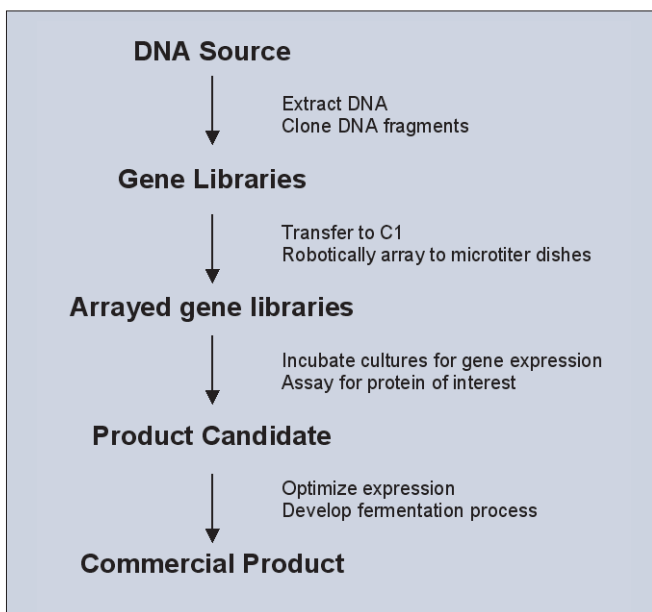


Figure 2. Overview of C1 integrated "gene-discovery to product manufacturing" system

OVERVIEW

An integrated protein & enzyme product-development system

The C1 gene discovery engine utilizes Zymark (now Caliper LifeSciences, Inc.) robots for the preparation and assay of gene expression libraries in C1 (*Figure 1*). The requisite genes can be from various sources—genomic or cDNA from individual organisms, DNA isolated from environmental samples, or individual gene variant sets generated in the laboratory. As summarized in *Figure 2*, gene libraries are transferred to C1, and individual clones are transferred to the wells of microtiter dishes using Caliper LifeSciences' Allegro™ system. After incubation of the cultures for growth and gene expression, cultures are assayed, using Caliper's Staccato™ robotic system, for enzymes and proteins of interest. Once those enzymes and proteins are discovered, expression is further optimized in C1, and a fermentation process is developed, allowing large-scale manufacturing of the product of interest.

The C1 fungal host has differentiating advantages, making it capable of functioning as an integrated discovery through manufacturing system for enzyme and protein products derived from DNA from eukaryotic sources. As a eukaryote, C1 is expected to faithfully express genes from other eukaryote sources. C1 also performs posttranslational modifications characteristic of many eukaryotic proteins and enzymes—for example, glycosylation, with no evidence of the hyperglycosylation described for yeast and other commonly used filamentous fungi. Efficient intron splicing would allow access to portions of the biodiverse gene pool not accessible to other screening systems, such as those using bacterial and yeast hosts. Since more than 90% of the Earth's species are eukaryotic by some estimates, the C1 system is expected to be able to discover genes and gene products that the bacterial and yeast systems will not be able to.

As an organism that was originally developed for the production of extracellular enzymes, C1 is capable of producing proteins and enzymes at high yields inexpensively and in large volume, using commonly used, low-cost, and chemically defined media. However, the advantages of C1 over other fungal expression systems is its ability to be both readily compatible with automated liquid-handling systems, and its ability to be grown in very large industrial-scale fermentors (e.g., 150,000 liters) at low viscosity. Critical features of C1 are summarized in *Table 1*, with comparison to other production systems used in the industry.

C1 has a culture morphology, unique among fungi, that confers

Table 1. Comparison of protein discovery and expression systems

Host System	Bacterial	Yeast	Plant	Insect Cell	Mammalian Cell	Other Fung	C1
Intron Processing	No	Limited	✓	✓	✓	✓	✓
Eukaryotic gene expression	Limited	Limited	✓	✓	✓	✓	✓
HTS Compatibility	✓	Limited	No	No	No	No	✓
Proper Glycosylation	No	No	No	✓	✓	No	TBD
Optimized output for manufacturing	✓	✓	No	No	Limited	✓	✓
Dual usage in HTS and manufacturing	Limited	Limited	No	No	No	No	✓

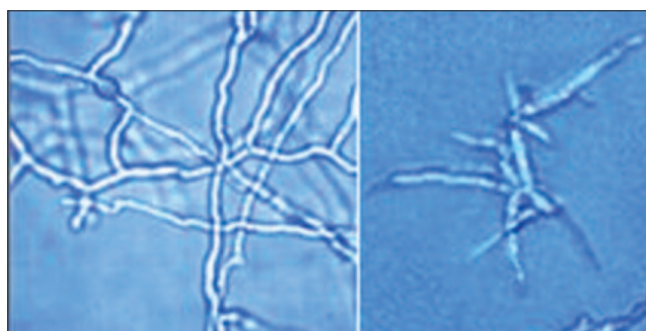


Figure 3. Fungal morphology. At left is a microscopic image of a typical filamentous fungus. The filamentous nature of this organism results in highly viscous cultures, surface matting, and clumping. A C1 "propagule" is shown at right. In addition to other advantages (see text), nonfilamentous growth of C1 allows transfer of propagules from plate to plate in an HTS environment for gene expression library compression and replication.

the ability not only to grow in up to 384-well microtiter dishes but for those microvolume cultures to be robotically transferred from well to well for culture replication. The morphology of C1 leads to the formation of individual mycelial fragments, referred to as transferable elements, or "propagules" (*Figure 3*). The formation of these propagules results in nonviscous growth in culture, allowing adequate aeration in microtiter wells. In addition, formation of propagules results in lack of pelleting, surface matting, and aerial sporulation. This eliminates canula tip clogging in robotic liquid-handling systems. The lack of aerial sporulation, in addition, prevents well-to-well cross-contamination. Of some fifteen species of fungi tested, none exhibited the combination of efficient propagule formation as well as lack of tip clogging that C1 did.

The morphology and physiology of the C1 strain also confers advantages in the area of large-scale manufacturing of enzymes and other protein products. The fragmented nature of the culture promotes high-level production and secretion of these products.

The lower viscosity of the culture also allows the use of fermentation parameters, for example, high feed and aeration rates, that would otherwise be unmanageable. The ability of the strain to grow and produce protein/enzyme at neutral pH offers the additional advantage of allowing the production of pH-labile products. Most other commercially viable yeast and fungal production systems operate in the acidic pH range.

Conclusions & prospects

The C1 gene expression system is currently in late-stage development. About 15 genes, including many human genes, have been expressed in C1. Two enzyme products developed using the C1 system have already been launched for industrial applications, and several others are in various stages of development. In safety and toxicity tests with the C1 organism and an enzyme product from C1, no adverse effects attributable to the organism or the product were found. Based on these test results, a GRAS Notice filing is in progress with the FDA. To further improve the versatility and yield of the C1 host to produce diverse enzymes and proteins from DNA sourced from various eukaryotes, sequencing of the genome of the fungal organism is also under way.

The gene discovery system is also in the final stage of validation. Current activities are involved with optimization of gene discovery from libraries of fungal genes. These studies will be expanded to the screening of DNA/cDNA libraries for other eukaryotic organisms. In addition, the screening system is anticipated to be ideally suited to the screening of evolved gene variants

for engineered proteins with improved functional properties. In this context, the C1 system is expected to be uniquely advantageous for many eukaryotic and secreted proteins.

The C1 system is expected to have additional utility for discovering and developing new diagnostic enzymes, proteins, and reagents for the healthcare, chemicals, and food industries. One potential novel use could be in the discovery and development of new antifungal compounds and fungicides. A filamentous fungus that operates in an HTS setting is especially well suited for such research, to: (i) identify and isolate genes involved in the disease process through mode-of-action studies; and (ii) screen compound libraries to identify highly specific compounds against fungal pathogens. Until now, such a fungus has been unavailable, necessitating the use of yeast, with the corresponding limitations as previously described; the C1 system will make such compromises unnecessary.

Having an integrated system such as that described here is expected to eliminate bottlenecks in the product and manufacturing development process for novel enzymes and proteins needed by various industries. Using expression as a criterion for discovery eliminates a potential bottleneck in obtaining sufficient quantities of material needed for laboratory and field testing of enzymes for industrial applications, as well as preclinical and clinical testing of biopharmaceuticals. Eliminating this bottleneck is expected to contribute to a decrease in incompatible leads, a higher probability of success in R&D, and shorter R&D timelines for bringing new products to market. 