FEASIBILITY OF HANSENULA AS AN EXPRESSION SYSTEM

By

Amit Midha (031509)

Neha Ahuja (031547)

Pankaj Sharma (031549)

Submitted in partial fulfillment of the Degree of Bachelor of Technology

DEPARTMENT OF Biotechnology and Bioinformatics

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY-WAKNAGHAT

JUNE-2007

CERTIFICATE

This is to certify that the work entitled, "**EASIBILITY OF HANSENULA AS AN EXPRESSION SYSTEM**" submitted by *Amit Midha, Neha Ahuja, Pankaj Sharma* in partial fulfillment for the award of degree of Bachelors of Technology in ***Bioinformatics*** of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Place: Waknaghat, Solan

Date: 02, June, 2007

Gyan

[Dr.Gyan P. Mishra]

ACKNOWLEDGEMENT

We would like to express our sincere gratitude to our guide **Dr. Gyan P. Mishra** whose able guidance, tremendous support and continuous motivation made this project work possible.

We would also like to show appreciation to **Dr. Anil Kant** for the invaluable time and assistance he gave us with the project. A special thanks to **Dr. Chauhan** for his extremely valuable inputs. We would also like to thank our families and friends without whose cooperation this project could not have been possible.

Place : Waknaghat

Date : 02 June, 2007

(Amit Midha, Neha Ahuja, Pankaj Sharma)

CONTENTS

List of Figures

List of Tables

Abstract

Feasibility and Market Size Determination are the key areas of focus. There are many yeast expression systems in the market for various therapeutic and research purposes. A comparison of three major yeast expression systems has been presented, highlighting the superiority of one over the other. This study shows that the recombinant protein industry, with a market value of $32 billion in 2003 was expected to rise by almost 13% in another six years. In 2004, this industry sector included more than 110 companies that were involved in the discovery, development, and marketing of rDNA products. These companies had a pipeline of more than 80 therapeutics in clinical development and a combined portfolio of 73 marketed products.The *Hansenula polymorpha* expression system has a huge market potential due to its application in both pharmaceutical and non-therapeutic areas. From vaccines to animal feed and food ingredients, chemical industry to agriculture, it has a wide application range.This feasibility study compares the Hansenula expression technology with all other expression systems available in the market. We have done technology analysis and highlighted the advantages, disadvantages and discussed the Hansenula Technology as an upcoming expression system for Recombinant Protein production.

INTRODUCTION

The discovery and development of recombinant DNA technology has formed the cornerstone of the modern medical biotechnology industry. With a market valued at almost $32 billion in 2003, the changing dynamics of the recombinant therapeutic proteins market will have a significant impact on numerous companies and therapy areas.The need for suitable expression systems for highly efficient and reliable production of pharmaceutical and industrial proteins is increasing continuously due to ever-growing new and generic target proteins. The selection of an appropriate expression system is a crucial decision. The considerable potential of yeast has been demonstrated in several commercial areas aside from traditional food products, e.g., oxido-reduction and/or enantio- and regioselective reactions in organic chemistry; total biosynthesis from simple carbon sources; production of technical enzymes such as lipases and esterases, and low cost products such as feed enzymes. Furthermore, yeast cell material can be a source of several substances beneficial to health or can be used in cosmetic applications (e.g., glycans, ceramides). The species best established in industrial production procedures are *S. cerevisiae, K. lactis, P. pastoris, and H. polymorpha.*

Yeasts are attractive hosts for the production of heterologous proteins. Unlike prokaryotic systems, their eukaryotic subcellular organization enables them to carry out many of the post-translational folding, processing and modification events required to produce "authentic" and bioactive mammalian proteins. In addition, they retain the advantages of a unicellular microorganism, with respect to rapid growth and ease of genetic manipulation. Other than this a number of noteworthy advances in the over-expression of recombinant proteins in yeasts have enabled the secretion of correctly folded, glycosylated or even 'humanized' (and thus pharmacologically active) proteins. The frequent exclusion of low-priced products derived from natural plant or animal sources is due to strict requirements for consistent product quality, in addition to high purity and safety issues with respect to increasingly emerging diseases such as BSE.

During past two decades hansenula polymorpha has drawn attention as a promising host for the production of heterologus protein. The increasing popularity can be attributed to the advantages it offers over the traditional Saccharomyces cerevisiae as it has very strong tightly regulated promoters besides this it has a strong frquence of non homologus recombination. It has high yields, short and flexible fermentations, cost effective, easy and inexpensive processing, secure and reliable production. Proteins with molecular mass higher than 30 kDa are retained in the cytoplasm sac of *Saccharomyces cerevisiae,* whereas *Hansenula polymorpha* efficiently secretes proteins with a molecular mass of above 150 kDa, like glucoamylase.

The two species of methylotrophic yeast *Hansenula polymorpha* and *Pichia pastoris.* Although these two species are close relatives, there are some important distinctions between the species and the expression systems developed in them that should be considered when selecting the system for a specific application. The *Hansenula polymorpha* expression system has a huge market potential due to its application in both pharmaceutical and non-therapeutic areas. From vaccines to animal feed and food ingredients, chemical industry to agriculture, it has a wide application range.Like other yeasts it is a microorganism that can be cultured in large fermenters to high cell densities within a short time. It is a safe organ-ism in not containing pyrogens, pathogens or viral inclusions. It can release compounds into a culture medium as it has all the components required for secretion (this is for instance not the case with bacteria like *Escherichia coli*). It can provide attractive genetic components for an efficient production of proteins.

It must contain several genetic elements: 1. A selection marker, required to select a transformed strain from an untransformed background –this can be done if for instance such an element enables a deficient strain to grow under culturing conditions void of a certain compound like a particular amino acid that cannot be produced by the deficient strain). 2. Certain elements to propagate and to target the foreign DNA to the chromosome of the yeast (ARS and/or rDNA sequence).

This report evaluates the market opportunities for *Hansenula* yeast expression system. The attractiveness of the *H. polymorpha* platform is commercially exploited by several biotech companies for the development of production processes.

The objective of the project are:

1. Comparing hansenula expression system with other technologies available in the market and analysing its advantages and drawbacks over other expression systems in the market.

2. Market size identification and feasibility study for hansenula expression system.

REVIEW OF LITERATURE

EXPRESSION SYSTEM

An expression system consists, minimally, of a source of DNA and the molecular machinery required transcribing the DNA into mRNA and translating the mRNA into protein using the nutrients and fuel provided.

Common expression systems include: Bacteria (such as E.coli), yeast (such as S.cerevisiae), plasmid, artificial chromosomes phage (such as lambda), cell lines or virus (such as baculovirus, retrovirus, and adenovirus) (www.wikipedia.com).

PROTEIN EXPRESSION

Recently, numerous developments have been made to improve the production of soluble and active proteins in heterologous expression systems. These include modifications to the expression constructs, the introduction of new and/or improved pro- and eukaryotic expression systems, and the development of improved cell-free protein synthesis systems. Generally, protein expression systems can be categorized into the following groups namely prokaryotic expression system and eukaryotic expression system. (G. M. Bhopale* and R. K. Nanda, 2004)

Prokaryotic Expression Systems:

E.coli expression system uses hosts for the production of heterologous proteins and its genetics are far better characterized than those of any other microorganism. To clone the gene of interests into a variety of E. coli expression vectors with different expression tags or fusion proteins and express them in a basic E. coli strain. To clone the gene of interests into a regular E. coli expression vector and express it in a variety of E. coli hosting strains

Insect and mammalian cell cultures

Insect cells are a higher eukaryotic system than yeast and are able to carry out more complex post-translational modifications. They also have the best machinery for the folding of mammalian proteins and, therefore, give you the best chance of obtaining soluble protein when you want to express a protein of mammalian origin. The most commonly used vector system for recombinant protein expression in insect is

baculovirus, although baculoviral also can be used for gene transfer and expression in mammalian cells.

Advantages of Baculovirus-assisted insect cell expression is that it is optimal for glycosylated protein expression in a cost-effective manner. There are many advantages for heterologous gene expression. Heterologous cDNA is expressed well. Proper transcriptional processing of genes with introns occurs but is expressed less efficiently. As with other eukaryotic expression systems, baculovirus expression of heterologous genes permits folding, post-translational modification and oligomerization in manners that are often identical to those that occur in mammalian cells. The insect cytoplasmic environment allows proper folding and S-S bond formation, unlike the reducing environment of the E. coli cytoplasm. Post-translational processing identical to that of mammalian cells has been reported for many proteins. These include proper proteolysis, N- and O-glycosylation, acylation, amidation, carboxymethylation, phosphorylation, and prenylation. Proteins may be secreted from cells or targeted to different subcellular locations. Single polypeptide, dimeric and trimeric proteins have been expressed in baculoviruses. Finally, expression of heterologous proteins is under the control of the strong polyhedrin promoter, allowing levels of expression of up to 30% of the total cell protein.

Deficiencies of insect cells are seen as an inefficient processing and an impairment of the folding and secretion capacity due to baculovirus infection, a high, in part baculovirus- encoded, protease activity and the resulting necessity to routinely employ protease inhibitors in the culture media or to develop protease deficient vectors, an insufficient expression strength and deviations of the posttranslational modification pattern, which could act immunogenically. For optimization, the construction of new innovative vectors and the coexpression of chaperones, foldases and folding factors such as canexin have been suggested, as has the broader development and application of alternative systems like Drosophila. Preferably applied in pharmaceutical production processes are mammalian systems like CHO cells and BHK cells.

YEAST EXPRESSION SYSTEM

Yeast is a eukaryotic organism and has some advantages and disadvantages for protein expression as compared to E. coli. One of the major advantages is that yeast

cultures can be grown to very high densities, which makes them especially useful for the production of isotope labeled protein for NMR.

The two most used yeast strains are *Saccharomyces cerevisiae* and the methylotrophic yeast *Pichia pastoris*. Various yeast species have proven to be extremely useful for expression and analysis of eukaryotic proteins. These yeast strains have been genetically well characterized and are known to perform many posttranslational modifications. These single-celled eukaryotic organisms grow quickly in defined medium, are easier and less expensive to work with than insect or mammalian cells, and are easily adapted to fermentation. Yeast expression systems are ideally suited for large-scale production of recombinant eukaryotic proteins.

Advantages includes high yield, high productivity , chemically defined media, product processing similar to mammalian cells ,stable production strains, durability ,lower protein production cost.

Reason for high level of protein production is adequate copies of vector (10-100 copies per cell), suitable promoters, proper inducible system, targeted cellular location. (What's in Store for Yeast Biotechnology? – The Joint 3rd Swiss–Czech and BioTech2005 Symposium in Retrospect; 2005)

CHARACTERSTICS

High cell densities

When yeast is grown with the high-cell-density fermentation technology, unprecedented levels of cell mass per liter of fermentation fluid are produced. The system has attained dry-cell-weight densities exceeding 100 gram/liter and continuous fermentation productivities of 10 to 12 grams of recombinant protein/liter/hour.

Controllable process

The growth medium that feeds yeast is completely defined. It consists of a simple, inexpensive formulation. The carbon source is fed to the fermentor at a rate designed to achieve maximum cell density while maintaining optimal production of foreign protein. This process minimizes any toxic effects the foreign protein might have on the yeast.

Generations of stability

Expression of foreign genes is achieved by integration of foreign DNA into the chromosomal DNA of the host genome. The integrated DNA is stable for many generations; all cells can produce the protein. In contrast, plasmid-based systems require selective pressure on plasmids to maintain the foreign DNA. Cells that lose the plasmid cannot produce the desired foreign protein.

Durability

The Yeast Expression System requires no special handling. It was developed to withstand the adverse conditions of large scale, continuous fermentors. This feature makes yeast able to survive unexpected disruptions in the fermentation process.

Maximum Value

High per-cell expression levels combined with high cell-density growth of yeast translates into greater quantities of recombinant protein per fermentor volume. This reduces production costs by increasing the amount of product per fermentation run. Protein purification is another cost-saving area. The yeast system can secrete protein into the medium, so the broth that enters purification contains a higher concentration of the desired protein. Pure protein is recovered with higher yield and lower cost.

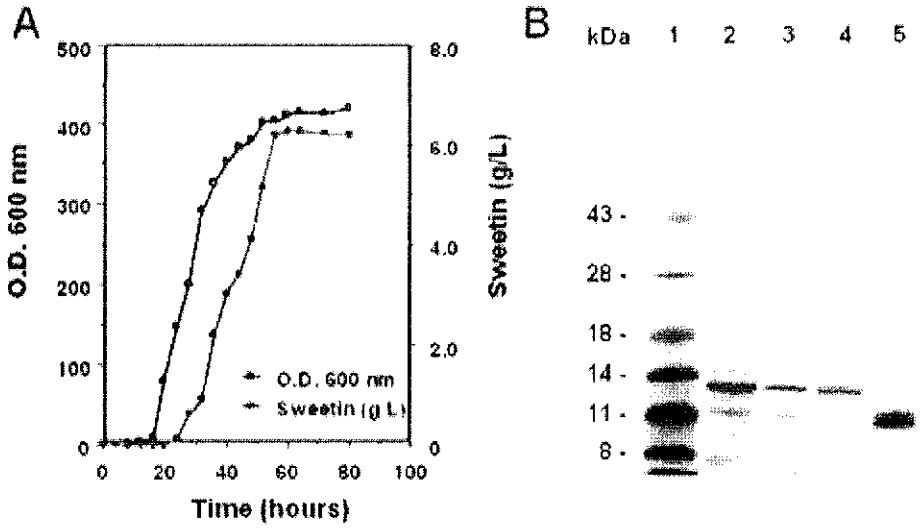


Figure 1. Recombinant protein production in yeast.

MAMMALIAN EXPRESSION SYSTEM:

Usage of mammalian expression

The proteins produced in the mammalian expression system have the best structural and functional features that are usually most close to their cognate native form and can satisfy the following application needs or utility namely transgene expression, biochemical analyses, assay standards, functional studies of the protein (in vitro and ex vivo), structural studies, including protein crystallization, protein structure and NMR, protein-protein interaction experiments, enzyme kinetics, immunogen for antibodies development, proteomic and phenomics study, drug target discovery and validation, cell line development, drug screening, and in vitro model system,animal studies, including in vivo functional and ADME, PK/TK and safety studies, physiology and pathology studies, diagnostic application, therapeutic application, prophylactic (vaccine) development, protein engineering and mutagenesis studies.

Membrane protein expression

Integral membrane protein expression in mammalian cells is not difficult if one is trying to accomplish expression at low physiological levels. For example, integral membrane protein expression has been used for decades for drug and biological discovery. However, over-expression of integral membrane proteins presents a number of different challenges like they produce a large quantity of membrane protein for purification etc.

RECOMBINANT PROTEINS

Gene recombinant pharmaceuticals are continuously gaining an increasing importance in medicine and are expected to help cure diseases which are not yet treatable today. Some 30 compounds (Table 1), with an actual market volume of U.S. $ 50–60 billion, have already been introduced into therapy and about 300 compounds are estimated to be in development worldwide. The expression systems employed for their manufacture have to meet key criteria, like consistent product quality and cost-effectiveness. Of particular interest with respect to the latter issue are expression systems enabling the secretion of correctly folded proteins into the culture broth. Such secretory systems offer advantages in terms of simple and fast product-purification procedures and the avoidance of costly cell rupture, denaturation and refolding

processes. This article gives an overview on expression systems currently employed in the pharmaceutical industry, evaluates the most prominent secretory systems among the prokaryotes, yeasts and filamentous fungi as well as insect and mammalian cell cultures with respect to their suitability for pharmaceutical manufacture by discussing their potential productivity and their physiological properties and outlines optimization strategies which are presently applied to render secretory systems more efficient and competitive. (Alex K Pavlou & Janice M Reichert, Recombinant protein therapeutics—success rates, market trends and values to 2010 (December 2004)).

Table 1 Overview on the currently worldwide commercialized recombinant pharmaceuticals and the expression systems employed for their production. The substances are not listed strictly alphabetically but are partially grouped according to therapeutic areas. Antibodies, which are mostly manufactured by hybridoma cell line systems are not considered. Data were extracted from the European patent data base (esp@cenet; http://de.espacenet.com) and the IDdb3 data base (http://www.iddb3.com). (*BHK* Baby hamster kidney, *CHO* chinese hamster ovary)

Product	Company	System
Blood coagulation factors (VII, VIII, IX)	Novo-Nordisk/Bayer/Centeon	BHK cells
	Genetics Baxter/Centeon/Wyeth	CHO cells
Calcitonin	Unigene	*Escherichia coli*/CHO cells
DNase (cystic fibrosis)	Roche	CHO cells
Erythropoetin	Janssen Cilag/Amgen/Boehringer	CHO cells
Darbepoetin	Amgen	CHO cells
Follicle stimulating hormone (follitropin)	Serono/Organon	CHO cells
Luteinization hormone	Serono	CHO cells
Gonadotropin	Serono	CHO cells
Glucagon	Novo-Nordisk	*Saccharomycex cerevisiae*
Glucocerebrosidase (Gaucher disease)	Genzyme	CHO cells
Growth hormones (somatotropines)	Pharmacia & Upjohn/Lilly/ Novo-Nordisk/Ferring/Genentech	*E. coli*
	Serono	Mouse cell line
	Serono/Bio-Technology General Corp	CHO cells
Eutropin (human growth hormone derivative)	LG Chemical	*Sac. cerevisiae*
Growth factors (GCSF, GMCSF)	Novartis/Essex/Amgen/Roche	*E. coli*
	Chugai Pharmaceuticals	CHO cells
Platelet derived growth factor (PDGF)	Janssen-Cilag	*Sac. cerevisiae*
PDGF-agonist	Zyno/Genetics	*Sac. cerevisiae*
Hepatitis B vaccine	GlaxoSmithKline	*Sac. cerevisiae*
	Rhein-Biotech	*H. polymorpha*
Hirudin	Aventis/Novartis	*Sac. cerevisiae*
Insulin and muteins	Aventis/Lilly/Berlin-Chemie	*E. coli*
Insulin	Bio-Technology General Corp	*E. coli*
	Novo-Nordisk	*Sac. cerevisiae*
Interferon alpha and muteins	Roche/Essex/Yamanouchi	*E. coli*
Interferon beta	Schering	*E. coli*
	Biogen/Serono	CHO cells
Interferon gamma (mutein)	Amgen/Boehringer	*E. coli*
Interleukin 2	Chiron	*E. coli*
Oprelvekin (interleukin 11-agonist)	Wyeth	Human cell line ROMI 8866
OP-1 (osteogenic, neuroprotective factor)	Curis/Striker	*E. coli*
Tissue plasminogen-activator	Genentech/Roche/Boehringer	CHO cells
Recombinant plasminogen-activator	Genentech/Roche/Boehringer	*E. coli*
Stem cell factor	Amgen	CHO cells
Tumor necrosis factor	Boehringer	*E. coli*

Prokaryotic (bacteria) or eukaryotic (yeast, mammalian cell culture) systems are generally used as a host for the production of usable quantities of the desired rDNA

product2–4. Most of the rDNA products approved by FDA are being produced using these systems. Bacteria such as Escherichia coli are widely used for the expression of rDNA products. They offer several advantages due to high level of recombinant protein expression, rapid growth of cell and simple media requirement. However, there are some limitations such as intracellular accumulation of heterologous proteins, the potential for product degradation due to trace of protease impurities and production of endotoxin. Yeast such as Saccharomyces cerevisiae, Hansenulla polymorpha and Pichia pastoris are among the simplest eukaryotic organisms.

However, cost of production of the products using these systems is high because of slow growth and expensive nutrient media. The choice of expression system can influence the character, quantity and cost of a final product.

Production and purification of rDNA products

A general process for the production of rDNA products is illustrated in Figure 1. The first step is isolation of the identified gene that is responsible for expression of the desired product. After isolation and characterization of the human gene, it is inserted into small circular pieces of DNA called plasmid. The recombinant plasmid is inserted into a bacterial yeast or cultured animal cell. Clones of transformed host cell are isolated and those that produced the protein of interest in the desired quantities are preserved under suitable condition as a master cell bank. The cell banks are characterized and properly maintained for use in subsequent transformation procedures. The cell bank should be periodically tested for cell viability, genetic and phenotypic stability. As manufacturing needs arise, cells from working cell can be scaled up to produce the product in roller bottles and/or fermentors. Fermentors are generally used for growth of E. coli or yeast. Mammalian cells are often grown in roller bottles. Inoculation of host cells that contained an expression vector is added to defined volume of medium in either fermentors or roller bottles. The cells are allowed to grow until the nutrients in the medium are depleted or excreted by the products reaching inhibitory levels. By providing a balanced mixture of nutrients and/or chemicals to neutralize accumulating growth inhibitor, product yield in the medium or cell density can be improved. At the end of the run, the host cells are harvested and the recombinant

product is isolated from culture medium or cells. (F. R. Schmidt, Recombinant expression systems in the pharmaceutical industry. (July, 2004))

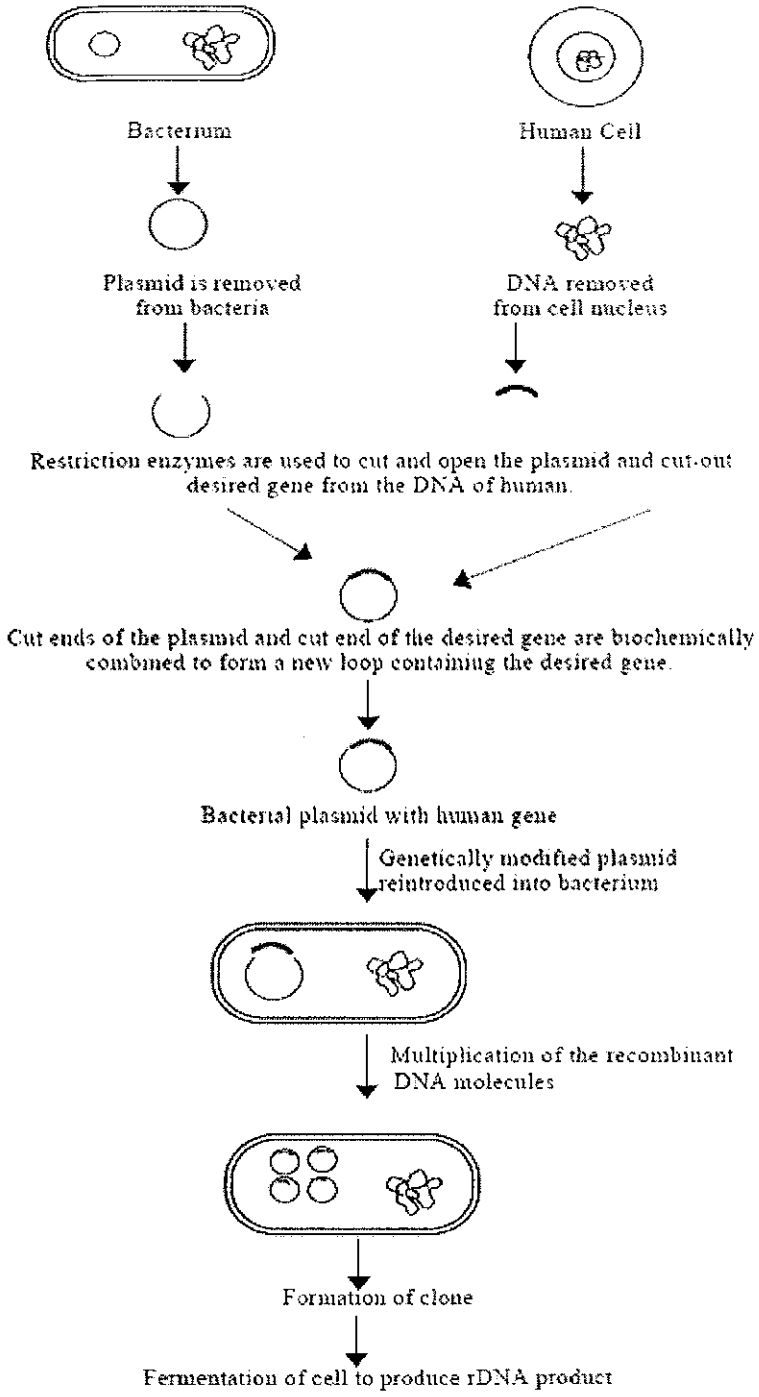


Figure 2. Recombinant DNA Technology for production of human therapeutics.

Purification is an important aspect in the production of rDNA products. The overall goal of purification is to bring as much product with as little loss as possible. A general purification process is presented in Figure 2.

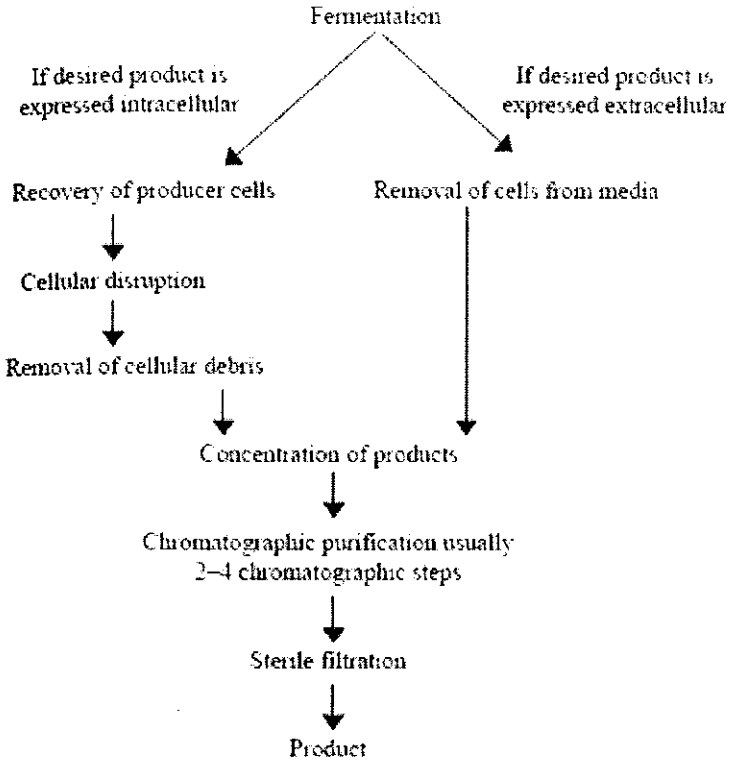


Figure 3. General Process for purification of rDNA products.

This step is normally achieved by centrifugation or filtration. The cells are disrupted or lysed and cell debris is removed by centrifugation, leaving behind a dilute solution of crude desired product. If mammalian or yeast system is used, the desired product can be obtained directly from the medium. Nowadays, ultrafiltration has become the method of choice of concentration of products12. There are several different methods that can be used for purification of rDNA products, but only chromatographic purification methods are generally used (Table 1)13–15. A combination of two to four different chromatographic techniques is generally employed in a typical downstream processing procedure. Gel filtration and ion exchange chromatography are the most common. Affinity chromatography is employed wherever

possible, as it has high biospecificity and one can achieve a high degree of purification. Appropriate attention needs to be given to validation of the purification process such as column loading capacity and column regeneration. The purification process must be validated to ensure that it is adequate to remove extraneous substances such as chemicals used in purification, column contaminations, endotoxin, residual cellular proteins and viruses. The reproducibility of the purification process with respect to its ability to remove specific contaminants should also be determined. Further, columns should also be validated regarding leaching of legends (e.g. dye, affinity legends, etc.) throughout the expected lifespan of the column. The evidence of purity of the purified product should be established. Physico-chemical, biological and immunological characterizations of purified product should also be obtained using a wide range of analytical tests. Characterization tests of products may include mainly amino acid composition, peptide mapping, electrophoretic assays (SDS–PAGE, Western blot, isoelectric focusing), HPLC, immunoassay, endotoxin test, potency determination test, etc.

Gene expression

Gene expression, or simply expression, is the process by which a gene's DNA sequence is converted into functional proteins.

Any step of gene expression may be modulated, from the transcription step to post-translational modification of a protein. Gene regulation gives the cell control over structure and function, and is the basis for cellular differentiation, morphogenesis and the versatility and adaptability of any organism. Gene regulation may also serve as a susbstrate for evolutionary change, since control of the timing, location, and amount of gene expression can have a profound effect on the functions (actions) the gene in the organism. Non-protein coding genes (e.g. rRNA genes, tRNA genes) are not translated into protein.

The expression of many genes is known to be regulated after transcription, so an increase in mRNA concentration need not always increase expression. Nevertheless, the expression of many genes at once may be assessed with DNA microarray technology, which can provide a relative measure of the cellular concentration of different messenger RNAs; often thousands at a time. While the name of this type of

assessment is actually a misnomer, it is often referred to as expression profiling. Such expression profiles may be an indicator of response of the cells to certain exposures or events. A more sensitive and more accurate method of assessing the relative expression of individual genes is real-time polymerase chain reaction or RT-PCR. With a carefully constructed standard curve RT-PCR can produce an absolute measurement such as in number of copies of mRNA per nanolitre of homogenized tissue, or in number of copies of mRNA per total poly-adenosine RNA. Protein expression levels can be estimated by a number of means. One method involves fusing the gene sequence of the desired protein to that of another gene which can serve as a reporter protein, such as the green fluorescent protein or the enzyme beta-galactosidase. The expression level of these reporter proteins can be directly quantified using standard techniques. This technique is very powerful, but may be limited by possible changes in the functional behaviour of the expressed fusion construct relative to the natural protein. Less sophisticated methods of measuring protein expression include "Western" blotting, immunoassay, and functional (e.g. biochemical) assays. The pattern of detection of a gene or gene product may be described using terms such as facultative, constituative, circadian, cyclic, housekeeping, or inducible. (bcs.whfreeman.com/lehninger)

Modification of DNA

Chemical modification of DNA

Methylation of DNA is a common method of gene silencing. DNA is typically methylated by methyltransferase enzymes on cytosine nucleotides in a CpG dinucleotide sequence (also called "CpG islands"). Analysis of the pattern of methylation in a given region of DNA (generally a promoter) can be achieved through a method called bisulfite mapping. Methylated cytosine residues are unchanged by the treatment, whereas unmethylated ones are changed to uracil. The differences are analyzed in sequencing gels. Abnormal methylation patterns are thought to be involved in carcinogenesis.

Regulatory protein is a term used in genetics to describe a protein involved in regulating gene expression. It is usually bound to a regulatory binding site which is sometimes located near the promotor although this is not always the case. Regulatory proteins are often needed to be bound to a regulatory binding site to switch a gene on,

(activator) or to shut off a gene (repressor). Generally, as the organism grows more sophisticated, their cellular protein regulation becomes more complicated and indeed some human genes can be controlled by many activators and repressors working together.

HANSENULA EXPRESSION SYSTEM – TECHNOLOGY FOCUS

The technology discussed is focused on the prime standard in yeast expression technology, Hansenula polymorpha. The advantages of this technology are best proven by the number of different products in the global market. Both pharmaceutical and non-therapeutic products are efficiently and affordably manufactured from recombinant Hansenula. The technology is applied for high-level secretion of recombinant proteins, as well as for intra-cellular production of soluble proteins. High strain and product stability, unmet productivity and process economy as well as the absence of pathogens and pyrogens make Hansenula a reliable and accepted system for a number of commercial products and applications. Productivities are typically ranging from 0.5 to more than 10 g/l. Manufacturing costs are much less than for other systems applied. Hansenula derived pharmaceutical and feed/food products have been registered in more than 60 countries and have been approved by WHO and FDA.

FEATURES OF HANSENULA

Genetics: High stability of strains; multi-copy strains with up to 120 copies per cell; co-expression of up to 3 different genes; vectors w/o antibiotic resistance marker; stable high yields.

Fermentation: Wide range of cultivation conditions and it requires simple, inexpensive media without methanol; short fermentation times: 50 hrs; Up-scaling to 30 m^3 achieved: short and flexible fermentations

Product: Low levels of impurities; high productivity: 0.5 - 13.5 g/L; high stability ; correct folding and processing; cost effective and easy.

Reliability: Approved Hansenula derived products; high batch to batch reproducibility; strong patent protection; no allergenic potential; established technology

Approaches & Tools

Gene design and expression optimization (transcription/translation)

Increased expression level by engineered non-translated regions; strain engineering;

vector engineering, fermentation design; protein release, improvement of expression/secretion at high level,Availability of new tools (vectors and strains).

Hansenula Technology Platform

High stability of multi-copy (120 per cell) strains; Co-expression of different genes, Fermentation: robust (w/o complex additives),short (50 hrs), cheap (no MeOH), large scale (30 m^3); High productivities: 0.5 - 13.5 g/L ;Correct folding, disulfide bonding and processing; Intracellular production of soluble proteins; No allergenic potential.

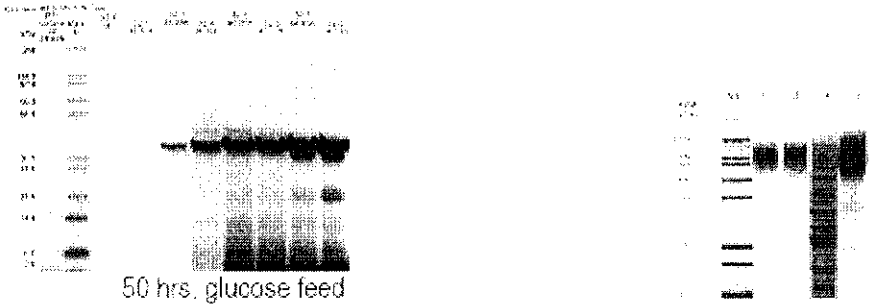


Figure 4. Proteins bands (Electrophoresis results)

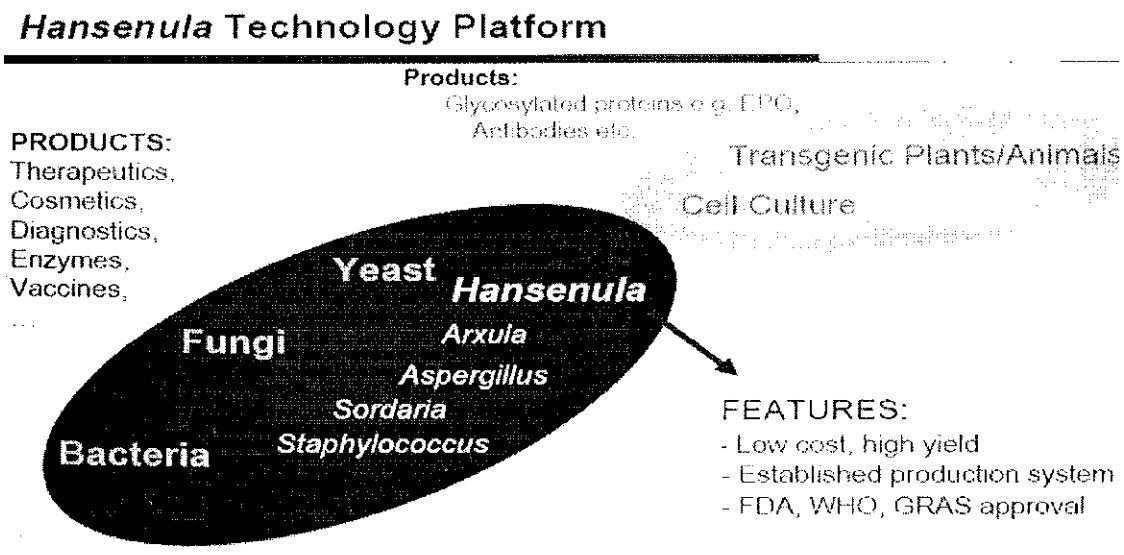


Figure 5. Technology Focus

Hansenula Characteristics

The growing demand for a microbial expression system that circumvents the complications associated with E. coli has led to the development of a proprietary expression system in the methylotrophic yeast Hansenula polymorpha.

Methylotrophic Yeast

The four known genera of methylotrophic yeast (Hansenula, Pichia, Candida, and Torulopsis) share a common metabolic pathway that enables them to use methanol as a sole carbon source (see figure). In a transcriptional regulated response to methanol induction, several of the enzymes are rapidly synthesized at high levels. Since the promoters controlling the expression of these genes are among the strongest and most strictly regulated yeast promoters, methylotrophic yeast have become very attractive as hosts for the industrial production of recombinant proteins. The cells themselves can be grown rapidly to high densities, and the level of product expression can be regulated by simple manipulation of the medium. (Benz I, Schmidt A (2002) Never say never again: protein glycosylation in pathogenic bacteria. Mol Microbiol 45:267– 276).

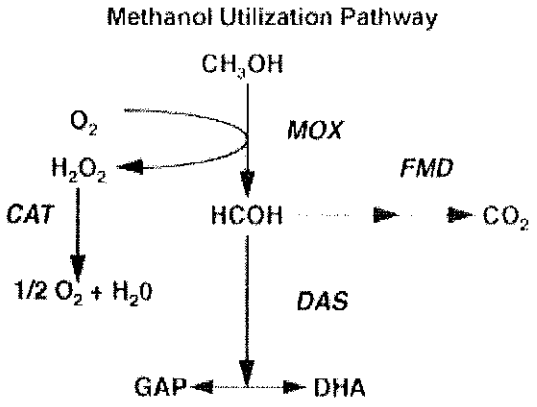


Figure 6. Methanol Utilization Pathway.

Hansenula polymorpha is amethylotrophic yeast with unusual characteristics. It is used as a protein factory for pharmaceuticals.

Hansenula polymorpha (Pichia angusta) belongs to a limited number of methylotrophic yeast species (yeasts that can grow on methanol). The range of methylotrophic yeasts includes Candida boidinii, Pichia methanolica, Pichia pastoris and Hansenula polymorpha. H. polymorpha is taxonomically a species of the Saccharomycetaceae family. The leading taxonomy monographs follow a recent proposal to merge the genera Pichia and Hansenula and to re-name H. polymorpha as Pichia angusta. However, many scientists desire to maintain the popular name H. polymorpha. Three basic *H. polymorpha* strains with unclear relationships and of independent origins exist identified in the fifties in soil samples, the gut of insects or in spoiled concentrated orange juice. They exhibit different features and are applied to basic research and to recombinant protein production: Strain CBS4732 (CCY38-22-2; ATCC34438, NRRL-Y-5445) strain DL-1 (NRRL-Y-7560; ATCC26012) strain NCYC495 (CBS1976; ATAA14754, NRLL-Y-1798) strains CBS4732 and NCYY495 can be mated whereas strain DL-1 cannot be mated with the other two. Strains CBS4732 and DL-1 are employed for recombinant protein production, strain NCYC495 is mainly used for the study of nitrate assimilation.

The entire genome of strain CBS4732 has completely been sequenced. H. polymorpha is a thermo-tolerant microorganism with some strains growing at temperatures of 50 °C and more. The organism is able to assimilate nitrate and can grow on a range of carbon sources in addition to methanol. Cells grown under conditions of elevated temperature accumulate a sugar named trehalose (this sugar is usually found in insects) as thermo-protective compound. It was shown that trehalose synthesis is not required for growth under these conditions, but for acquisition of thermotolerance. The synthetic steps for trehalose synthesis have been detailed for H. polymorpha, and TPS1, the key enzyme gene of this pathway, has been isolated and characterized. All methylotrophic yeasts share an identical methanol utilization pathway (Fig. 1) Growth on methanol is accompanied by a massive proliferation of cell organelles named peroxisomes in which the initial enzymatic steps of this pathway take place. H. polymorpha is model organism to study all aspects of peroxisomal functions

and the underlying molecular biology. During growth on methanol key enzymes of the methanol metabolism are present in high amounts. An especially high abundance can be observed for enzymes called MOX (methanol oxidase), FMDH (formate dehydrogenase), and DHAS (dihydroxyacetone synthase). Their presence is regulated at the transcriptional level of the respective genes. In the related species C. boidinii, P. methanolica, and P. pastoris this gene expression strictly depends on the presence of methanol or methanol derivatives, whereas in H. polymorpha strong expression is elicited by appropriate levels of glycerol or under conditions of glucose starvation. H. polymorpha produces glycoproteins with two types of sugar chains, N- and O-linked glycans are attached to protein. Studies on the structure of N-linked chains have revealed a certain average length (Man8-12GlcNAc2) with terminal alpha-1,2-linked mannose residues, and not with allergenic terminal alpha-1,3-linked mannose residues as found in other yeasts, especially in the baker's yeast Saccharomyces cerevisiae.

Features of the Expression Vectors

Plasmids have been designed as shuttle vectors for propagation in E. coli and thus contain an ori sequence and a selection marker (typically the B-lactamase gene). Although the plasmids possess autonomously replicating sequences (ARS) for chromosomal-independent amplification, they are integrated into the H. polymorpha chromosome by non-homologous recombination.

The expression cassette itself consists of either the MOX or the FMD promoter, a multiple insertion site, and a MOX terminator. For proteins to be secreted, a variety of leader sequences is available; these include the S. cerevisiae MFα1, S. occidentalis GAM1, and Carcinus maenas hyperglycemic hormone sequences. Alternatively, proteins can be targeted to the peroxisomes by inserting the universal S/A/C-K/R/H-L tripeptide motif at the C-terminus. Once genes are integrated, they show extraordinary mitotic stability with no detectable loss or rearrangement of plasmid DNA after 800 generations of growth in the absence of selection. A number of nutritional and antibiotic markers are available for selection, as are the appropriate strain variants.

Productivity

As with any expression system, the product yield varies depending on the specific application. Expression levels of more than 10 g/L have been achieved with

some products; typical yields range from 0.5 to several g/L. In addition to plasmid copy number, factors that can affect product yield include efficiency of secretion, protein stability, cell density, and some of the salient features of the organism itself. H. polymorpha is a very efficient secretor, although low amounts of endogenous proteins are secreted. The efficiency is somewhat dependent on the specific sequences of the leader and of the protein itself; thus, two identical constructs with different target genes may show different expression levels.

The ability to test a variety of leader sequences is an important feature of this system, because it allows for the optimization of the leader for each specific application. Hansenula cells can be grown quite rapidly to high cell densities (150 g/L, dry weight) with typical fermentation times of 100-150 hr. Generally, proteins secreted from H. polymorpha are unlikely to be exposed to significant proteolysis; there is virtually no evidence of C-terminal truncation of the type often encountered in S. cerevisiae.

Post-Translational Modifications

The glycosylation capability of all yeast, including H. polymorpha, is restricted to the high mannose type of structure. However, the hyperglycosylation observed in Saccharomyces (in which 40 or more mannose residues are added onto the core) is a very rare occurrence in H. polymorpha. The organism contains a signal protease with Kex2-like recognition sequence, and proteins are found to be efficiently and accurately cleaved upon secretion. For other enzymatic modifications that are not native to the host, it may be possible to engineer a strain expressing the desired enzyme as well as the product of interest.

Advantages of the Hansenula System

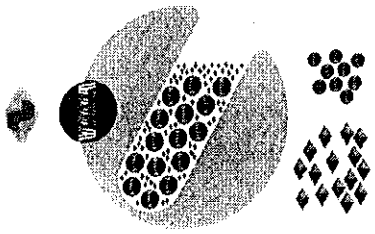


Figure 7. Hansenula Strain

Stable Strains Multi-copy strains with up to 150 copies per cell,Co-expression of up to 4 different genes.

Short, Flexible Fermentations Wide range of cultivation conditions, Inexpensive media without methanol.

Cost-Effective Product Recovery Low levels of impurities; High productivity: 13.5 g/L obtained, High product stability, low protease activity

Reliable, Secure Process High batch-to-batch reproducibility, Strong patent protection, proven technology.

Commercial Successes of Hansenula

A number of products currently on the market have proven the value of Hansenula technology, including a recombinant Hepatitis B vaccine produced from Hansenula. It is the first registered pharmaceutical product derived from methylotrophic yeast world-wide. Hansenula also holds the record in yeast derived-protein production, with a productivity of more than 13 g/L in phytase production. Several other industrial products are marketed by licensees making use of the economically advantageous features of Hansenula. In 2002, a Hansenula-derived recombinant enzyme received GRAS status from the FDA.

Using the Hansenula Technology

Hansenula polymorpha requires relatively simple cultivation and scale-up processes. High product yields are achieved through high gene copy integration, fast growth to high cell density, and an exclusive library of strong promoters. Hansenula works well for either single or multi-step biocatalytic conversions. With this system you can produce high concentration of recombinant product using low-cost culture medium. Research licenses are accompanied by intense training and are uncoupled from commercialization. Commercial licenses are also available with terms negotiable according to business application.

Biotechnological applications of Hansenula polymorpha

Hansenula polymorpha with its unusual characteristics provides an excellent platform for the gene technological production of proteins, especially of pharmaceuticals like insulin for treatment of diabetes, hepatitis B vaccines or IFNalpha-2a for the treatment of hepatitis C. Derivatives of both CBS4732 and DL-1 are

employed in the production of such recombinant compounds. Further yeasts employed for this application are *Pichia pastoris*, *Arxula adeninivorans* (see wickipedia enzyclopedia) and *Saccharomyces cerevisiae* and others (see wickipedia Yeast expression platforms). Why is *H. polymorpha* applied to this production? Like other yeasts it is a microorganism that can be cultured in large fermenters to high cell densities within a short time. It is a safe organ-ism in not containing pyrogens, pathogens or viral inclusions. It can release compounds into a culture medium as it has all the components required for secretion (this is for instance not the case with bacteria like Escherichia coli). It can provide attractive genetic components for an efficient production of proteins. In Fig. 2 the general design of a vector (a genetic vehicle to transform a yeast strain into a genetically engineered protein producer). It must contain several genetic elements: 1. A selection marker, required to select a transformed strain from an untransformed background –this can be done if for instance such an element enables a deficient strain to grow under culturing conditions void of a certain compound like a particular amino acid that cannot be produced by the deficient strain). 2. Certain elements to propagate and to target the foreign DNA to the chromosome of the yeast (ARS and/or rDNA sequence).

A segment responsible for the production of the desired protein compound a so-called expression cassette. Such a cassette is made up by a sequence of regulatory elements, a promoter that controls, how much and under which circumstances a following gene sequence is transcribed and as a consequence how much protein is eventually made. This means that the segment following the promoter is variable depending on the desired product – it could be a sequence determining the amino acids for insulin, for hepatitis *B vaccine* or for interferon. The expression cassette is terminated by a following terminator sequence that provides a proper stop of the transcription. The promoter elements of the *H. polymorpha* system are derived from genes that are highly expressed, from instance from the MOX gene, the FMD gene or the TPS1 gene mentioned before. They are not only very strong, but can also be regulated by certain addition of carbon sources like sugar, methanol or glycerol.

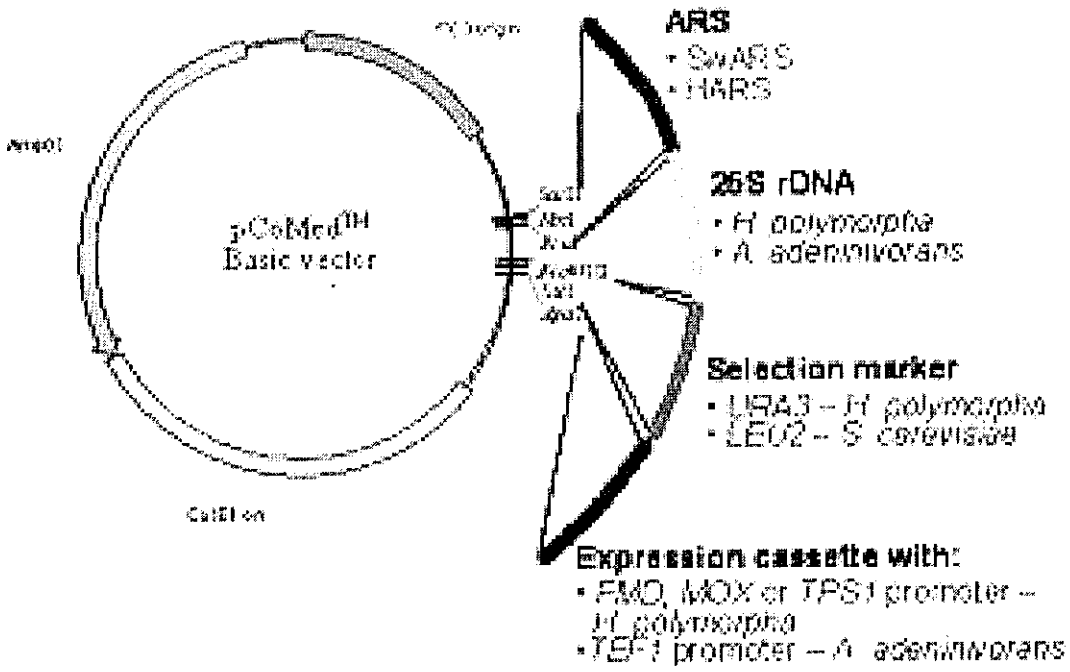


Figure. 8. Design and functionality of CoMed vector system. The CoMed basic vector contains all E. coli elements for propagation in the E. coli system and a MCS for integration of ARS, rDNA, selection marker and expression cassette modules. For this purpose, ARS fragments are flanked by SacII and BcuI restriction sites, rDNA regions by BcuI and Eco47III restriction sites, selection markers by Eco47III and SalI restriction sites and promoter elements by SalI and ApaI restriction sites.

The attractiveness of the H. polymorpha platform is commercially exploited by several biotech companies for the development of production processes, among others by PharmedArtis, located in Aachen, Germany and the Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK). One of the premier expression systems in use today, Hansenula polymorpha. Worldwide exclusive licensing rights for this yeast technology covering various application fields are held by Artes Biotechnology of Germany

Hansenula polymorpha for System Versatility

The extraordinary versatility of the Hansenula expression system lies in the ability to express multiple proteins within the same cell. A vector can be designed with

two or more expression cassettes in a tandem arrangement; subsequent transformants will always have the genes present in equimolar ratios. This approach has been used successfully for the production of human hemoglobin. The different expression cassettes can also be placed in different plasmids and introduced in successive transformations. Further flexibility is thus derived from the ability to select the optimal gene ratio for the system under investigation. This opens up innumerable possibilities in both recombinant protein production and in biocatalysis. It is particularly well suited for engineering multiple-step bioconversions, since different enzymes can be expressed in the ratios leading to optimal product yield. The following examples highlight some of the recent successes using this system.

Hansenula as a Biocatalyst

Glycolate oxidase is a peroxisomal enzyme which has been used for the biocatalytic production of glyoxylic acid from glycolate. In order to avoid further oxidation of the glyoxylate product, the hydrogen peroxide generated during the reaction must be removed by a catalase. Hansenula polymorpha was engineered to perform this bioconversion by co-expression of spinach glycolate oxidase (GO) and S. cerevisiae catalase T (CTT1). A strain bearing approximately 30 copies of integrated plasmid with the GO gene behind the FMD promoter was transformed with a second plasmid bearing the CTT1 gene behind the same promoter. After selection by G418 resistance, strains with high CTT1 activity were scored for desired characteristics. CTT1 copy number ranged from 2-25 copies; all double-transformants showed excellent mitotic stability, with no detectable change in the integrated DNA after 800 generations of growth under non-selective conditions. The strain showing the highest of both enzymatic activities was selected for fermentation. The cells were harvested, permeabilized, and then used in a bioconversion reaction. When ethylenediamine was included in the reaction, yields of glyoxylate exceeded 97%. Cells could easily be recovered by centrifugation and reused in as many as 25 successive reactions.

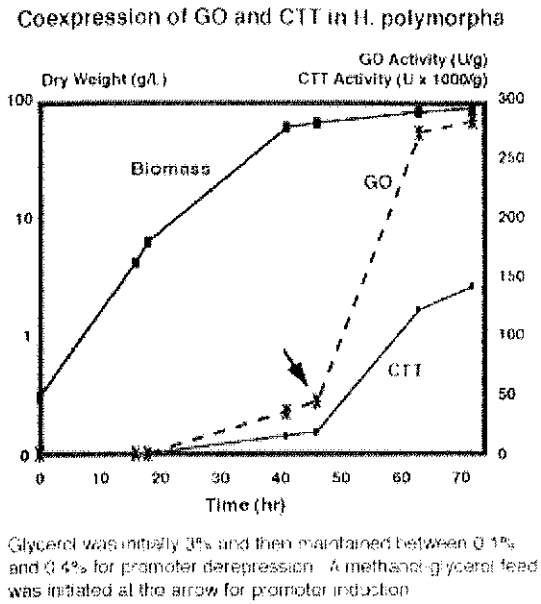
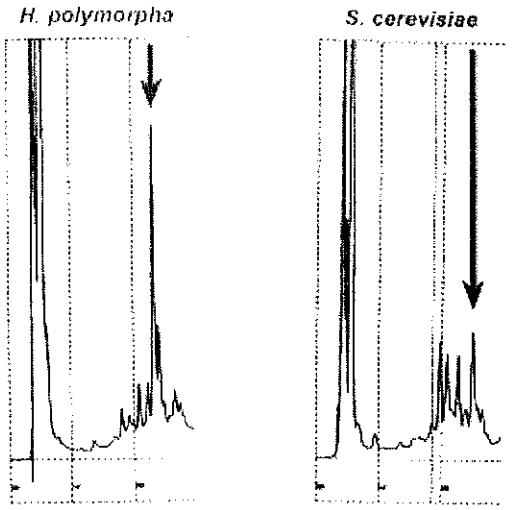


Figure 9. Coexpression Curve.

Correct Processing of Recombinant Hirudin in Hansenula

Hirudin is a potent and specific inhibitor of thrombin and has gathered considerable interest because of its potential as a therapeutic and/or diagnostic agent. To test the suitability of H. polymorpha as an expression host, the hirudin sequence was fused to the prepro segment of MFα1, GAM1 with an added KEX2 site, or CHH, each downstream of the MOX promoter. Selected strains were fermented, and the secreted hirudin was analyzed for the presence of variants by HPLC. All constructs directed the secretion of hirudin, and the protein synthesized with the GAM1 or CHH prepro sequence was authentically processed. When the MFα1 prepro sequence was used, approximately 13% of the hirudin had a 1 amino acid N-terminal extension. In all 3 cases the amount of full-length hirudin was 70-90% of the total secreted, depending on the fermentation condition. The species that normally contaminates full-length hirudin in S. cerevisiae could not be detected. BASF/Knoll AG is currently evaluating the H. polymorpha-derived hirudin in Phase III clinical trials.



HPLC Chromatogram illustrating various forms of recombinant hirudin in *H. polymorpha* and *S. cerevisiae*. Properly processed hirudin is indicated by the arrows

Figure 10.HPLC Chromatography graph.

Use of Hansenula for Hepatitis B Vaccine Production

Serum-derived Hepatitis B particles consist of 90% S, 5% M, and 5% L surface antigens. Since the S-antigen has been shown to carry antigenic sites important for developing immune responses in humans, an effective vaccine composed of virus-like particles consisting of only the S-antigen was developed using the H. polymorpha expression system. High level intracellular expression of S-antigen particles was achieved using H. polymorpha strains that stably maintained several copies of the S-antigen expression plasmid integrated into the genome. The H. polymorpha-derived vaccine consisting of purified S-antigen particles has been produced by Korean Green

Cross Corporation and has been approved for use in humans by the World Health Organization.

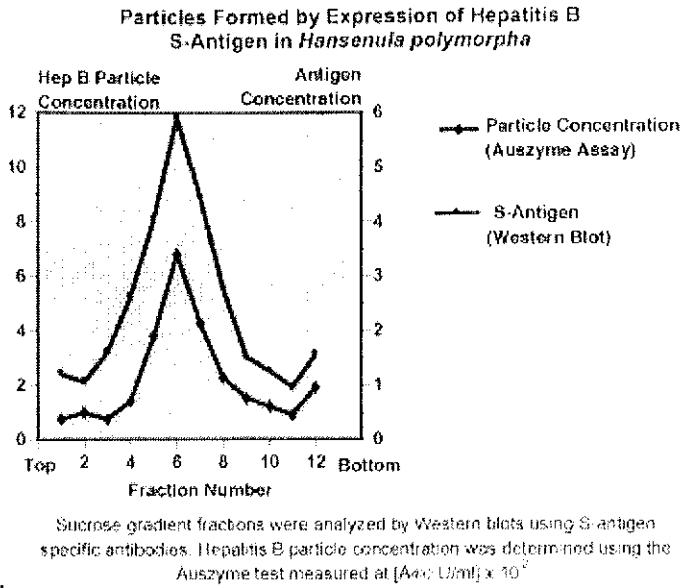


Figure.11. Particle Curve.

Designing Hansenula polymorpha as a Biocatalyst for the Oxidation of α-Hydroxy Acids

Two potential industrial applications of the enzyme glycolate oxidase (GO) are its use in converting glyoxylate to glycolic acid and pyruvate to lactate. Technical Application Note No. 101 summarizes a series of experiments examining an efficient biocatalytic process for glycolic acid and lactate production developed using a strain of Hansenula polymorpha which expresses high levels of spinach glycolate oxidase enzyme.

Hansenula products

Enzymes: Hexose oxidase (FDA);Lipase (FDA);Phytase (approved by EFSA in Q1/06).

Generics: Hepatitis B vaccine; Hirudin; Interferon alpha 2a; Insulin (Wosulin)

Biotransformation: Glycolate oxidase; Mannitol Dehydrogenase

Feasibility Study and Market Size Determination for Hansenula Yeast Expression System

Yeast has been used by mankind since ancient times. Its considerable potential has been demonstrated in several commercial areas aside from traditional food products, e.g., oxido-reduction and/or enantio- and regioselective reactions in organic chemistry; total biosynthesis from simple carbon sources; production of technical enzymes such as lipases and esterases, and low cost products such as feed enzymes. Furthermore, yeast cell material can be a source of several substances beneficial to health or can be used in cosmetic applications (e.g., glycans, ceramides). The species best established in industrial production procedures are S. cerevisiae, K. lactis, P. pastoris, and H. polymorpha.

This report (1) gives an overview of the US market for recombinant proteins and enzymes, (2) evaluates major expression systems used for recombinant protein production with respect to the companies using them, and (3) outlines the market opportunities for Hansenula expression systems in the US and Canada.

US Market: Growth and Opportunities for Recombinant Proteins

The discovery and development of recombinant DNA technology has formed the Cornerstone of the modern medical biotechnology industry. With a market valued at almost $32 billion in 2003, the changing dynamics of the recombinant therapeutic proteins market will have a significant impact on numerous companies and therapy areas. Between 2001 and 2003, this blockbuster market share increased from 51.8% to 64.3% and is forecast to remain above 58% in 2010. Twelve brands will lead market growth over the next few years. The leading brands are expected to grow from $20M in 2004 to $30M in 2010, covering 57% of the total market. The recombinant protein market will continue to grow from $35M in 2004 to $42M in 2006, and reach $52M in 2010 (Figure 1).

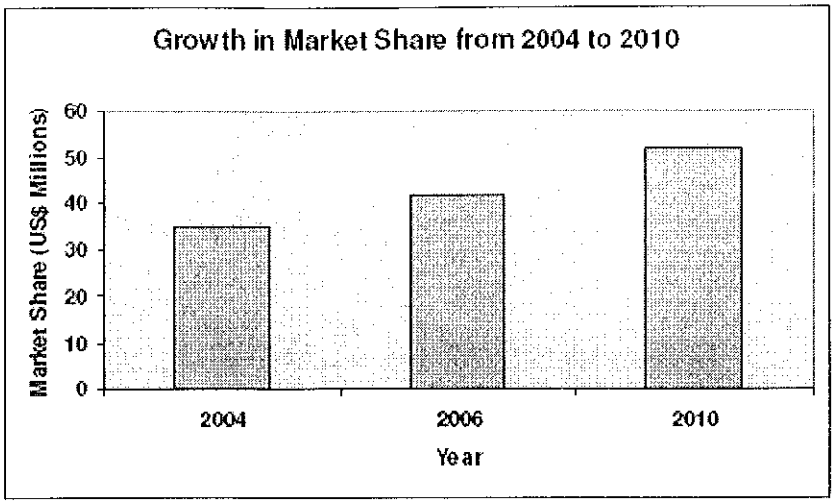


Figure 12. Market Share Graph.

The rDNA market could, in several key therapy areas such as oncology, CNS disease, infectious diseases, and AIID, face strong competition from other technological platforms such as antibodies (e.g., Johnson & Johnson's Remicade or Abbott's Humira vs. Amgen's Enbrel in arthritis, Genentech's antibody Raptiva vs. Biogen IDEC's fusion protein Amevive in psoriasis) or small-molecule drugs (e.g., Teva's oral copaxone vs. Biogen's Avonex and Serono's Rebif). For this reason, companies may attempt to diversify risk by investing in new technological platforms. For example, the merger between Biogen and IDEC united a leading rDNA biotech specialist with an antibody expert, creating a hybrid portfolio with blockbusters or strong income earners from two different technological platforms.

Market for the yeast expression systems:

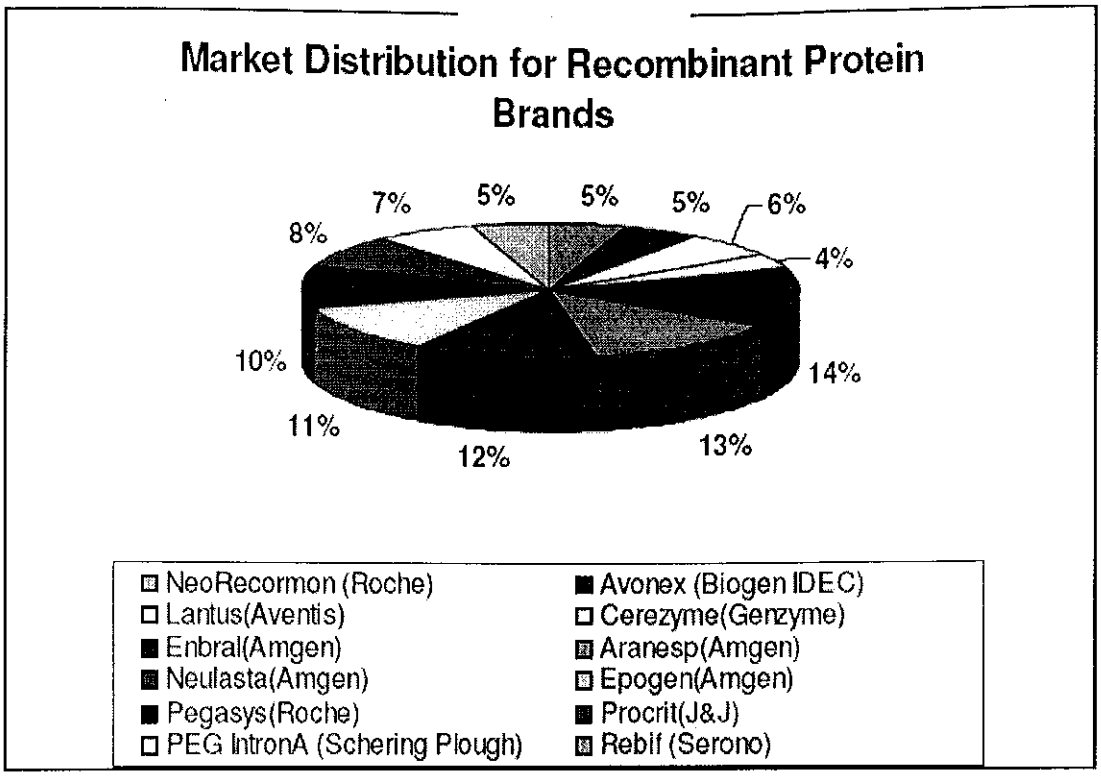


Figure 13 shows the world-wide distribution of market sub-segments generated by industry's leading recombinant protein brands in the coming six years. The US captures 80% of the total market.

Comparative Study based on data analyzed on technical and economical terms :

Comparison of Various Yeast Expression Systems

The various means by which protein is produced in any industry is by prominent expression and secretion systems like prokaryotes, yeasts, and filamentous fungi as well as insect and mammalian cell cultures. There are several reasons why yeast technology has an edge over other technologies for production of protein.A numbers of noteworthy advances in the over-expression of recombinant proteins in yeasts have enabled the secretion of correctly folded, glycosylated or even 'humanized' (and thus pharmacologically active) proteins.The frequent exclusion of low-priced products derived from natural plant or animal sources is due to strict requirements for consistent product quality, in addition to high purity and safety issues with respect to increasingly

emerging diseases such as BSE.The expected expiry of numerous patents on biopharmaceuticals in the near future, which may open the market to bio-generica.

The three main yeast expression systems used for industrial purposes are: *Saccharomyces cerevisiae, Pichia pastoris* and *Hansenula polymorpha.* Saccharomyces cerevisiae is the best genetically characterized eukaryotic organism and is still the prevalent yeast species in pharmaceutical production process; however, Pichia pastoris, first employed by Phillips Petroleum for single-cell protein production, is one of the most frequently used for protein expression. Proteins with molecular mass higher than 30 kDa are retained in the cytoplasm sac of Saccharomyces cerevisiae, whereas Hansenula polymorpha efficiently secretes proteins with a molecular mass of above 150 kDa, like glucoamylase.

The major differences in the three expression system are outlined in Table 1. The table discusses various characteristics like secretion efficiency, the fermentation time, the protease activity etc.

Table2. Expression System Comparison.

Characteristics	*S. cerevisiae*	*P. pastoris*	*H. polymorpha*
Secretion Efficiency	+	++	++
Protease Activity in SecretorySecretory Vesicles	High	Low	Low
Hyperglycosylation	More heterogeneous	Less	Less
Requirement of Explosion Proof Equipment	No	Yes	No
Methylotroph	No	Yes	Yes
Fermentation Time	More than 250 hrs	150-200 hrs	50-70 hrs
Glycosylation	Allergenic potential	No Allergenic potential	No Allergenic potential

Competitors and Competition

As discussed with different expression systems, their use and applications are in various fields. Compared to Pichia and Hansenula, the Saccharomyces system differs in both application and level of expression. While Pichia and Hansenula have many similarities, the latter shows several advantages such as: multicopy strains, stability over a long generation time, the possibility to stably co express different genes, short fermentation times and fermentation without methanol. The major challenge is to approach companies working with Pichia and to convince them to change to the Hansenula technology.

Companies That Manufacture Proteins which could equally be Expressed in Hansenula

The first category identifies companies currently manufacturing proteins which could easily be expressed in the Hansenula expression system. The profile of five such companies is presented in the report in the later section.These are the companies which cater to the needs of both therapeutic and non-therapeutic areas. We has identified some major areas of focus in this wide category. Figure 3 shows 260 companies in the initial database. There are 45 (17%) companies into enzyme production that can be used in various areas like beverage, detergent, pesticide, fertilizers etc, 65 (26%) working in biofuel production, 20 (8%) engaged in diagnostics, 48 (18%) focusing on Animal feed and Agriculture, 37 (14%) companies into vaccines and 45(17%) into recombinant proteins.

Companies with Recombinant Proteins in Their Pipeline

The second category identifies the companies with recombinant proteins in their pipeline .The difference in these companies and the companies in category one is that these companies have pipelines with drugs in the discovery or pre-clinical stage. There are 25 companies in the initial study but this number is expected to reach 75 in the next stage of mapping. A brief profile of five companies from this category is provided in the report.

Future Prospects/ Additional Market

The impressive ability to "cut-and-paste" genes from one microorganism to another, thereby mass-producing specific enzymes or desired chemical products, has led to an entirely new biotechnology business sector. This market lies not only in the field of therapeutics and vaccines, but also includes bioplastics, semisynthetic drugs, and modified vegetable oils for processed foods, biodiesel, biosurfactants, and commodity chemical feedstocks. A new energy law includes a federal renewable fuels standard that requires 7.5 billion gallons of ethanol per year to be blended with gasoline by 2012. Currently, 12 billion gallons of ethanol per year are produced worldwide with 4 billion made in the U.S-- nearly all from cornstarch. The global bioethanol market is thus expected to grow to nearly 30 billion gallons annually by 2020, according to the Renewable Fuels Association.

The major focus of this project has been market size determination and a feasibility study for Hansenula technology.

1. Research and Development Services for the application of suitable expression systems. These include:

a. Hansenula Feasibility Studies

b. System Screening Services.

2. Licenses (research or commercial as relevant) for various technologies.These include:

a. Expression systems such as Hansenula, Aspergillus, Sordaria and Arxula.

b. Process technologies such as the CTAB technology for protein release.

c. Process technologies for recombinant products, e.g. Hirudin.

The bulk of products soon to go off-patent give a large market that can be exploited by Many companies using Hansenula technology now and in the near future. During 2002-04, as many as 20 biotech drugs have lost their patent rights in the US and the US patents for 13 biotech products expired in 2005.It is estimated that drugs worth $60 billion will go off patent by the year 2010.

Technolgy Comaprison

Hansenula Expression system Vs Other Expression systems

Table 3. Comparison of Different Expression Systems

Characteristics	E. coli	Yeast	Insect cells	Mammalian cells
Cell Growth	Rapid (30 Min)	Rapid (90 Min)	Slow (18-24 H)	Slow (24 H)
Complexity of Growth Medium	Minimum	Minimum	Complex	Complex
Cost of Growth Medium	Low	Low	High	High

Expression Level	High	Low - High	Low - High	Low - Moderate
Extra cellular Expression	Secretion to Periplasm	Secretion to Medium	Secretion to Medium	Secretion to Medium
Protein Folding	Refolding Usually Required	Refolding May Be Required	Proper Folding	Proper Folding
N-linked Glycosylation	None	High Mannose	Simple, No Sialic Acid	Complex
O-linked Glycosylation	No	Yes	Yes	Yes
Phosphorylation	No	Yes	Yes	Yes
Acetylation	No	Yes	Yes	Yes
Acylation	No	Yes	Yes	Yes
gamma-Carboxylation	No	No	No	Yes
Yield (mg) (per liter culture)	50-500	10-200	10-200	0.1-100
Success Rate (%) (soluble or functional)	40-60	50-70	50-70	80-95
Project Cost	Low	Low	Middle	High
Recommended Use	Antigen protein, Protein standards, Functional proteins	Proteins with glycosylation, Vaccine, Secreted form, Alternative to insect cell system	Proteins with glycosylation, Assay standards, Secreted form, Alternative to yeast system	Functional study, PTM study, Assay standards, Characterization
Advantage	Simple,	Simple, low	Relatively	Natural protein

	robust, lowest cost, highest yield	cost, good for certain proteins	higher yield, better PTM	configuration, best PTM
Disadvantage	Least PTM[a]	Longer time, less PTM L	Longer time, higher cost	Highest cost, lower yield

Key distinctions between *H. polymorpha* and *P. pastoris*

The two species of methylotrophic yeast that have been developed for commercial-scale recombinant protein production are Hansenula polymorpha and Pichia pastoris. Although these two species are close relatives, there are some important distinctions between the species and the expression systems developed in them that should be considered when selecting the system for a specific application. Enzymes of the P. pastoris methanol utilization pathway, including alcohol oxidase, are rapidly and strongly induced in response to methanol induction. In H. polymorpha, the enzymes can be induced either by methanol or by glycerol depression. This appears to be a feature of the organism itself since the AOX1 promoter of P. pastoris (the analogue of the H. polymorpha MOX promoter) is induced by glycerol derepression in H. polymorpha but not in P. pastoris. This is particularly significant for applications involving large-scale production, since it circumvents the potential hazards associated with the use of methanol.

The plasmids used for expression in P. pastoris are designed for homologous recombination and integration at the AOX or HIS4 locus, whereas those used in H. polymorpha integrate by non-homologous recombination at random loci. The number of plasmid copies integrated in the P. pastoris system is limited, usually to <10, whereas up to 150 copies have been integrated using the H. polymorpha system. Since protein expression is correlated with gene dosage in these organisms, it is possible to achieve significantly higher expression in H. polymorpha despite the fact that the AOX promoter of P. pastoris is the strongest promoter. It is also possible to achieve more control over the level of production by regulating the copy number. Furthermore, when plasmids are integrated at the AOX locus, the gene encoding the alcohol oxidase is disrupted; once the cells are transferred to methanol their growth is significantly

retarded. The growth rate of H. polymorpha remains rapid before and after induction. Finally, H. polymorpha is a more thermotolerant organism than is P. pastoris, easily withstanding temperatures up to 43°C.

RESOURCES AND METHODS

The data was collected by using web tools, databases and tele-calling methods.

To begin with, a comparative study of different types of expression systems was conducted. The different technologies were studied in detail by visiting different websites by using relevant keywords to retrieve the most relevant results.

The second step was to make a database of companies by using different search criterions and which can be probable buyers of the Hansenula expression technologies. This is called market size identification.

Then the database was divided in six different categories viz Enzyme Producing, Biofuel and Agriculture, Diagnostics, Animal Feed and Agriculture, Vaccines, Bulk protein Production.

The next step was to reach the probable buyers of the technology by running email campaigns and tele-calling to get an idea of the expression system technologies that the mapped companies use and why do they feel the technologies they use are the most suitable to them and what are the various criterions they consider to select an expression system.

Based on the technical study and the feedbacks obtained from different companies the valid comparison criterions were laid down for comparing our technology with others prevalent in the market and we came up with a report addressing its feasibility to be used as a successful expression system.

RESULTS AND DISCUSSIONS

The two species of methylotrophic yeast that have been developed for commercial-scale recombinant protein production are Hansenula polymorpha and Pichia pastoris. Although these two species are close relatives, there are some important distinctions between the species and the expression systems developed in them that should be considered when selecting the system for a specific application.

Why is Hansenula Better

Enzymes of the *P. pastoris* methanol utilization pathway, including alcohol oxidase, are rapidly and strongly induced in response to methanol induction. In H. polymorpha, the enzymes can be induced either by methanol or by glycerol derepression. This appears to be a feature of the organism itself since the AOX1 promoter of P. pastoris (the analogue of the H. polymorpha MOX promoter) is induced by glycerol derepression in H. polymorpha but not in P. pastoris. This is particularly significant for applications involving large-scale production, since it circumvents the potential hazards associated with the use of methanol.

Other advantages

The plasmids used for expression in P. pastoris are designed for homologous recombination and integration at the AOX or HIS4 locus, whereas those used in H. polymorpha integrate by non-homologous recombination at random loci. The number of plasmid copies integrated in the P. pastoris system is limited, usually to <10, whereas up to 150 copies have been integrated using the H. polymorpha system. Since protein expression is correlated with gene dosage in these organisms, it is possible to achieve significantly higher expression in H. polymorpha despite the fact that the AOX promoter of P. pastoris is the strongest promoter. It is also possible to achieve more control over the level of production by regulating the copy number. Furthermore, when plasmids are integrated at the AOX locus, the gene encoding the alcohol oxidase is disrupted; once the cells are transferred to methanol their growth is significantly retarded. The growth rate of H. polymorpha remains rapid before and after induction.

Finally, H. polymorpha is a more thermotolerant organism than is P. pastoris, easily withstanding temperatures up to 43°C. We conclude from the comparison that

Hansenula is at par with mammalian cell expression system as far as technology is concerned but at economical front mammalian system is quite costly and at some gene expression prove to be a waste of resources while at the same time Hansenula proves to be leading the race on technology as well as economical and cost front.

Market Analysis

These companies manufacture proteins which could be expressed in Hansenula. These companies were short listed and contacted and agreed to look forward towards Hansenula as promising technology for their future use. (www.canbiotech.com).

Innogenetics	www.innogenetics.com	*Saccharomyces cerevisia*
Curagen corporation	www.curagen.com	*Saccharomyces cerevisia*
Globeimmune	www.globeimmune.com	*Saccharomyces cerevisia*
Chiron Corporation	www.chiron.com	*Pichia pastoris*
Zymogenetics	www.zymogenetics.com	*Pichia pastoris*
Novozymes	www.novozymes.com	*Pichia pastoris*
Medeva Pharma	www.ucb-group.com	*Pichia pastoris*
Smith Kline Biologicals	www.gsk-bio.com	*Hansenula polymorpha*
E.I. Dupont De Nemours	www2.dupont.com	*Pichia pastoris* & *Hansenula polymorpha*
Pfizer	www.pfizer.com	*Hansenula polymorpha*
Merck & co	www.merck.com/product	*Saccharomyces cerevisia*
Techlab, inc	www.techlab.com	*Saccharomyces cerevisia*
Schering-Plough	www.schering-plough.com	*Saccharomyces cerevisia*

Table:4 different companies using different yeast expression systems

Table 4 shows the players working with one or more of these three expression systems. Those using Pichia, e.g., Chiron Corporation and Medeva Pharma, or Saccharomyces have to be approached specifically and to be convinced about the well-defined Hansenula advantages. Companies already applying other yeasts are not competitors but rather challenges. Competitors are those offering other systems, such as Invitrogen and RCT for the Pichia, New England Biolabs and DSM for the K. Lactis system. Several preliminary conclusions can be drawn from this interim report and are presented in light of the various research categories.

The first category identifies companies which currently manufacture proteins that have already been expressed yeast and which could just as well expressed in Hansenula expression system. This market has a lot of potential with few limitations to growth. The major reason is the extensive application areas, e.g., the chemical industry, enzymes, animal feed, human proteins, vaccines etc.. The second category identifies companies that have recombinant proteins in their pipeline. Apart from what Hansenula has already produced, there are other therapeutic areas that can be explored. This technology can be used by such companies for studies in gene optimization, vector cloning, transformation/integration, screening, genomic characterization and preliminary high-cell density fermentation.

Finally, the number of therapeutic drugs going off patent gives rise to the generic market, offering substantial opportunities, both now and in the future.

CONCLUSION

This report evaluates the market opportunities for various products and services offered by the company; especially the Hansenula yeast expression system.

Technical Feasibility and Market Size Determination are the key areas of focus. There are many yeast expression systems in the market for various therapeutic and research purposes. A comparison of three major yeast expression systems has been presented, highlighting the superiority of one over the other. This study shows that the recombinant protein industry, with a market value of $32 billion in 2003 was expected to rise by almost 13% in another six years. In 2004, this industry sector included more than 110 companies that were involved in the discovery, development, and marketing of rDNA products. These companies had a pipeline of more than 80 therapeutics in clinical development and a combined portfolio of 73 marketed products.

Hansenula Expression system is better in following ways:

Stable Strains Multi-copy strains with up to 150 copies per cell,Co-expression of up to 4 different genes.

Short, Flexible Fermentations Wide range of cultivation conditions, Inexpensive media without methanol. Cost-Effective Product Recovery Low levels of impurities; High productivity: 13.5 g/L obtained, High product stability, low protease activity

Reliable, Secure Process High batch-to-batch reproducibility, Strong patent protection, proven technology. The Hansenula polymorpha expression system has a huge market potential due to its application in both pharmaceutical and non-therapeutic areas. From vaccines to animal feed and food ingredients, chemical industry to agriculture, it has a wide application range. We conclude from the study that Hansenula polymorpha can be used as a better expression system considering the various parameters that are necessary to make an expression system feasible and successful in the market.

BIBLIOGRAPHY

Alex K Pavlou & Janice M Reichert, Recombinant protein therapeutics—success rates, market trends and values to 2010 (December 2004).

Anné J, Mellaert L van (1993) Streptomyces lividans as host for heterologous protein production. FEMS Microbiol Lett 114:121–128.

Bassford PJ, Silhavy TJ, Beckwith JR (1979) Use of gene fusion to study secretion of maltose-binding protein into Escherichia coli periplasm. J Bacteriol 139:19–31 bcs.whfreeman.com/lehninger.

Benz I, Schmidt A (2002) Never say never again: protein glycosylation in pathogenic bacteria. Mol Microbiol 45:267–276.

F. R. Schmidt, Recombinant expression systems in the pharmaceutical industry. (July, 2004).

Recombinant DNA expression products for human therapeutic use G. M. Bhopale* and R. K. Nanda july 2004.

What's in Store for Yeast Biotechnology? – The Joint 3rd Swiss–Czech and BioTech2005 Symposium in Retrospect. (2005).

www.antibodydirectory.com

www.canbiotech.com

www.wikipedia.com