IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

GENENTECH, INC. and CITY OF HOPE, *Plaintiffs*, v. AMGEN INC., *Defendant*.	)	C.A. No. 17-1407-CFC (CONSOLIDATED)
GENENTECH, INC., *Plaintiff and Counterclaim Defendant*, v. AMGEN INC., *Defendant and Counterclaim Plaintiff*.	)	C.A. No. 18-924-CFC

APPENDIX TO GENENTECH'S LETTER-BRIEF CONCERNING CONSTRUCTION OF "FOLLOWING FERMENTATION" AND SUPPORTING DECLARATION OF DR. HANSJÖRG HAUSER

Bödeker et al., Production of recombinant factor VIII from perfusion cultures: I. Large-scale fermentation, Animal Cell Technology (1994) ("Bödeker 1994")	Appx234
Ozturk et al., Real-time Monitoring of Protein Secretion in Mammalian Cell Fermentation: Measurement of Monoclonal Antibodies Using a Computer-Controlled HPLC System (BioCad/RPM), *Biotechnol Bioeng*., 48:201-206 (1995) ("Ozturk 1995")	Appx240
Kemp G., O'Neil P., *Large-Scale Production of Therapeutic Antibodies: Considerations for Optimizing Product Capture and Purification*, in: Subramanian G. (eds) Antibodies (2004) ("Kemp 2004")	Appx246
Dwivedi, *Validation of Cell Culture-Based Processes and Qualification of Associated Equipment and Facility*, in: Ozturk, Cell Culture Technology for Pharmaceutical and Cell-Based Therapies ("Dwivedi 2006")	Appx272
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Kaufmann et al., *Influence of low temperature on productivity, proteome and protein phosphorylation of CHO cells*, Biotechnol Bioeng., 63(5): 573-582 (1999)	Appx344
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Roobol et al., *ATR (ataxia telangiectasia mutated- and Rad3-related kinase) is activated by mild hypothermia in mammalian cells and subsequently activates p53*, Biochem J., 435(2):499-508 (2011)	Appx360
Hunt et al., *Low-Temperature Pausing of Cultivated Mammalian Cells*, Biotechnol Bioeng., 89(2):157-63, (2004)	Appx374
Yoon et al., *Effect of Low Culture Temperature on Specific Productivity and Transcription Level of Anti-4-1BB Antibody in Recombinant Chinese Hamster Ovary Cells*, Biotechnol Prog., 19(4):1383-6 (2003)	Appx381
Roobol et al., *Biochemical insights into the mechanisms central to the response of mammalian cells to cold stress and subsequent rewarming*, FEBS J., 276(1):286-302 (2009)	Appx385

CELL CULTURE TECHNOLOGY FOR PHARMACEUTICAL AND CELL-BASED THERAPIES

edited by
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Validation of Cell Culture-Based Processes and Qualification of Associated Equipment and Facility

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INTRODUCTION

Why validate? Though validation is a well-accepted and recognized cGMP requirement in today's Pharma business, this question is often posed during the product or process development (PD) activities in a start-up or even in an established company. In a nutshell, validation is not only a regulatory requirement, but it makes "good business sense." Validated processes assure production of quality product, batch after batch, and ultimately result in fewer headaches down the road in terms of fewer deviations during production, quality assurance (QA) discrepancy investigations, adverse events from the field, and regulatory observations (483s and its global equivalent) during regulatory inspections. In addition, they improve cost effectiveness in terms of preventing process failures, lot rejections, re-processing of salvageable lots, and attaining maximum plant capacity. Moreover, a sound and thorough validation strategy not only assures the production of top quality products, but also builds confidence and provides peace of mind to its customers. It also boosts the morale of the company employees and help build a sound and trustworthy relationship and track record with the regulatory agencies. The latter may come as a blessing for a company's future dealings with the regulatory agencies.

The term process validation originated in 1983 when the Food and Drug Administration (FDA) expanded the cGMP guidelines to cover demonstration of process consistency/reproducibility, but the guidelines were not finalized until 1987 (1). These guidelines were originally intended to be adopted by all drug product and biological manufacturers, but were later extended to the medical device and diagnostic manufacturers and to the blood collection/distribution/users and blood product manufacturers (2,3). Though originally intended only for the finished drug

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product, these regulations have been recently extended to bulk drugs and bulk biologicals (4).

The original definition of the term process validation was described by the FDA as ''Establishing a documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its predetermined specifications and quality attributes.''

In practice, process validation (process performance qualification, PPQ) is more complicated than the simple definition stated above and is only one element of the overall validation process. It is a culmination of all other validation studies, such as equipment qualification (installation qualification, IQ; operational qualification, OQ; and performance qualification, PQ), computer qualification (IQ and OQ), utilities and facilities qualification (IQ, OQ, and PQ) cleaning validation (PQ), environmental qualification (PQ), and analytical qualification (PQ), all covered under a validation master plan (VMP) written for each new technology, process, or a product.

The invention of recombinant DNA technology in the late 1970s and its widespread application to eukaryotic and prokaryotic cells for developing unique medical applications/treatments resulted in the establishment of a new field known as ''Genetic Engineering'' today (5,6). These developments opened the floodgates for innovation that resulted in the establishment of many biotechnology companies worldwide. Of these, more than 50% of the biotechnology companies are working on cell culture technology for producing pharmaceutical and cellular therapies.

Due to continued innovation in this field the application of process validation concepts and guidelines are becoming increasingly complex, challenging, and difficult to understand by technical professionals, regulatory auditors, and cGMP compliance enforcers working in the pharmaceutical and biotechnology-related organizations. Since it is impossible to cover all aspects of process validation for the numerous biotechnology-derived products in this chapter, an attempt will be made only to provide a simplified version of the regulatory requirements that are needed for licensing cell culture-derived pharmaceuticals and cellular therapies. This chapter is intended to provide a bird's-eye view of the regulatory requirements for process validation to entrepreneurs before they plan for building a new manufacturing plant and expect to obtain licensure for a product (Product License Application, PLA) or a biologic (Biologic License Application, BLA) or a drug (New Drug Application, NDA) from regulatory agencies. This chapter is expected to prepare them well before they begin that challenging, eventful, exhausting, memorable, and ultimately rewarding journey.

APPROACH AND RATIONALE

The innumerable amount of research and development studies conducted on a large number of medical products has enabled us to understand that the quality attributes for any given product are not an unexpected output. But, are largely dependent on the process parameters used during their production. Therefore, the control of quality attributes for any biological or pharmaceutical product is in our hands; and with the development of new technologies, quality attributes for the new products can now be built into the manufacturing process. In this respect, the process design in relation to the respective product quality attributes has become crucial for the development and licensing of the medical and pharmaceutical products (7–10).

Since the breakthrough in genetic engineering a few decades ago, numerous medical, biological, pharmaceutical, and diagnostic products and applications

based on cell culture technology have been invented. They are based on microbial fermentation (eukaryotic and prokaryotic), hybridoma technology, and tissue regeneration. Even plant cell technology is being evaluated to produce medical and therapeutic products for human use. The examples of the cells used for this purpose are: bacteria (*Escherichia coli*), fungi (*Aspergillus*, *Saccharomyces*), mammalian cells (CHO, BHK, myeloma, melanoma, hybridoma, etc.), insect cells (*Drosophila*), and plant cells (tobacco, spinach, etc.). The majority of the products are secreted by the cells in the spent medium (harvest) by applying the rDNA technologies and manipulation of respective genes in the cells. The examples of recombinant products derived from these technologies are: erythropoietin (rEPO), anti-hemophilic factor (rFVIII), tissue plasminogen activator (rTPA), growth factors (EGF, TGF, PDGF, TNF, etc.), hormones (Insulin, LH, FSH, etc.), interferons (IF-1, IF-2, etc.), interleukins (IL-2, IL-4, IL-6, etc.), monoclonal antibodies (mAbs), and other enzymes and proteins (cerezyme, galactosidase, etc.). Some of the products are expressed in the inclusion bodies within cells and the cells therefore must be lysed to extract the products out (insulin, EGF, etc.). Epithelial cells, neuroblastoma, osteoblastoma and cartilage cells are being grown in laboratories and used as medical devices for a number of treatments (burns, tissue implant, tissue regeneration, etc.). A number of monoclonal antibodies are being generated from bacterial, mammalian, and plant cell technologies for the treatment of cancer, autoimmune diseases, and other immunological disorders.

A general approach to streamline validation concepts and policies has been evolving over the last number of years. These efforts have resulted in better understanding of the requirements for the validation by the industry professionals. For the purposes of clarity and better understanding this article will employ the newly emerging approach on validation concepts (11,12). Accordingly, qualification of all equipment and systems (design qualification, DQ; installation qualification, IQ; operational qualification, OQ; and performance qualification, PQ) will be referred as ''Equipment Qualification'' and not as ''Validation.'' The term ''Validation'' will be used only for ''Process Validation'' studies that are related with the studies (with or without active ingredient) at the small-scale (lab-scale) or full production scale (process validation, PV or PPQ).

The variety of cell culture technologies and many different approaches to use them as pharmaceutical products or medical devices makes the task of building the quality attributes in the manufacturing process very challenging. This also makes the task of process validation more difficult as generic models of process validation cannot be used, and every process validation study needs to be devised from scratch based on the technology being used. For example, the level of impurities (DNA, host cell contaminating proteins, etc.) may be substantially less in the starting material where the product is secreted out in the spent medium (harvest) as compared to the product that is expressed intracellularly such as in the inclusion bodies. Therefore, the design of the manufacturing process and the resultant process validation studies would be very different for the two approaches to isolate and purify the product(s).

The possible impurities and contaminants in a cell culture-based product are: intact cells, adventitious agents [bacteria, fungi, mycoplasma, viruses, transmissible spongiform encephalitis (TSE)/bovine spongiform encephalitis (BSE)], endogenous retroviruses, host cell nucleic acids and proteins, foreign proteins (from raw materials and microbial contaminations), endotoxins, and contaminating process chemicals (13). A validated process, therefore, must demonstrate effective removal, inactivation, or reduction of these impurities and contaminants to acceptable levels.

Though it is preferable to perform process validation studies at full-scale operational level, it is not always possible to perform them at manufacturing scale due to practical limitations (e.g., virus and nucleic acid reduction studies may require huge amounts of model viruses and nucleic acids). In such cases, scaled-down bench-level studies are acceptable as long as all process input parameters are kept the same as in the full-scale and the output parameters are comparable to the full-scale (14). Whenever this approach is used, demonstration and justification of the acceptability of the scaled-down model should be performed prior to formal process validation.

PROCESS DEVELOPMENT

Development of a Defined Process

The critical steps for the development of a defined process are outlined in Fig. 1. We will examine below the requirements for developing a reliable and reproducible process for a cell culture derived product. The definition of a defined process may be summarized as "a process that provides a high degree of assurance that it will consistently produce a product meeting its predetermined specifications and quality attributes." This definition seems simple and doable (in the beginning phase of a project) but becomes difficult to achieve when all the details for a cell culture-based product are brought into consideration. Adequate confidence must be built by doing sufficient experimentation and development work to demonstrate that the process can consistently produce a product of pre-specified quality. Range finding (feed stream) studies should be performed for every critical and noncritical process parameter (15), and operational set-points must be established after completion of the range finding studies. Worst-case studies (upper and lower ranges) should be performed during the development phase, (as it is much easier to do them during development than during actual production). Alert and action levels (limits) for out-put parameters (test results and specifications) must be established with adequate justification. In-process and final product specifications (acceptance criteria) must be defined clearly with sound scientific justifications.

The success of a well executed project depends on a well written process development (PD) report with sufficient details for every aspect of the process and a well executed transfer of technology from the R&D department to the operations department. The R&D personnel not only adequately transfer the technology, but must provide training to production personnel in every aspect of the process. The role of the R&D personnel does not end here, they should actively monitor the process after successful process validation by applying the statistical tools such as statistical process control. Post-validation process data must be analyzed to ensure that the process performs within the established boundaries. Process capability (Cpk) calculations must be performed on the post-validation process data to evaluate process performance. The process data should also be analyzed by applying other statistical tests, such as Student's *t*-test, to determine confidence intervals on process performance. A 95% confidence interval is generally acceptable for process validation studies. Many companies, however, run their production processes at 98% confidence interval or up to ± 6 SD of the validated process parameters. These analyses demonstrate whether the process is in control and build confidence for running the process on a consistent basis.

The importance and relevance of good PD work that eventually pays off many fold must be emphasized here. It is generally acknowledged that many pharmaceutical

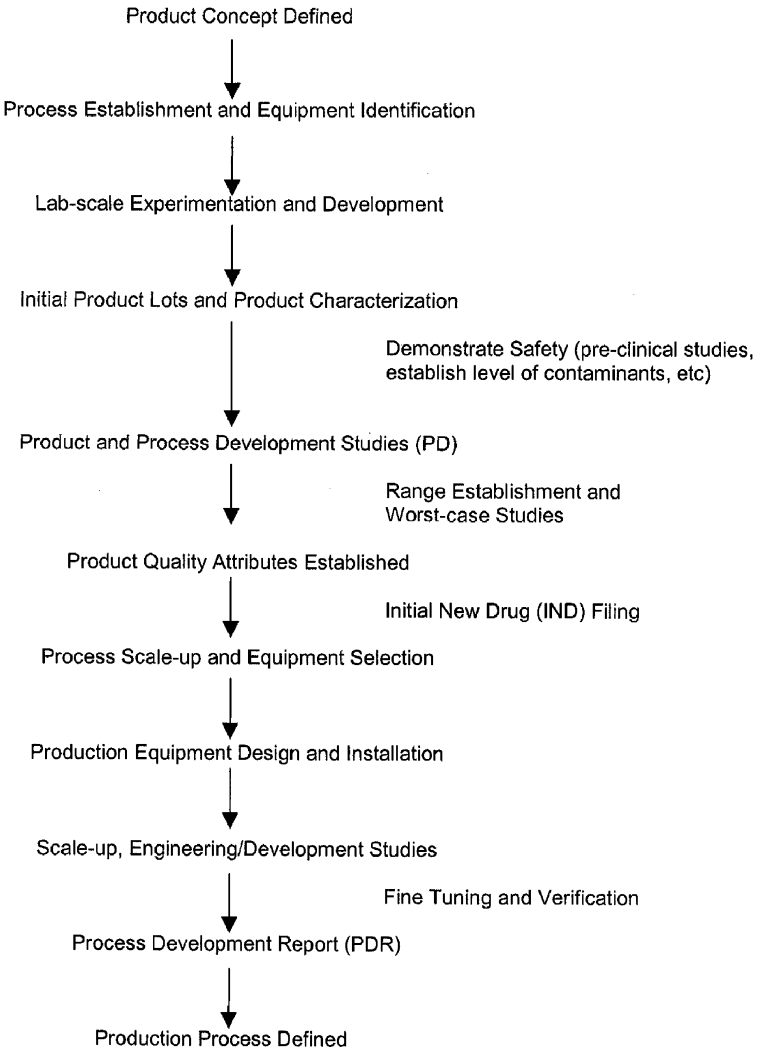

Figure 1 Critical steps for developing a defined process.

and biotechnology organizations shy away from doing comprehensive PD work as they are in a rush to reach the marketplace. In our competitive world of today, timing is key for making or breaking of an organization. Often what we do not realize is that there are no short cuts and eventually (sooner or later) we have to do the required PD work. The smart approach, therefore, would be to perform all required PD work before process validation, rather than during process validation or after completion of a process validation project. In the latter case, the validation projects generally become confusing, cost a great deal of money, and delay project completion (16).

A poorly developed process will typically allow only narrow ranges for operational parameters and may result in the rejection of large amounts of otherwise good

in-process material produced slightly outside the narrow process ranges developed. Extension of the process ranges or scale-up of manufacturing processes after initial validation requires time consuming regulatory review and approvals, repetition of all the work performed previously, and almost always turns into a costly validation project. It prevents pioneering organizations from taking leadership positions in the marketplace due to limited product supplies. It may lead and encourage competitors to enter the field and snatch the leadership position from the organizations that developed the product at the first place. It is a lesson many organizations learn, albeit late.

Process Development Report

The importance of well-executed PD work and a well-written process development report (PDR) cannot be emphasized enough. PD and PDRs are the key components of a successful technology transfer from R&D to manufacturing (8). The success or failure of a process validation project greatly depends on the quality and details of the PD work performed and the quality of PDRs in terms of their content, clarity, and completeness. Poorly written reports often cause a great deal of frustration for all involved, result in unnecessary delays, impact project schedule, and even lead to ultimate failure of a project. Many organizations perform excellent PD work, but lack in writing clear and complete reports. Ideal PDRs should contain the following information in as much detail as possible:

- Objective and definition of a process/product
- Scope and rationale
- Process description
- Process flow chart
- Materials and methods
- Equipment and facilities
- Utilities and accessories
- HVAC and environmental requirements
- Process input and output parameters (critical and noncritical)
- In-process testing and acceptance criteria
- Product specifications
- Calibration and preventive maintenance
- Other process requirements
- Result and discussion
- Conclusion
- References

Process Parameters

It is paramount that all process operating parameters (input parameters) that affect product quality attributes (output parameters) are established clearly during the PD phase of a new process, product, or a technology. This is accomplished typically by performing studies at lower and upper limits of the operating ranges generally referred to as the worst-case studies, crash studies or feed-stream studies. Some studies are performed up to the edge of failure and then stepped back to the ranges where process performance is acceptable. These studies can be simulated or performed with active ingredient or product derived from starting material generated during PD phase of the project. These studies can also be performed by generating starting material by artificially setting the parameters to the upper and lower limits of

the range. The process parameters are generally classified as critical process parameters and noncritical process parameters.

Critical Process Parameters

By definition the critical process parameters are "those operating parameters that directly influence the quality attributes of the product being produced." For example, temperature and pH in a fermenter are considered critical operating parameters as they have a direct influence on the viability of the organism and the chemical or biological activity of the product being produced. Other parameters that may be considered critical for fermentation processes are: cell viability, media conductivity, glucose concentration, oxygen and air uptake rates, and cell density in the production vessel or device.

Noncritical Process Parameters

The noncritical process parameters are "those operating parameters that have no direct influence on the quality attributes of the product being produced." For example, cell age and media flow rates in a fermenter are considered as noncritical operating parameters as they have no direct influence on the viability of the organism or the activity of the product being produced. Other parameters that may be considered noncritical for fermentation processes are: cell density in the inoculum, cell productivity, agitation rate, perfusion rate, and cell osmolality in the production vessel or device.

Cell Culture and Fermentation Process

A number of different approaches have been used to exploit cell culture technology and develop pharmaceutical products and medical devices, for example expression of the molecule of interest by cells through genetic manipulation or the use of cells as such for treating certain medical conditions. Of these, the technology based on product expression through genetic manipulation is most common. Commercial fermentation processes and bioreactor technologies have been developed in the last several decades to state of the art production of pharmaceutical agents of interest. The introduction of rEPO, rTPA, rFVIII, rInsulin, rHGH, rPDGF, etc. to treat many medical problems would have not been developed without these advances in the technologies. Figure 2 depicts a flow diagram for a typical fermentation process. We will discuss below the steps involved in the development of a commercial cell culture process in the light of process validation. Of special interest here is the establishment of critical and noncritical process parameters that will be verified during the process validation phase.

Cell Line Development

Once a clone has been selected for commercial development it is crucial that the nutritional requirements for the cell line must be defined. The cell line may need to be adapted for growth in certain cases, such as the expression and production of a product in a serum-enriched or serum-free media. The following nutritional requirements in terms of their concentration (% or molarity) or amounts (g/L or PPM or PPB as appropriate), and growth conditions must be established:

- Chemically defined growth medium
- Need for protein/serum/plasma or a protein-free media

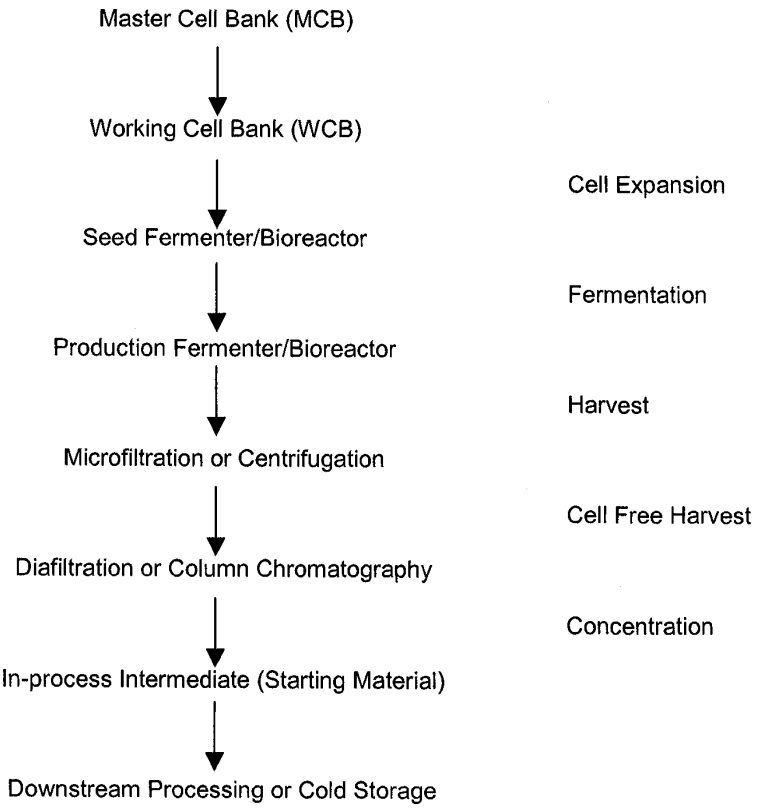

Figure 2 Typical fermentation process flow diagram.

- Requirements for vitamins or fatty acids
- Requirements of any special chemicals
- Requirements of growth factors or hormones, etc.
- Optimizatipon and maintenance of appropriate pH and ionic strength
- Requirements of oxygen, carbon dioxide or other gases
- Optimizatipon and maintenance of appropriate temperature
- Frequency of media changeover
- Frequency of harvesting of the cell line or product

Cell Line Characterization

The cell line must be fully characterized (17,18) for the absence of objectionable organisms or contaminants as follows:

- Absence of bacteria or spores
- Absence of fungi and mycoplasma
- Screening for adventitious viruses (nonretroviral)
- Screening for species-specific viruses (nonretroviral)
- Retrovirus contamination from other species

- Absence of other objectionable agents such as prions and TSE/BSE
- Phenotype/genotype characterization
- Identity and genetic stability

Master Cell Bank (MCB) Preparation

After selection of the appropriate clone for production purposes, the clonal cells should be expanded to appropriate fermentation scale, preferably to production scale fermentation, and used to prepare an MCB in appropriate sized vials for storage in liquid nitrogen. After preparation, the MCB should be scaled-up to evaluate its life cycle and productivity. The process parameters that need to be established at this stage are: cell concentration and viability for preparing MCB, cell volume in the vial, purity and amount of the preserving agent such as DMSO, storage temperature, and acceptable cell recovery and viability after thawing of the MCB vial.

Working Cell Bank (WCB) Preparation

The MCB is typically expanded up to seed or production scale fermentation to prepare the WCB in appropriate sized vials or bags suitable for storage at $-70^{\circ}C$ or colder. The WCB should also be scaled-up to production scale fermentation to evaluate its productivity and other growth conditions, and it should be fully characterized as outlined in ''Approach and Rationale'' to ascertain that it is free of objectionable organisms and contaminants. The process parameters that should be established at this stage are: cell concentration and viability for preparing WCB, cell volume in the vial or bag, purity and amount of the preserving agent such as DMSO, storage time and temperature, and acceptable cell recovery and viability after thawing of the WCB vial or bag.

Cell Expansion and Seed Preparation

Procedures (SOPs, BPRs, etc.) should be prepared that describe in detail all the steps for the expansion of cells starting from WCB through preparation of the seed for inoculation of the final-scale production device (fermenter, bioreactor, bag or bottle or vessel). The cell expansion procedure may require only a few steps or may have a number of steps before a seed is ready for inoculation of the production device. In addition, the seed may be used immediately to inoculate a production vessel or it may be stored further until use. Therefore, it is important to evaluate the process and identify the critical and noncritical process parameters for each process independently.

The examples of the process parameters that may be established at this stage are: thawing time and temperature, volume of media and size of flask or bottle for initial cell growth, time and temperature for initial cell growth, media pH, conductivity, temperature, glucose concentration, oxygen and air uptake rates, cell viability and cell recovery at different stages, cell density for scale-up to the final seed vessel (bottle or bag or fermenter or bioreactor), cell density and cell viability in the seed to be used for inoculation of the production device (bottle or bag or vessel or fermenter or bioreactor), and storage time and temperature for the seed (inoculum).

Production Scale Fermentation

Fermentation at the production scale may be carried out in a vessel (fermenter or bioreactor), bottle, or a bag depending on the product type. The product may be

the cells themselves, for which efficient cell growth may be critical, or the product may be a biochemical entity (enzyme, protein, hormone, etc.) expressed either in the cells intracellularly retained in the inclusion body or secreted out of the cell in the spent medium. In the latter case, the stability of the molecule in the spent medium should be explored as storage time and temperature for the harvest will be critical for the stability of the product. The process parameters required to be established for the fermentation are: cell viability and cell density in the inoculum, media pH, conductivity, temperature, glucose concentration, oxygen and air uptake rates, cell productivity, cell life span, agitation rate, perfusion rate, cell density, cell viability, and cell osmolality in the production vessel or device.

Continuous fermentation (perfusion). Continuous fermentation, where the product is generally secreted in the spent medium, is the most efficient and commonly employed technology for the production of biopharmaceuticals today. The main concept of this technology is to keep the cells alive as long as they produce a quality product. The number of days the cells are kept in a fermenter (fermenter days) varies depending on the cell type and established time period (weeks or months) for producing a quality product. In this approach the cells are expanded to desired optimum concentration and induced to adhere to coated (with proteins such as collagen) or noncoated acrylic beads where they can survive for many months as long as their nutritional needs are met. Fresh medium is introduced (perfused) and spent medium (harvest) is removed from the fermenter on a continuous basis. A number of cell sedimentation devices (conical, incline or plate settlers) are used to separate the cells from harvest. The cells are returned to the fermenter and the harvest is collected in a harvest tank or bag. The fermenters used in this technology are typically smaller in size (50–2500 L), as continuous perfusion of media allows sufficient volume of harvest collected on a daily basis.

Since the equipment used is more complex and the fermenter cycle is typically long (months), the validation effort is more rigorous for this technology. Establishment of acceptable fermenter days requires full cell characterization (see Cell Line Characterization) at the beginning (early), middle, and end (late) of fermentation to demonstrate that the cell characteristics do not change over time. In addition, product quality attributes are evaluated for the product derived from early, middle, and late stages of fermentation. These activities are performed and established during PD phase and confirmed during formal process validation (PPQ).

Batch fermentation. This nonperfusion technology is employed for products that are either secreted in the spent medium or expressed intracellularly. The cells or the harvest is collected for the isolation and purification of the product depending on the expression of the product in the cells or in the spent medium. The fermenter cycle is generally short (days) for batch fermentation process than for continuous fermentation process (weeks or months). This technology is most efficient for products that are expressed intracellularly where cell mass expansion is critical for productivity. It is less efficient for cell secreted products as the cost of operation is high. The fermenters used in this technology are typically larger in size (500–25,000 L), as it is a batch operation that allows collection of cells or harvest only once per fermenter cycle. Since this technology does not use cell sedimentation devices and fermenter cycle is short (days), the validation effort for batch fermentation process is less rigorous.

Cell mass expansion. This technology is similar to batch fermentation process except the main objective of the fermentation is to expand cell mass. It is used mainly for the products that are expressed intracellularly or where the cells themselves are used for medical treatment (as a medical device), and the cell mass is critical for

productivity. The fermenter cycle is short (days) for this technology than for continuous fermentation process (weeks or months). The fermenter sizes used in this technology vary depending on the requirements of the cell mass. Since the equipment used is simpler and the fermenter cycle is short (days), the validation effort for this fermentation process is less rigorous.

Though validation of fermentation process may be simpler for medical devices using biologically active cells, process validation for their formulation, storage, and delivery are more complex. Since mammalian cells are more fragile than protein molecules, their storage without impacting their quality attributes are more challenging. Demonstration of biological activity retention for a heterologus cell-based product during production, distribution, and storage is a daunting task. Cell characterization studies (see Cell Line Characterization) may have to be performed more rigorously after formulation, storage, and end of shelf life of these devices. In addition, the level of impurities and contaminants would also require rigorous investigation during these stages. Moreover, an assurance that the biological activity and safety do not impair and adverse reactions do not increase during these stages also needs to be demonstrated.

New approaches and future of cell-based therapies. The manipulation of cell culture technologies to generate unique therapies and medical treatments has just begun. Further development of these technologies would be essential for their impromptu use in new ways to treat diseases. Mammalian cells are being evaluated for grafting, transplantation, tissue regeneration, and organ culture. Stem cells are being developed into numerous cell-based treatments/cure for many diseases such as cancer, HIV, Alzheimer's, Parkinson's, etc. Gene therapy is expected to be the ultimate cure for many diseases in the 21st. century. This field is expected to grow exponentially in the next 25 years and bring numerous challenges for cell culture scientists. A number of microbial hosts (bacteria, plasmids, viruses, etc.) are being evaluated as carriers or vehicle for gene therapy products. For a successful gene therapy product it is crucial that it is free of any side effects, is long lasting, and is fully effective. To accomplish these goals the gene therapy products would have to be pure, free from undesirable components, easy to use, effectively targeted to desirable site, effective transformation and expression of desired genes, complete correction or deletion of defective genes, and they must prove to increase longevity. Cell culture scientists would definitely address all these issues and develop appropriate technologies to attain desired results. However, imagination for validation of all these diverse technologies and processes is mind-boggling today. New approaches to validate these technologies, production equipment, and production processes would have to be developed to meet yet to be established regulatory requirements.

Product Isolation, Separation, and Concentration

The procedures for the isolation of the product, its separation from impurities and contaminants, and its concentration by different technologies, depending on the product type, are performed after the fermentation process is complete. The cell separation techniques such as centrifugation or microfiltration may be applied to concentrate cells for product recovery or remove cells from the harvest that contains the product. The parameters that may be critical for process validation for this process are: cell concentration and viability in the fermenter effluent (spent media with or without cells), storage time and temperature for the effluent, centrifugation speed or microfiltration rate for cell separation, cell separation time and temperature, and

storage time and temperature for the concentrated in-process intermediate (IPI, starting material).

In-Process Intermediate (IPI) or Product Preparation and Stabilization

The IPI may be the cell suspension or cell extract or concentrated harvest fluid depending on the product type. Some of the processes require stabilization of the IPI for storage prior to further processing of the product. In these cases a formulating agent may be added to the concentrated IPI prior to its storage in the cold. The parameters that should be considered for process validation here are: the purity and concentration of formulating agent, mixing of the formulating agent with the IPI, freezing time and temperature for the formulated IPI, and storage time and temperature for the formulated IPI.

For medical device applications, the IPI (cell suspension/tissue) may be the final product that may require cleaning and removal of impurities, formulation for stabilization, and preparation of the product for clinical use. In such cases, the parameters that need to be established for process validation are: amount of contaminants (DNA, proteins, etc.) in the final product, cell/tissue morphology, genetic characterization of the cells/tissue, amount of formulating agent, storage time and temperature, and shelf life of the product.

Downstream Process Development

The pharmaceutical or therapeutic proteins expressed in cells are further purified from the IPI generated during the fermentation. The purification steps may involve microfiltration/diafiltration, salt or solvent fractionation, and column chromatography (ion exchange, hydrophobic interaction, affinity, gel filtration, etc.). The examples of critical process parameters that should be established here are: pH, conductivity, salt or solvent residues, membrane life cycle, column operation parameters (equilibration, load, wash, elution, regeneration, storage, cleaning, life-cycle, etc.), impurities and contaminant clearance (DNA, microbes, viruses, proteins, etc.), and hold times and temperature for in-process material and equipment. There is a whole battery of process parameters that should be established for process validation for these operations. It is beyond the scope of this book chapter to go into details of these parameters, but they are discussed in more detail elsewhere (19).

Bulk Formulation, Stabilization, and Final Packaging

The purified in-process material (bulk) is typically formulated for stabilization and either stored until further processing or filled and/or freeze-dried depending on the mode of application for the product. These operations are generally performed in the clean room environments (class 100) that require detailed and cumbersome PQ studies. At this stage, the product is characterized in detail and every lot of the product is evaluated against pre-established specifications and quality attributes. These include: product amount (units or weight), purity, strength, pH, ionic strength, amount of trace metals, amount of impurities and contaminants (DNA, proteins, microbial load, endotoxins, etc.), amount of residual reagents (solvents, polymers, chemicals, etc.), amount of formulating agents (excipients, sugars, etc.), sterility, storage time and temperature, and product shelf life. The process parameters governing these quality attributes should be considered as critical

parameters during process validation studies. The filled or lyophilized product vials are inspected for container closure integrity and labeled appropriately. The product coming from validation runs is placed on stability to demonstrate that the product is stable during its entire shelf life. In addition, shipping studies are performed to demonstrate that the packaging is shatter-proof and the product is stable during shipping. All appropriate process parameters covering these quality aspects of the process should be established and verified during the process validation studies.

Process Validation

Process validation projects are complex and cover a great deal of details. The critical steps for performing process validation studies are depicted in Fig. 3. The process validation begins with the receipt of a PD report from the R&D arm of an organization, and ends with the approval of a process validation report and applicable SOPs/BPRs by the QA arm of the organization. A well-written PD report with adequate details of the developed process greatly facilitates process validation. In addition, efficiency and success of a process validation depends heavily on the extent and quality of PD work and/or engineering runs performed on associated equipment (see Process Validation Protocols and Final Reports for details). Prior to formal process validation, processes should be run rigorously on associated equipment to gain valuable experience. The experience gained is well worth as it saves time and resources during the formal validation stage by efficiently resolving any unforeseen problems, discrepancies or deviations. In no event, the engineering runs or PD studies be left for performance during the formal process validation stage (PPQ phase). Successful completion of a process validation study requires good coordination among the responsible departments such as production/manufacturing, quality control, QA, engineering, R&D, regulatory affairs, etc. We will discuss below the types of processes that are generally covered under process validation projects (PV or PPQ).

- Manufacturing procedures for fermentation, product isolation, purification, formulation, sterilization, filling, and freeze drying of products
- Microfiltration, ultrafiltration, and sterile filtration
- Cleaning procedures for equipment and processes (clean-in-place, CIP)
- Lifecycle determinations for chromatographic resins, membranes and filters
- Impurity/contaminant clearance (DNA, viruses, host cell proteins, etc.) studies
- Impurity/contaminant inactivation (virus, endotoxins, TSE/BSE, etc.) studies
- Sterilization and steam-in-place (SIP) systems
- Critical utilities such as water for injection (WFI). (PPQ is performed to demonstrate process reproducibility/consistency and product quality)
- Environmental qualification for facilities (EQ). (EQ is a combination of air handling, equipment/facility cleaning, sanitation, gowning, and environmental monitoring)
- Re-processing of process intermediates, bulk, and final product
- Revalidation of processes due to major change control requests (CCRs)

Unlike equipment re-qualification, revalidation of a production process is currently not a mandatory cGMP requirement, but must be considered after implementation of a large number of CCRs or as soon as a process shift is noticed. The

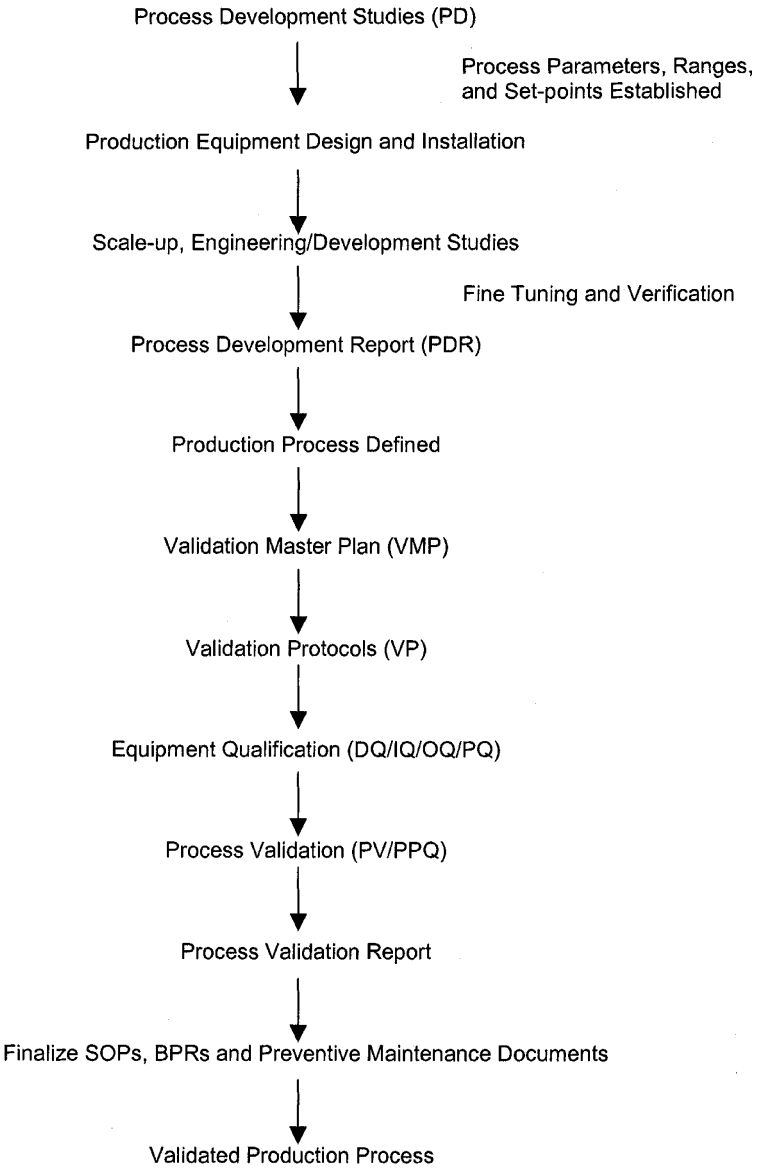

Figure 3 Critical steps leading to process validation.

purpose of the process revalidation should still be to demonstrate that the implementation of CCRs or an observed shift in the process has not affected quality attributes of the final product. Even if no major changes were implemented or process has not shifted, process revalidation should still be considered to demonstrate that the process is running within controlled limits, at some appropriate time intervals after original process validation (after 100/ 500/1000 lots or 2/5/10 years, as appropriate).

Life Cycle of Process Validation

Process validation is a continuous process, it does not end after the sign-off of the original process validation report. Figure 4 illustrates the life cycle of process validation by keeping the process and product quality attributes in mind. It is not uncommon for companies to initiate change control requests within days or weeks after the completion of the original process validation. Many companies have made major process changes within one year of the original process validation.

Since cell culture and fermentation processes are continually evolving, changes are being made to manufacturing processes on a regular basis by many companies. In fact, a number of second-generation products are derived from the same cell lines, except the cell lines have been adapted to produce the product in a protein-free (serum/plasma) media.

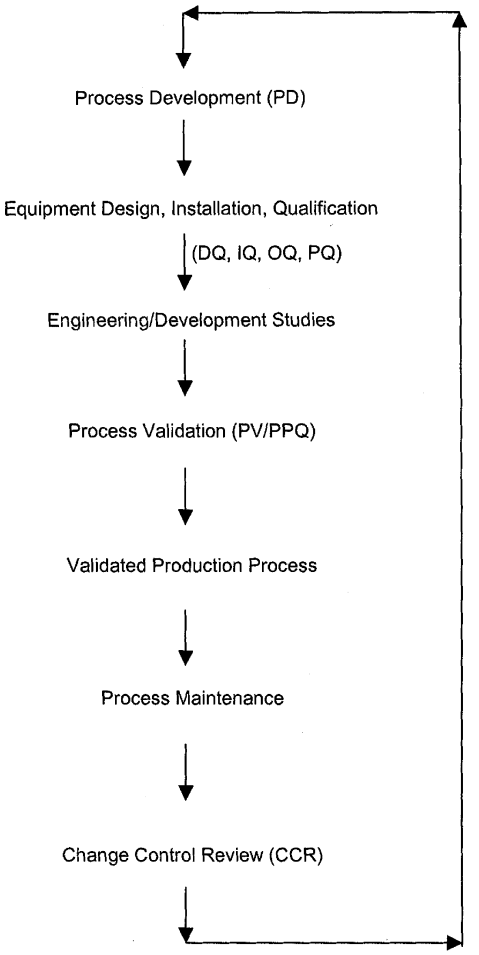

Figure 4 Life cycle of process validation.

Though such changes improve the product quality and safety tremendously, the process validation task is nevertheless the same. Such changes often lead to the construction of a new manufacturing plant and complete re-validation of the production process. Therefore, the life cycle of a manufacturing process after original validation is mainly dependent on the volume of change control activity.

Though regulatory agencies have not made a guideline yet for process re-validations, they have been advising drug/biological manufacturers for some time to evaluate the need for process re-validation at some regular intervals. Many companies do not feel the need for process re-validations based on the activities that are covered under their change control management programs. However, it may be prudent to perform process re-validation after a major process change or after a number of small changes to assess the cumulative effect of many change control requests. One possible mechanism may be to perform process re-validations after every 100/200/500/1000 production runs or, 2/5/10 years or sooner, as justified by the evaluation of the historical process input and output parameters, specifications, set-points, action limits, or other observations that suggest that the validated process may have shifted.

VALIDATION OF THE MANUFACTURING PROCESSES AND ASSOCIATED EQUIPMENT QUALIFICATION

Validation SOPs

Establishment of sound validation SOPs and strict adherence to them is crucial for the success of any validation project in a cell culture-derived drug/biological manufacturing organization. These SOPs are typically written by validation professionals and are approved by the responsible groups (e.g., operations, engineering, validation, research, PD, QC, QA, and regulatory affairs) that have a stake in the validation projects. The SOPs should be established for the required validation functions, as listed below:

- Site or facility validation policy and management
- Validation master plan and final report
- Validation requirements for DQ, IQ, OQ, PQ, PD, PPQ, EQ, and RQ validation protocols
- Design of worst-case studies for OQ, PD, and PPQ protocols
- Determination of acceptance criteria for validation protocols
- Design of prospective or concurrent validation studies, and retrospective data analysis
- Write a validation protocol and a validation final report
- Revision of a validation plan, protocol or a final report
- Execution of IQ and OQ protocols
- Execution of engineering and PD protocols (studies)
- Execution of PV or PPQ protocols (studies)
- Validation of analytical methods, assays, and procedures
- Performance of validation studies on clearance and/or inactivation of impurities contaminants
- Performance of filter qualifications and sterile filtration validations
- Performance of cleaning validation studies on equipment, accessories, and processes

- Performance of sanitation and sterilization validation studies
- Performance of validation studies on automated systems (computers, DCS, and PLCs)
- Performance of environmental qualification (EQ) studies
- Review, verification and analysis of validation data and documents
- Documentation of validation discrepancies and deviations
- Performance of re-qualification (RQ) studies on validated equipment, systems and processes
- Establishment of a validated lifecycle (for cells, resins, filters, membranes, etc.)
- Requirements for maintaining a equipment or a process in a validated state
- Display of validation status (labels) for validated equipment and systems
- Training and certification program for personnel involved in validation projects

In addition to these, many other SOPs may be established for performing specific validation functions depending on the need of the equipment, system or process (e.g., determination of equipment surface finish, calibration of instruments and thermocouples, determination of agitation rates, determination of HETP and Af on chromatography columns). The required SOPs are typically identified and established by qualified validation professionals depending on the need of an organization.

Validation Master Plan

It is paramount that a detailed VMP should be written before implementation of any new or unlicensed process for the production of a cell culture derived drug/biological product (11,12,20–22). The VMP provides details of an organization's plans for carrying out all validation activities on production equipment, systems, and processes. The plan should provide details of equipment, facilities, utilities, raw materials, storage times and temperatures, environmental requirements, production processes, critical and noncritical process parameters, process set points, SOPs and BPRs, in-process and final product sampling, analytical assays and methods, QC testing, in-process testing and acceptance criteria, and product release specifications. The VMPs should be approved by all responsible stakeholders or senior management of a company (operations/manufacturing, QA, QC, engineering, PD, and regulatory affairs), and should contain the following information at a minimum:

- Objective
- Scope and rationale
- Process and product design
- Description of manufacturing facility
- Equipment description and qualification (DQ/IQ/OQ/PQ)
- Process description and qualification (PQ/PPQ)
- Description of utilities and supplies
- Description of automated systems
- Equipment cleaning (CIP/COP) and sanitation (SIP)
- HVAC and EQ
- Process parameters and set points
- In-process testing and product specifications
- Analytical methods and procedures
- Manufacturing procedures and batch records

- Responsibilities
- Execution plan and schedule
- Documentation and training
- Modification and change control
- Preventive maintenance
- References
- Attachments
- Facility diagram
- Process flow diagram
- List of validation protocols
- List of PDRs
- List of engineering reports
- Project schedule and Gantt charts
- Other pertinent documents

Equipment Qualification

A great deal of information is available in the literature, web sites of the regulatory agencies (FDA, EMEA, ICH), professional societies (PDA, ISPE, AAPS, etc.), various seminars and symposia, and from professional consultants for the qualification of process equipment. The key components of equipment qualifications are covered in the following validation studies:

- Design qualification (DQ)
- Installation qualification (IQ)
- Operational qualification (OQ)
- Performance qualification (PQ)

Equipment qualification studies should be performed for all process equipment (fermenters/bioreactors, chromatography, microfiltration/ ultrafiltration systems, tanks and vessels, autoclaves, CIP and SIP systems, freeze dryers, etc.), utility equipment (WFI, clean steam, solvent delivery, etc.), supply equipment and accesories (gases, filters, raw materials, etc.), automated systems (computers, DCS, PLCs, etc.), and critical facility equipment (HVAC, warehouse, storage chambers, shipping containers, etc.). It is beyond the scope of this chapter to cover details of equipment qualifications. The readers can review selected references (11,12,22,23) to learn more about equipment qualifications.

Equipment Engineering Runs and Process Development Studies

After completion of the DQ, IQ, OQ and PQ studies on the equipment, it is prudent to perform equipment engineering runs and PD studies (pre process validation studies, also known as trial runs) to ascertain that the developed process is scalable to the production scale. These studies confirm that the process can perform effectively within the ranges established at the small-scale or previously developed scale. These studies also help in ensuring that the formal process validation studies would not run into major discrepancies, deviations, or failures. They also provide an opportunity to fix any problems that may have been ignored previously. Furthermore, they provide a chance to develop or fine tune acceptance criteria and specifications for the formal process validation studies down the road.

These studies are performed under approved PD protocols (see Cell Culture and Fermentation Process). Typically three runs are performed at the production

scale by following the SOPs and BPRs written for the process. All sampling and testing is performed as in the process validation studies except that some tests may not be required (have no chance of failure due to scale of operation). PD final reports are prepared after review and analysis of all results and associated data. Appropriate conclusions are drawn and recommendations for formal process validation studies are made in the final reports. The examples of these studies are equipment load studies (autoclaves, depyrogenation ovens, viral inactivation tanks, pasteurization, column chromatography, cleaning validations, etc.), mock runs (process runs without active ingredient), partial load studies (part load with active ingredient and remaining load with an excipient), and full process runs with active ingredient. In conclusion, these studies provide peace of mind that the formal process validation would be un-eventful or would be completed with minimum difficulties.

Process Validation Protocols (PV or PPQ) and Final Reports

Process validation protocols are written to demonstrate that the production processes are reproducible, are in control, and consistently produce a product of predefined specifications and quality attributes. The protocols are also used to demonstrate impurity/contaminant clearance, validation of operating ranges, equipment cleaning, and establishment of lifecycles for chromatographic resins, filters, and membranes. It is indisputable that well written process validation protocols are instrumental for efficiency and success of process validation studies. The protocols should contain details of the production process and equipment to be used, SOPs and BPRs to be used, critical and noncritical process parameters, process set-points and action limits, sampling and QC testing, analytical methods and assays to be used, and in-process and final product specifications. The process validation protocols should be designed to incorporate worst-case studies based on the acceptable level of risk for the process and the product. It is important that all pre-requisites (e.g., DQ/IQ/OQ/PQ on equipment or a system, approved SOPs/BPRs, instrument calibration, and personnel training) are completed before execution of a process validation protocol.

After execution of the validation protocols and completion of all testing, validation final reports should be written with complete details of protocol execution (BPRs and sample tables), test results, discrepancies and deviations, modifications or change control requests, passage/failures against acceptance criteria, statistical data analysis, preventive measures and maintenance, validated critical and noncritical process parameters, process set-points and action/alert limits, supporting data, and conclusions. The validation protocols (and final reports after completion of the study, see below) should be reviewed and/or approved by all responsible parties such as manufacturing, PD, engineering, quality control, QA, validation, and regulatory affairs (optional), as appropriate, prior to its execution. A typical outline of a process validation protocol and final report (validation package) are given below:

- Approval signatures page
- Objective
- Scope and rationale
- Process description
- Acceptance criteria
- Responsibilities
- References

- Prerequisites
- Validation procedures or test functions
- Result, data analysis and discussion
- Validated critical and noncritical process parameters
- List of SOPs and BPRs
- List of supporting data and documentation
- List of discrepancies and deviations
- Conclusion

The validation final reports must contain all results obtained during the execution of a process validation protocol. All deviations should be described along with justifications for their acceptance. The results must be evaluated against the pre-established acceptance criteria, product specifications, and quality attributes. The data should also be evaluated statistically and confidence intervals for the data should be calculated to demonstrate process robustness. Process capability (Cpk) calculations should also be performed to demonstrate process reproducibility. The final report should draw a scientifically sound conclusion based on the results obtained during the study. Ranges and set-points for all validated critical and non-critical process parameters should be established. In addition, alert and action limits for validated process parameters should also be established wherever applicable. Moreover, a plan to monitor process parameters during production should be devised, and the historical data should be evaluated statistically at pre-established time periods (yearly or after every 100 lots or other suitable interval). Process capability (CpK) calculations should also be repeated on historical data to demonstrate process reproducibility. The in-process action and alert limits and product specifications should be re-evaluated and tightened, wherever possible, after complete data analysis at pre-established time periods.

Raw Materials

The quality of raw materials plays a major role in attaining quality attributes for the final product and indirectly affects success or failure of process validation. The critical raw materials for a cell-derived product during fermentation are: basal media, purified water or WFI, salts and buffering agents, oxygen and carbon dioxide, amino acids, vitamins, glucose, serum or plasma proteins (animal or human), and other nutrients such as hormones or growth factors. It is essential to establish specifications for all raw materials used in the production process, and ensure that quality attributes of all incoming raw material lots are met against the established specifications. Any changes made to the specifications for raw materials should be evaluated through change control management and process validation(s) performed wherever necessary. In addition, FIFO (first in first out) procedures should be established for approved/released incoming raw material lots.

Facilities

All equipment installed in a facility must be qualified (DQ, IQ, OQ and PQ, wherever applicable). The typical facility related equipment includes HVAC, cold/freezer freezer rooms, cooling towers and heat exchangers, chemical/solvent tanks and distribution system, and waste treatment and disposal systems. The facility cleaning procedures are established based on the requirements for each classification (class

100, 1000, 10,000, and 100,000; or grade A, B, C, or D), and must be validated under the facility EQ. Emergency power systems should also be qualified (DQ, IQ, and OQ) to demonstrate that uninterrupted power supply is available to critical production equipment (cold rooms, freezers, freeze dryers, etc.).

Utilities

The critical utility systems must be qualified (DQ, IQ, OQ, and PQ). The examples of utilities that are used in a typical cell culture based production facility are potable water, purified water, WFI, plant steam and clean steam systems, gas distribution systems, CIP and SIP systems, and electricity supply systems.

Production Equipment

Besides equipment qualification (DQ, IQ, OQ, and PQ), typical production equipment used in a cell culture-based facility must be validated (PPQ). The production equipment includes media and buffer preparation equipment, media filtration equipment, media storage tanks, media and buffer distribution system, seed fermenters or bioreactors, production-scale fermenters or bioreactors, aseptic transfer equipment, cell settlers, heat exchangers, pumps and agitation systems, gas sparging equipment, harvest tanks, microfiltration and ultrafiltration systems, centrifuges, and initial capture and concentration equipment. These validation studies may be performed separately or may be combined in the process validation study for a new process, product or technology.

Instruments

Though all instruments associated with equipment systems are generally covered in the equipment qualification, any stand-alone instrument must be qualified separately. In addition, all instruments must be calibrated at the time of their qualification and must remain on a regular calibration schedule after their qualification. The established calibration schedule must be justified and scientifically sound. All test instruments used to measure a specific parameter during validation must also be calibrated.

Analytical Equipment, Instruments, and Methods

All analytical equipment and instruments must be qualified (DQ, IQ, OQ, and PQ), and associated methods, procedures or assays must be validated (PPQ). During this phase of validation, the procedures must be evaluated for precision, accuracy, repeatability, and variability. The results must be evaluated statistically to demonstrate confidence intervals for each sampling condition. The instrument to instrument, operator to operator, and intra- or inter-assay variabilities must be established.

Distributed Control Systems (DCS) and Programmable Logic Controllers (PLCs)

All automated systems (computers, DCS and PLCs) must be qualified (DQ, IQ, OQ, and PQ), and associated software must be tested for its intended function, use, and

its lifecycle (24–26). The validation aspects of these systems are verified and confirmed during the process validation phase of the validation activity.

Cleaning and Disinfecting

The cleaning of the equipment including the CIP systems must be qualified (DQ, IQ, OQ, and PQ), and the procedures must be validated (PD, PV, and PPQ). The cleaning validation studies must demonstrate that the process residues and cleaning agent residues are removed to the acceptable levels after the cleaning (27). The acceptable levels for the residues must be established by actual scientific data or sound scientific knowledge (28–31). Appropriately validated assays to test the residues must be used. In addition, all cleaning and disinfecting agents must be qualified or approved for use. The cleaning requirements for each step of the process must be established per appropriate guidelines established by regulatory agencies (27–29). Since the final rinse in cleaning processes for biological products is performed by WFI, many companies use the quality attributes of WFI as acceptance criteria for rinse samples. In addition to rinse sampling, cleaning agent residues and process residues (protein, fatty acids, nucleic acids, raw material components, etc.) are tested by surface swabbing and evaluated against pre-established acceptance criteria. The typical assays used for cleaning validations are pH, conductivity, TOC, microbial load, endotoxin, protein assays/analysis, and other assays for specific residues. A cleaning monitoring program should be established to maintain the equipment in a validated state (31).

Standard Operating Procedures (SOPs) and Batch Production Records (BPRs)

The appropriate SOPs and BPRs must be drafted, reviewed, and approved prior to beginning of process validation studies. These procedures may be modified, if needed, with appropriate justifications during validation as long as the last validation runs (three or more) are performed after making the modifications and all validation acceptance criteria are met. The modifications to the procedures during validation should be made through the change control system of the organization. It is important that all changes made during validation are fully incorporated in the SOPs and BPRs prior to approval of the final validation package.

Personnel and Training

The success of a process validation project depends solely on two things—first an effective and detailed technology transfer program, and second an effective and detailed training program for the new processes or technologies. Therefore, it is crucial that the organizations establish a very effective and practical technology transfer function, and an effective and practical training program for the operations personnel. The initial training is typically provided by the R&D arm of the organization (or whoever developed the process and has the most knowledge or experience). The training program and procedures must be well documented and must follow cGMP guidelines.

Documentation

The regulatory agencies have stated in no uncertain terms that the lack of documentation (even if the work was performed) would be interpreted as if no work was

performed. Therefore, the importance of documentation cannot be emphasized enough. The following documents need to be in place for the purposes of process validation:

- An approved validation master plan/report, if applicable.
- An approved validation protocol/report/final package.
- Original or copies of all approved specifications or acceptance criteria.
- Original or copies of all PD reports.
- Original or copies of all approved process validation parameter documents.
- Original copies of all prerequisite sheets completed during validation.
- Original copies of all validation attachments/execution documents.
- Original or copies of all SOPs/BPRs employed during validation execution.
- Original or copies of all QC test reports.
- Original or copies of all other test reports.
- Original or copies of all raw data, or location of all archived raw data.
- Original or copies of all supporting data related to validation.
- Original or copies of all change control requests implemented during validation.
- Original or copies of all deviation reports encountered during validation.
- Original or copies of all corrective action reports, if applicable.
- Original or copies of all SOPs/BPRs/documents revised during or as a result of validation.
- Original or copies of all other documents related to validation.

Preventive Maintenance

Maintenance of validation post-licensure is as important as original validation, as it transforms into cGMP compliance after licensure of the production process. To maintain a process in the validated state, it is crucial that procedures for preventive maintenance (PM) be established prior to original validation, modified as appropriate during validation, and are followed thoroughly and timely after validation. In addition, the processes should be monitored regularly with respect to process parameters observed during production. The historical process parameters data should be evaluated at some fixed intervals (annually or sooner if needed) with respect to validated parameters and be tightened or loosened (in principle) as appropriate and justified by the data based on sound scientific principles and policies. The historical data must be evaluated by applying appropriate statistical methods and calculation for process capability (Cpk). The procedures for the following activities should be established as needed:

- Evaluation of production equipment, parts, and accessories.
- Calibration program for all instruments used in production and testing.
- Equipment re-qualification and process re-validation program.
- Preventive maintenance program for equipment, facilities, and processes.
- Change control program.
- Evaluation of validated process parameters.

Change Control

A change control system must be instituted to document all changes made to validated production processes (32). The change control activity begins after the installation of the equipment systems and continues throughout the lifecycle of the process

or product. The change control requests (CCRs) should be initiated prior to making the change, except in emergency situations, and must be reviewed and approved by all involved groups. The impact on validated systems and processes must be assessed and any identified validation work must be completed before closing the CCR. If the change is minor, little or no validation work may be required, however if the change is major, full revalidation may be required. The US FDA published a guideline in July 1997 (33) that requires notification of all changes to the agency depending on the extent of the change in the following manner:

Major changes require submission and approval of a supplement prior to the distribution of the affected product. *Moderate* changes require submission of a supplement at least 30 days (CBE30) prior to the distribution of the affected product. *Minor* changes do not require any submission prior to the distribution of the affected product, but must be documented in the annual report.

MANUFACTURING PLANT QUALIFICATION

The manufacturing plant qualification and licensing requires additional validation, testing, and documentation besides equipment qualification, utilities/facilities qualification, process validation, and establishment of PM and change control programs. Successful completion of the following items is key to the licensure of a manufacturing plant for a cell culture-derived pharmaceutical or therapeutic or diagnostic product:

Plant Design and Construction

The manufacturing plant must be designed and constructed per appropriate local, state and federal regulations and bylaws. The plant must be built by keeping the product and personnel flow in mind. In general, the product flow should be unidirectional. All required essential utilities (power, water, waste disposal, sewer, etc.) and facilities (warehouse, receiving, shipping, etc.) must be planned for and built into the manufacturing plant. Location and installation of all production equipment, process utilities, facilities, and support systems must be designed and procured per requirement of the production process. Additional details for manufacturing plant design and construction are available elsewhere (8,20,21).

Validation Master Plan

A VMP must be written, approved by the management, executed, and revised/updated as needed starting with the site selection and plant construction and ending with the licensure of the manufacturing plant (see Process Development Report Section for the details that must be included in the VMP).

Equipment Installation

The production equipment, utilities/facilities equipment, and all support system equipment as designed for the process must be procured from quality manufacturers and installed per manufacturer's recommendations in the manufacturing plant. All essential utilities (power, water, disposal, etc.) and supplies (gases, solvents, etc.) must be available prior to the installation of equipment systems. Equipment check-

outs (ECOs) must be performed to evaluate safe and normal operation of the equipment prior to equipment qualification. It is also essential that the equipment life cycle be established at this phase to ensure that the equipment performs as designed during the entire life of the equipment.

Equipment Qualification

Please refer to Equipment Engineering runs and Process Development Studies, and Process Validation Protocols and Final Reports Sections for details on equipment qualification. It is crucial that ECOs and dry runs (trial/engineering runs) are performed during the equipment qualification phase to ensure that the equipment operates within the ranges as designed for the process and certified by the manufacturer.

Equipment Performance Qualification

These studies are performed during the qualification phase of the equipment to ensure that the equipment delivers the desired output for the manufacturing process such as flow rates, temperature, pH, conductivity, agitation rates, sparging rates, cell retention, bioburden reduction, endotoxin removal, depyrogenation, sterile filtration, sterilization, etc. Equipment loading studies (autoclave loads, vial washer loads, depyrogenation loads, stopper processing loads, chemical inactivation tank loads, etc.) are performed during this phase to ensure that the equipment loads are processed appropriately to meet all quality attributes desired in the qualified loads. It is essential to evaluate which of the equipment needs to be placed on equipment re-qualification program to ensure that equipment delivers desired quality attributes in the processed loads during the entire life of the equipment.

Heat, Ventilation, and Air Conditioning (HVAC)

The qualification of HVAC system (DQ, IQ, OQ, and PQ) for the manufacturing plant must be completed during the validation phase. This includes qualification of the air handlers, HEPA filters, distribution piping, and associated equipment. The PQ on the HVAC system must demonstrate that the adequate air flow and particle levels (viable and nonviable) are achieved per specification (Federal Standard 209E or European ISO Standard or equivalent) for the different manufacturing environments (class 100, 1000, 10,000, and 100,000; or grade A, B, C, D by European standards).

Cleaning and Sanitation

Effective equipment and facility cleaning and sanitation procedures play a major role in maintaining equipment in a validated state and maintaining its life cycle, and by doing so, assures maintenance of product quality attributes (27–31). Cleaning and sanitation procedures should be developed that can effectively clean equipment product contact surfaces, working and processing areas, work surfaces, floor and wall surfaces, and drainage and disposal systems. The cleaning/sanitation procedures must be validated by using appropriate acceptance criteria. The specifications or acceptance criteria for cleaning and sanitation should be established on a case by case basis by consideration of the manufacturing process, processing time, and

in-process materials used. Hold times for process equipment after cleaning must be established and demonstrated (qualified). PD studies should be performed to evaluate effectiveness of the cleaning agents and procedures and establishment of acceptance criteria before PPQ studies are performed. The typical tests used to demonstrate cleaning of equipment product contact surfaces are visual examination, microbial load, endotoxin level, pH, conductivity, level of residual process impurities, level of residual cleaning agents, TOC, and any other appropriate test for a residue. The typical tests used to demonstrate effective sanitation procedures are visual examination, levels of viable particles, level of nonviable particles, and absence of objectionable organisms. The following cleaning and sanitation procedures should be established at a minimum:

- Procedures for cleaning inner and outer surfaces of equipment.
- Procedures for cleaning of chromatography columns, filters and membranes
- Recipes and methods for CIP/COP systems.
- Manual cleaning procedures.
- Cleaning/sanitation procedures for work surfaces, floors, and walls.
- Cleaning/sanitation procedures for drains and disposal systems.
- Cleaning/sanitation procedures for utilities (WFI, gases, steam, etc.)

Gowning and Personal Safety

Appropriate and protective gowning is essential for preventing the product from getting contaminated by human interaction and also to safeguard humans from any undesirable effects due to exposure of finished products, in-process materials, process chemicals, and supplies or accessories used during production. The personal safety devices used are lab coats or gowns, eye/hearing protection devices, gloves, face shields, and chemical or solvent handling devices. Clear procedures should be written for effective use of gowning and personal safety devices.

Environmental Qualification

In addition to qualification of HVAC, equipment/facility cleaning, and gowning; an EQ should be performed to demonstrate clean manufacturing environment as a combination of an effective HVAC system, effective equipment cleaning procedures, effective gowning procedures, and effective facility cleaning procedures at dynamic and at rest conditions. The acceptance criteria for the EQ for class 100,000, 10,000, 1000, and 100 (grade A, B, C, and D by European Standard) are different and are derived from Federal Standard 209E and other US and European regulations. The typical documentation and testing performed during EQ are as follows:

- Number and gowning status of the operators present during dynamic conditions.
- Number and gowning status of the operators present during rest conditions.
- Swab testing of operator's gowns for presence of viable and nonviable particles.
- Verification of air flow changes/hour and air flow rates during testing.
- Testing of viable and nonviable particles in the air. Testing of viable and nonviable particles in swab samples from work surfaces, floor surfaces, and walls.
- Verification of all cleaning performed during the testing period.

- Presence/absence of objectionable organisms in the environment.
- Discrepancy investigations and implementation of corrective actions.
- Establishment of an environmental monitoring program.

Preventive procedures to check the presence of insects, pests, reptiles, and rodents should also be employed.

Capacity Evaluation

Though not a regulatory requirement yet, regulatory agencies sometimes request that a production capacity evaluation be performed for each manufacturing facility that requires licensure. Capacity evaluation should be performed by verifying the capacity of the equipment, utilities, supplies, facilities, raw materials, in-process materials, and final product production. The following items should be evaluated for the capacity assessment of the manufacturing facility:

- Capacity for manufacturing areas in terms of space and production for equipment such as seed and production fermenters, media/buffer tanks, harvest tanks, filtration/microfiltration, ultrafiltration/diafiltration, chromatography columns and skids, in-process material and bulk storage tanks, filling machines, lyophilizers, vial washers and processors, stopper washer and processors, cold rooms and freezer rooms, refrigerators and freezers, warehouse capacity, storage of raw materials and process intermediates, and storage of quarantine and released product.
- Capacity for processing/storage of potable water, purified water, WFI, plant steam, clean steam, CIP, SIP, cooling towers, heat exchangers, gases, etc.
- Capacity or production/storage of critical raw materials such as plasma, serum, hormones, peptides or proteins, affinity-matrix for columns, etc. used in the production.
- Capacity in terms of processing supplies such as autoclave loads, depyrogenation oven loads, vial/stopper processor loads, filters, etc.
- Capacity in terms of cleaning of equipment and turnaround time.
- Capacity for maintaining appropriate manufacturing environment
- Capacity in terms of personnel for working space, production schedule training, document archival, etc. performed in an orderly and a normal way.

A capacity report should be prepared and approved by responsible departments after evaluation of the above listed and any other requirements for capacity. The capacity report should clearly demonstrate the plant capacity in terms of product produced per day/week/month/year based on the capacity of each equipment, process, schedules, and trained personnel.

Access and Security

The manufacturing areas should be accessible only to qualified and authorized personnel. Adequate security procedures should be established to demonstrate that the manufacturing facility is secure and not accessible to unauthorized people. Regulatory agencies have been paying special attention to this issue lately due to recent product counterfeiting incidents in generic drug companies (34).

Building Licensure

A BLA is submitted to the FDA or its equivalent EMEA or other global regulatory agencies, after completion of the above listed and other required activities and documentation, for licensure of the manufacturing facility. A pre-approval inspection (PAI) is often performed by the regulatory agencies to verify that the information submitted to them is accurate and complete. The FDA and other global regulatory agencies have indicated recently that they may make PAI optional, if the previous compliance or system based inspections of the applying company have been satisfactory. Upon successful inspection or verification of the information submitted, a BLA may be approved by the regulatory agencies permitting shipping of the approved product produced from the manufacturing facility to its customers.

SUMMARY

In summary, process validation for a new cell culture derived-product, process, technology, or a new manufacturing facility should be carried out with a lot of careful planning and brainstorming along with a pinch of passion and dedication to details. Often process validation projects end up in tremendous delays and exorbitant costs due to poor planning, unrealistic goals and schedules, and inexperience of the assigned staff. It is a very expensive activity and should be carried out with good planning and caution. Validation activities take time on their own and often cannot be sped up no matter how many resources are poured into it. Albeit challenging and tough, it is an unforgettable journey that more often than not results in a joyful regulatory approval of a new product, process, technology, or a new manufacturing facility. Once taken successfully, one craves for this journey again and again and again.

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(19) **United States**

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Porro et al. (43) **Pub. Date: Jun. 21, 2007**

(54) **INCREASE IN STRESS TOLERANCE WITH ASCORBIC ACID DURING FERMENTATION**

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(21) Appl. No.: **11/546,951**

(22) Filed: **Oct. 12, 2006**

Related U.S. Application Data

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(30) **Foreign Application Priority Data**

Apr. 7, 2006 (US)..... PCT/US06/12854

Publication Classification

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C12N 1/21	(2006.01)
C12N 15/74	(2006.01)

(52) **U.S. Cl.** **435/139**; 435/252.3; 435/254.2; 435/483

(57) **ABSTRACT**

A method of increasing stress tolerance in recombinant organisms that have been engineered for industrial production is described. Stress tolerance is increased by making L-ascorbic acid available to the recombinant organism, either by exogenous addition to the culture medium or by endogenous production from D-glucose by the recombinant organism. To enable endogenous production, the recombinant organism is transformed with a coding region encoding a mannose epimerase (ME), a coding region encoding an L-galactose dehydrogenase (LGDH), and a D-arabinono-1, 4-lactone oxidase (ALO). The recombinant organism may be further transformed with a myoinositol phosphatase (MIP).

Figure 1

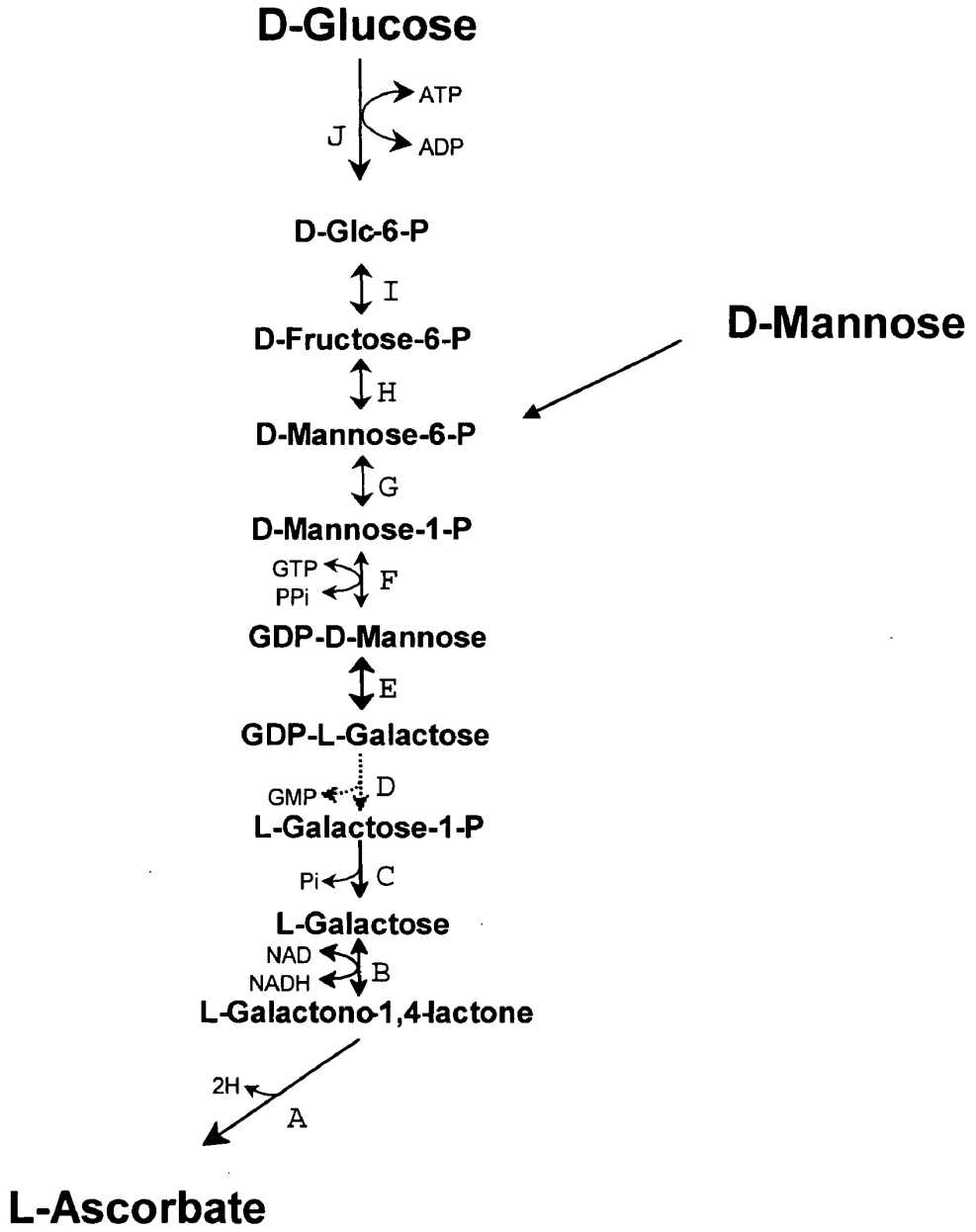

D-Glucose
ATP
J
ADP
D-Glc-6-P
I
D-Fructose-6-P
D-Mannose
H
D-Mannose-6-P
G
D-Mannose-1-P
GTP
PPi
F
GDP-D-Mannose
E
GDP-L-Galactose
GMP
D
L-Galactose-1-P
Pi
C
L-Galactose
NAD
NADH
B
L-Galactono-1,4-lactone
2H
A
L-Ascorbate

Figure 2A

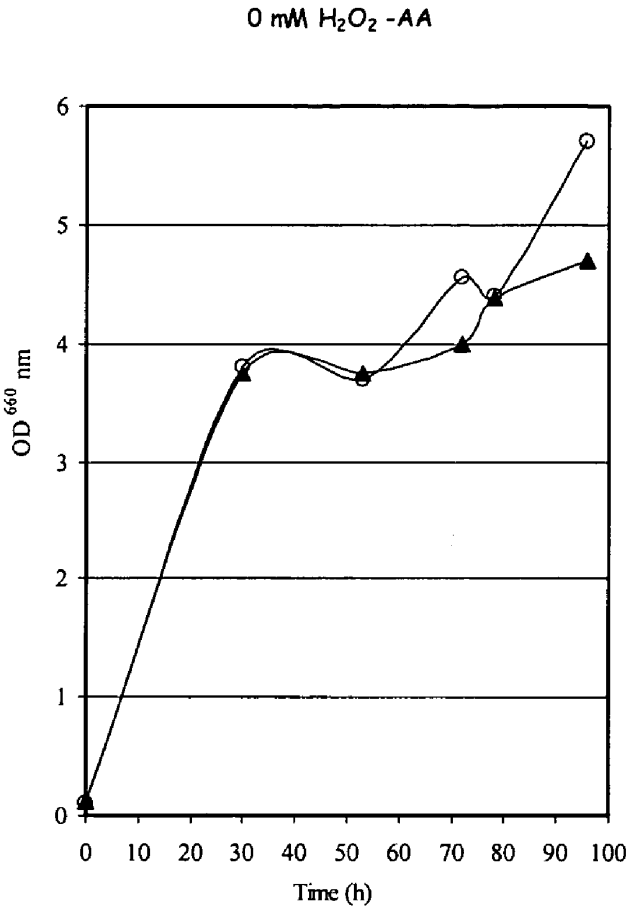

0 mM H_2O_2 -AA
OD^{660} nm
0
1
2
3
4
5
6
0 10 20 30 40 50 60 70 80 90 100
Time (h)

Figure 2B

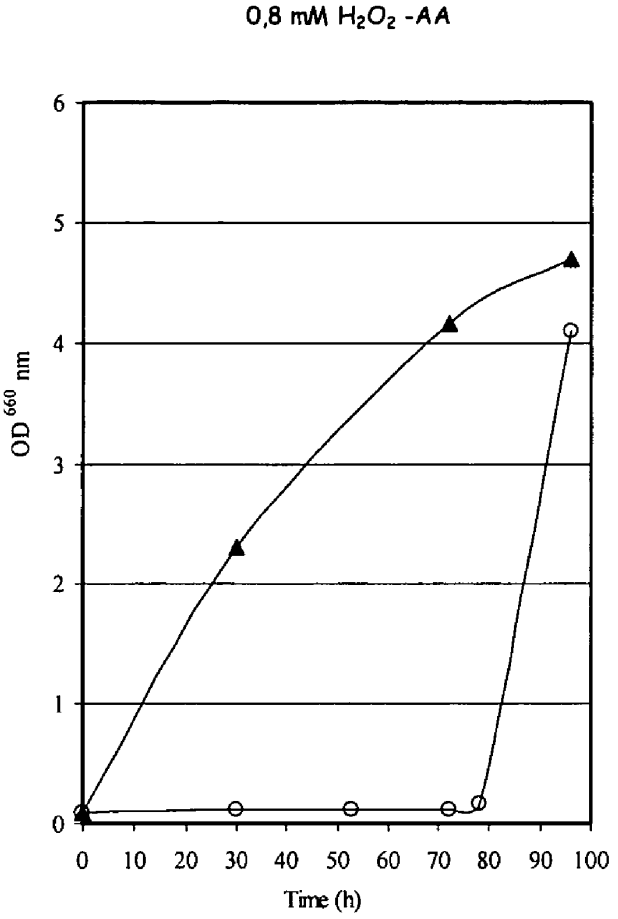

0,8 mM H_2O_2 -AA
OD 660 nm
0
1
2
3
4
5
6
0 10 20 30 40 50 60 70 80 90 100
Time (h)

Figure 2C

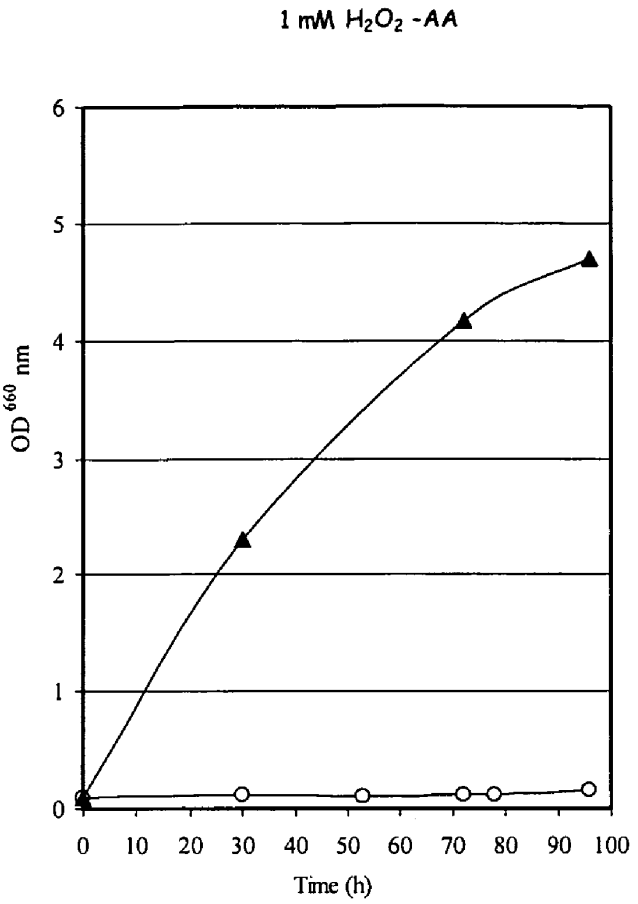

1 mM H_2O_2 -AA
OD^{660} nm
0
1
2
3
4
5
6
0 10 20 30 40 50 60 70 80 90 100
Time (h)

Figure 3A

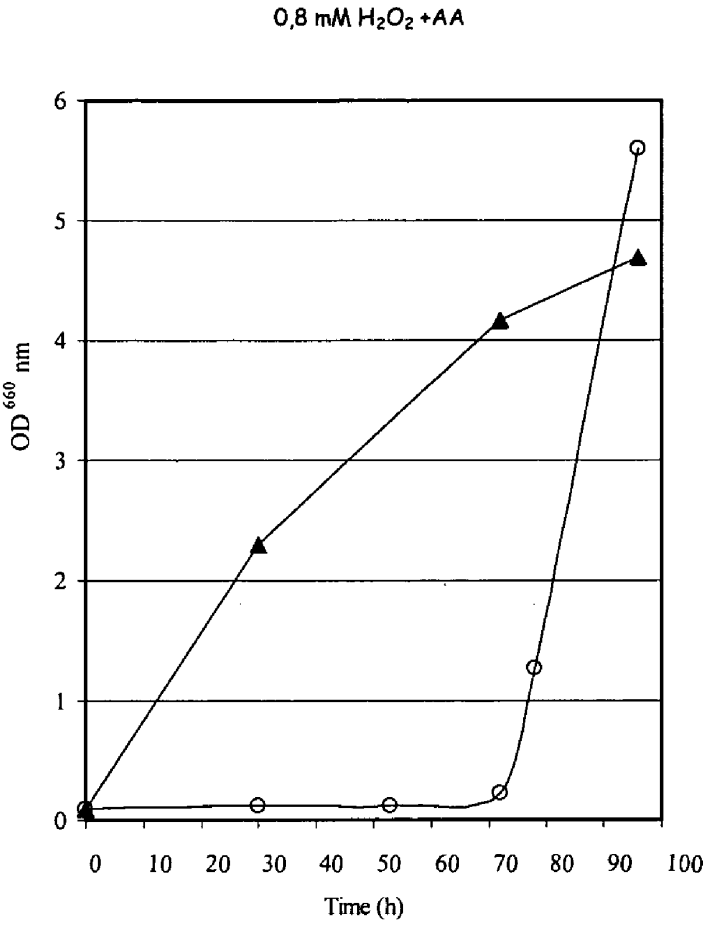

0,8 mM H_2O_2 +AA
OD 660 nm
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70
80
90
100
Time (h)

Figure 3B

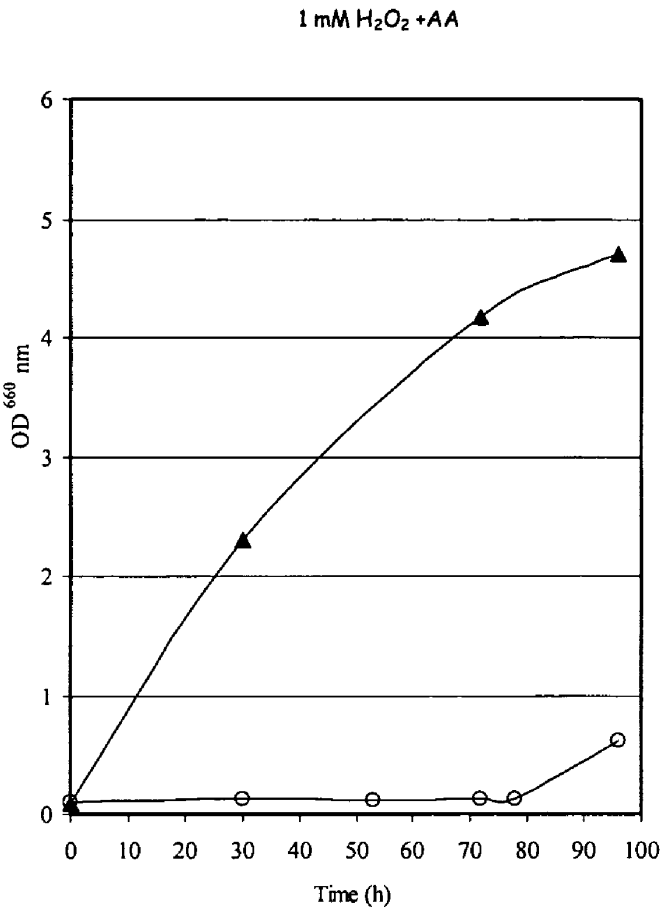
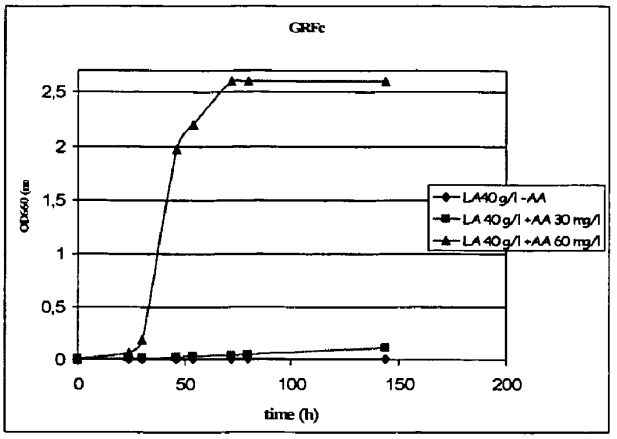
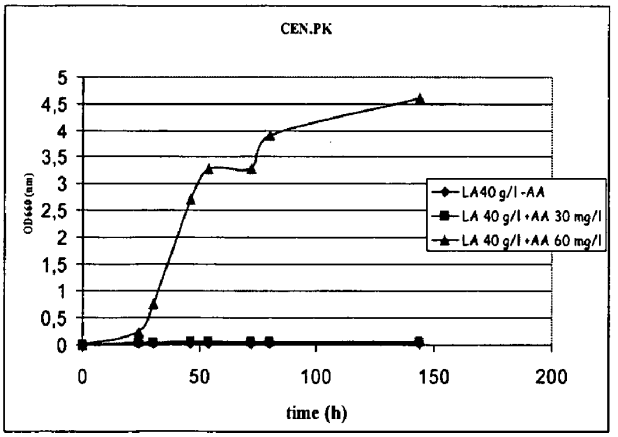

1 mM H_2O_2 +AA
OD^{660} nm
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70
80
90
100
Time (h)

Figure 3C


GRFe
2,5
2
1,5
1
0,5
0
0
50
100
150
200
time (h)
LA40 g/l -AA
LA 40 g/l +AA 30 mg/l
LA 40 g/l +AA 60 mg/l


CEN.PK
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
0
50
100
150
200
time (h)
LA40 g/l -AA
LA 40 g/l +AA 30 mg/l
LA 40 g/l +AA 60 mg/l

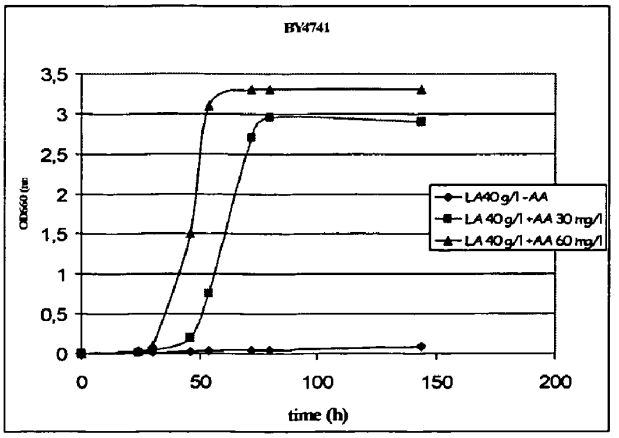

BY4741
3,5
3
2,5
2
1,5
1
0,5
0
0
50
100
150
200
time (h)
LA40 g/l -AA
LA 40 g/l +AA 30 mg/l
LA 40 g/l +AA 60 mg/l

Figure 4A

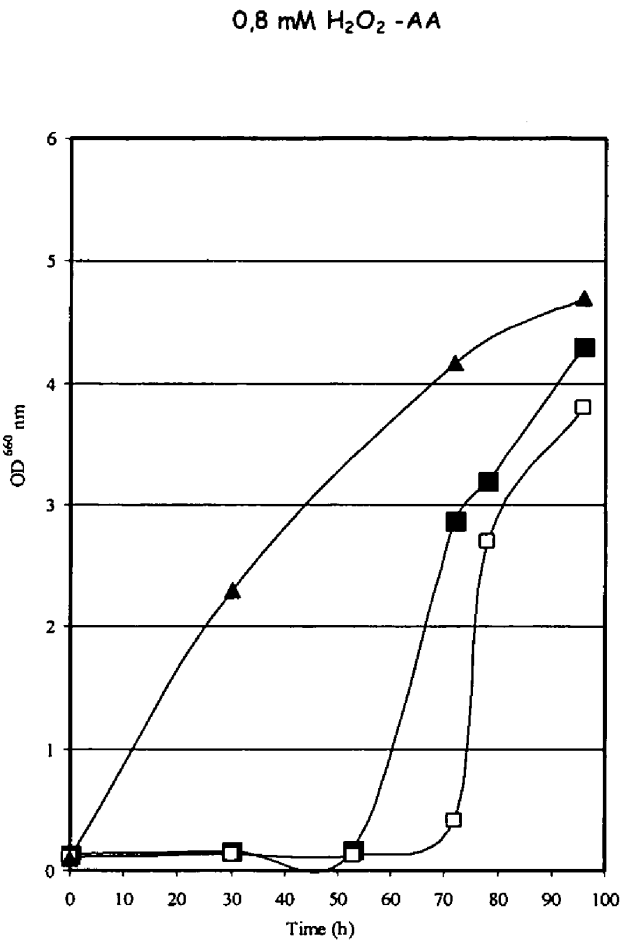

0,8 mM H_2O_2 -AA
OD^{660} nm
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70
80
90
100
Time (h)

Figure 4B

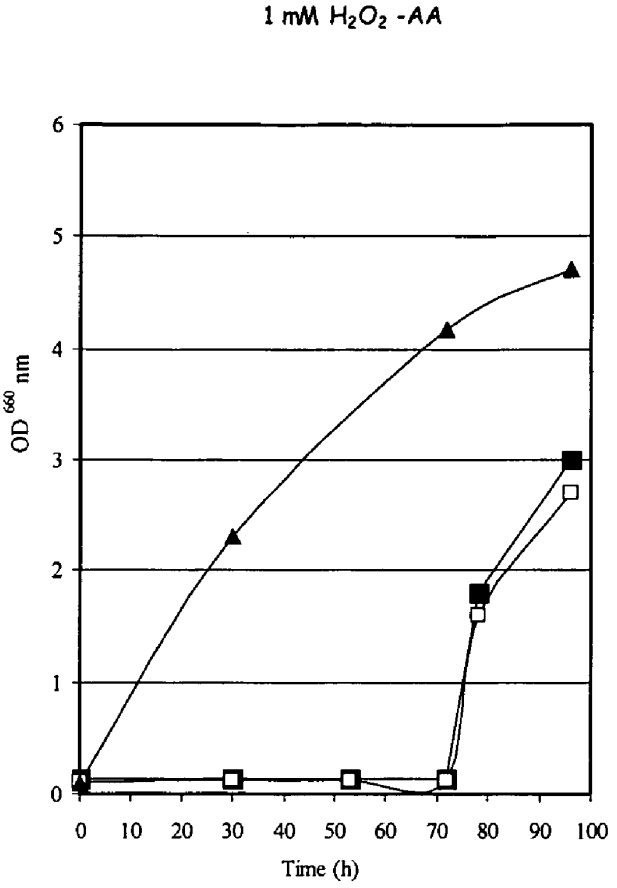

1 mM H_2O_2 -AA
OD^{660} nm
0
1
2
3
4
5
6
0 10 20 30 40 50 60 70 80 90 100
Time (h)

Figure 5A

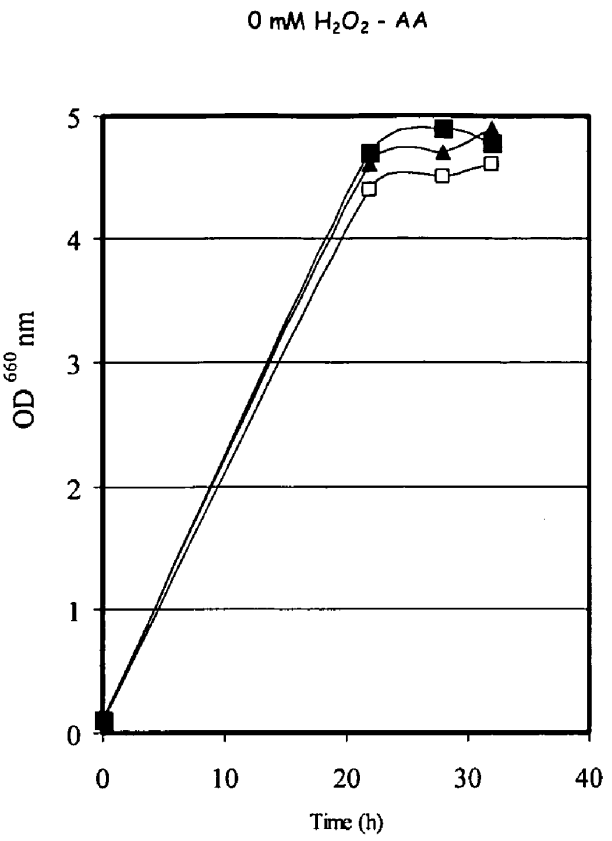


Figure 5b

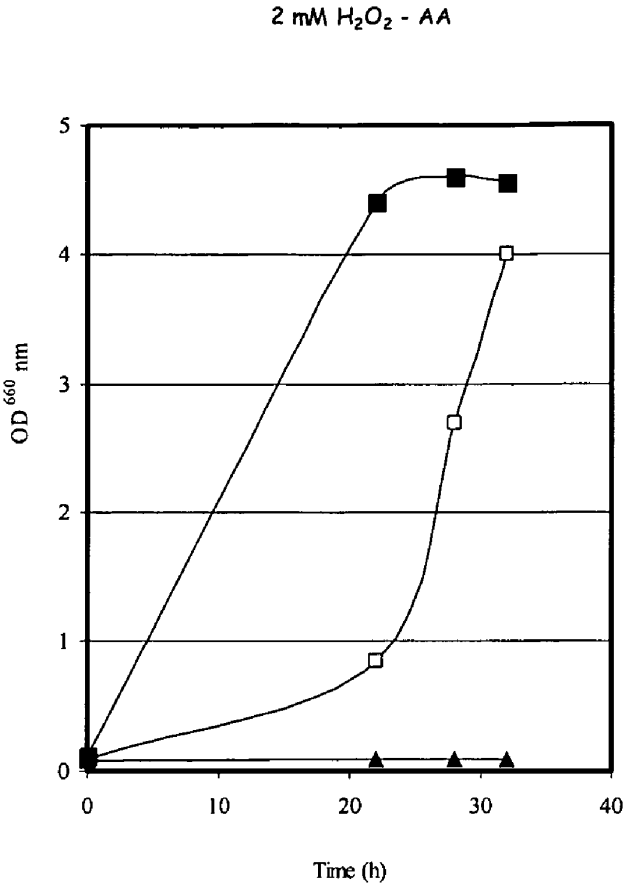


Figure 6

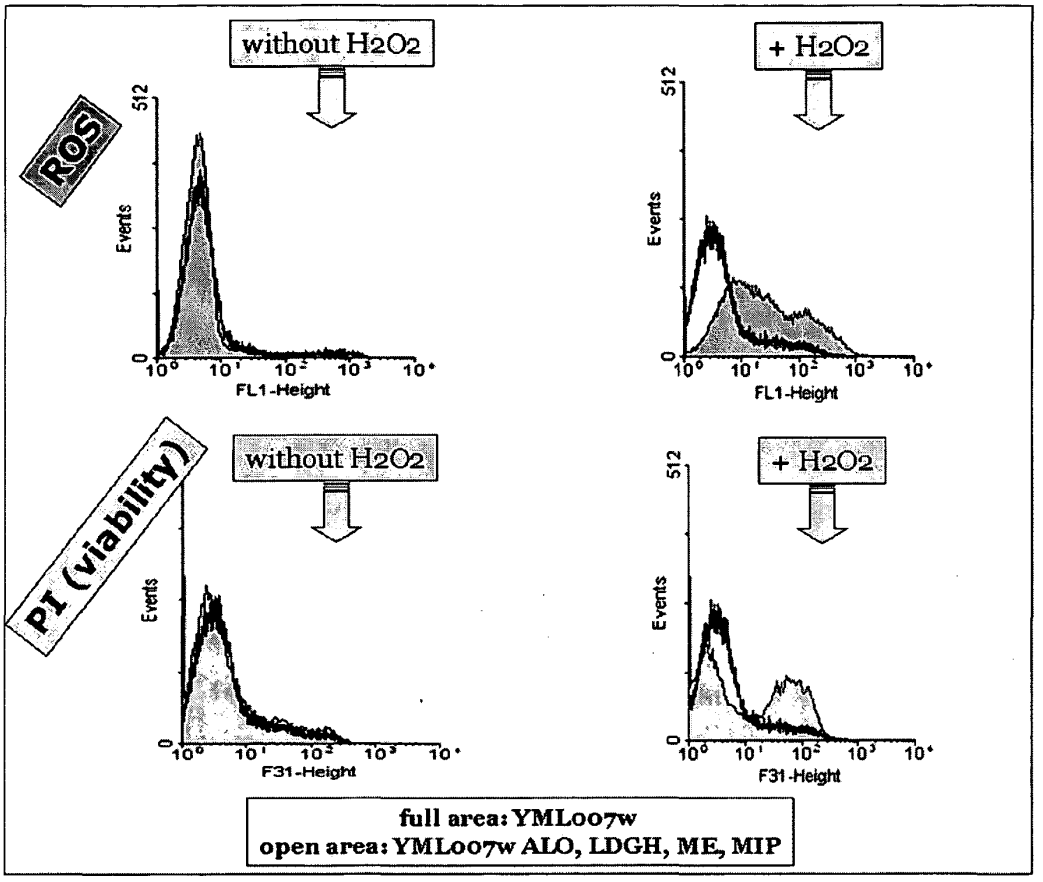
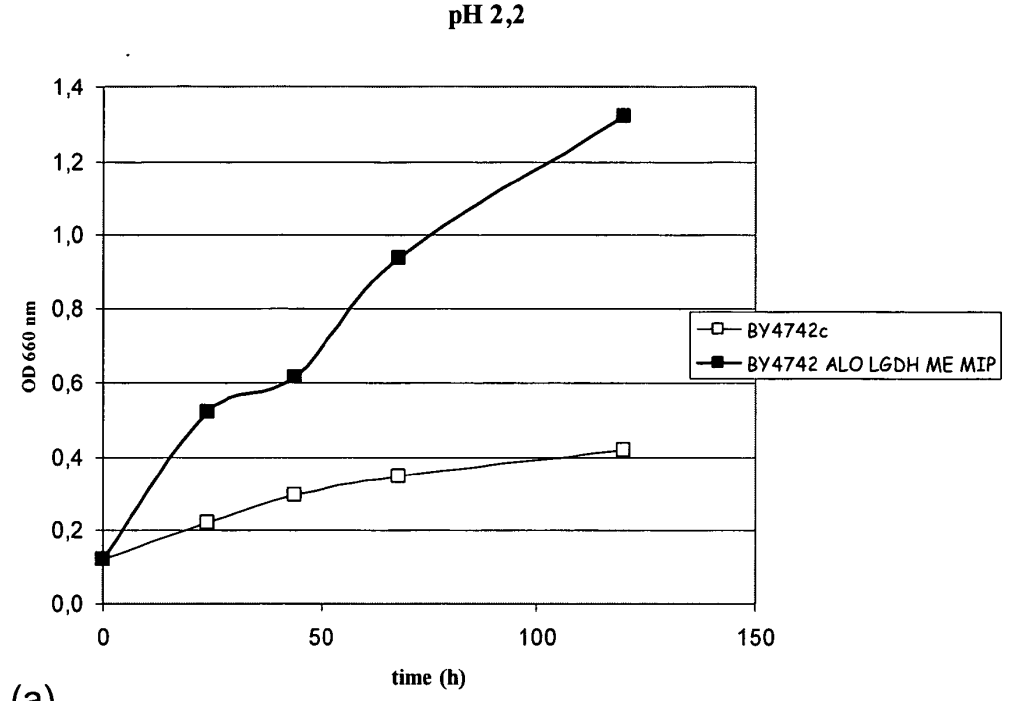
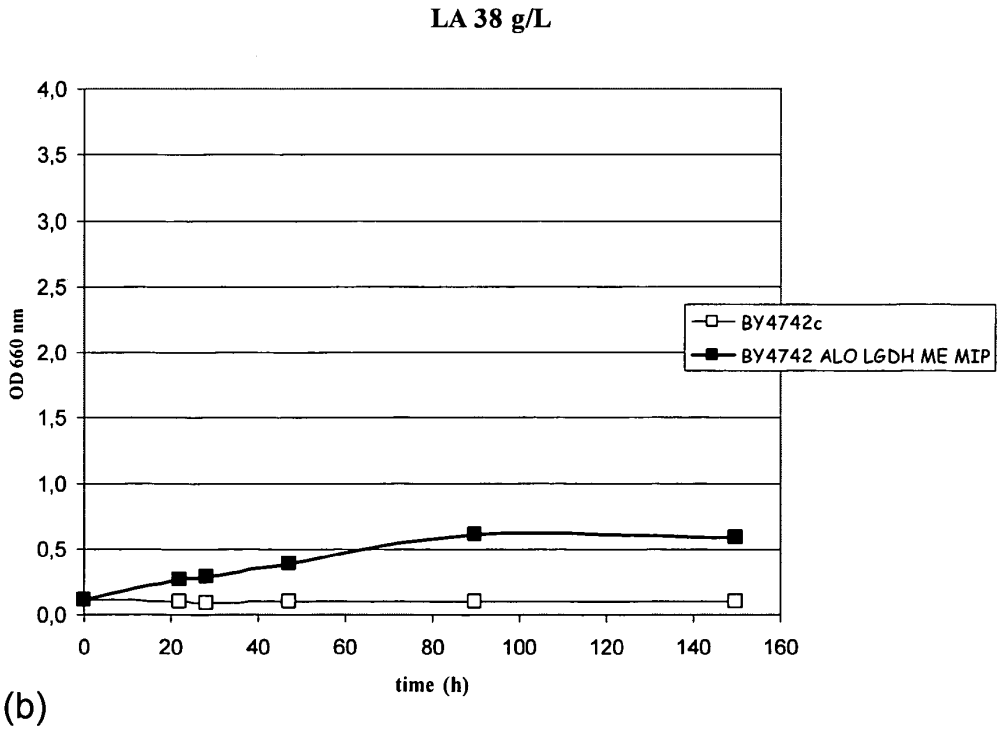

without H2O2
+ H2O2
ROS
PI (viability)
Events
512
0
FL1-Height
F31-Height
full area: YML007w
open area: YML007w ALO, LDGH, ME, MIP

Figure 7

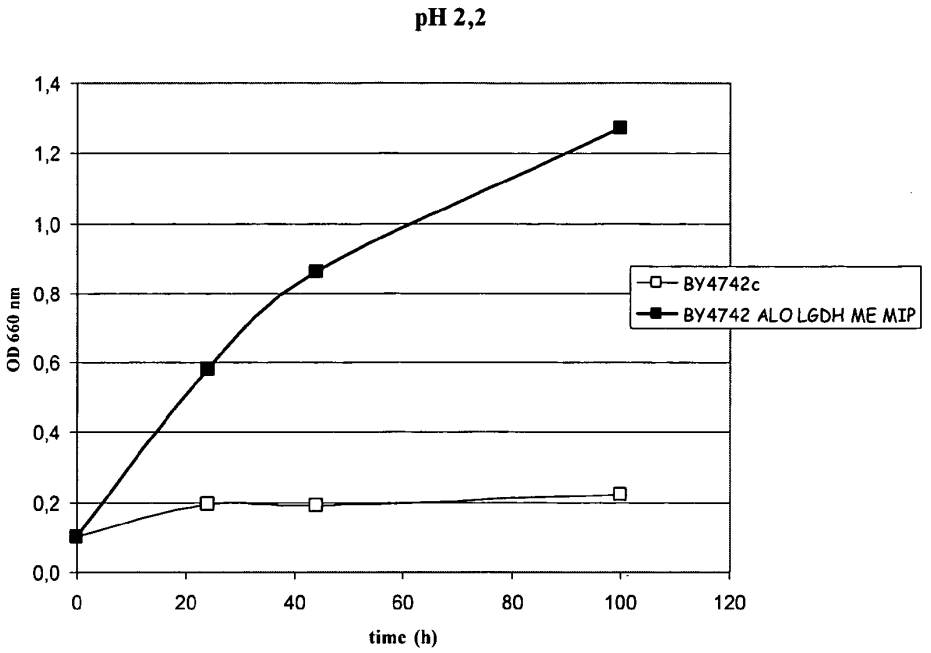

pH 2,2
OD 660 nm
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0
50
100
150
time (h)
BY4742c
BY4742 ALO LGDH ME MIP

(a)

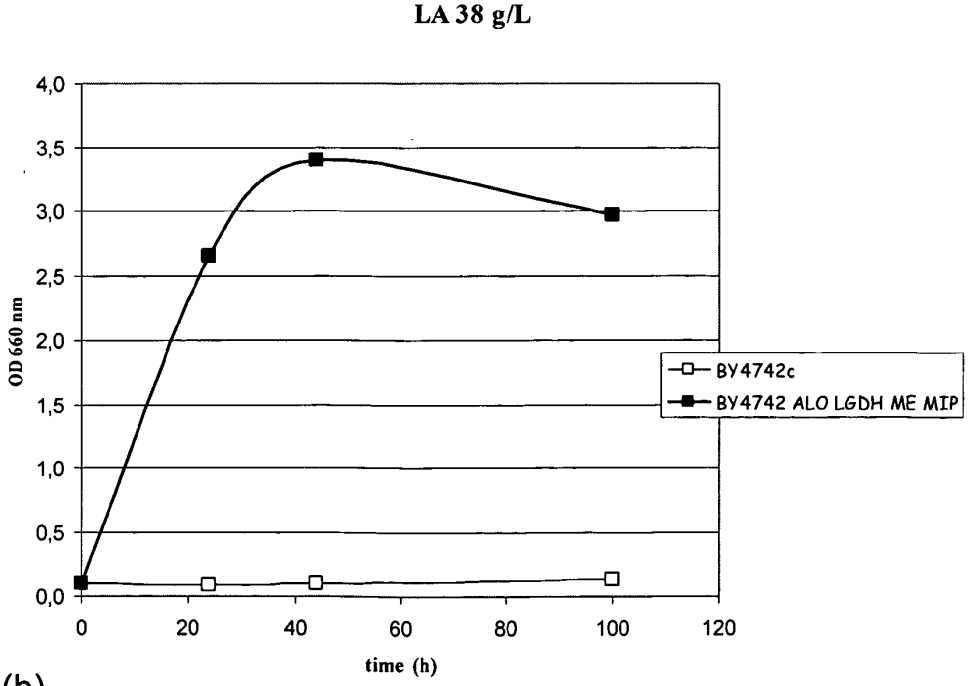

LA 38 g/L
OD 660 nm
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0
20
40
60
80
100
120
140
160
time (h)
BY4742c
BY4742 ALO LGDH ME MIP

(b)

Figure 8

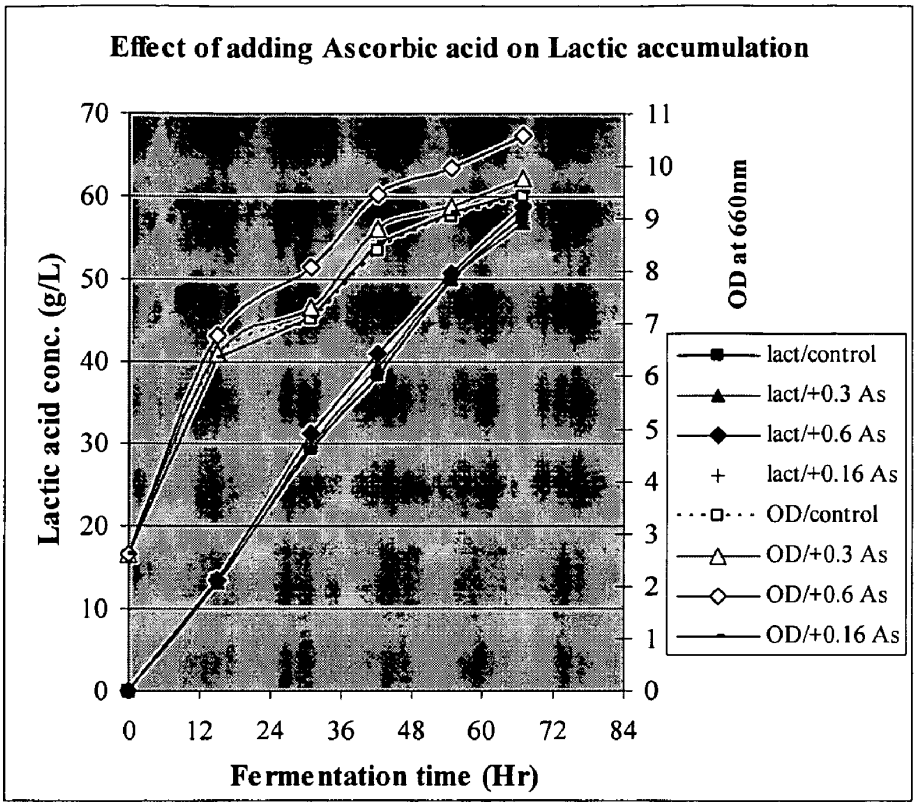

pH 2,2
OD 660 nm
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0
20
40
60
80
100
120
time (h)
BY4742c
BY4742 ALO LGDH ME MIP

(a)


LA 38 g/L
OD 660 nm
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0
20
40
60
80
100
120
time (h)
BY4742c
BY4742 ALO LGDH ME MIP

(b)

Figure 9


Effect of adding Ascorbic acid on Lactic accumulation
Lactic acid conc. (g/L)
OD at 660nm
Fermentation time (Hr)
0
10
20
30
40
50
60
70
0
1
2
3
4
5
6
7
8
9
10
11
0
12
24
36
48
60
72
84
lact/control
lact/+0.3 As
lact/+0.6 As
lact/+0.16 As
OD/control
OD/+0.3 As
OD/+0.6 As
OD/+0.16 As

INCREASE IN STRESS TOLERANCE WITH ASCORBIC ACID DURING FERMENTATION

RELATED APPLICATIONS

[0001] This application is a continuation-in-part of U.S. Ser. No. 11/105,162, filed on Apr. 13, 2005, which is incorporated herein by reference. This application claims priority from U.S. Ser. No. 11/105,162 and from PCT/US06/012854, filed on Apr. 7, 2006, also incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The present invention relates generally to the field of increasing stress tolerance in organisms used for industrial production. More particularly, it relates to a process for making L-ascorbic acid available to organisms during industrial production.

BACKGROUND

[0003] Microorganisms and cells can be easily grown on an industrial scale and are frequently employed in the commercial production of compounds such as organic acids, amino acids, vitamins, polyols, solvents, biofuels, therapeutics, vaccines, proteins, and peptides. Both prokaryotic and eukaryotic microorganisms are today easily and successfully used for the production of heterologous proteins as well as for the production of natural or engineered metabolites. Among prokaryotes, *Escherichia coli* and *Bacillus subtilis* are often used. Among eukaryotes, the yeasts, *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, are often used. However, in an industrial process, wherein the organism is used as a means for production, stress on the organism typically leads to lower or zero production of the product, lower or zero productivity, a lower or zero yield of the product, or two or more thereof. Bacteria, yeast, other fungi, cultured animal cells, and cultured plant cells show similar responses to stress. (Close, D. C., et al., *Oxidative Stress, Exercise, and Aging*, H. M. Alessio, A. E. Hagerman, Eds. (2006), pp. 9-23; Sugiyama, K., et al., (2000), J Biol. Chem. 275, 15535-15540; Mongkolsuk, S. and Helmann, J. D. (2002), Molecular Microbiology 45, 9-15). Techniques for minimizing stress would therefore be useful for improving industrial production by these organisms.

[0004] Stresses may have cellular (internal or intracellular) origins, environmental (external or extracellular) origins, or both. Classical examples of the internally-originating stresses include protein and metabolite overproduction (in terms of weight/volume) and protein and metabolite overproductivity (in terms of weight/volume per unit time), among others. Examples of externally-originating stresses include high osmolarity, high salinity, oxidative stress, high or low temperature, non-optimal pH, presence of organic acids, presence of toxic compounds, and macro- and micronutrient starvation.

[0005] Stress is typically caused by stressors (or stimuli). Stressors are negative influences on a cell that require the cell to dedicate more effort to maintain equilibrium than is required in the absence of the stressor. This greater effort can lead to a higher or lower metabolic activity, lower growth rate, lower viability, or lower productivity, among other effects. Stressors are agents of a physical, chemical or biological nature that represent a change in the usual intracellular or extracellular conditions for any given life form. It follows that while a specific condition (e.g., a temperature of 65° C.) may be stressful (or even lethal) to a certain species that normally lives at 37° C., it may be optimal for a thermophilic organism.

[0006] At the cellular level, stress can damage DNA, lipids, proteins, membranes, and other molecules and macromolecules, induce apoptosis (programmed cell death), cell necrosis and cell lysis, and impair cell integrity and cell viability. These effects are often mediated by the generation of reactive oxygen species (ROS).

[0007] ROS can be generated through both intracellular and extracellular stimuli. The majority of endogenous ROS are produced through leakage of these species from the mitochondrial electron transport chain. In addition, cytosolic enzyme systems, including NADPH oxidases and by-products of peroxisomal metabolism, are also endogenous sources of ROS. Generation of ROS also can occur through exposure to numerous exogenous agents and events including ionizing radiation, UV light, chemotherapeutic drugs, environmental toxins, and hyperthermia. Oxidative damage caused by intracellular ROS can result in DNA base modifications, single- and double-strand DNA breaks, and the formation of apurinic/apyrimidinic lesions, many of which are toxic and/or mutagenic. Therefore, the resulting DNA damage may also be a direct contributor to deleterious biological consequences (Tiffany, B. et al., (2004) Nucleic Acids Research 32, 3712-3723).

[0008] One example of an industrial process known to be hampered by stress responses is the production of lactic acid by bacteria or yeast. During a typical lactic acid fermentation, the accumulation of lactic acid in the medium also causes a drop in pH of the medium. The stress of low pH is amplified by the ability of the organic free acid to diffuse through the membrane and dissociate in the higher pH of the cytoplasm. The accumulation of lactic acid inhibits cell growth and metabolic activity. The toxicity of these stresses is mediated at least in part by reactive oxygen species. As a result, the extent of lactic acid production is greatly reduced by the accumulation of lactic acid in the medium.

[0009] The addition of $Ca(OH)_2$, $CaCO_3$, NaOH, or NH_4OH to the fermentation medium to neutralize the lactic acid and to thereby prevent the pH drop is a conventional operation in industrial processes to counteract the negative effects of free lactic acid accumulation. These processes allow the production of lactate(s) by maintaining the pH at a constant value in the range of about 5 to 7, which is well above the pKa of lactic acid (3.86).

[0010] However, this neutralization procedure has major disadvantages. Additional operations are required to regenerate free lactic acid from its salt and to dispose of or recycle the neutralizing cation, which adds expense to the process. The added operations and expense could be lessened if free lactic acid could be accumulated by organisms growing at low pH values. To this end, the use of recombinant yeast that are engineered for industrial production of free lactic acid, and, in particular, recombinant yeast from strains showing greater tolerance for extreme environmental conditions have been described. Engineered strains of recombinant yeast functionally transformed with a gene for lactate dehydrogenase (LDH) in the genera *Saccharomyes, Zygosaccharomyces, Torulaspora*, and *Kluveromyces* have been produced as

described in U.S. Pat. Nos. 6,429,006 and 7,049,108. While these recombinant strains show improved efficiency of lactic acid production at low pH, they are still adversely affected by stresses. In addition, it may be necessary to use organisms or strains that are less tolerant of extreme environmental conditions for the industrial production of specific compounds.

[0011] Ascorbic acid is a known antioxidant that is produced in all higher plants and many higher animals. Ascorbic acid has been shown to modulate the heat shock response in yeast through an effect on ROS(C. Moraitis and B. P. G. Curran. (2004), *Yeast* 21, 313-323), and to improve cell viability and reduce proteolysis of the end product of high cell-density fermentation (Xiao, A. et al. (2006), Appl. Microbiol. Biotechnol. 72, 837-844). These effects suggest that ascorbic acid could improve stress tolerance in general in organisms utilized for industrial production.

[0012] We have shown that recombinant yeast that are functionally transformed to produce L-ascorbic acid, the biologically active enantiomer, from D-glucose produce lower levels of ROS and exhibit improved growth and viability under conditions of low pH, oxidative stress, and in the presence of high concentrations of lactic acid. (Branduardi, P., et al., International Specialised Symposium on Yeast. ISSY25, Systems Biology of Yeast—From Models to Applications. "L-ascorbic acid production from D-glucose in metaboloic engineered *Saccharomyces cerevisiae* and its effect on strain robustness." Hanasaari, Espoo, Finland, Jun. 21, 2006).

[0013] Accordingly, it would be advantageous to industrial fermentation processes if the organisms and cells used for industrial production could endogenously produce L-ascorbic acid from D-glucose.

SUMMARY OF THE INVENTION

[0014] The present invention relates to a method of increasing stress tolerance in a recombinant organism that is engineered for industrial production of at least one product. The method comprises making L-ascorbic acid available to the recombinant organism.

[0015] In one embodiment, ascorbic acid is made available by functionally transforming the recombinant organism with a coding region encoding a mannose epimerase (ME), a coding region encoding an L-galactose dehydrogenase (LGDH), and a coding region encoding a D-arabinono-1,4-lactone oxidase (ALO). In a further embodiment, the functionally transformed, recombinant organism is further functionally transformed with a coding region encoding a myoinositol phosphatase (MIP).

[0016] In another embodiment, the L-ascorbic acid is made available by culturing the recombinant organism in culture medium containing an effective amount of L-ascorbic acid.

DESCRIPTION OF THE DRAWINGS

[0017] FIG. **1** shows the main plant pathway for the synthesis of L-ascorbic acid from D-glucose.

[0018] FIG. **2** shows the optical density at 660 nm of BY4742 (▲) and YML007w (yap1 mutant strain) (○) yeast in the absence (FIG. **2***a*) and presence (FIGS. **2***b*-**2***c*) of oxidative stress. Yap1 activates genes required for the response to oxidative stress; deletion of this gene leads to the observed phenotype.

[0019] FIG. **3** shows the impact of two stressors on yeast growth. FIGS. **3***a*-**3***b* show the optical density at 660 nm of BY4742 wt (▲) and YML007w (○) yeast in the presence of H_2O_2 in medium +/−ascorbic acid. FIG. **3***c* shows the optical density at 660 nm of wild type yeast GRFc, CEN.PK 113-5D, and BY4741 in the presence of 40 g/l lactic acid and zero, or increasing levels of ascorbic acid.

[0020] FIG. **4** shows the optical density at 660 nm of BY4742 wt (▲); YML007w expressing ALO, LDGH and ME (□); and YML007w expressing ALO, LDGH, ME and MIP (■) yeasts in the presence of oxidative stress (FIGS. **4***a*-**4***b*).

[0021] FIG. **5** shows the optical density at 660 nm of wild type GRFc (▲); GRF18U expressing ALO, LDGH and ME (□); and GRF18U expressing ALO, LDGH, ME and MIP (■) yeast strains in the absence (FIG. **5***a*) and presence (2 mM of H_2O_2) of oxidative stress. (FIG. **5***b*).

[0022] FIG. **6** shows ROS (upper panels) and viability (bottom panels) determination by flow cytometric analyses of *S. cerevisiae* cells producing (YML007w ALO, LDGH, ME, MIP, open area) or not producing (YML007w, full area) ascorbic acid when grown in minimal glucose medium in the presence (right) or absence (left) of hydrogen peroxide.

[0023] FIG. **7** shows growth curves of strains BY4742c (□) and BY4742 ALO, LDGH, ME, MIP (■) inoculated in minimal glucose medium at pH 2.2 (a), or in minimal glucose medium pH 3.0 containing 38 g/l of lactic acid (b).

[0024] FIG. **8** shows growth curves of strains BY4742c (□) and BY4742 ALO, LDGH, ME, MIP (■) that were first grown for 24 h in minimal glucose medium under nonlimiting conditions, and then transferred to minimal glucose medium at pH 2.2 (a), or to minimal glucose medium pH 3 containing 38 g/l of lactic acid (b).

[0025] FIG. **9** shows growth curves, as measured by OD660, and lactic acid production by *S. cerevisiae* strain NRRL Y-30696 grown in minimal glucose medium containing 2.78 g/L $CaCO_3$ and increasing concentrations of ascorbic acid (AA). 0 g/L AA (□), 0.16 g/L AA (+), 0.3 g/L AA (▲), or 0.6 g/L (♦)

DETAILED DESCRIPTION

[0026] The present invention relates to a method of increasing stress tolerance in recombinant cells or organisms that have been engineered for the industrial production of products such as organic acids, amino acids, vitamins, polyols, solvents, biofuels, therapeutics, vaccines, proteins, and peptides by increasing the available amount of ascorbic acid.

[0027] A "recombinant" cell or organism is one that contains a nucleic acid sequence that is not naturally occurring in that cell or organism, or one that contains an additional copy or copies of an endogenous nucleic acid sequence, wherein the nucleic acid sequence is introduced into the cell or organism or into an ancestor cell thereof by human action. Introduction of the gene into the cell or organism is known as "transformation" and the recipient organism or cell is said to be "transformed." Recombinant DNA techniques are

well-known to those of ordinary skill in the art, who will also understand how to choose appropriate vectors and promoters for the transformation of particular organisms or strains. (For example, see methods in Sambrook, J. and Russell, D. W., *Molecular Cloning: A Laboratory Manual,* 3rd Edition, Cold Spring Harbor Laboratory Press, 2001). Very basically, a coding region of the homologous and/or heterologous gene is isolated from a "donor" organism that possesses the gene. The recombinant organism, as well as the donor, may be a prokaryote, such as a bacterium, or a eukaryote, such as a protozoan, alga, fungus, plant, or animal.

[0028] In one well-known technique, a coding region is isolated by first preparing a genomic DNA library or a cDNA library, and second, identifying the coding region in the genomic DNA library or cDNA library, such as by probing the library with a labeled nucleotide probe that is at least partially homologous with the coding region, determining whether expression of the coding region imparts a detectable phenotype to a library microorganism comprising the coding region, or amplifying the desired sequence by PCR. Other techniques for isolating the coding region may also be used.

[0029] Methods for preparing recombinant nucleotides and transferring them into a host organism are well-known to those of ordinary skill in the art. Briefly, the desired coding region is incorporated into the recipient organism in such a manner that the encoded protein is produced by the organism in functional form. That is, the coding region is inserted into an appropriate vector and operably linked to an appropriate promoter on the vector. If necessary, codons in the coding region may be altered, for example, to create compatibility with codon usage in the target organism, to change coding sequences that can impair transcription or translation of the coding region or stability of the transcripts, or to add or remove sequences encoding signal peptides that direct the generated protein to a specific location in or outside the cell, e.g., for secretion of the protein. Any type of vector, e.g., integrative, chromosomal, or episomal, may be used. The vector may be a plasmid, cosmid, yeast artificial chromosome, virus, or any other vector appropriate for the target organism. The vector may comprise other genetic elements, such as an origin of replication to allow the vector to be passed on to progeny cells of the host carrying the vector, sequences that facilitate integration into the host genome, restriction endonuclease sites, etc. Any promoter active in the selected organism, e.g., homologous, heterologous, constitutive, inducible, or repressible may be used. An "appropriate" vector or promoter is one that is compatible with the selected organism and will generate a functional protein in that organism. The recombinant organism thus transformed is referred to herein as being "functionally transformed."

[0030] The recombinant cells and organisms of the invention can be obtained by any method allowing a foreign DNA to be introduced into a cell, for example, transformation, electroporation, conjugation, fusion of protoplasts or any other known technique (Spencer J. F. et al. (1988), Journal of Basic Microbiology 28, 321-333). A number of protocols are known for transforming yeast, bacteria, and eukaryotic cells. Transformation can be carried out by treating the whole cells in the presence of lithium acetate and of polyethylene glycol according to Ito H. et al. ((1983), J. Bacteriol., 153:163), or in the presence of ethylene glycol and dimethyl sulphoxyde according to Durrens P. et al. ((1990) Curr. Genet., 18:7). An alternative protocol has also been described in EP 361991. Electroporation can be carried out according to Becker D. M. and Guarente L. ((1991) Methods in Enzymology, 194:18). The use of non-bacterial integrative vectors may be preferred when the yeast biomass is used at the end of the fermentation process as stock fodder or for other breeding, agricultural or alimentary purposes.

[0031] The transformed organism is propagated in an appropriate culture medium. Culturing techniques and specialized media are well known in the art. For industrial production, the organism is preferably cultured in an appropriate medium in a fermentation vessel.

[0032] Organisms frequently utilized for industrial production are yeast and bacteria. Yeast to be transformed can be selected from any known genus and species of yeast. Yeast species are described by N. J. W. Kreger-van Rij, ("The Yeasts," (1987) *Biology of Yeasts*, A. H. Rose and J. S. Harrison, Eds. London: Academic Press, Chapter 2) In one embodiment, the yeast genus is selected from the group consisting of *Saccharomyces, Zygosaccharomyces, Candida, Hansenula, Kluyveromyces, Debaromyces, Nadsonia, Lipomyces, Torulopsis, Kloeckera, Pichia, Schizosaccharomyces, Trigonopsis, Brettanomyces, Cryptococcus, Trichosporon, Aureobasidium, Lipomyces, Phaffia, Rhodotorula, Yarrowia*, and *Schwanniomyces*. In another embodiment, the yeast is selected from *S. cerevisiae* strains, including GRF18U, W3031B, BY4742 (MATα; his3; leu2, lys2; ura3, EuroScarf Accession No. Y10000); *Z. bailii* ATCC 60483; *K. lactis* PM6-7A; BY4741 (MATα; his3; leu2; met15; ura3, Euroscarf Accession No. Y00000), CEN.PK 113-5D (MATα ura3-52; cir+), and yeast strains engineered to produce lactic acid, including NRRL Y-30696, NRRL Y-30698, NRRL Y-30742; *K. lactis* PM6-7/pEPL2, PMI/C1[pELP2]; *Zygosaccharomyces bailii* ATTC36947/pLAT-ADH, ATCC60483/pLAT-ADH.

[0033] Yeast have been widely utilized in the production of products. Yeast biomass is an important product as cultures for development of food products as well as a nutrient rich food and feed component. Genetic engineering has broadened the value of yeast production systems providing a route to organic acids (Porro, D. et al. (2002), U.S. Pat. No. 6,429,006); vitamins (Shiuan, D., US2003/0104584); polyols (Geertman, J. M, et al., (2006) Metabolic Engineering, June 30:(Epublication); biofuel (Ho, N. W. Y. and Tsao, G. T. (1998), U.S. Pat. No. 5,789,210); (Bosman, F., et al. (2006) U.S. Pat. No. 7,048,930); proteins (Gerard, G. F., et al. (2006). U.S. Pat. No. 7,115,406); and peptides (Lee, S. Y., et al., Lett. Appl. Microbiol (2003), 36, 121-128.).

[0034] Bacteria to be transformed can be selected from any known genus and species of the Eubacteria or the Archaea (also encompassed herein by the term, "bacteria"). Bacteria are cataloged at the NCBI Taxonomy website: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Taxonomy. In one embodiment the bacteria can be selected from the genera *Bacillus, Escherichia, Lactobacillus, Lactococcus, Pseudomonas*, or *Acetobacter.*

[0035] Bacteria have been widely utilized to produce industrial products. The natural range of available products has been extended by mutagenesis and screening and further by genetic engineering. Bacteria provide routes to organic acids (WO2006/083410); amino acids (WO2005/090589);

vitamins (Santos, et al., Abstracts of Papers, 232nd ACS National Meeting, San Francisco, Calif., United States, Sep. 10-14, 2006, BIOT-243); polyols (Dunn-Coleman, N. S., et al. (2006) U.S. Pat. No. 7,074,608); solvents (Harris, L. M., et al. (2001), Journal of Industrial Microbiology & Biotechnology 27, 322-328); biofuels (Ingram, L. O. and Zhou, S. WO2000/071729); therapeutics (Pizza, M., et al. (2006) U.S. Pat. No. 7,115,730); proteins (Gerard, G. F., et al. (2006) U.S. Pat. No. 7,115,406); and peptides (Knapp, S., et al. (1992) U.S. Pat. No. 5,159,062).

[0036] Filamentous fungi are widely utilized to produce organic acids (Bizukojc, M. and Ledakowicz, S., Process Biochemistry (2004), 39, 2261-2268.); and proteins (Wang, L., et al., (2003) Biotechnology Advances 23, 115-129). Filamentous fungi to be transformed can be selected from any known genus and species. Fungi are cataloged at the NCBI Taxonomy Website: http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=4751.

[0037] In one embodiment the filamentous fungi can be selected from the genera *Rhizopus, Aspergillus*, or *Trichoderma.*

[0038] In one embodiment of the invention, the recombinant organism is functionally transformed with coding regions that encode a mannose epimerase (D-mannose:L-galactose epimerase; ME), L-galactose dehydrogenase (LGDH); and D-arabinono-1,4-lactone oxidase (ALO). These coding sequences enable the recombinant organism to produce enzymes necessary for the endogenous production of L-ascorbic acid from D-glucose. As a result of transformation with ME, LGDH, and ALO, and endogenous production of L-ascorbic acid, the organism shows increased tolerance to stress when compared with a strain of the same organism that cannot produce L-ascorbic acid.

[0039] An ME is any GDP-mannose-3,5-epimerase (5.1.3.18), that is any enzyme that catalyzes the conversion of GDP-mannose to GDP-L-galactose (FIG. **1**). An exemplary ME is encoded by the sequence listed as SEQ ID NO:1.

[0040] In one embodiment, the ME has at least about 95% identity with SEQ ID NO:1. "Identity" can be determined by a sequence alignment performed using the ClustalW program and its default values, namely: DNA Gap Open Penalty=15.0, DNA Gap Extension Penalty=6.66, DNA Matrix=Identity, Protein Gap Open Penalty=10.0, Protein Gap Extension Penalty=0.2, Protein matrix=Gonnet. Identity can be calculated according to the procedure described by the ClustalW documentation: "A pairwise score is calculated for every pair of sequences that are to be aligned. These scores are presented in a table in the results. Pairwise scores are calculated as the number of identities in the best alignment divided by the number of residues compared (gap positions are excluded). Both of these scores are initially calculated as percent identity scores and are converted to distances by dividing by 100 and subtracting from 1.0 to give number of differences per site. We do not correct for multiple substitutions in these initial distances. As the pairwise score is calculated independently of the matrix and gaps chosen, it will always be the same value for a particular pair of sequences."

[0041] In another embodiment, the recombinant organism transformed with the coding sequences for ME, LGDH, and ALO is further functionally transformed with a coding region encoding a myoinositol phosphatase (MIP). An MIP is any myoinositol phosphatase (3.1.3.25), that also catalyzes the conversion of L-galactose-1P to L-galactose. L-galactose-1-phosphatase has been annotated as inositol/myoinositol monophosphatase galactose-1-phosphatase and may be referred to as MIP/VTC4 (Conklin, P. L. et al. (2006) J. Biol. Chem. 281, 15662-70). In one embodiment, the MIP has at least about 95% identity with SEQ ID NO:2. Identity is determined as described above.

[0042] In another embodiment, the recombinant organism is further transformed with a coding region encoding an enzyme selected from L-galactono-1,4-lactone dehydrogenase (AGD), D-arabinose dehydrogenase (ARA) or L-gulono-1,4-lactone oxidase (GLO), as described, for example, in U.S. Pat. No. 6,630,330, which is incorporated herein by reference.

[0043] Although the pathway for the production of ascorbic acid in plants is shown in FIG. **1**, the present invention is not limited to the enzymes of the pathways known for the production of L-ascorbic acid intermediates or L-ascorbic acid in plants, yeast, or other organisms. (Examples of known L-ascorbic acid pathways in plants and animals are described in Conklin, P. L., et al. (2006), J. Biol. Chem. 281, 15662-15670; and in Valpuesta, V. and Botella, M. A. (2004) Trends in Plant Science 12, 573-577). One of ordinary skill in the art will understand that increasing flux through any pathway resulting in L-ascorbic acid biosynthesis will result in production of higher levels of L-ascorbic acid. This can be accomplished by increasing the levels of enzymes in the pathway that are limiting.

[0044] The coding regions for any of the desired enzymes may be isolated from any source or may be chemically synthesized. Following transformation with the coding regions for ME, LGDH, and ALO, (with or without the coding region for MIP), the recombinant organism is cultured in medium containing a carbon source that can be converted to L-ascorbic acid, such as D-glucose.

[0045] When the recombinant organism for industrial production is a eukaryotic organism, it is important to ensure that each of the enzymes used to produce ascorbic acid is appropriately compartmentalized in the eukaryotic cell. This is accomplished by including sequences encoding targeting labels in the recombinant vector. These types of sequences are disclosed, for example, in Alberts, B., et al., *Molecular Biology of the Cell,* 4th Edition, New York: Garland Science Publ., 2002, pages 659-710.

[0046] With respect to the invention, "production" means the process of making one or more products using a recombinant organism. Production can be quantified at any moment in time after commencement of the process by determining the weight of a product produced per weight or volume of the medium on which the recombinant organism's growth and survival is maintained, or weight or volume of the recombinant organism's biomass. "Productivity" means the amount of production, as quantified above, over a given period of time (e.g., a rate such as g/L per hour, mg/L per week, or g/g of biomass per hour). "Yield" is the amount of product produced per the amount of substrate converted into the product. This definition of "yield" also applies to endogenous production of L-ascorbic acid.

[0047] Stress tolerance, as used herein, may manifest as a decrease in the negative impact of stress on the organism,

such as a decline in the production of ROS or a positive effect on productivity, yield, or production. An increase in stress tolerance can be measured by a number of parameters, for example, as an increase in growth rate, an increase in cell density, a decrease in the inhibition of productivity, an increase in viability, an increase in metabolism, or an increase in yield, productivity, or production. An "effective amount" of L-ascorbic acid is an amount of L-ascorbic acid present in the culture medium that gives rise to an improvement in stress tolerance as measured by any of these parameters, when compared with stress tolerance of the organism grown in medium that does not contain L-ascorbic acid.

[0048] As shown in FIGS. **2-5**, yeast transformed with coding sequences for ME, LGDH, and ALO, or with this group of coding sequences plus a coding sequence for MIP, have greater stress tolerance than yeast that are not so transformed. FIG. **7** shows that endogenously produced L-ascorbic acid correlates with increased tolerance to low pH and oxidative stresses. This increased stress resistance can manifest as one or more of increased growth rate of the transformed organism, increased viability of the transformed organism, or increased production by the transformed organism.

[0049] We also show, in FIG. **3**, that the addition of L-ascorbic acid to the fermentation medium improves stress tolerance, in particular, tolerance to low pH and oxidative stress. Accordingly, in one embodiment of the invention, the available amount of ascorbic acid is increased by adding L-ascorbic acid to the fermentation medium. Exogenous L-ascorbic acid may be added to cultures that do or do not produce L-ascorbic acid endogenously.

[0050] Though not wishing to be bound by a single theory, we suggest that the increased stress tolerance results from an increase in antioxidant levels (specifically, L-ascorbic acid) and a reduction in the levels of endogenous reactive oxygen species (ROS) in the organism, imparting greater resistance to oxidative stress, as shown in FIG. **6**. The increased stress tolerance makes organisms that endogenously produce ascorbic acid particularly suitable for industrial production. Such organisms include plant and animal cells that produce ascorbic acid either naturally or through genetic engineering. (e.g., organisms described in Valpuesta, V. and Botella, M. A. (2004) Trends in Plant science 9, 573-577 and genetically engineered plant and animal cells.)

[0051] Organisms with increased stress tolerance that are to be used for industrial production may be created by any methods known to those of skill in the art for engineering recombinant organisms. The organism may be co-transformed with the necessary coding regions for production of L-ascorbic acid (i.e., ME, LGDH, ALO+/−MIP) and the coding sequences for the industrial product that the organism will produce. The organism may first be engineered to express the L-ascorbic acid coding sequences and then subsequently be transformed with coding regions for the industrial product. Alternatively, the organism may first be engineered to produce the industrial product and subsequently be transformed with the coding regions for production of L-ascorbic acid.

[0052] Endogenous production of L-ascorbic acid by the recombinant organism is particularly useful if the recombinant organism is cultivated under conditions of osmotic, pH, temperature, or oxidative stress. Osmotic stress is a condition in which the organism or cell encounters a difference in osmolarity from the optimal osmolarity defined for the respective microorganism. For example, in the yeast *S. cerevisiae*, an osmolarity greater than 500 mOsmol leads to a stress response.

[0053] A pH stress occurs if an organism or strain of organism encounters a difference in pH value from the optimal pH value for that strain of more than one to three pH units. For example, in the wild type strain of the yeast *S. cerevisiae*, the typical optimal pH for performance of bioprocesses is 5.0. A pH of less than 4.0 or more than 6.0 may cause a stress response in this strain that can affect the transcription of pH sensitive genes.

[0054] Temperature stress is a condition in which the organism encounters a cultivation temperature different the optimal temperature value for growth or production for a particular organism. In the yeast, *S. cerevisiae*, a temperature at or above 32° C. can cause stress responses. For the bacterium *E. coli*, a temperature at or above 38° C. can lead to stress responses.

[0055] Oxidative stress is a general term used to describe the steady state level of oxidative damage in a cell, caused by the reactive oxygen species (ROS). This damage can affect a specific molecule or the entire organism. Reactive oxygen species, such as free radicals and peroxides, represent a class of molecules that are derived from the metabolism of oxygen and exist inherently in all aerobic organisms. Oxidative stress results from an imbalance between formation and neutralization of pro-oxidants. Animal cells, as well as single-celled organisms, can be exposed to significant oxidative stress during standard cell culture conditions.

[0056] Endogenous production of L-ascorbic acid is also particularly useful in a cell or organism if it is subjected to stress due to overproduction of a metabolite or a protein. Such stresses may be indicated, for example, by the upregulation of genes related to the UPR (unfolded protein response), which is known in the art. (Foti, D. M., et al. (1999) J. Biol. Chem. 274, 30402-30409).

[0057] In one embodiment, the recombinant organism may be a yeast that has been engineered to produce and secrete lactic acid. The applications of lactic acid and its derivatives encompass many fields of industrial activities (e.g., chemistry, cosmetics, and pharmacy), as well as important aspects of food manufacture and use. Furthermore, today there is growing interest in the production of such an organic acid to be used directly for the synthesis of biodegradable polymer materials.

[0058] Lactic acid may be produced by chemical synthesis or by fermentation of carbohydrates using single-celled organisms. The latter method is now commercially preferred because organisms have been developed that produce exclusively one isomer, as opposed to the racemic mixture generated by chemical synthesis. The most important non-recombinant industrial organisms currently used to produce lactic acid, such as species of the genera *Lactobacillus, Bacillus*, and *Rhizopus*, produce L(+)-lactic acid. Production by fermentation of D(−)-lactic acid or mixtures of L(+)- and D(−)-lactic acid are also known.

[0059] During a typical lactic acid fermentation, the accumulation of lactic acid in the medium is detrimental to

metabolic activity. In addition, the accumulation of lactic acid lowers the pH of the medium, which also inhibits cell growth and metabolic activity. As a result, the extent of lactic acid production is reduced as the lactic acid product accumulates.

[0060] Methods for the construction of recombinant yeasts expressing at least one copy of a lactate dehydrogenase (LDH) gene, which shifts the glycolytic flux towards the production of lactic acid, have been described in U.S. Pat. Nos. 6,429,006 and 7,049,108, both of which are incorporated herein by reference. These references report that lactic acid can be produced by metabolically modified yeasts belonging to the genera of *Kluyveromyces, Saccharomyces, Torulaspora* and *Zygosaccharomyces*. While any yeast species could be used, these species are preferred because these strains can grow and/or metabolize at very low pH, especially in the range of pH 4.5 or less. In addition, genetic engineering methods for these strains are well-developed, and these strains are widely accepted for use in food-related applications.

[0061] The yield of lactic acid can be increased by increasing copy numbers of the LDH gene in each yeast. Higher yields (>80% g/g) of lactic acid may be obtained from these engineered yeast strains if both the ethanolic fermentation pathway and the use of pyruvate by mitochondria are replaced by lactic fermentation. The recombinant yeast can also be transformed to overexpress a lactate transporter, for example, the JEN1 gene encoding for the lactate transporter of *S. cerevisiae*, can to ensure secretion of the product.

[0062] The expression of a LDH gene in yeast strains allows the production of lactic acid at acid pH values so that the free acid is directly obtained and the cumbersome conversion and recovery of lactate salts are minimized. In this invention, the pH of the fermentation medium may initially be higher than 4.5, but will decrease to a pH of 4.5 or less, preferably to a pH of 3 or less at the termination of the fermentation.

[0063] The gene coding for LDH may be from any species (e.g., mammalian, such as bovine, or bacterial), and it may code for the L(+)-LDH or the D(−)-LDH. Alternatively, both types of LDH genes may be expressed simultaneously. In addition, any natural or synthetic variants of LDH DNA sequences, any DNA sequence with high identity to a wild-type LDH gene, any DNA sequence complementing the normal LDH activity may be used.

[0064] The co-expression of ascorbic acid in a lactic acid producing microorganism to improve the stress tolerance and robustness of that organism could be accomplished by introduction of ME, LGDH, ALO, and, optionally, MIP. The transformation of the yeast strains could be carried out by means of either integrative or replicative plasmid or linear vectors. In a particular embodiment of the invention, the recombinant DNA is part of an expression plasmid which can be of autonomous or integrative replication.

[0065] For the production of lactic acid, the recombinant yeast strains that endogenously produce ascorbic acid and produce and secrete lactic acid would be cultured in a medium containing a carbon source, D-glucose, and other essential nutrients. The lactic acid would be recovered at a pH of 7 or less, preferably at a pH of 4.5 or less, and even more preferably at a pH of 3 or less. Because the pH of the culture medium would be reduced, less neutralizing agent would be required. The formation of lactate salt would be correspondingly reduced and proportionally less regeneration of free acid would be necessary in order to recover lactic acid.

[0066] Because the recombinant yeast are more stress tolerant due to the endogenous production of L-ascorbic acid, the yeast cells separated from the lactic acid product could be utilized again as seed microorganisms for a fresh lactic acid fermentation. In addition, the yeast cells could be continuously separated and recovered during the lactic acid fermentation, and hence, the fermentation could be carried out continuously at low pH with less severe effects of pH and oxidative stress on yeast viability, production, productivity, and yield.

[0067] The following definitions are provided in order to aid those skilled in the art in understanding the detailed description of the present invention.

[0068] "Ascorbic acid" as well as "ascorbate" as used herein, refers to L-ascorbic acid.

[0069] "Ascorbic acid precursor" is a compound that can be converted by an organism of the present invention, either directly or through one or more intermediates, into L-ascorbic acid.

[0070] "Amplification" refers to increasing the number of copies of a desired nucleic acid molecule or to increase the activity of an enzyme, by whatsoever means.

[0071] "Codon" refers to a sequence of three nucleotides that specify a particular amino acid.

[0072] "DNA ligase" refers to an enzyme that covalently joins two pieces of double-stranded DNA.

[0073] "Electroporation" refers to a method of introducing foreign DNA into cells that uses a brief, high voltage DC charge to permeabilize the host cells, causing them to take up extra-chromosomal DNA.

[0074] "Endonuclease" refers to an enzyme that hydrolyzes double stranded DNA at internal locations.

[0075] "Engineered for industrial production" refers to a recombinant organism that has been genetically modified to produce an industrial product.

[0076] Enzyme 1.1.3.37, D-arabinono-1,4-lactone oxidase, refers to a protein that catalyzes the conversion of D-arabinono-1,4-lactone+O_2 to D-erythroascorbate+H_2O_2. The same enzyme due to broadness of substrate range catalyses the conversion of L-galactono-1,4-lactone+O_2 to L-ascorbic acid+H_2O_2. Erroneously the same enzyme is referred to as L-galactono-1,4-lactone oxidase (enzyme 1.1.3.24) (Huh, W. K. et al. (1998), Mol. Microbiol. 30, 895-903)

[0077] Enzyme 1.3.2.3, L-galactono-1,4-lactone dehydrogenase, refers to a protein that catalyzes the conversion of L-galactono-1,4-lactone+2 ferricytochrome C to L-ascorbic acid+2 ferrocytochrome C.

[0078] Enzyme 1.1.3.8, L-gulono-1,4-lactone oxidase, refers to a protein that catalyzes the oxidation of L-gulono-1,4-lactone to L-xylo-hexulonolactone which spontaneously isomerizes to L-ascorbic acid.

[0079] Enzyme GDP-mannose-3,5-epimerase (5.1.3.18), refers to a protein that catalyzes the conversion of GDP-mannose to GDP-L-galactose.

[0080] Enzyme myoinositol phosphatase (3.1.3.23), refers to a protein that catalyzes the conversion of L-galactose-1P to L-galactose. L-galactose-1-phosphatase has been annotated as inositol/myo-inositol monophosphatase galactose-1-phosphatase and may be referred to as MIP/VTC4 (Conklin, P. L. (2006) J. Biol. Chem. 281, 15662-70).

[0081] Other enzymes of interest, and their classification numbers, are as follows:

GDP-Mannose 3,5-epimerase	5.1.3.18
L-Galactono-1,4-lactone dehydrogenase	1.3.2.3
UDP-Glucuronate 4-epimerase	5.1.3.6
L-Gulono-1,4-lactone oxidase	1.1.3.8
Myoinositol 1-P monophosphatase	3.1.3.25
UDP-Glucose 4-epimerase	5.1.3.2
D-arabinose 1-dehydrogenase (NAD)	1.1.1.116
D-arabinose 1-dehydrogenase (NADP)	1.1.1.117

[0082] The term "expression" refers to the transcription of a gene to produce the corresponding mRNA and translation of this mRNA to produce the corresponding gene product, i.e., a peptide, polypeptide, or protein.

[0083] The term "fermentation" refers to a process in which organisms growing in a liquid or solid medium produce an industrial product. As used herein, the term does not refer exclusively to non-oxidative metabolism.

[0084] The phrase "functionally linked" or "operably linked" refers to a promoter or promoter region and a coding or structural sequence in such an orientation and distance that transcription of the coding or structural sequence may be directed by the promoter or promoter region.

[0085] The phrase "functionally transformed" refers to an organism that has been transformed with an exogenous nucleic acid and is capable of producing a functional protein or peptide encoded by that amino acid.

[0086] The term "gene" refers to chromosomal DNA, plasmid DNA, cDNA, synthetic DNA, or other DNA that encodes a peptide, polypeptide, protein, or RNA molecule, and regions flanking the coding sequence involved in the regulation of expression.

[0087] The term "genome" encompasses both the chromosomes and plasmids within a host cell. Encoding DNAs of the present invention introduced into host cells can therefore be either chromosomally integrated or plasmid-localized.

[0088] "Heterologous DNA" refers to DNA from a source different than that of the recipient cell.

[0089] "Homologous DNA" refers to DNA from the same source as that of the recipient cell.

[0090] "Hybridization" refers to the ability of a strand of nucleic acid to join with a complementary strand via base pairing. Hybridization occurs when complementary sequences in the two nucleic acid strands bind to one another.

[0091] The term "medium" refers to the chemical environment of the organism, comprising any component required for the growth of the organism and one or more precursors for the production of ascorbic acid. Components for growth and precursors for the production of ascorbic acid may or may be not identical.

[0092] "Open reading frame (ORF)" refers to a region of DNA or RNA encoding a peptide, polypeptide, or protein.

[0093] "Plasmid" refers to an extra chromosomal, replicatable piece of DNA.

[0094] "Polymerase chain reaction (PCR)" refers to an enzymatic technique to create multiple copies of one sequence of nucleic acid. Copies of DNA sequence are prepared by shuttling a DNA polymerase between two amplimers. The basis of this amplification method is multiple cycles of temperature changes to denature, then re-anneal amplimers, followed by extension to synthesize new DNA strands in the region located between the flanking amplimers.

[0095] The term "promoter" or "promoter region" refers to a DNA sequence, usually found upstream (5') to a coding sequence, that controls expression of the coding sequence by controlling production of messenger RNA (mRNA) or other functional RNAs, (e.g., tRNAs, rRNAs, sRNAs), by providing the recognition site for RNA polymerase and/or other factors necessary for start of transcription at the correct site.

[0096] A "recombinant cell" or "transformed cell" is a cell that contains a nucleic acid sequence not naturally occurring in the cell or an additional copy or copies of an endogenous nucleic acid sequence, wherein the nucleic acid sequence is introduced into the cell or an ancestor thereof by human action.

[0097] The term "recombinant vector" or "recombinant DNA or RNA construct" refers to any agent such as a plasmid, cosmid, virus, autonomously replicating sequence, phage, or linear or circular single-stranded or double-stranded DNA or RNA nucleotide sequence, derived from any source, capable of genomic integration or autonomous replication, comprising a nucleic acid molecule in which one or more sequences have been linked in a functionally operative manner. Such recombinant constructs or vectors are capable of introducing a 5' regulatory sequence or promoter region and a DNA sequence for a selected gene product into a cell in such a manner that the DNA sequence is transcribed into a functional mRNA, which may or may not be translated and therefore expressed.

[0098] "Restriction enzyme" refers to an enzyme that recognizes a specific sequence of nucleotides in double stranded DNA and cleaves both strands; also called a restriction endonuclease. Cleavage typically occurs within the restriction site or close to it.

[0099] "Selectable marker" refers to a nucleic acid sequence whose expression confers a phenotype facilitating identification of cells containing the nucleic acid sequence. Selectable markers include those, which confer resistance to toxic chemicals (e.g. ampicillin, kanamycin) or complement a nutritional deficiency (e.g. uracil, histidine, leucine).

[0100] "Screenable marker" refers to a nucleic acid sequence whose expression imparts a visually distinguishing characteristic (e.g. color changes, fluorescence).

[0101] "Transcription" refers to the process of producing an RNA copy from a DNA template.

[0102] "Transformation" refers to a process of introducing an exogenous nucleic acid sequence (e.g., a vector, plasmid, or recombinant nucleic acid molecule) into a cell in which that exogenous nucleic acid is incorporated into a chromosome or is capable of autonomous replication. A cell that has undergone transformation, or a descendant of such a cell, is "transformed" or "recombinant."

[0103] "Translation" refers to the production of protein from messenger RNA.

[0104] "Unit" of enzyme refers to the enzymatic activity and indicates the amount of micromoles of substrate converted per mg of total cell proteins per minute.

[0105] "Vector" refers to a DNA or RNA molecule (such as a plasmid, cosmid, bacteriophage, yeast artificial chromosome, or virus, among others) that carries nucleic acid sequences into a host cell. The vector or a portion of it can be inserted into the genome of the host cell.

[0106] The term "yield" refers to the amount of industrial product or L-ascorbic acid produced by the recombinant organism, as (molar or weight/volume) divided by the amount of precursor consumed (molar or weight/volume) multiplied by 100.

[0107] List of Abbreviations:

Asc L-ascorbic acid (vitamin C)

AGD L-galactono-1,4-lactone dehydrogenase (without signaling peptide)

ALO D-arabinono-1,4-lactone oxidase

ARA D-arabinose dehydrogenase

Gal L-galactono-1,4-lactone

Gul L-gulono-1,4-lactone

LGDH L-galactose dehydrogenase

ME Mannose epimerase

MIP Myoinositol phosphatase

RGLO L-gulono-1,4-lactone oxidase

TCA trichloroacetic acid

TPI triosephosphateisomerase

EXAMPLES

[0108] The following examples are included to demonstrate particular embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Materials and Methods

[0109] 1. Determination of Ascorbic Acid

[0110] Ascorbic acid was determined spectrophotometrically following the method of Sullivan, M. X. et al. (1955), Assoc. Off. Agr. Chem., 38, 514-518). The sample (135 μl) was mixed in a cuvette with 40 μl of H_3PO_4 (85%). Then 675 μl of α,α'-Bipyridyl (0.5%) and 135 μl $FeCl_3$ (1%) were added. After 10 min the absorbance at 525 nm was measured. In some experiments, the identity of the ascorbic acid was confirmed by HPLC (Tracer Extrasil Column C8, 5 μM, 15×0.46 cm, Teknokroma, S. Coop. C. Ltda. # TR-016077; Eluent: 5 mM cetyltrimethylammonium bromide, 50 mM KH_2PO_4 in 95/5H_2O/Acetonitrile; Flow rate: 1 ml min^{-1}, Detection UV @ 254 nm) with pure L-ascorbic acid (Aldrich, A9,290-2) as standard.

[0111] 2. Amplification of Specific Gene Sequences

[0112] To amplify specific gene sequences, PfuTurbo DNA polymerase (Stratagene #600252) was used on a GeneAmp PCR System 9700 (PE Appl. Biosystems, Inc.). Standard conditions used were: 400 μM dNTP, 0.5 μM primers, 0.5 mM $MgCl_2$ (in addition to the buffer), and 3.75 U Pfu per 100 μl reaction.

[0113] The sequences of the genes used have been publicly reported via Genbank, as follows, except for MIP. The MIP sequence listed as SEQ ID NO:4 differed from the Genbank sequence, accession no. NM_111155, by two translationally silent point substitutions: at bp271, A (NM_111155) to T (SEQ ID NO:4); at bp 685, T (NM_11155) to G (SEQ ID NO:4).

Gene	Genbank accession no(s).	SEQ ID NO:
ME	AY116953	3
MIP	n.a.	4
ALO	U40390, AB009401	5, 6
LGDH		7

[0114] The following program was used for amplification of ALO:

94° C.	5 min		
94° C.	45 s	}	33 cycles
50° C.	30 s		
72° C.	1 min 40 s		
72° C.	7 min		
4° C.	To completion		

[0115] The following program was used for amplification of LGDH:

94° C.	5 min		
94° C.	45 s	}	33 cycles
56° C.	30 s		
72° C.	1 min 40 s		
72° C.	7 min		

-continued

4° C.	To completion	

[0116] The following program was used for amplification of ME:

94° C.	5 min	
94° C.	15 s	} 30 cycles
50° C.	30 s	
72° C.	1 min 30 s	
72° C.	7 min	
4° C.	To completion	

[0117] The following program was used for amplification of MIP:

94° C.	5 min	
94° C.	15 s	} 28 cycles
59.8° C.	30 s	
72° C.	45 s	
72° C.	7 min	
4° C.	To completion	

[0118] Template DNA for LGDH, ME, and MIP: 50 ng plasmid cDNA library pFL61 *Arabidopsis* (ATCC #77500 (Minet M. et al. (1992), Plant J. 2, 417-422)). Template DNA for ALO: 50 ng genomic DNA from *S. cerevisiae* GRF18U, extracted using a standard method. PCR products were blunt-end cloned into the EcoRV site of pSTBlue-1 using the perfectly blunt cloning kit from Novagen Inc. (#70191-4).

Oligonucleotides used		Gene amplified
SEQ ID NO:8:	tttcaccatatgtctactatcc	ALO
SEQ ID NO:9:	aaggatcctagtcggacaactc	(yeast)
SEQ ID NO:10:	atgacgaaaatagagcttcgagc	LGDH
SEQ ID NO:11:	ttagttctgatggattccacttgg	(plant)
SEQ ID NO:12:	gcgccatgggaactaccaatggaaca	ME
SEQ ID NO:13:	gcgctcgagtcactcttttccatca	(plant)
SEQ ID NO:14:	atccatggcggacaatgattctc	MIP
SEQ ID NO:15:	aatcatgcccctgtaagccgc	(plant)

[0119] 3. Plasmid Construction

[0120] The naming convention used herein is that pSTBlue-1 containing, for example, ALO in the sense direction regarding its multiple cloning site (MCS) was designated pSTB ALO-1. In a further example, pSTBlue-1 containing ALO in the antisense direction regarding its MCS was designated pSTB ALO-2, and so on.

[0121] Inserts were cloned using either the pYX series (R&D Systems, Inc.) or the centromeric expression plasmids pZ_3 and pZ_4 (P. Branduardi, et al. The Yeast *Zygosaccharomyces bailii*: a New Host for Heterologous Protein Production, Secretion and for Metabolic Engineering Applications, FEBS Yeast Research, FEMS Yeast Res. (2004) 4, 493-504). Standard procedures were employed for all cloning purposes, (Sambrook, J. and Russell, D. W., *Molecular Cloning: A Laboratory Manual,* 3rd Edition, Cold Spring Harbor Laboratory Press, 2001).

pSTB LGDH-1	EcoRI	pYX022	pH LGDH	HIS 3 (marker)
pSTB ALO-1	EcoRI	pYX042	pL ALO	LEU 2 (marker)
pSTB ME-1	EcoRI	pZ_3	pZ_3 ME	Kan^r (marker)
pSTB ME-1	EcoRI	PZ_4	PZ_4 ME	Hph^r (marker)
pSTB MIP-1	EcoRI	pYX012	pU MIP	URA 3 (marker)

[0122] For all the work performed below, the yeast control strains were transformed with the corresponding empty vectors.

[0123] 4. Yeast Cultivation and Examination:

[0124] Yeast strains used were *S. cerevisiae* GRF18U (Brambilla, L. et al., 1999, FEMS Microb. Lett. 171, 133-140), *S. cerevisiae* GRFc (Brambilla et al. 1999 FEMS Microb. Lett. 171: 133-140), *S. cerevisiae* BY4742 (MATα; his3; leu2, lys2; ura3, EuroScarf Accession No. Y10000), *S. cerevisiae* YML007w (BY4742; MATα; his3; leu2, lys2; ura; YML007w::KanMX4 (the yap1 deleted strain) EuroScarf Accession No. Y10569); CEN.PK 113-5D (MATα ura3-52; cir+) (see, for example, VanDijken et al. (2000) Enzyme Microb. Technol. 26, 706-714); and BY4741 (MATα; his3; leu2; met 15; ura3, Euroscarf Accession No. Y00000), or strains derived from them through transformation with the different developed plasmids. All strains were cultivated in shake flasks in minimal medium (0.67% w/v YNB (Difco Laboratories, Detroit, Mich. #919-15), 2% w/v glucose or mannose, with addition of the appropriate amino acids or adenine or uracil, respectively, to 50 mL^{-1}) and/or the appropriate antibiotic (G418 or hygromicin to 500 mg/l and 400 mg/l, respectively) under standard conditions (shaking at 30° C.). The initial optical density at 660 nm was about 0.05 for ascorbic acid determination, and 0.1 for the kinetics of the recovery from oxidative stress.

[0125] Cells were recovered by centrifugation at 4000 rpm for 5 min at 4° C., washed once with cold distilled H_2O, and treated as follows: for determination of intracellular ascorbic acid, cells were resuspended in about 3 times the pellet volume of cold 10% TCA, vortexed vigorously, kept on ice for about 20 min, and then the supernatant was cleared from the cell debris by centrifugation.

[0126] 5. Yeast Transformation:

[0127] Transformation of yeast cells was performed by the standard LiAc/ss-DNA/PEG method (Gietz, R. D. and Schiestl, R. H. (1996), Transforming Yeast with DNA, Methods in Mol. and Cell. Biol.).

[0128] Experimental Results

[0129] 6. Expression of *Arabidopsis thaliana* ME, MIP, LDGH and *S. cerevisiae* ALO in GRF18U

[0130] The genes encoding *A. thaliana* ME, *S. cerevisiae* ALO, *A. thaliana* LGDH, and *A. thaliana* MIP were placed

under the control of the TPI (triosephosphateisomerase) promoter each on its own integrative plasmid, except ME, which was sub-cloned in a centromeric plasmid. Two or more of the genes were integrated into *S. cerevisiae* GRF18U and BY4742. Each gene was integrated at a unique locus.

[0131] FIG. **1** provides a schematic representation of the current understanding of the physiological biosynthetic pathway leading from D-glucose to L-ascorbic acid in plants. The following enzymes are involved: A, L-galactono-1,4-lactone dehydrogenase (1.3.2.3), B, L-galactose dehydrogenase, C, myoinositol phosphatase (3.1.3.23), D, pyrophosporylase, E, GDP-mannose-3,5-epimerase (5.1.3.18), F, mannose-1-phosphate guanylyltransferase (2.7.7.22), G, phosphomannomutase (5.4.2.8), H, mannose-6-phosphate isomerase (5.3.1.8), I, glucose-6-phosphate isomerase (5.3.1.9), J; hexokinase (2.7.1.1).

[0132] In the pathway shown in FIG. **1**, ALO catalyzes reaction A, LGDH catalyzes reaction B, ME catalyzes reaction E, and MIP catalyzes reaction C.

[0133] Wild-type yeast cells are known to produce GDP-mannose (reactions F-J in FIG. **1**) and to transport it to the endoplasmic reticulum.

[0134] The table below shows the conversion of D-Glucose and D-Mannose to ascorbic acid by *S. cerevisiae* GRFc (control), or *S. cerevisiae* GRF18U transformed with (i) ALO and LDGH; (ii) ALO, LDGH and ME; or (iii) ALO, LDGH, ME and MIP. Cells were grown on mineral medium (2% glucose or mannose, 0.67% YNB) starting from an OD^{660} of 0.05. After 24 hours of growth, ascorbic acid was determined. While both the wild-type GRFc and GRF18U cells transformed with ALO and LGDH did not accumulate ascorbic acid, cells transformed with ALO, LDGH and ME, or ALO, LDGH, ME and MIP, respectively unexpectedly accumulated considerable amounts (i.e. greater than background levels) of ascorbic acid.

[0135] Transformed yeast were batch grown on glucose- or mannose-based media:

Expressed gene	Total (ascorbate plus erythroascorbate) on glucose-containing media	Total (ascorbate plus erythroascorbate) on mannose-containing media
Wt (control)	0.0205	0.0220
ALO, LGDH (control)	0.0210	0.0221
ALO, LDGH, ME	0.0302	0.0332
ALO, LDGH, ME, MIP	0.0450	0.0296

(Total (ascorbate plus erythroascorbate) values are mg/OD^{660} of Biomass/L)

[0136] The values determined in the control strain indicate the production of erythroascorbate normally produced by wild type yeasts.

[0137] We conclude that the yeast endogenously possesses activities which can nonspecifically catalyze reactions from GDP-L-galactose to L-galactose (see FIG. **1**). Specifically, though not to be bound by theory, we conclude that GDP-L-galactose spontaneously hydrolyses to L-galactose-1-P and that a nonspecific phosphatase catalyzed the conversion of L-galactose-1-P to L-galactose, which was then converted to L-ascorbic acid by LGDH and ALO. MIP provided superior catalysis of L-galactose-1-P to L-galactose than did the putative nonspecific phosphatase (ALO, LGDH, ME, MIP vs. ALO, LGDH, ME).

[0138] We did not observe any ascorbic acid accumulation in the medium.

[0139] 7. Sensitivity to Oxidative Stress

[0140] FIG. **2** shows that YML007w yeast hosts are particularly sensitive to oxidative stress. Yap1p activates genes required for the response to oxidative stress; deletion of this gene leads to the observed phenotype (Rodrigues-Pousada C A, et al. (2004) FEBS Lett.

[0141] 567, 80-85)

[0142] The following yeast strains have been analyzed:

[0143] BY4742 (▲).

[0144] YML007w (○)

[0145] FIG. **2**A. The yeast strains were grown on mineral medium (2% glucose, 0.67% YNB) starting from an OD^{660} of 0.1.

[0146] FIG. **2**B. The yeast strains were grown on mineral medium (2% glucose, 0.67% YNB) starting from an OD^{660} of 0.1 in the presence of 0.8 mM of H_2O_2.

[0147] FIG. **2**C. The yeast strains were grown on mineral medium (2% glucose, 0.67% YNB) starting from an OD^{660} of 0.1 in the presence of 1.0 mM of H_2O_2.

[0148] The two strains grew in the absence of H_2O_2 (FIG. **2**A) while growth of the YML007w yeast host was strongly delayed in medium containing 0.8 mM of hydrogen peroxide (FIG. **2**B) and completely impaired in the medium containing 1 mM of hydrogen peroxide (FIG. **2**C).

[0149] 8. Effect of Ascorbic Acid in Media on Stress Tolerance

[0150] FIG. **3** shows that the growth sensitivity of YML007w yeast, as shown in FIG. **2**, can be rescued by adding ascorbic acid to the medium, and that the effect of ascorbic acid in the medium on robustness is concentration dependent and can be optimized for different yeast strains.

[0151] FIG. **3**A. The yeast strains were grown on minimal medium (2% glucose, 0.67% YNB) starting from an OD^{660} of 0.1 in presence of 0.8 mM of H_2O_2. Ascorbic acid was added at T=0 at a final concentration of 15 mg/L. BY4742 (▲); YML007w (○).

[0152] FIG. **3**B. The yeast strains were grown on mineral medium (2% glucose, 0.67% YNB) starting from an OD^{660} of 0.1 in presence of 1.0 mM of H_2O_2. Ascorbic acid was added at T=0 at a final concentration of 15 mg/L. BY4742 (▲); YML007w (○).

[0153] FIG. **3**C. Three yeast strains (GRFc, BY4741, and CEN.PK 113-5D) were grown in 2×YNB medium (2% glucose, 1.34% YNB), containing lactic acid at 40 g/l, pH3. Ascorbic acid was added to the medium at the concentrations shown. The data demonstrate that the negative effects of lactic acid on growth can be overcome by exogenous ascorbic acid, and that the effect of ascorbic acid is dose dependent.

[0154] 9. Effect of Endogenous Ascorbic Acid on Sensitivity to Oxidative Stress

[0155] FIG. **4** shows that the growth defects of the YML007w yeast hosts can be rescued following expression of ALO, LDGH, ME, and MIP.

[0156] The following yeast strains have been analyzed:

[0157] BY4742 (▲)

[0158] YML007w expressing ALO, LDGH and ME (□)

[0159] YML007w expressing ALO, LDGH, ME and MIP (■)

[0160] FIG. **4**A. The yeast strains were grown on minimal medium (2% glucose, 0.67% YNB) starting from an OD^{660} of 0.1 in presence of 0.8 mM of H_2O_2.

[0161] FIG. **4**B. The yeast strains were grown on minimal medium (2% glucose, 0.67% YNB) starting from an OD^{660} of 0.1 in presence of 1.0 mM of H_2O_2.

[0162] Endogenous production of ascorbic acid "rescued" the yeast from stress-induced growth inhibition in a manner similar to that obtained by adding ascorbic acid to the culture medium (see FIG. **3**).

[0163] 10. Effect of Endogenous Ascorbic Acid on Robustness of GRF Yeast Strains

[0164] FIG. **5** shows that the wild type GRF yeast strain is sensitive to fermentative stress conditions (stress condition induced by adding 2 mM of H_2O_2); surprisingly, the recombinant yeast strains producing ascorbic acid show a strong robustness, indicating an increased tolerance to stress. The following yeast strains were analyzed: GRFc (closed triangle); GRF18U expressing ALO, LDGH and ME (open square); and GRF18U expressing ALO, LDGH, ME and MIP (closed square).

[0165] FIG. **5**A. The yeast strains were grown on mineral medium (2% glucose, 0.67% YNB) starting from an OD660 of 0.1.

[0166] FIG. **5**B. The yeast strains were grown on mineral medium (2% glucose, 0.67% YNB) starting from an OD660 of 0.1 in presence of 2.0 mM of H_2O_2. The wild type strain does not consume glucose.

[0167] All the strains used in this experiment bear the same auxotrophic complementation and the same antibiotic resistance cassettes (that are necessary for the expression of the different heterologous genes), so that it was possible to use the same media for all of them, either the ones expressing 3 or 4 heterologous genes or the wild type strain.

[0168] For this experiment, as a classical example of stress, we challenged wild type yeast cells with H_2O_2. As expected, wild type cells grow well in the absence of H_2O_2 (FIG. **5**A), but the same yeast cells do not grow in the presence of the H_2O_2 (FIG. **5**B). It is generally accepted that this external stressor leads to damage to DNA, damage to lipids, damage to proteins, and damage to membranes, among other subcellular structures, and ultimately leads to a loss of cell viability and cell integrity. Therefore, it is not surprising that the presence of this stressor leads to zero production, zero productivity and zero yield of the product (in this case, wild type yeast biomass), as shown in FIG. **5**B.

[0169] By the transformation of wild type GRF yeast with (i) LGDH, ALO, and ME or (ii) LGDH, ALO, ME and MIP, the recombinant yeast produced ascorbic acid, as described above, whereas wild type yeasts do not naturally produce ascorbic acid. Surprisingly, the bioprocess based on these recombinant yeasts showed a high production, high productivity, and a high yield of the product, yeast biomass (FIG. **5**B). Values for production, productivity, and yield are greater than 0.00 in the recombinant yeast (values for the control strain).

[0170] This experiment shows the two recombinant GRF yeast strains are more tolerant to stress than wild type GRF yeast, and may therefore be more suitable for certain industrial processes. Though not to be bound by a single theory, we consider it likely the recombinant yeast are less sensitive to diverse stressors, possibly through both direct scavenging of reactive oxygen species (ROS) by ascorbic acid and interference by ascorbic acid with unwanted stress reactions, such as apoptosis, cell death, viability loss, and loss of cell integrity.

[0171] 11. Effect of Endogenous Ascorbic Acid on ROS and Viability

[0172] The *S. cerevisiae* strains YML007w and YML007w transformed to express ALO, LDGH, ME, and MIP were grown in minimal glucose medium with or without addition of H_2O_2. Each culture was then split into two, and one was stained with dehydrorodamine for the detection of reactive oxygen species (ROS), the other was stained with propidium iodide for viability determination. Samples were then analyzed with a flow cytometer and compared. FIG. **6** demonstrates a correlation between ascorbic acid production and reduction in ROS formation, as well as reduction of the fraction of nonviable cells.

[0173] 12. Effect of Endogenous Ascorbic Acid on Sensitivity to Low pH and Lactic Acid

[0174] The *S. cerevisiae* strains BY4742c and BY4742 transformed to express ALO, LDGH, ME, MIP were inoculated in minimal glucose medium, minimal glucose medium at low pH (2.2), or minimal glucose medium at pH 3.0 containing 38 g/l of lactic acid. FIG. **7** shows growth curves for BY4742c (open squares) and the same yeast background transformed to produce ascorbic acid (dark squares) in minimal glucose medium, pH 2.2 (FIG. **7***a*), and in minimal glucose medium containing 38 g/l lactic acid, pH 3.0 (FIG. **7***b*). In the transformed yeast strain producing ascorbic acid, peak levels of cells at low pH are approximately three-fold greater and peak levels of cells in medium containing lactic acid are approximately five-fold greater compared with the non-transformed strain.

[0175] The same experiment was conducted after the two yeast strains were grown for about 24 hours in minimal glucose medium and then inoculated in minimal glucose medium at low pH (2.2), or minimal glucose medium at pH 3.0 containing 38 g/l of lactic acid. The results are shown in FIG. **8**. At low pH, the transformed strain producing lactic acid showed more than a six-fold increase in peak cell numbers compared with the non-transformed strain (FIG. **8***a*). In medium containing lactic acid, the non-transformed strain showed no increase in growth, whereas the transformed yeast strain producing ascorbic acid showed exponentional growth with an approximately 3.5 fold increase at peak levels (FIG. **8***b*).

[0176] 13. Effect of Exogenous Ascorbic Acid on Growth of Lactic Acid Producing Yeast m850.

[0177] *S. cerevisiae* strain NRRL Y-30696 was inoculated in minimal glucose medium and 2.78 g/L $CaCO_3$ or minimal glucose medium with 2.78 g/L $CaCO_3$ and 0.16, 0.3, or 0.6 g/L ascorbic acid. OD660 (open symbols) and lactic acid (closed symbols) were monitored with time. The pH dropped in each case to 2.5 at 67 hours. FIG. **9** shows that growth, as measure by OD660, increased with increasing ascorbic acid, 0 g/L (O), 0.16 g/L (+), 0.3 g/L (▲), or 0.6 g/L (♦), while lactic acid production was equivalent at each level.

[0178] 14. Construction of a Yeast Strain Co-Producing Lactic Acid and Ascorbic Acid.

[0179] *S. cerevisiae* NRRL Y-30696 (Y-30696) has previously been engineered to produce lactic acid. The ability to co-produce a low level of endogenous ascorbic acid could be introduced by integrating the genes required for ascorbic acid production into Y-30696. As shown above, production of significant endogenous L-ascorbic acid can be achieved by the expression of sequences encoding ME, LGDH, and ALO+/−MIP. One or more of these genes, functionally coupled to an appropriate promoter, could be added to the L-LDH bearing plasmid of Y-30696, while additional genes, coupled to appropriate promoters, could be introduced at the sites of the deleted PDC genes. Methods for these steps are known in the art, and are found in Sauer, M., et al. (2004) Applied Environmental Microbiology 70, 6086-6091.

[0180] While the compositions and methods and yeast strains of this invention have been described in terms of particular embodiments, it will be apparent to those of skill in the art that variations may be applied without departing from the concept, spirit and scope of the invention.

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What is claimed is:

1. A method of increasing stress tolerance in a recombinant organism that is engineered for industrial production of at least one product comprising functionally transforming the recombinant organism with a coding region encoding a mannose epimerase (ME), a coding region encoding an L-galactose dehydrogenase (LGDH), and a coding region encoding a D-arabinono-1,4-lactone oxidase (ALO), whereby the recombinant organism is enabled to produce ascorbic acid endogenously.

2. The method of claim 1, wherein the recombinant organism is further functionally transformed with a coding region encoding a myoinositol phosphatase (MIP).

3. The method of claim 1, wherein the recombinant organism is further functionally transformed with a coding region encoding an enzyme selected from the group consisting of L-galactono-1,4-lactone dehydrogenase (AGD), D-arabinose dehydrogenase (ARA), and L-gulono-1,4-lactone oxidase (GLO).

4. The method of claim 1, wherein the recombinant organism produces lactic acid.

5. The method of claim 1, wherein the recombinant organism is an organism selected from the group consisting of bacteria, yeast, filamentous fungi, and animal cells.

6. The method of claim 1, wherein the recombinant organism is a yeast belonging to a genus selected from the group consisting of *Saccharomyces, Zygosaccharomyces, Candida, Hansenula, Kluyveromyces, Debaromyces, Nadsonia, Lipomyces, Torulopsis, Kloeckera, Pichia, Schizosaccharomyces, Trigonopsis, Brettanomyces, Cryptococcus, Trichosporon, Aureobasidium, Lipomyces, Phaffia, Rhodotorula, Yarrowia*, and *Schwanniomyces*.

7. The method of claim 5, wherein the recombinant organism is a yeast selected from the group consisting of *S. cerevisiae* strain GRF18U; *S. cerevisiae* strains W3031B, BY4741, BY4742, CEN.PK 113-5D and YML007w; *K. lactis* strain CBS2359; *Z. bailii* strain ATCC 60483; *S. cerevisiae* strains NRRL Y-30696, NRRL Y-30698, NRRL Y-30742; *K. lactis* strains PM6-7/pEPL2, PMI/C1[pELP2]; *Z. bailii* strains ATTC36947/pLAT-ADH, ATCC60483/pLAT-ADH.

8. The method of claim 5, wherein the recombinant organism is a bacterium of a genus selected from the group consisting of *Bacillus, Escherichia, Lactobacillus, Lactococcus, Pseudomonas*, and *Acetobacter*.

9. The method of claim 5, wherein the recombinant organism is a bacterium selected from the group of bacterial strains producing lactic acid consisting of *Bacillus coagulans, Lactobacillus helveticus, Lactobacillus delbrueckii, Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus pentosus*, and *Streptococcus thermophilus*.

10. The method of claim 5, wherein the recombinant organism is a filamentous fungus of a genus selected from the group consisting of *Aspergillis, Rhizopus*, and *Trichoderma*.

11. The method of claim 5, wherein the recombinant organism is a filamentous fungus selected from the group consisting of *Aspergillus kawachii, Aspergillus nidulans, Aspergillus niger, Aspergillus oryzae, Rhizopus arrhizus, Rhizopus microsporus, Rhizopus oryzae, Trichoderma harzianum, Trichoderma reesei*, and *Trichoderma viride*.

12. The method of claim 1, wherein the ME has at least about 95% identity with SEQ ID NO:1.

13. The method of claim 2, wherein the MIP has at least about 95% identity with SEQ ID NO:2.

14. The method of claim 1, wherein the recombinant organism is a yeast, and wherein the yeast is engineered to produce at least one product selected from the group consisting of organic acids, amino acids, vitamins, polyols, solvents, biofuels, therapeutics, vaccines, proteins, and peptides.

15. The method of claim 1, wherein the recombinant organism is a yeast, and wherein the yeast is engineered to produce organic acids.

16. The method of claim 1, wherein the recombinant organism is a yeast, and wherein the yeast is engineered to produce lactic acid.

17. The method of claim 1, wherein the recombinant organism is a bacterium and wherein the bacterium is engineered to produce at least one product selected from the group consisting of organic acids, amino acids, vitamins, polyols, solvents, biofuels, therapeutics, vaccines, proteins, and peptides.

18. The method of claim 1, wherein the recombinant organism is a bacterium and wherein the bacterium is engineered to produce organic acids.

19. The method of claim 5, wherein the recombinant organism is a bacterium and wherein the bacterium is engineered to produce lactic acid.

20. The method of claim 1, wherein the recombinant organism is a filamentous fungus and wherein the filamentous fungus is engineered to produce at least one product selected from the group consisting of citric acid, lactic acid, and enzymes.

21. A method of increasing stress tolerance in a recombinant organism that is engineered for industrial production of at least one product, comprising culturing the recombinant organism in a medium containing an effective amount of ascorbic acid.

22. The method of claim 21, wherein the effective amount of L-ascorbic acid is 0.005 to 2.0 grams/liter.

23. The method of claim 21 wherein the effective amount of L-ascorbic acid is 0.015 to 0.1 gram/liter.

24. The method of claim 21, wherein the recombinant organism is engineered for the industrial production of lactic acid.

25. The method of claim 21, wherein the recombinant organism is a bacterium, a yeast, a filamentous fungus, or an animal cell.

26. A method of increasing stress tolerance in an organism that produces lactic acid comprising culturing the organism in a medium containing 0.005 to 2.0 grams/liter of ascorbic acid.

* * * * *

Influence of Low Temperature on Productivity, Proteome and Protein Phosphorylation of CHO Cells

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Abstract: Proliferation of mammalian cells can be controlled by low cultivation temperature. However, depending on cell type and expression system, varying effects of a temperature shift on heterologous protein production have been reported. Here, we characterize growth behavior and productivity of the Chinese hamster ovary (CHO) cell line XM111-10 engineered to synthesize the model-product-secreted alkaline phosphatase (SEAP). Shift of cultivation temperature from 37°C to 30°C caused a growth arrest mainly in the G1 phase of the cell cycle concomitant with an up to 1.7-fold increase of specific productivity. A low temperature cultivation provided 3.4 times higher overall product yield compared to a standard cultivation at 37°C. The cellular and molecular mechanisms underlying the effects of low temperature on growth and productivity of mammalian cells are poorly understood. Separation of total protein extracts by two-dimensional gel electrophoresis showed altered expression levels of CHO-K1 proteins after decrease in cultivation temperature to 30°C. These changes in the proteome suggest that mammalian cells respond actively to low temperature by synthesizing specific cold-inducible proteins. In addition, we provide the first evidence that the cold response of mammalian cells includes changes in postranslational protein modifications. Two CHO proteins were found to be phosphorylated at tyrosine residues following downshift of cultivation temperature to 30°C. Elucidating cellular events during cold exposure is necessary for further optimization of host-cell lines and expression systems and can provide new strategies for metabolic engineering. *Biotechnol Bioeng* **63**: 573–582, 1999.

INTRODUCTION

All living cells show a distinct response to external stresses such as temperature, pressure, osmotic stress, and oxygen deprivation. The most common cellular response includes changes in gene expression resulting in the synthesis of specific sets of stress proteins. The effect of heat shock on prokaryotic and eukaryotic cells has been extensively studied (for reviews see Craig et al., 1985; Hightower and Hendershot, 1997). Heat-shock stress leads to transient induction of heat-shock proteins (HSPs), a group of proteins that is highly conserved in all organisms from bacteria to mammals, many of which act as molecular chaperones for protein folding.

Recent studies focusing on the response of cells to a decrease in temperature led to the discovery of cold-inducible proteins. However, unlike HSPs, cold-shock proteins are not conserved among all species (for a review see Thieringer et al., 1998). In *Escherichia coli*, a temperature shift from 37°C to 10°C leads to transient inhibition of most protein synthesis. Growth is suppressed for a fourth period during which cells adapt to resume growth (Jones et al., 1987). During this lag phase, strong induction of the bacterial cold-shock proteins occurs. These cold-shock proteins have diverse functions and include, for example, the ribosome-associated initiation factor IF-2 (Jones et al., 1987) and the recombination factor RecA (Walker et al., 1984). The cold-inducible bacterial proteins CspA, CspB, and CspG share the cold-shock domain (CSD) as a common motif and are thought to function as RNA/DNA chaperones, assisting translation and replication at low temperatures (Jiang et al., 1997; Thieringer et al., 1998). The CSD is also present in eukaryotic Y-box transcription factors; however, Y-box proteins do not demonstrate cold inducibility (Wolffe et al., 1994).

Eukaryotic organisms also exhibit changes in gene expression in response to low temperature. *Saccharomyces cerivisiae* cells shifted from 30°C to 10°C growth with a shorter doubling time after a 1-h-growth lag (Kondo et al., 1992). Four different proteins were found to be synthesized at elevated levels after downshift of temperature. For example, the level of the NSR1 protein increased three-fold upon cold shock. This protein acts as an auxiliary factor for ribosome biosynthesis at low temperatures (Kondo et al., 1992).

Furthermore, response to low temperature has been reported in plants and many plant genes were shown to be induced by cold stress (Shinozaki and Yamaguchi-Shinozaki, 1996; Jaglo-Ottosen et al., 1998; Baker et al., 1994; Thomashow, 1994). These changes in gene expression are triggered by at least five signal-transduction pathways in the plant cell (Shinozaki et al., 1996). A variety of

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functions have been described for the products of cold-inducible plant genes, including RNA-binding and chaperone activity (Carpenter et al., 1994).

The effects of low temperature on physiology, growth, and gene expression in mammalian cells have not been extensively studied. A temperature shift from 37°C to 30–33°C prolongs the total generation time of cultured mammalian cells (Rao and Engelberg, 1965; Watanabe and Okada, 1967). Recent cloning of the murine cold-inducible RNA-binding protein CIRP provided first evidence for up-regulation of a mammalian gene product in response to low temperature (Nishiyama et al., 1997). It is important to note that overexpression of CIRP in mouse cells at 37°C caused accumulation of cells in the G1 phase of the cell cycle (Nishiyama et al., 1997). Likewise, the transcript level of RBM3, another member of the glycine-rich RNA-binding protein family, was reported to be significantly higher in human cells after temperature shift to 32°C (Danno et al., 1997).

Mammalian cell lines are widely used as host-production-cell lines in bioprocesses to produce pharmaceutically important proteins such as blood-clotting factors, monoclonal antibodies, growth factors, cytokines, and trombolytics. Overall heterologous protein production, product quality, and cell viability are critical parameters in optimization of mammalian cell-culture-production processes. In this respect, low-temperature cultivation has been discussed as a step to improve batch-culture performance (Bloemkolk et al., 1992; Giard et al., 1982; Reuveny et al., 1993; Sureshkumar and Mutharasan, 1991; Weidemann et al., 1994). Although these reports agree that a decrease in cultivation temperature leads to prolonged culture viability, the effects of low temperature on heterologous protein production of mammalian cells varied among different studies (Bloemkolk et al., 1992; Furukawa and Ohsuye, 1998; Giard et al., 1982; Reuveny et al., 1986; Sureshkumar and Mutharasan, 1991; Weidemann et al., 1994). For example, cultivation of hybridoma cells at low temperatures resulted in the loss of specific monoclonal antibody productivity (Sureshkumar and Mutharasan, 1991) while CHO cells secreting human recombinant antithrombin-III (ATIII) did not demonstrate changes in specific cellular productivity after temperature downshift to 33°C (Roessler et al., 1996). In this case, a lengthened production phase resulted in higher ATIII product yields. In summary, lowering culture temperature generally suppresses cell growth but its effect on cellular productivity is variable among different cell lines and expression systems.

Temperature is a process parameter by which the proliferation rate in production processes can be controlled. Cold-induced growth arrest was found to be G1-phase specific in CHO-batch cultures (Moore et al., 1997). Previously, we demonstrated that controlling proliferation of mammalian cells by overexpression of the growth inhibitory protein $p27^{KIP}$ leads to higher specific production of secreted alkaline phosphatase (SEAP) (Fussenegger et al., 1997; 1998; Muzar et al., 1998). p27 is a member of the CIP/KIP family of cell-cycle inhibitors (CKIs). This protein acts as a negative regulator of cyclin-dependent kinases (CDKs), reaching its maximum-inhibitory activity in the G1-phase of the cell cycle (see Hengst and Reed, 1998 for a review). Several cell lines were constructed by stable transfection of CHO-K1 cells with a dicistronic-expression-plasmid encoding p27 and SEAP under the control of the cytomegalovirus-derived tetracycline-regulatable promoter $P_{hCMV*-1}$. Overexpression of p27 led to accumulation of cells in the G1-phase and growth-arrested cells exhibited enhanced SEAP production compared to proliferating cells.

In this study, we investigated the effect of low-cultivation temperature on productivity of recombinant CHO cells and molecular changes that accompany a temperature shift from 37°C to 30°C. We characterized growth behavior and productivity of the cell line XM111-10 at low temperature. This cell line was constructed by engineering CHO cells to synthesize SEAP under the control of the P_{CMV*-1}-based expression system. To acquire initial information on the molecular responses to low-temperature cultivation, protein patterns of CHO cells shifted to low temperature were determined by using two-dimensional gel electrophoresis and compared to the proteome at 37°C. Furthermore, assays were conducted to check if changes in tyrosine phosphorylation might play a role in the cold response of CHO cells.

MATERIALS AND METHODS

Cell Culture

Chinese hamster ovary cells (CHO-K1, ATCC:CCL 61) were cultured in FMX-8 medium (Dr. Messi, Cell Culture Systems, Zurich, Switzerland) supplemented with 10% fetal calf serum (FCS, Boehringer Mannheim, Lot Nr. 14713602) and 100 IU/mL penicillin at 37°C in a humidified atmosphere of 5% CO_2 in air. The cell line XM111-10 derives from the CHO-cell line XMK1-9 (Fussenegger et al., 1997; Mazur et al., submitted). XMK1-9 cells constitutively express the tetracycline-responsive transactivator (tTA) necessary for tetracycline-repressible gene expression (Gossen and Bujard, 1992). The pMF111 expression construct encoding the secreted alkaline phosphatase (SEAP) under the control of the tetracycline-repressible promoter $P_{hCMV*-1}$ was stably transfected into XMK1-9 cells to give the CHO-cell line XM111-10 (Fussenegger et al., 1997; Mazur et al., 1998). XM111-10 cells were cultured in 10% serum-enriched FMX-8 medium supplemented with 100 IU/mL penicillin, 400 μg/mL G418, and 6 μg/mL puromycin. The cells were cultivated in the absence of tetracycline to allow SEAP transcription at the maximum level. For temperature-shift experiments, cells were grown at 37°C until the culture reached exponential growth phase and were then shifted to 30°C in a humidified atmosphere of 5% CO_2 in air. After indicated times, cells were harvested for 2-D electrophoresis-sample preparation or to obtain whole cell extracts for Western blot analysis. The growth curve of XM111-10 cells

was determined by counting the cells in a 25 cm^2 cell culture flasks (T25) every 24 h. The cultivation was stopped after cells reached the stationary/death phase. For each day an initial cell population of 2×10^5 cells was seeded in triplicate into T25 flasks.

At 24-h time intervals, the cells were completely detached with cell dissolvation solution (SIGMA) and diluted 1:1000 with filter-purified Casyton buffer. The cell number was determined as mean values of six independent readings taken by a Casy 1 cell counter (Schaerfe System, Reutlingen, Germany).

SEAP and Metabolic Assays

Five hundred μL of culture supernatant were incubated for 5 min at 65°C to inactivate endogenous phosphatase activity. Cell debris were separated in a subsequent centrifugation step for 2 min at 10,000*g*. Four hundred μL of the supernatant and 500 μL 2 × SEAP buffer (20 m*M* *L*-homoarginine, 2*M* diethanolamine pH 9.8, 1 m*M* $MgCl_2$) were transferred to a half-micro cuvette. The enzymatic reaction was initiated by adding 100 μL of 120 m*M* *p*-nitrophenylphosphate (SIGMA 104 phosphatase substrate). The increase in light absorbance at 405 nm which accompanies the hydrolysis of *p*-NPP was measured at appropriate time intervals, and is a direct proportional measure for SEAP concentration (Berger et al., 1988). One mU is defined as the amount of SEAP which will hydrolyze 1.0 m*M* of *p*-NPP per min, and this equals an increase of 0.04 A_{405} units per min.

To assess the metabolic activities of XM111-10 cells in low temperature vs. 37°C cultivations, we measured the specific glucose uptake of cultures under both conditions. Initial populations of 2.5×10^5 cells were seeded into T75 flasks, and glucose concentrations were measured in the culture supernatants using an automated enzyme analysis system according to the manufacturer's protocol (Synchron CX5CE, Fullerton, CA, Beckman).

Flow Cytometric Analysis

To quantify changes in cell-cycle distribution after temperature downshift cells were stained with propidium iodide to measure the DNA content by flow-cytometric analysis (FACS). Cells were detached with trypsin-EDTA, washed twice with ice-cold PBS and counted using the coulter counter as described above. Subsequently, 1×10^6 cells were resuspended in 500 μL cold PBS. To this suspension, 12 μL RNase A (10 mg/mL), 60 μL propidium iodide (50 μg/mL in 50 m*M* sodium citrate, pH 7.6) and 115 μL lysis buffer (0.75 % Nonidet P40; 5 m*M* EDTA; 5 m*M* EGTA; 0.1× YOPRO buffer) were added (5× YOPRO buffer: 100 m*M* sodium citrate, pH 4.0; 134 m*M* NaCl).

Cells were lysed on ice in the dark for 30 min prior to FACS analysis. Propidium iodide-mediated fluorescence is proportional to the cellular DNA content based on the stoichiometric affinity of this DNA intercalating dye. All FACS analyses were performed on a FACStar^{Plus} using the Cell Quest^{TM} software (Becton-Dickinson, San Jose, CA).

Two-Dimensional Gel Electrophoresis

At indicated time points, cells were harvested by scraping into cold-cell dissolvation solution (SIGMA) and washed three times with ice-cold PBS. After counting, cells were resuspended in 50 μL Z-buffer (10 m*M* Tris HCl (pH 6.8), 2% β-mercaptoethanol (BME), and 1% Nonidet P40). The cell suspension was sonified at 4°C for 120 s at 85% output (Branson Sonifier 450). The resulting lysates were heated to 95°C for 5 min to denature all proteins, and then immediately stored at 80°C until the first-dimension gel was loaded. Prior to loading, samples were mixed with an equal volume of solution C (9*M* urea, 2% BME, 2% NP-40, 0.8% BioLyte (pH3-10) carrier ampholytes (BioRad, Hercules, California), and 0.002% bromophenol blue). Two samples were mixed with a quantity of 2-D gel standards (BioRad) to determine approximate molecular weights and pIs of protein spots.

Equilibration, size separation, detection, and gel analysis were all performed as described previously (Lee et al., 1996). The gels were all silver-stained for protein detection. Isoelectric focusing was slightly different from the previously described protocol, and was performed as follows (old values in brackets): pH 3–10-nonlinear strips were used instead of the linear strips; the rehydration solution contained 0.333% [0.385%] BioLyte [pH 3–10], and 0.167% BioLyte [pH 5–7] (instead of 0.166% ServaLyte [pH 3–10]); the heat-transfer fluid between the DryStrip tray and the alignment card was Plusone DryStrip cover fluid (Pharmacia Biotech, Brussels, Belgium) instead of paraffin oil; the gels were run for a total of 71750 [72750] V h, with the voltage ramped linearly from 500 to 3500 V during the first 5 h [3 h] and maintained for 15.5 h [17.5 h] at 3500 V.

Western Blot Analysis

For analysis of tyrosine phosphorylated proteins, cells were solubilized in NP-40 extraction buffer (50 m*M* Hepes, pH 7.4, 150 m*M* NaCl, 25 m*M* β-glycero-phosphate, 25 m*M* NaF, 5 m*M* EGTA, 1 m*M* EDTA, 1% NP-40, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 m*M* PMSF, 1 m*M* sodium orthovanadate) for 10 min on ice. The lysates were clarified by centrifugation at 16,000*g* for 15 min. Protein concentrations were determined by Bradford assay. Seventy μg of total protein were subjected to SDS-PAGE (7.5% gel) and then blotted onto polyvinylidene difluoride membranes. After blocking with 20% horse serum (Gibco BRL) in TTBS (50 m*M* Tris, pH 7.5, 150 m*M* NaCl, 0.05% Tween-20), filters were probed with a phosphotyrosine-specific monoclonal antibody (Druker et al., 1989; Upstate Biotechnology, clone 4G10). Proteins were visualized with peroxidase-coupled secondary antibody using the ECL detection system (Amersham).

RESULTS

Growth Behavior and Morphology of XM111-10 Cells at 30°C

In CHO-K1 derived XM111-10 cells, secreted alkaline phosphatase (SEAP) is expressed in a tetracycline-dependent manner under the control of the $P_{hCMV*-1}$ promoter (Mazur et al., 1998; Mazur et al., submitted). In the present study, cells were grown without tetracycline in the culture medium to obtain SEAP secretion at the maximum level. To characterize the growth behavior of XM111-10 at different temperatures, we compared a low-temperature cultivation to the standard 37°C cultivation. Cultures were initiated by seeding 2×10^5 cells into T25 flasks, and cells were counted every 24 h for a period of 5 d. In the low-temperature cultivation, cells were shifted 48 h post-seeding from 37°C to 30°C. After the decrease in temperature growth of XM111-10 cells was almost completely arrested (Fig. 1), leading to a four times higher cell count in the 37°C culture compared to the 30°C culture at day 5 (72 h post-shift). CHO-K1 wild-type cells showed a similar growth behavior when exposed to low temperature (data not shown). After 5 d at 30°C, XM111-10 cells show changed morphology; cells were somewhat elongated compared to the round cells grown at 37°C (Fig. 2).

Cell-Cycle Distribution of XM111-10 Cells at 30°C

To further investigate the growth suppression of XM111-10 cells at 30°C, we quantified the cell-cycle distribution during a temperature shifted process by flow cytometric analysis. Cells were grown to early exponential phase and then shifted to 30°C. Cells of independent cultivations were harvested at indicated time points to survey the time course of growth inhibition at low temperature.

Prior to temperature downshift, 51% of the cells were in S phase, 36% were found in G1 phase, and 13% in G2/M (Fig. 3). When cells were harvested 48 h post-shift, 68% of the cells were in G1 vs. 23% in S and 10% in G2/M phase of the cell cycle. Exposure of XM111-10 cells to low temperature for 72 h resulted in accumulation of 79% of cells in G1 while 11% remained in S phase and 10% in G2/M (Fig. 3). These data clearly demonstrate that G1 phase is prolonged when XM111-10 cells are grown at 30°C. Accumulation of cells in G1 occurs within 2–3 d of cultivation, and cells were shown to be viable for at least 1 week post-shift, a time scale that makes a low-proliferation bioprocess feasible.

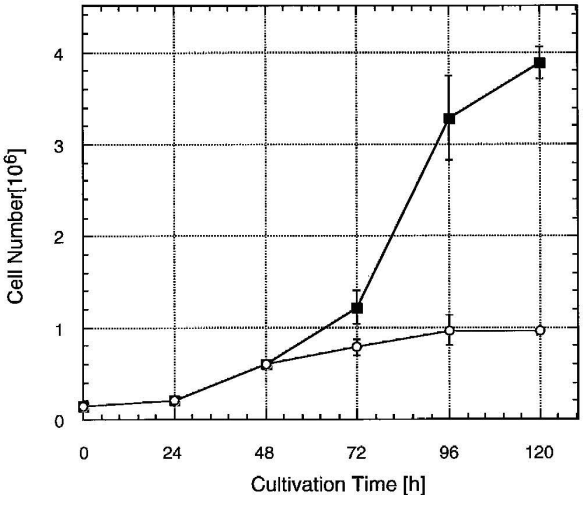

Figure 1. Comparison of growth behavior of XM111-10 cells grown at different temperatures. Cells were seeded at an initial cell density of 2×10^5 cells into T25 flasks and the cell number was measured daily for a period of 5 d. Low temperature cultivations were shifted from 37°C to 30°C 48 h post-seeding (○). Standard cultivations were grown for the entire period at 37°C (■).

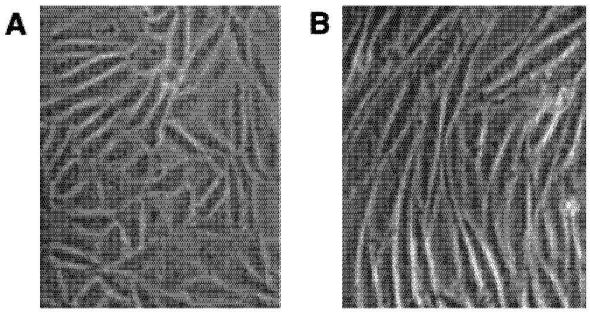

Figure 2. Morphology of XM111-10 cells at 37°C (A) and 5 d post-shift to 30°C (B).

Specific SEAP Productivity of XM111-10 Cells at 30°C

In the absence of tetracycline XM111-10 cells constitutively synthesize SEAP and secrete it into the culture medium (Mazur et al., submitted). To assess the influence of low temperature on $P_{hCMV*-1}$-directed expression and secretion of SEAP, we quantified the amount of SEAP in the culture medium for cultivations with and without temperature shift (see Fig. 1). Specific SEAP productivity values were obtained by normalizing the increase of product protein in the medium within a 24 h interval to 1×10^6 cells. When cells were grown at 37°C for the entire cultivation, specific SEAP productivity reached its peak values between 48 h and 72 h post-seeding (Fig. 4). This value was set to 100% and was determined to be 540 mU day^{-1} $(1 \times 10^6 \text{ cells})^{-1}$. On culture day 5, cells produced fivefold less SEAP. The specific productivity of cells grown at 30°C after 48 h increased up to 168 ± 3% compared to the 37°C productivity maximum, remaining at 154 ± 5% for another 24 h (Fig. 4). On culture-day 5, specific productivity at 30°C was eight times higher compared to the value obtained for the same day at 37°C.

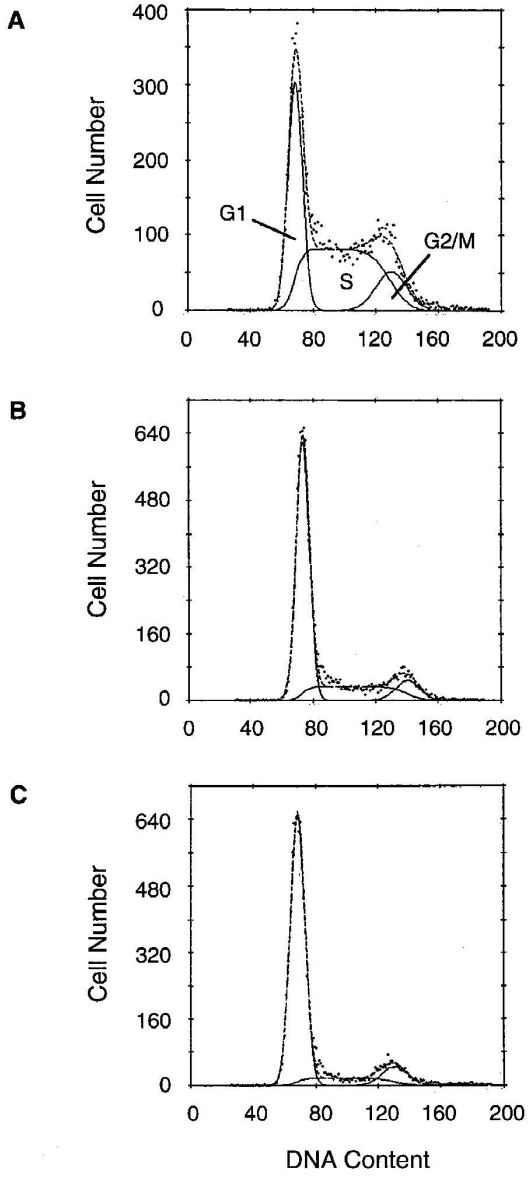

Figure 3. Cell-cycle distribution of XM111-10 cells in temperature-shift cultivations. Cells were grown at 37°C for 48 h and subsequently, temperature shifted to 30°C. Cells were harvested prior to shift (A), 48 h post-shift (B) and 72 h post-shift (C), stained with propidium iodide and analyzed for their DNA content by flow cytometry.

SEAP Production in a Temperature-Shifted-Batch Cultivation

The increase of specific SEAP productivity seen in XM111-10 cells grown at 30°C prompted us to determine the maximal product yield that could possibly be obtained at low temperature. We, therefore, shifted cells at higher densities to low temperature which resulted in a greater number of producing cells and terminated the cultivation only after some cells have started to enter death phase.

To quantify overall production 1×10^5 cells were seeded per T25 flask and grown at 37°C for 72 h before cultures were shifted to 30°C. SEAP accumulation in the medium was assayed and compared to the value obtained for the 37°C standard cultivation (Fig. 5). When cells were grown at 37°C, the maximum cell number was reached after 120 h. Cultures were terminated after 144 h when they entered death phase. In contrast, cultivation at 30°C prolonged the overall cultivation time up to 9 d. The maximum product amount for the 37°C process was set to 100% (154 mU/mL). In the low temperature cultivations SEAP accumulated up to 342 ± 4% after 9 culture days (Fig 5). Thus, approximately 3.5-fold higher final product titer is obtained with CHO XM111-10 cells in 30°C cultures.

Proteome Analysis of CHO Cells Grown at Different Temperatures

To survey the cold response of the CHO-cell proteome, we applied two-dimensional gel electrophoresis. CHO-K1 cells were grown at 37°C and shifted to 30°C after reaching exponential growth phase. Protein extracts from cells (5×10^6 cells per gel) harvested prior to shift and 48 h post-shift were compared. Proteins were visualized by silver-staining. In additional experiments, we separated 2-D standards (Biorad) alone and added to the samples. Superposition of these standard gels with the gels obtained for different temperatures provided the molecular weight and isoelectric point (pI) values marked in Figure 6. Computer-aided image analysis to compare 2-D electrophoresis patterns was performed using Melanie™ software. Care was taken to consider the relative overall darkness of certain gel regions to

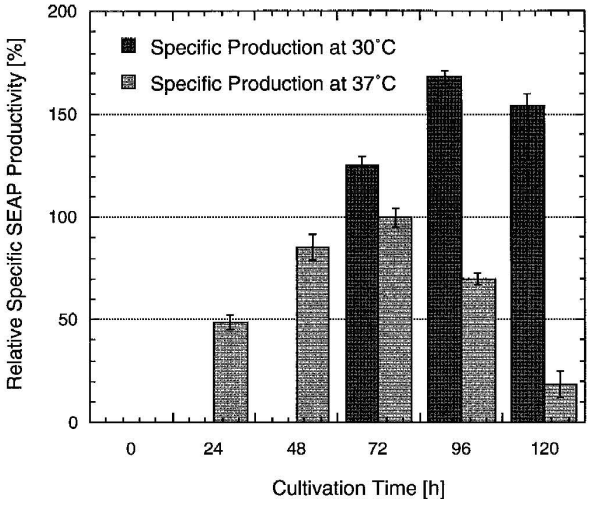

Figure 4. Relative-specific-SEAP productivity of XM111-10 cells at 30°C. The productivity data correspond to the growth behavior shown in Figure 1. SEAP concentration in the medium was measured every 24 h, and the daily increase was normalized to 1×10^6 cells to give specific productivity. The maximum specific productivity during standard cultivation at 37°C was set to 100% (540 mU d^{-1} (1×10^6 cells)$^{-1}$).

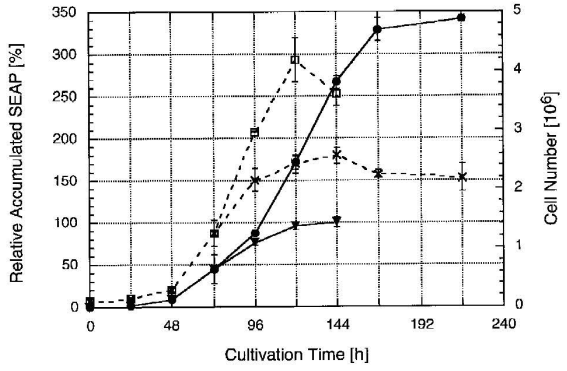

Figure 5. Cell proliferation and SEAP production in a temperature-shift-batch cultivation. XM111-10 cells were seeded at an initial cell density of 1×10^5 cells into T25 flasks, and the cell number was measured daily. Low-temperature cultivations were shifted from 37°C to 30°C 72 h post-seeding (✕). Standard cultivations were grown for the entire period at 37°C (□). Simultaneously, the SEAP concentration was measured in low temperature (●) and 37°C cultures (▼). The final SEAP yield of the 37°C cultivation was set to 100% (154 mU/mL).

eliminate effects caused by staining differences. Cold exposure clearly changed the overall protein expression patterns (Fig. 6). Spots that show significant changes in intensity after 48 h of cold exposure are highlighted in Fig. 6B. Six spots appeared darker on the 2-D image of extracts from the shifted cells. Three spots were detected with lower intensity on the same gel image. In one case, a spot clearly visible on the 37°C gel (Fig. 6B) was completely undetectable in extracts from cold-exposed cells. The changes in spot intensities seen in the 2-D images represent altered levels of a specific set of proteins when CHO cells are exposed to low temperature. In addition, we compared our gel images to a CHO-K1 reference protein map to match previously identified proteins (Lee et al., 1996). We were able to determine the location of HSP 90, HSC 70, HSP 60, β-Actin, α-Tubulin, and β-Tubulin (Fig. 6A). None of these proteins was subject to up- or downregulation due to cold exposure. However, our experiments demonstrate that low-temperature exposure of CHO cells changes the expression level of other specific proteins.

Influence of Low Temperature on Tyrosine-Phosphorylated Proteins in CHO Cells

Many signals are transmitted from the cell surface to the nucleus by kinase cascades, which trigger gene expression as a response to external stimuli. To obtain further insight into molecular pathways governing cold response of mammalian cells we surveyed the tyrosine-phosphorylation pattern in CHO cells when exposed to low temperature. CHO-K1 cells were grown at 37°C to exponential growth phase and subsequently shifted to 30°C.

Because both cell–cell contact and low temperature lead to growth suppression and may result in similar phosphorylation events, we included control extracts from cells which were kept at 37°C for an additional 24 h. We were thereby able to distinguish between the effect of cell–cell contact and low temperature on tyrosine-phosphorylated proteins. Proteins of CHO-K1 cells at 30°C were extracted at 24 h, 48 h, and 72 h post-shift. Extracts were examined for the presence of tyrosine-phosphorylated proteins by Western blot analysis using a phosphotyrosine-specific monoclonal antibody (Druker et al., 1989). Prior to the shift, five strong phosphotyrosine-containing bands were visible (Fig. 7, lane 1). In control cells at 37°C 24 h post-shift, increased tyrosine phosphorylation of a protein of approximately 160 kDa was observed (Fig. 7, lane 2). An increase in intensity of this band was also evident when extracts from cells grown at 30°C for 48 and 72 h were blotted (Fig. 7, lanes 4 & 5). However, two tyrosine-phosphorylated bands of approximately 120 and 140 kDa present also in control cells at 37°C demonstrated a more dramatic increase when cells were shifted to 30°C (Fig. 7, lane 2 vs. lane 5). A strong band corresponding to an approximately 40 kDa ty-

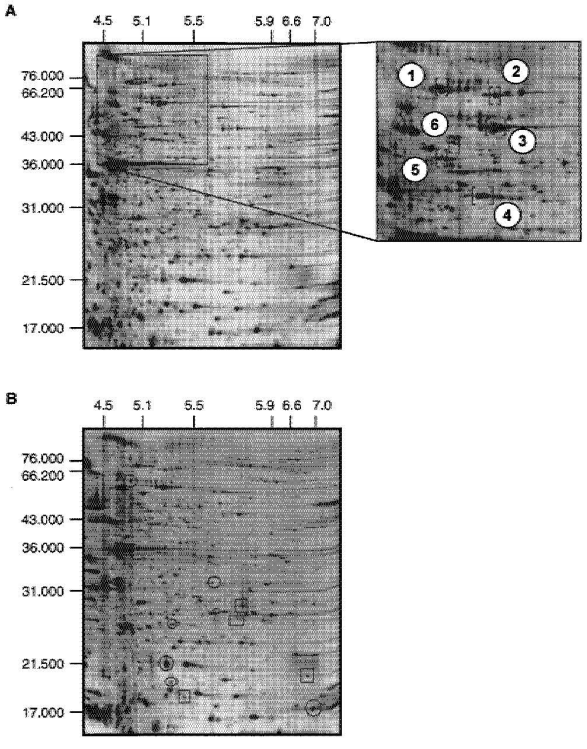

Figure 6. Alterations in the proteome of CHO cells following shift to low temperature. Whole-cell extracts of 5×10^6 CHO cells grown at 37°C (A) and 48 h post-temperature shift to 30°C (B) were separated by 2-D gel electrophoresis, and proteins were visualized by silver-staining. Spots that show significant changes in intensity after 48 h of cold exposure are highlighted in Figure 6B. Spots that appeared darker on the 2-D image are highlighted by ovals, while rectangles mark spots that were detected with lower intensity. Mobilities of coelectrophoresed-size markers are indicated on the left. pI marker values are indicated on the horizontal axis. Numbers in the enlarged gel section (A) refer to proteins identified by comparison to a CHO-K1 reference map (Lee et al., 1996): HSP 90 **(1),** HSC 70 **(2),** HSP 60 **(3),** β- and γ- Actin **(4)** β-Tubulin **(5)** and α-Tubulin **(6).**

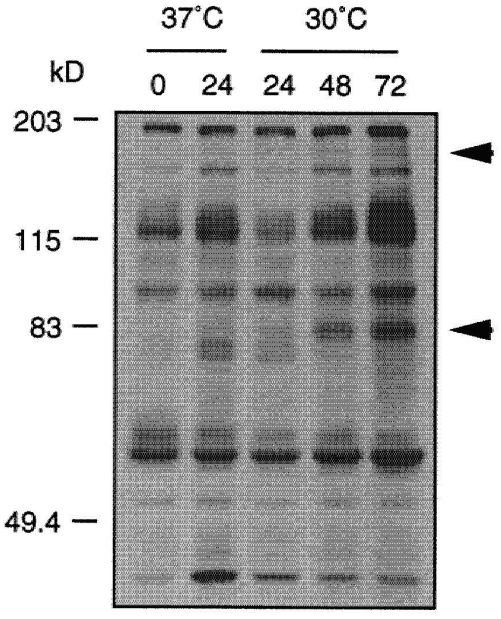

Figure 7. Influence of low temperature on tyrosine-phosphorylated proteins in CHO cells. Cells were harvested at indicated times (h) after temperature shift from 37°C to 30°C. The time point 37°C 0 h (lane 1) corresponds to the initial point of the 30°C culture. 70 μg of samples were separated by 7.5% SDS-PAGE. Membranes were probed with a phosphotyrosine-specific monoclonal antibody. Mobilities of coelectrophoresed size markers are indicated on the left. Arrows indicate low temperature specific bands.

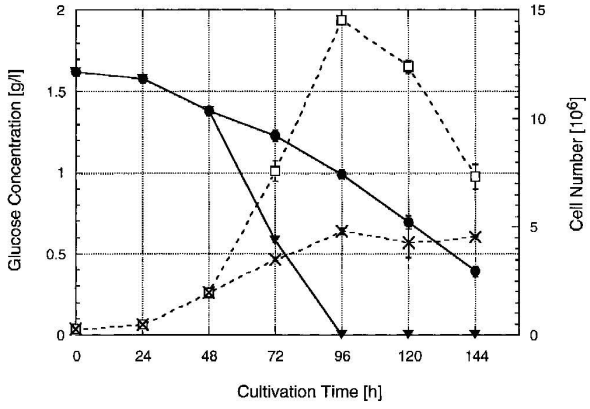

Figure 8. Cell proliferation and glucose consumption in a temperature-shift-batch cultivation. CHO cells were seeded at an initial cell density of 2.5×10^5 cells into T75 flasks and the cell number was measured daily. Low temperature cultivations were shifted from 37°C to 30°C 48 h post-seeding (✕). Standard cultivations were grown for the entire period at 37°C (□). Simultaneously, the glucose concentration was measured in low temperature (●) and 37°C cultures (▼).

rosine-phosphorylated protein occurred at 37°C 24 h post-shift, but was only weakly induced at 30°C. Interestingly, an approximately 80 kDa protein-demonstrated phosphorylation on tyrosine at low temperature which steadily increased during the 72 h time course. Similarly, but to a lower extent, a 180 kDa protein showed elevated levels of phosphotyrosine after 72 h at 30°C (Fig. 7, lanes 3–5). On the other hand, no tyrosine phosphorylation of these two proteins was observed when cells were harvested at 37°C 24 h post-shift (lane 2), indicating that these phosphorylation events are specific to cells grown at low temperature.

Glucose Consumption of CHO Cells in Low-Temperature Cultivations

To exclude medium depletion as a possible factor in the appearance of the two tyrosine-phosphorylation specific bands (Fig. 7), we quantified the glucose concentration in the culture medium for cultivations with and without temperature shift. Cultures were initiated by seeding 2.5×10^5 cells into T75 flasks and cells were counted every 24 h for a period of 6 d. In the low-temperature cultivation, cells were shifted 48 h post-seeding from 37°C to 30°C. The concentration of glucose in the culture medium was measured simultaneously (Fig. 8). The glucose concentration in culture medium 72 h post-shift to 30°C was 0.69 g/L compared to 0.58 g/L when cells grew for another 24 h at 37°C. These data indicate that medium depletion does not play a role in the induction of two tyrosine-phosphorylated proteins at low temperature seen in our immunoblot experiments (Fig. 7).

DISCUSSION

CHO cells are widely used for large-scale production of human recombinant proteins for therapeutic use. Production of heterologous proteins at low temperature is discussed as an approach to improve mammalian batch-culture performance. In this study, the CHO K-1 derived-cell line XM111-10, engineered to produce SEAP under the control of the cytomegalovirus derived P_{hCMV^*-1} promoter, was used as a model system to investigate the influence of low-cultivation temperature on heterologous protein production. We characterized the growth behavior of XM111-10 cells at low temperature by shifting cells to 30°C at different cultivation stages. In all experiments, the growth rate decreased significantly after temperature shift to 30°C, with growth suppression occurring more rapidly when cells were shifted at low densities. These results emphasize that temperature can be used as a means to control proliferation of mammalian cells.

FACS analysis showed that approximately 80% of cells accumulated in the G1 phase of the cell cycle 72 h after shift to 30°C. The G1/S transition is the most important restriction point in the mammalian cell cycle and its control is essential in many cellular processes such as embryonic development and cancer. It is tempting to speculate that cells respond actively to the decrease in temperature by halting cell-cycle progression at the G1/S boundary before DNA replication occurs. However, we found 10% of the cells to

reside in the G2/M phase of the cell cycle 72 h post-shift, indicating that cold-induced growth arrest might not only occur at a singular check point.

We next compared the specific SEAP productivity of XM111-10 cells following shift to 30°C to the maximum productivity value of cells grown at 37°C for the entire cultivation period. Our data show that in addition to increasing specific productivity for our system, low temperature prolongs cultivation time in batch cultures, and thereby further increases the overall amount of SEAP accumulated. According to previous studies, low temperature does not generally have a positive effect on recombinant-protein production and can, in some cases, result in decreased productivity (Bloemkolk et al., 1992; Giard et al., 1982; Reuveny et al., 1986; Sureshkumar and Mutharasan, 1991; Weidemann et al., 1994). Recently, enhanced C-terminal α-amidating enzyme production was observed in CHO cells grown at 30°C (Furukawa and Kazuhiro, 1998). These data are consistent with our findings and support the hypothesis that diverging results regarding productivity at low temperatures might be explained by different host-cell lines and/or promoters used.

Recently, we showed elevated SEAP production in $p27^{KIP1}$ overexpressing CHO cells (Fussenegger et al., 1998, Mazur et al., 1998; Mazur et al., submitted). Similar to cold exposed XM111-10 cells, these cells exhibit their increase in productivity while residing in the G1 phase of the cell cycle. Expression of the *seap* gene was driven by the $P_{hCMV*-1}$ promoter in both cases. $P_{hCMV*-1}$ consists of a minimal promoter sequence derived from the human cytomegalovirus (CMV) promoter IE combined with a multiple-tet operator site encoded in Transposon 10 of *E. coli*. In the absence of tetracycline the transactivator (tTA), which is constitutively expressed in XM111-10 cells, binds the tet-operator sequences and thereby, activates the promoter. Constitutive tTA expression in XM111-10 cells is driven by the full-length cytomegalovirus (CMV) promoter. Interestingly, it was recently reported by several groups that human cytomegalovirus infection inhibits cell-cycle progression, including the transition from G1 to S (Bresnahan et al., 1996; Dittmer and Mocarski 1997; Jault et al., 1995; Lu et al., 1996). Presumably, host-cell proliferation is blocked prior to S phase to provide a favorable environment for expression of viral genes. This could explain high activity of the CMV-derived $P_{hCMV*-1}$/tTA expression system in cells that reside in the G1 phase of the cell cycle. However, it remains to be investigated whether elevated transcription is responsible for the increase of SEAP secretion seen in cold exposed or p27 overexpressing CHO cells.

A temperature-shift process divides cultivation into a growth phase at 37°C and a low-proliferation phase at 30°C. Sometimes it is desirable to induce gene expression at a certain stage of the cultivation, for instance, when a gene product is expressed that inhibits growth of the host cell. In this case, separation of the process into a proliferation and a production phase would be the ideal solution. We expect our model system to be especially suitable for establishing such a biphasic bioprocess because product synthesis in XM111-10 cells can be completely suppressed by addition of tetracycline to the medium (Mazur et al., submitted). After an initial proliferation-phase withdrawal of tetracycline from the medium accompanied by a temperature downshift will suppress growth and initiate SEAP production. Similar processes can be established with cells engineered to coexpress growth inhibitors such as p27 together with the product gene in a tetracycline regulatable manner. However, a major problem in controlling proliferation in a production process by genetic engineering is the occurrence of mutants which escape growth suppression (Mazur et al., submitted). Mutation or loss of the genes necessary for proliferation control will provide a substantial growth advantage to mutant cells which can lead to a high number of non-producing cells and subsequent loss in product yield. On the contrary, no fast growing mutants were observed when proliferation of XM111-10 cells was controlled by low temperature.

The molecular response of mammalian cells to low temperatures between 27°C and 32°C is not well-understood. The discovery of the glycine-rich RNA-binding protein CIRP provided first evidence for induction of specific proteins in cold-exposed murine fibroblasts (Nishiyama et al., 1997). However, it still remains to be elucidated whether mammalian cells generally respond in an active manner by induction of cold-specific proteins. To test whether distinct proteins are subject to up- and downregulation due to a drop of cultivation temperature, the CHO proteome was analyzed by 2-D polyacrylamide electrophoresis of whole cell extracts from CHO cells grown at 37°C and from cells that were shifted for 48 h to 30°C. The 30°C 2-D gel images showed 10 spots with significantly higher or lower intensity compared to the 37°C standard. These experiments demonstrate that some proteins do, indeed, exhibit altered expression levels in mammalian cells after temperature downshift.

Stress signals must be transduced into biochemical changes to alter gene expression patterns. Such transduction is commonly mediated, in part, by a number of intracellular kinase and phosphatase enzymes. One group among them are the stress-activated protein kinases (SAPKs) or alternatively the c-jun *N*-terminal kinases (JNKs) and p38 MAP kinase (the mammalian homologue of yeast HOG1) (Paul et al., 1997). These kinases are activated in response to a number of cellular stresses including inflammatory cytokines, bacterial endotoxins, ischaemia/cellular ATP depletion, and heat and chemical shock. In stress-kinase pathways the signal is transduced by altering the phosphorylation state of tyrosine residues of target proteins. We used a phosphotyrosine-specific monoclonal antibody (Druker et al., 1989) to investigate the role of tyrosine phosphorylation in the cold response of CHO cells. Western blot analysis showed several tyrosine-phosphorylated proteins to be induced between 48 h and 72 h post-shift to 30°C. To distinguish between phosphorylation caused by contact inhibition in confluent cultures and temperature-specific effects we included extracts from cells grown another 24 h at 37°C (37°C 24 h).

Comparison of immunoblotted extracts revealed low-temperature-specific tyrosine phosphorylation of two proteins of approximately 180 kD and 80 kD, respectively. Additional experiments showed that 48 h and 72 h after the shift to low-temperature glucose concentration is higher than in the medium of the control cells (37°C 24 h) indicating that it is unlikely that medium depletion plays a roll in the induction of the two phosphorylated proteins.

To our knowledge, this report provides the first evidence for low-temperature-specific changes at a post-translational level in mammalian cells. The two proteins demonstrating cold-induced tyrosine phosphorylation may represent key regulators in mammalian cold response and remain to be identified. An important cellular event after decrease in cultivation temperature seems to be suppression of cell growth, preferably by halting the cell cycle in G1. It will be of great interest to identify the molecules which trigger this active-cellular response.

In summary, low-temperature cultivation is a means to control cell proliferation and can serve as a first step to establish two-stage mammalian bioprocesses. The decrease in temperature prolongs culture viability which is thought to be a result of delayed onset of apoptosis (Moore et al., 1997). Furthermore, temperature shift can lead to an increase in specific productivity and overall product yield. Determining molecular aspects of this stress response may, therefore, provide important information useful to further improve animal cell-culture performance.

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Hypothermia Causes a Reversible, p53-Mediated Cell Cycle Arrest in Cultured Fibroblasts

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Normal human fibroblasts grown in cell culture undergo a reversible growth arrest when incubated at 28°C. During incubation at 28°C, levels of p53 and p21 rise in these cells and cell cycle analysis shows that they have undergone a cell cycle arrest. To examine the importance of p53 in mediating this arrest, mouse embryo fibroblasts that are either wild-type or that are defective in p53 were also subjected to hypothermia. Only those cells with wild-type p53 undergo a cell cycle arrest, indicating that p53 has a role in mediating this response. Because many tumor cells have defective p53, this suggests that hypothermia may increase the selective toxicity of chemotherapeutic agents for tumor cells.

Key words: Hypothermia; Cell cycle arrest; p53 protein; Fibroblasts

Both hyper- and hypothermia have had wide applications in a variety of medical procedures and therapeutic regimens. Hyperthermia sensitizes tumor cells to ionizing radiation and to radiation combined with chemotherapy [reviewed in (1,2)]. The protective effect of mild hypothermia against neuronal damage following ischemia is well established (3,4) and has been attributed to hypothermia-modulated expression of specific immediate-early genes induced by ischemia (5,6).

The response of mammalian cells to elevated temperature as a single modality has been studied extensively during the past decade at the cellular and molecular levels. Progress has been made in understanding the role of heat shock proteins (HSPs) in transient thermotolerance, in permanent heat resistance [for review see (7)], and in protection against stress-induced apoptosis (8,9). In contrast to elevated temperatures, the present knowledge of molecular and biochemical consequences of downshifts in temperature comes primarily from studies of the cold shock response in *Escherichia coli* (10–12). Although the effect of low temperatures on growth and cell cycle progression was noted in mammalian cells as early as 1965 (13), the first known mammalian cold-inducible protein was identified only recently (14).

As reported here, we have found that hypothermia induces a cell cycle arrest and increases levels of p53 protein in normal human fibroblasts. Studies with mouse embryo fibroblasts have shown that in contrast to p53 wild-type cells, cells that are deficient in p53 fail to arrest at low temperature, indicating that hypothermia-induced cell cycle arrest of mammalian cells requires functional p53 protein.

MATERIALS AND METHODS

Cell Lines

The normal human diploid fibroblast cell line, AG01522, was obtained from the Aging Cell Repository, Coriell Institute for Medical Research (Camden, NJ) at a population doubling level (PDL) of 15 (PDL: number of cumulative population doublings from primary culture). Human fibroblasts were cultured as monolayers in minimum essential medium (MEM) supplemented with nonessential amino acids (ICN Pharmaceuticals, Costa Mesa, CA). Mouse embryonic fibroblast cells, wild-type for p53 and p53 null mutants, both at passage 2, were obtained from Stephen N. Jones, Department of Cell Biology, University of Massachusetts Medical Center (15,16). These cells were grown as monolayers in Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Gaithersburg, MD). Penicillin and streptomycin at concentrations of 100 IU/ml each and 15% heat-inactivated fetal calf serum were added to all media.

Culture Conditions and Viability Determinations

Cells were plated at a density of 2×10^4 cells/cm^2, and temperature experiments were performed with parallel sets of cells incubated at either 37°C or 28°C in humidified atmospheres with 5% or 4.6% CO_2, respectively. Cells were harvested by trypsinization for viability determinations by the trypan blue exclusion (TBE) assay. In order to include cells that may have been detached from the monolayer, the medium above the monolayer was collected and centrifuged prior to tryp-

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[2]Abbreviations used: BrdU, 5-bromo-2′-deoxyuridine; FACS, fluorescence activated cell sorting.

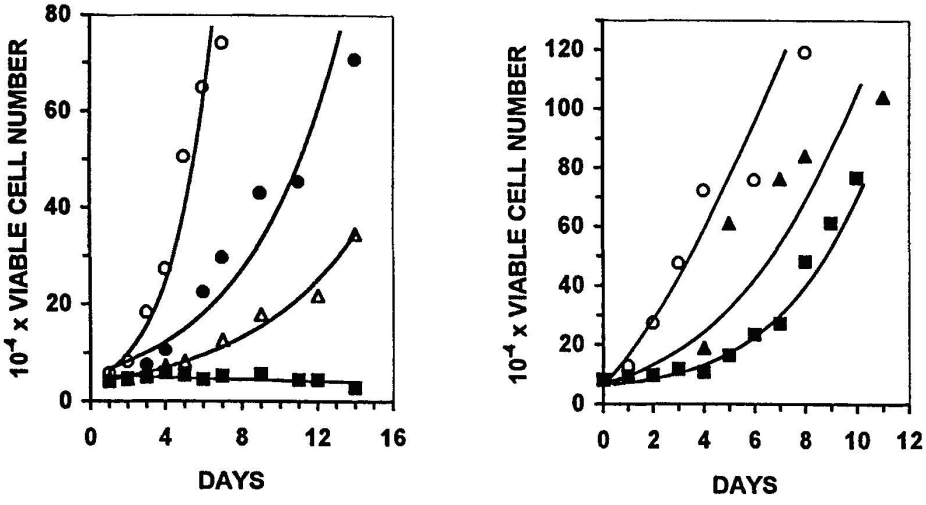

Figure 1. Effect of hypothermia on growth of normal human AG01522 fibroblasts. Left panel: cells were incubated continuously at 37°C (○), 33°C (●), 31°C (△), or 28°C (■). Right panel: cells were either incubated continuously at 37°C (○), or grown at 28°C for 2 (▲) or 4 (■) days and then transferred to 37°C. The viable cell number was determined by the trypan blue exclusion assay.

sinization, and the pellet was combined with cells detached by trypsin.

Cell Cycle Analysis

Cell cycle distribution was determined as DNA content per cell by propidium iodide staining. Approximately 1×10^6 cells were fixed with ethanol and kept at 4°C for at least 24 h before analysis. Prior to staining, low molecular weight DNA was extracted by the procedure described by Hotz et al. (17). This extraction allows identification of cell populations with fractional, sub-G_1 DNA content indicative of DNA fragmentation and cell death. Ethanol-fixed cells were pelleted and incubated for 30 min in pH 7.8 buffer consisting of 50 mM Na_2HPO_4 (9 parts) and 25 mM citric acid (1 part), containing 0.1% Triton X-100. Cells were then stained for 30 min at room temperature in 10 mM piperazine-*N,N'*-bis(2-ethanosulfonic acid) (PIPES) buffer, pH 6.8, containing 0.1 N NaCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 20 μg/ml propidium iodide, and 50 μg/ml RNase. Cell cycle analysis was performed using a Becton-Dickinson FACScan flow cytometer (Mountain View, CA). At least 15,000 events were collected per sample; cell doublets and aggregates were electronically eliminated from analysis. The percentage of cells in each phase of the cell cycle was determined using Modfit software (Verity Software House, Topsham, ME).

Replicative DNA synthesis was determined as 5-bromo-2′-deoxyuridine ($BrdU^2$) incorporation following a slightly modified procedure described by Jones et al. (15). Cells were labeled in 13 μM BrdU for 2 h, har-

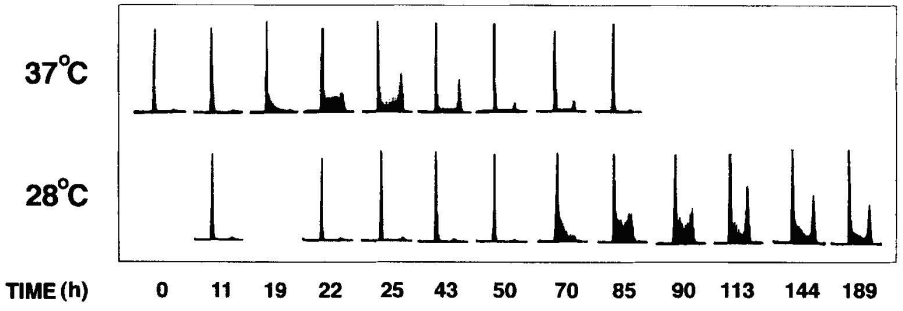

Figure 2. Effect of hypothermia on cell cycle progression in normal human AG01522 fibroblasts. Cells were plated at a density of 2×10^4 cells/cm^2 and incubated at either 37°C or 28°C; after staining with propidium iodide the distribution of DNA content was analyzed by FACS analysis. Top row: DNA profiles at 37°C; bottom row: DNA profiles at 28°C.

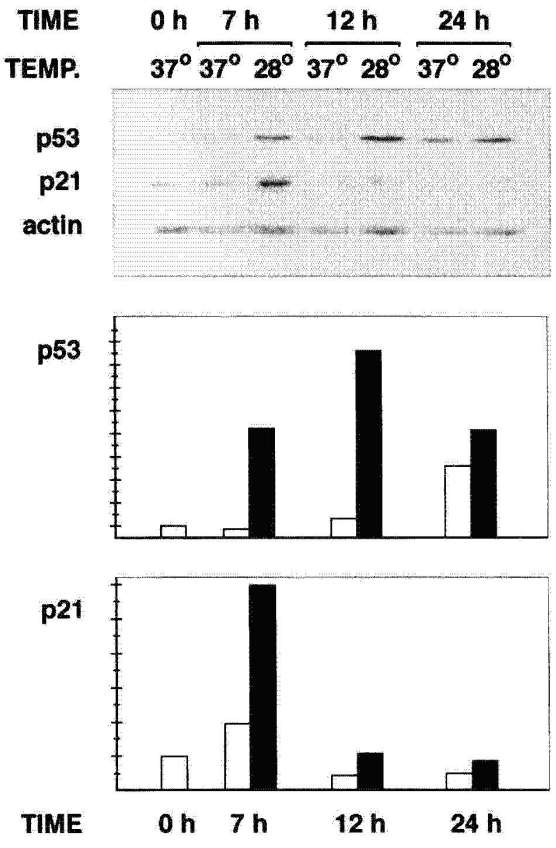

Figure 3. Levels of p53 and p21 in human fibroblasts grown at 37°C or at 28°C. Cells were plated at a density of 2×10^4 cells/cm^2 and incubated at either 37°C or 28°C. At the indicated times, cells were lysed and protein (50 µg) was separated by PAGE on 10% SDS gels. Western blot analyses (upper panel) were performed as described in the text. Levels of p53 (middle panel) and p21 (lower panel) were determined densitometrically. Plotted values are densities relative to β-actin. Open bars: 37°C; solid bars: 28°C.

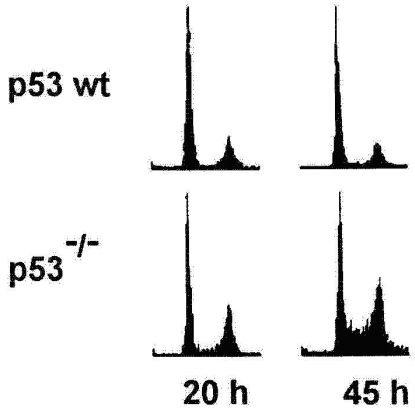

Figure 4. Effect of hypothermia on cell cycle progression in wild-type and p53-deficient mouse embryo cells. Cells were plated at a density of 2×10^4 cells/cm^2, incubated continuously at 28°C, and stained with propidium iodide for analysis of DNA content. Top row: wild type cells; bottom row: p53-deficient cells.

vested by trypsinization, fixed with 70% ethanol, and stored at −20°C until analysis. Samples were treated with 0.1 N HCl containing 0.5% Triton X-100 for 30 min at room temperature, boiled for 2 min, and rapidly cooled to denature DNA. Cells were then washed twice with 0.1 M $Na_2B_4O_7$ solution (pH 8.5), incubated with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibodies for 30 min (1:4 dilution; Caltag Laboratories, Burlingame, CA), and counterstained with propidium iodide as described.

Western Blot Analysis

For Western blot analysis, cells were harvested by trypsinization at either 37°C (for cells grown at 37°C) or at 28°C (for cells grown at 28°C), rinsed with PBS, and stored at −20°C until analysis. Samples were lysed on ice for 30 min in 50 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (Boehringer Mannheim, Indianapolis, IN), 100 KIU/ml aprotinin (Calbiochem, San Diego, CA), and 10 µg/ml leupeptin (Boehringer Mannheim). Cell lysates were centrifuged for 10 min at $15,600 \times g$ and the protein concentration in each supernatant was determined by the BioRad protein assay. Samples were boiled for 5 min in a loading buffer of 50 mM Tris-HCl, pH 6.8, containing 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.1% bromphenol blue, and 28 mM β-mercaptoethanol. Proteins (50 µg/lane) were resolved by electrophoresis in 10% polyacrylamide-SDS gels and transferred to a polyvinylidene fluoride membrane (BioRad, Hercules, CA) with a transfer buffer of 25 mM Tris-HCl containing 192 mM glycine and 10% v/v methanol. Biotinylated protein markers (New England Biolabs, Beverly, MA) were used as molecular weight standards. After transfer, the gel was stained with Coomassie blue as a control for loading and transfer. The membrane was probed with the p53-specific monoclonal antibody, Pab1800 (Oncogene Research Product-Calbiochem, Cambridge, MA), and then reprobed with the p21-specific antibody WAF1 (Ab-1) (Oncogene) and with the monoclonal anti-β-actin N350 antibody (Amersham Life Science, Arlington Heights, IL). Immunoreactive proteins were detected with alkaline phosphatase-conjugated anti-mouse immunoglobulin (IgG) and a chemiluminescent reagent (CDP-star) (New England BioLabs). After autoradiography, signals were quantitated by densitometric scanning with a Molecular Dynamics Personal Densitometer. Analysis was performed using the ImageQuant (version 1.1) program. Levels of p53 and p21 proteins were determined relative to the levels of β-actin.

RESULTS AND DISCUSSION

The effects of mild hypothermia on the growth of normal human fibroblast AG01522 cells are shown in

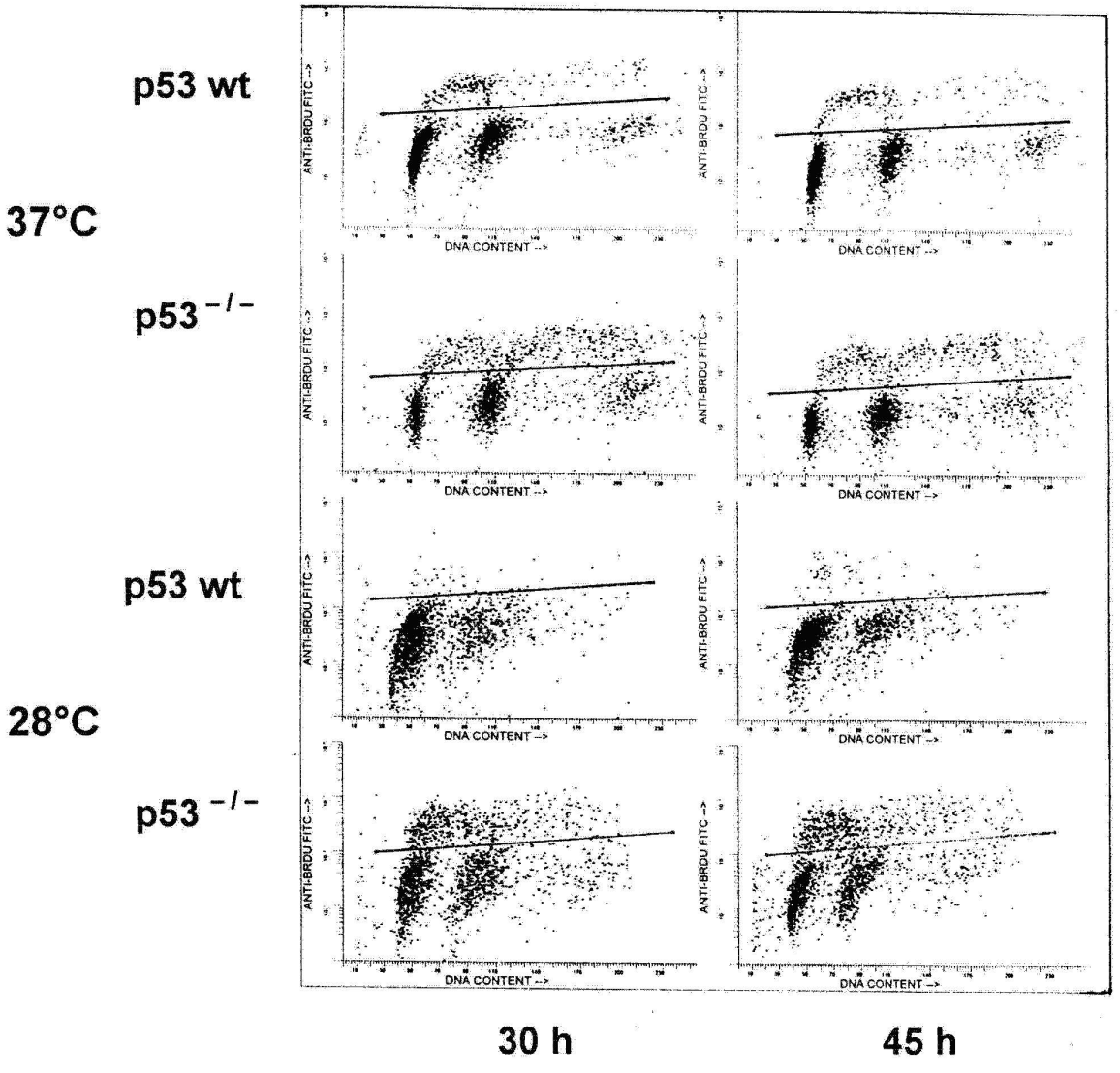

Figure 5. Effect of p53 status on cell cycle progression in mouse embryo fibroblasts incubated at 37°C or 28°C. Cells were plated at a density of 2×10^4 cells/cm^2 and incubated at either 37°C or 28°C. At the indicated times, the cells were labeled with BrdU for 2 h, fixed, stained with anti-BrdU-FITC and propidium iodide, and analyzed by flow cytometry. First row from the top: wild-type cells at 37°C; second row: p53-deficient cells at 37°C; third row: wild-type cells at 28°C; and bottom row: p53-deficient cells at 28°C.

Figure 1. The left-hand panel shows the growth of cells incubated at either 37°C, 33°C, 31°C, or 28°C. At 33°C and 31°C, the cell number increases more slowly than at 37°C, but at 28°C the cell number remains constant; there is no indication of cell death at the lower temperatures either by trypan blue exclusion criteria or from cell cycle analysis data. This suggests that the constant viable cell number in the population of AG01522 cells incubated at 28°C results from cell cycle arrest rather than from a balance between cell proliferation and cell death. The data in the right-hand panel of Figure 1 indicate that the growth arrest at 28°C is reversible; cells resume growth and proliferate with a normal growth rate after 2 or even 4 days at 28°C when the temperature is shifted back to 37°C.

We examined the effect of hypothermia on cell cycle progression by FACS analysis as shown in Figure 2. Fibroblasts were plated at a density of 2×10^4 cells/cm^2 and incubated continuously at either 37°C or 28°C; subsequently, cells were harvested and cell cycle analysis was performed as described. As seen from this figure, cell cycle progression into the S phase is delayed for at least 2 days in populations of AG01522 cells grown at 28°C. After 4 days, cells at 28°C start very slowly to

accumulate in the G_2/M phase and the fraction of cells in G_2/M increases on the expense of cells in G_1 from 12% on day 4 to 30% on day 8. A lack of increase in the percentage of cells in G_1 for the entire duration of the experiment suggests that cells do not progress into the next cell cycle. This is in agreement with the data on cell growth as determined by the TBE assay.

Cell cycle arrest is a cellular response common to many physiological stress conditions, but the pathways leading to arrest may be stress specific. G_1 arrest induced by DNA damaging agents is most often a manifestation of the p53-dependent transactivation of the cyclin-dependent kinase inhibitor, p21 (18–20). To examine the effects of mild hypothermia on cellular p53 and p21 protein levels in fibroblasts, Western blot analyses were performed with whole cell extracts from AG01522 cells incubated at either 37°C or 28°C. As shown in Figure 3, both p53 and p21 accumulated in cells incubated at 28°C. However, the time course for the accumulation of these two proteins was different. The highest level of p21, a threefold increase above the level at 37°C, was found in cells after 7 h of incubation at 28°C. The p53 levels peaked after 12 h and reached an eightfold increase above the level at 37°C. The levels of p21 protein had decreased before the cells were released from the G_1 block.

As suggested by other studies, the p53–p21 pathway may also be involved in cellular responses to elevated temperatures (21–23), and to cold shock (4°C for 60 min) (24). In contrast, hypoxia increases cellular p53 levels, but accumulation of cells in the G_1 phase by hypoxia is not p53 dependent (25). To test whether p53 is indeed required for the cell cycle arrest we observe at low temperature, cell cycle analyses were performed on wild-type and p53-deficient mouse embryo fibroblasts incubated at 28°C. The profiles of DNA content shown in Figure 4 indicate that p53-deficient cells progress through the cell cycle under hypothermic conditions whereas cells that are wild-type for p53 stay arrested for at least 2 days.

The requirement for p53 in cell cycle arrest by hypothermia was confirmed by measuring BrdU incorporation during the incubation of wild-type and p53-deficient mouse embryo fibroblasts at 28°C and 37°C. The results shown in Figure 5 demonstrate the suppression of replicative DNA synthesis in wild-type cells at 28°C for at least 45 h after plating. Even 65 h after plating (data not shown), fewer than 4% cells of the wild-type population incorporate BrdU at 28°C. In contrast, nearly 30% of the p53-deficient cell population undergo DNA synthesis at 28°C. One fraction of BrdU-incorporating p53-deficient cells has a higher than diploid DNA content, and probably reflects the DNA rereplication previously demonstrated for cells lacking p53 (26). A fraction of hyperdiploid cells is present in the populations of cells grown at both temperatures and is a growth characteristic of p53-deficient mouse embryo fibroblasts (27).

In conclusion, we have shown that mammalian cells undergo a reversible cell cycle arrest in response to mild hypothermia. Because p53 appears to be required for this arrest and many tumor cells lack functional p53, normal cells would presumably arrest while p53-deficient tumor cells would continue to cycle under hypothermic conditions. This hypothermia-induced cell cycle arrest could protect normal cells and might magnify the selective toxicity for tumor cells of antitumor agents that act on proliferating cells.

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online data

ATR (ataxia telangiectasia mutated- and Rad3-related kinase) is activated by mild hypothermia in mammalian cells and subsequently activates p53

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In vitro cultured mammalian cells respond to mild hypothermia (27–33 °C) by attenuating cellular processes and slowing and arresting the cell cycle. The slowing of the cell cycle at the upper range (31–33 °C) and its complete arrest at the lower range (27–28 °C) of mild hypothermia is effected by the activation of p53 and subsequent expression of p21. However, the mechanism by which cold is perceived in mammalian cells with the subsequent activation of p53 has remained undetermined. In the present paper, we report that the exposure of Chinese-hamster ovary-K1 cells to mildly hypothermic conditions activates the ATR (ataxia telangiectasia mutated- and Rad3-related kinase)–p53–p21 signalling pathway and is thus a key pathway involved in p53 activation upon mild hypothermia. In addition, we show that although $p38^{MAPK}$ (p38 mitogen-activated protein kinase) is also involved in activation of p53 upon mild hypothermia, this is probably the result of activation of $p38^{MAPK}$ by ATR. Furthermore, we show that cold-induced changes in cell membrane lipid composition are correlated with the activation of the ATR–p53–p21 pathway. Therefore we provide the first mechanistic detail of cell sensing and signalling upon mild hypothermia in mammalian cells leading to p53 and p21 activation, which is known to lead to cell cycle arrest.

Key words: ataxia telangiectasia mutated- and Rad3-related kinase (ATR), Chinese-hamster ovary cell (CHO cell), cold shock, hypothermia, lipidomics, metabolomics, p53.

INTRODUCTION

Under mildly hypothermic conditions (31–33 °C), mammalian cells proliferate slowly [1] and generally attenuate the processes of transcription and mRNA translation [2] (although protein folding may actually improve [3]), and the cell cycle proceeds at a much reduced rate [4]. However, below 30 °C, cells become arrested, predominantly in G_1 phase [5,6], normally the stage in the cell cycle when protein synthesis rates are optimal. Indeed, because of this, other strategies for inducing cell cycle arrest in late proliferative stage cultures of mammalian cells in an industrial sense have been investigated, including generation of cell lines with inducible expression of the general cyclin inhibitor p21 [7] and the addition of solvents, such as DMSO, to the growth medium, which also induces p21 expression [8]. However, exposure to mildly hypothermic conditions remains the most economic and most effective way of extending the productive life of cultured mammalian cells for large-scale recombinant protein production [9].

The slowing of the cell cycle at the upper range of mild hypothermia (31–33 °C) and its complete arrest at the lower range of mild hypothermia (27–28 °C) is regulated by the expression of p21 [10]. There are numerous examples (reviewed in [11]) of p21-induced cell cycle arrest protecting damaged or stressed cells from apoptosis, thus providing a time window within which the damage may be repaired or stress conditions removed. This is certainly the case for mildly cold-stressed cells, since they recover rapidly and fully on returning to 37 °C [12]. It is also well established that p21 induction in mildly hypothermic cells is subsequent to an increase in the stability and hence amounts of the tumour suppressor protein p53 [10,13] and to changes in the p53 isoform array [12], although the post-translational modification(s) generating these observed changes in p53 isoform pattern remain to be identified. Indeed, p53-deficient mammalian cells do not show cell cycle arrest at mildly hypothermic temperatures, confirming the key role of p53 in regulating this process upon cold shock [10,13]. However, the mechanism(s) by which p53 phosphorylation and amounts are unregulated upon mammalian cells being placed under hypothermic conditions, or how these conditions are sensed, are currently unknown.

In addition to cell cycle arrest and the general attenuation of transcription and translation, changes to the cell membrane composition are also observed when both prokaryotic [14] and eukaryotic [15] cells are exposed to hypothermic conditions. Essentially, cells respond to reduced temperature by increasing the polyunsaturated fatty acid content of membrane phospholipids, thereby maintaining the fluidity under hypothermic conditions, so-called homeoviscous adaptation [16]. At 37 °C, an increase in polyunsaturated fatty acid content of membrane phosphatidylcholines, induced by exposure to the Ca^{2+}-dependent phospholipase A_2 inhibitor BEL (bromoenol lactone), has been reported to arrest mammalian cells in G_1-phase by activation of the p53–p21 pathway [17]. This was subsequently shown to be mediated by phosphorylation of p53 at Ser^{15} by a member of

Abbreviations used: ATM, ataxia telangiectasia mutated; ATR, ATM- and Rad3-related kinase; BEL, bromoenol lactone; CHO, Chinese-hamster ovary; CIRP, cold-inducible RNA-binding protein; DMEM, Dulbecco's modified Eagle's medium; DNA-PK, DNA-dependent protein kinase; FBS, fetal bovine serum; mTOR, mammalian target of rapamycin; Mdm2, murine double minute 2; $p38^{MAPK}$, p38 mitogen-activated protein kinase; PC, principal component; PC-DFA, principal component-discriminant function analysis; PIKK, phosphoinositide 3-kinase-related kinase; qRT-PCR, quantitative real-time PCR; RNAi, RNA interference; siRNA, small interfering RNA; UTR, untranslated region.

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the PIKK (phosphoinositide 3-kinase-related kinase) family, ATR (ataxia telangiectasia mutated- and Rad3-related kinase) [17], a signalling pathway more usually associated with cell cycle arrest in response to compromised DNA replication [18]. Since there was no evidence of DNA damage in these cells with altered membrane composition, it was concluded that an increase in the ratio of polyunsaturated to saturated fatty acids in phosphatidylcholines in cell membranes independently activates the ATR–p53–p21 pathway [17]. Furthermore, the expression of the cold-shock protein CIRP [cold-inducible RNA-binding protein, also known as hnRNP (heterogeneous nuclear ribonucleoprotein) A18] is induced at mildly hypothermic temperatures in mammalian cells [19] and binds to the 3′-UTRs (untranslated regions) of certain transcripts, increasing their translation [20]. CIRP protein binds to the 3′-UTR of *ATR* mRNA, and overexpression of CIRP results in increased ATR protein levels [20].

In view of these reports and our own previous observations that changes in p53 isoform pattern are observed upon mildly hypothermic conditions in mammalian cells as are the detection of lipid droplets at lower temperature [12], we set out to investigate (i) whether p53 activation upon mild hypothermia was at least in part mediated through the ATR kinase signalling pathway, and (ii) whether mild hypothermia resulted in changes in lipid composition consistent with those previously reported to activate ATR (an increase in the ratio of polyunsaturated to saturated fatty acids), thus linking hypothermia-induced changes in membrane composition to hypothermia-induced cell cycle arrest. We show that p53 phosphorylation and activation in the commercially relevant CHO (Chinese-hamster ovary)-K1 cell line is mediated by the ATR–p53–p21 pathway and ATR signalling is thus a key pathway involved in p53 activation upon mild hypothermia, and, furthermore, that cold-induced changes in cell membrane lipid composition are associated with this. We therefore provide the first mechanistic detail of cell sensing and signalling upon mild hypothermia in CHO cells leading to p53 and p21 activation, which are known to subsequently result in cell cycle arrest.

EXPERIMENTAL

Cells and cell maintenance

CHO-K1 cells (originally sourced from the European Collection of Cell Cultures) were maintained in DMEM (Dulbecco's modified Eagle's medium)/F12 (Invitrogen), supplemented with 10 % (v/v) dialysed heat-inactivated FBS (fetal bovine serum) (PAA, catalogue no. A15–507), glutamine, glutamate, aspartate, nucleosides and non-essential amino acids (Invitrogen), at 37 °C in a 5 % CO_2 atmosphere as described previously [12]. HeLa (Ohio) cells (sourced from the A.T.C.C.) were maintained in DMEM supplemented with 10 % (v/v) FBS (PAA, catalogue no. A15–151), 2 mM glutamine and non-essential amino acids. ^{35}S-labelled amino acid incorporation into proteins was assessed as described in [12]. Exposure to mildly sub-physiological temperatures was undertaken in routine culture medium in appropriately regulated (±0.1 °C) incubators. Exposure to 15 μM BEL (Sigma) was for 6 h at 37 °C in normal growth medium. Caffeine (Sigma) was used at a final concentration of 2.5 mM, wortmannin (Sigma) at a final concentration of 20 μM, the p38 kinase inhibitor SP203580 (Calbiochem) at a final concentration of 10 μM, the ATM (ataxia telangiectasia mutated) inhibitor KU0055933 at a final concentration of 10 μM and the DNA-PK (DNA-dependent protein kinase) inhibitor NU7441 at a final concentration of 1 μM. Cells were exposed to these inhibitors for 30 min at 37 °C prior to transfer, without removal of the inhibitor, to mildly hypothermic conditions. For RNAi (RNA interference) knockdown, cells were transfected with validated siRNAs (small interfering RNAs) for Hs ATR (*Homo sapiens* ATR), *Hs_ATR_11* (Qiagen) and *Hs_ATR_12* (Qiagen), using HiPerfect reagent (Qiagen) as described in the manufacturer's instructions and a final siRNA concentration of 5 nM. When combining RNAi knockdown of ATR with inhibitor experiments, CHO-K1 cells were first exposed to ATR siRNA for 48 h at 37 °C, then 10 μM SP203580 was added for a further 30 min prior to transfer, in the continued presence of siRNA and SP203580, to 32 °C or 27 °C for 10 h.

Extraction of RNA, protein and lipids from cells

Total RNA was extracted from intact cells using the commercially available Qiagen RNeasy kit as per the manufacturer's instructions. Cell lysates for protein analysis were prepared by lysing PBS-washed cells into ice-cold extraction buffer, 20 mM Hepes/NaOH, pH 7.2, containing 100 mM NaCl, 1 % (w/v) Triton X-100, protease inhibitors (10 μg/ml leupeptin, 2 μg/ml pepstatin and 0.2 mM PMSF) and protein phosphatase inhibitors (50 mM NaF and 1 mM activated Na_3VO_4). For each lipid extraction, 5×10^6 CHO-K1 cells (60 % confluent) were washed with 10 ml of 0.8 % NaCl at the appropriate temperature and then scraped into 600 μl of solid-CO_2-chilled methanol, followed by extraction of lipids by vortex-mixing for 15 s with 600 μl of solid-CO_2-chilled chloroform and then freezing in liquid N_2 for 1 min before thawing on ice. This freeze–thaw procedure was repeated twice more. The chloroform extract was then washed twice by adding 900 μl of ice-cold water, vortex-mixing for 30 s followed by centrifugation at 16 000 ***g*** for 15 min and removal of the aqueous layer. Samples were stored at −80 °C prior to transportation (as solutions) on solid CO_2 to Manchester for lipid analysis.

qRT-PCR (quantitative real-time PCR) analysis of mRNA levels

Relative quantification of mRNA levels was undertaken by qRT-PCR using the Bio-Rad Laboratories iScript™ one-step kit as described in the manufacturer's instructions with the following primers: human ATR, Quantitect primer assay Hs_ATR_1_SG (Qiagen); CHO ATR, forward, 5′-GTTAATCCATGGTCGAGC-3′, reverse, 5′-TTGTCATAGTACTTGGCAAGG-3′; human actin, forward, 5′-CCGAGGACTTTGATTGCAC-3′, reverse, 5′-AGTGGGGTGGCTTTTAGGAT-3′; and CHO actin, forward, 5′-AGCTGAGAGGGAAATTGTGCG-3′, reverse, 5′-GCAACGGAACCGCTCATT-3′. Reactions were carried out using a Mastercycler® ep Realplex thermocycler (Eppendorf) programmed for a reverse transcription incubation at 50 °C for 10 min, followed by a 95 °C hold for 5 min and subsequent 40 cycles of 10 s at 95 °C and 20 s at 55 °C.

SDS/PAGE and immunoblot analysis

For SDS/PAGE analysis, 10 % separation gels were prepared as described by Laemmli [21], loading 20 μg of protein lysate per lane. SDS/PAGE-resolved polypeptides were transferred on to a nitrocellulose membrane, which was then blocked with 5 % (w/v) non-fat dried skimmed milk powder in 0.2 % Tween 20/TBS (Tris-buffered saline). Primary antibodies were sourced as follows: anti-ATR, Santa Cruz Biotechnology (N19); anti-p53, Dako (clone DO-7); anti-p53 phosphorylated at Ser^{15}, Cell Signaling Technology (#9284); anti-p21, Santa Cruz Biotechnology (C19); and anti-β-actin, Sigma (clone AC15). Horseradish peroxidase-conjugated secondary antibodies were detected by enhanced chemiluminescence using Hyperfilm ECL (GE Healthcare). Linearity of the antibody response over the

concentration range of the target protein had been established previously [12].

Immunofluorescence microscopy

PBS-washed CHO-K1 cells grown on 13 mm glass coverslips were fixed in 3 % (w/v) paraformaldehyde in PBS for 15 min at 37 °C or 27 °C, then permeabilized with 0.5 % Triton X-100 in PBS for 10 min at room temperature (21 °C). After blocking in 0.1 % Tween 20/PBS containing 3 % (w/v) BSA, coverslips were incubated with anti-ATR antibody (1:50 dilution) overnight at 4 °C. Further processing and detection were as described in [12].

MS analysis of lipids

Profiling of the lipid fraction of cell extracts was performed using DIMS (direct infusion MS [22]). Chloroform extracts (400 μl) were diluted in 600 μl of methanol. Samples were infused into an electrospray Thermo Fisher Scientific LTQ-Orbitrap XL mass spectrometer operating in negative-ion mode at a flow rate of 5 $\mu l \cdot min^{-1}$ for 1 min. Accurate mass data were acquired in the Orbitrap mass analyser operating at a mass resolution of 100 000 (at *m/z* 400) and with a scan time of 1.2 s. All mass spectra were averaged to provide a single mass spectrum for each sample that was passed forward for further data processing and analysis. All mass peaks were binned to the nearest nominal mass (defined as mass bins).

Data analysis of lipid profiles

Multivariate PC-DFA (principal component-discriminant function analysis) using cross-validation was performed to inspect the clustering of sample classes as detailed in [23]. All data were normalized to a sum of 1. A PCA (principal component analysis) model was then constructed [24] with three of six samples per class; the first ten PCs (principal components) were extracted and these accounted for 99.8 % of the total explained variance. Next, these PCs were used by the DFA algorithm [25] with the *a priori* knowledge of the six different treatments. In order to validate this PC-DFA model, it was tested by cross-validation by the projection of the three remaining samples, a process that allows cluster integrity to be assessed; that is to say, if the projected samples co-cluster with samples used to construct the PC-DFA model, then the groupings observed and their relationships are valid. All multivariate analyses were performed in MatLab (MathWorks). To define statistically significant differences, Kruskal–Wallis analysis of variance was performed. For those mass bins of statistical significance, further analysis was performed to determine the accurate lipid mass contributing to this statistical difference. Putative identification of lipids (as the deprotonated ion, sodiated or potassiated negatively charged adducts) was performed by matching to the accurate mass of lipids in the Lipid Maps database (http://www.lipidmaps.org/) with a mass accuracy less than 2 p.p.m.

RESULTS

p53 is phosphorylated at Ser^{15} when CHO-K1 cells are exposed to mildly hypothermic conditions

When CHO-K1 cells were transferred from 37 °C to 32 °C the subsequent growth rate was greatly reduced, and cells transferred to 27 °C ceased to proliferate (Figure 1A). Under both of these mildly hypothermic conditions, expression of p21 was induced and maintained throughout a 5 day period, whereas phosphorylation of p53 at Ser^{15} initially increased dramatically, but then decreased towards the end of the 5 day period (Figure 1B). Notably, the levels of both p53 phosphorylation and p21 expression were greater at 27 °C compared with 32 °C in line with the proliferation status of the cells at these two temperatures, and at 32 °C cell proliferation was observed towards the end of the 5 day period when Ser^{15} phosphorylation levels once again decreased (Figure 1B). A more detailed examination of the early period following the temperature shift to 27 °C (Figure 1C) clearly showed that the phosphorylation of p53 at Ser^{15} preceded a modest increase in p53 levels that, in turn, preceded the induction of p21. This

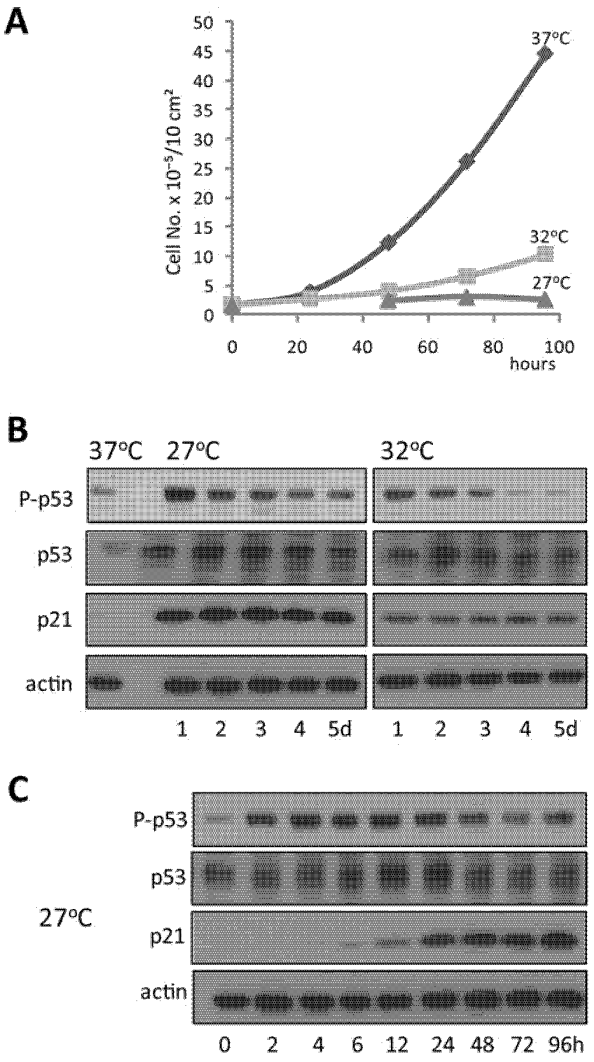

Figure 1 Mild hypothermia reduces cell proliferation and induces phosphorylation of p53 at Ser^{15} and subsequent p21 expression

(**A**) Growth curves of CHO-K1 cells maintained at the indicated temperatures. (**B**) Immunoblot detection of p53 phosphorylated at Ser^{15}, total p53 protein and p21 in lysates of CHO-K1 cells maintained at the indicated temperatures for the indicated number of days. (**C**) Immunoblot detection of p53 phosphorylated at Ser^{15}, total p53 protein and p21 in lysates of CHO-K1 cells maintained at 27 °C for the indicated number of hours. In (**B** and **C**), immunoblot detection of β-actin served as an indicator of lysate protein loading.